ISSN 0378-4347

VOL. 307 NO. 1 APRIL 13, 1984

(Biomedical Applications, Vol. 32, No. 1)

JOURNAL OF CHROMATOGRAPHY BIOMEDICAL APPLICATIONS

ELSEVIER

Scope. The *Journal of Chromatography* publishes papers on all aspects of chromatography, electrophoresis and related methods. Contributions consist mainly of research papers dealing with chromatographic theory, instrumental development and their applications. The section *Biomedical Applications,* which is under separate editorship, deals with the following aspects: developments in and applications of chromatographic and electrophoretic techniques related to clinical diagnosis (including the publication of normal values); screening and profiling procedures with special reference to metabolic disorders; results from basic medical research with direct consequences in clinical practice; combinations of chromatographic and electrophoretic methods with other physicochemical techniques such as mass spectrometry. In *Chromatographic Reviews,* reviews on all aspects of chromatography, electrophoresis and related methods are published.

Submission of Papers. Papers in English, French and German may be submitted, in three copies. Manuscripts should be submitted to: The Editor of *Journal of Chromatography,* P.O. Box 681, 1000 AR Amsterdam, The Netherlands, or to: The Editor of *Journal of Chromatography, Biomedical Applications,* P.O. Box 681, 1000 AR Amsterdam, The Netherlands. Review articles are invited or proposed by letter to the Editors and will appear in *Chromatographic Reviews* or *Biomedical Applications.* An outline of the proposed review should first be forwarded to the Editors for preliminary discussion prior to preparation. Submission of an article is understood to imply that the article is original and unpublished and is not being considered for publication elsewhere. For copyright regulations, see below.

Subscription Orders. Subscription orders should be sent to: Elsevier Science Publishers B.V., P.O. Box 211, 1000 AE Amsterdam, The Netherlands. The *Journal of Chromatography* and the *Biomedical Applications* section can be subscribed to separately.

Publication. The *Journal of Chromatography* (incl. *Biomedical Applications, Chromatographic Reviews* and *Cumulative Author and Subject Indexes, Vols. 101-150 and 276-300*) has 30 volumes in 1984. The subscription prices for 1984 are:

J. Chromatogr. (incl. *Chromatogr. Rev.* and *Cum. Indexes Vols. 101-150 and 276-300*) + *Biomed. Appl.* (Vols. 282-311):
Dfl. 4800.00 plus Dfl. 630.00 (postage) (total *ca.* US$ 2088.50)

J. Chromatogr. (incl. *Chromatogr. Rev.* and *Cum. Indexes Vols. 101-150 and 276-300*) only (Vols. 282-304):
Dfl. 4025.00 plus Dfl. 483.00 (postage) (total *ca.* US$ 1734.00)

Biomed. Appl. only (Vols. 305-311):
Dfl. 1225.00 plus Dfl. 147.00 (postage) (total *ca.* US$ 528.00).

Journals are automatically sent by airmail to the U.S.A. and Canada at no extra costs, and to Japan, Australia and New Zealand, with a small additional postal charge. Back volumes of the *Journal of Chromatography* (Vols. 1 through 281) are available at Dfl. 190.00 (plus postage). Claims for issues not received should be made within three months of publication of the issue. If not, they cannot be honoured free of charge. Customers in the U.S.A. and Canada wishing information on this and other Elsevier journals, please contact Journal Information Center, Elsevier Science Publishing Co. Inc., 52 Vanderbilt Avenue, New York, NY 10017. Tel. (212) 867-9040.

Abstracts/Contents Lists published in Analytical Abstracts, Biochemical Abstracts, Biological Abstracts, Chemical Abstracts, Chemical Titles, Current Contents/Physical, Chemical & Earth Sciences, Current Contents/Life Sciences, Deep-Sea Research/Part B: Oceanographic Literature Review, Index Medicus, Mass Spectrometry Bulletin, Referativnyi Zhurnal and Science Citation Index.

See page 3 of cover for Publication Schedule, Information for Authors and information on Advertisements.

0378-4347/84/$03.00

Printed in The Netherlands

VOL. 307, NO. 1 JOURNAL OF CHROMATOGRAPHY APRIL 13, 1984

(Biomedical Applications, Vol. 32, No. 1)

CONTENTS

(Abstracts/Contents Lists published in Analytical Abstracts, Biochemical Abstracts, Biological Abstracts, Chemical Abstracts, Chemical Titles, Current Contents/Physical, Chemical & Earth Sciences, Current Contents/Life Sciences, Deep-Sea Research/Part B: Oceanographic Literature Review, Index Medicus, Mass Spectrometry Bulletin, Pharmaceutical Abstracts, Referativnyi Zhurnal, and Science Citation Index)

(Continued overleaf)

Notes

JOURNAL OF CHROMATOGRAPHY

VOL. 307 (1984)

(Biomedical Applications, Vol. 32)

JOURNAL
of
CHROMATOGRAPHY

INTERNATIONAL JOURNAL ON CHROMATOGRAPHY,
ELECTROPHORESIS AND RELATED METHODS

BIOMEDICAL APPLICATIONS

Vol. 32 1984

ELSEVIER
AMSTERDAM — OXFORD — NEW YORK — TOKYO

J. Chromatogr., Vol. 307 (1984)

0378-4347/84/$03.00

Printed in The Netherlands

Journal of Chromatography, 307 (1984) 1—9
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2023

REVISED METHOD FOR THE QUANTITATIVE DETERMINATION OF 5-HYDROXYTRYPTAMINE IN HUMAN PLASMA BY GAS CHROMATOGRAPHY—MASS SPECTROMETRY—SELECTED ION MONITORING

SHIGEO BABA*, MASAHIRO UTOH and MASANOBU HORIE

Tokyo College of Pharmacy, 1432-1, Hachioji, Tokyo 192-03 (Japan)

and

YUKIO MORI

Gifu College of Pharmacy, 5-6-1, Mitahora-higashi, Gifu 502 (Japan)

(First received September 20th, 1983; revised manuscript received December 5th, 1983)

SUMMARY

To estimate the 5-hydroxytryptamine level in human plasma by gas chromatography—mass spectrometry—selected ion monitoring (GC—MS—SIM), a recovery method from plasma and derivatization conditions with pentafluoropropionic anhydride were investigated. By heating 5-hydroxytryptamine at 140°C for 4 h in the presence of pentafluoropropionic anhydride the peak intensity of derivatized 5-hydroxytryptamine increased more than three times in comparison with the reported method. There was a marked difference in the plasma levels of 5-hydroxytryptamine obtained using the GC—MS—SIM method compared to those obtained using fluorometric assay.

INTRODUCTION

5-Hydroxytryptamine (5-HT) is generally considered as a neurotransmitter in the brain and may play an important role in sleeping or learning mechanisms [1, 2]. To elucidate the relationship between the plasma level of 5-HT and mental retardation or hereditary disease, numerous studies on the determination of plasma 5-HT level in human have been performed [3—7]. There are, however, large variations in the measured values for plasma 5-HT among these

0378-4347/84/$03.00

reports. A fluorometric assay has been mainly used for the determination of 5-HT in biological samples. In this technique, however, it is difficult to correct for the recovery of 5-HT from plasma and to remove the interference of other compounds having the same fluorescence character as 5-HT. On the other hand, gas chromatography—mass spectrometry—selected ion monitoring (GC—MS—SIM) using deuterated 5-HT as an internal standard has also been applied to the quantitative determination of 5-HT [8, 9] because the instrumentation provides good sensitivity and specificity. However, in these reports, the analyzed samples were limited to urine and brain.

This present paper describes improved analytical methodology for the determination of 5-HT in human plasma by GC—MS—SIM. The method involves derivative formation for GC—MS—SIM analysis using deuterated 5-HT as an internal standard.

EXPERIMENTAL

Chemicals

5-HT[$\alpha,\alpha,\beta,\beta,$-$d_4$] creatinine sulphate (5-[^{2}H]HT) (98.0 atom %) and 5-HT[sidechain-^{14}C] creatinine sulphate (5-[^{14}C]HT) (58.0 mCi/mmol) were purchased from Merck Sharp & Dohm Canada and New England Nuclear, respectively.

Instrumentation

A Shimadzu GC-6 AM gas chromatograph was equipped with a synchronized accumulating radioisotope detector having five counting cells (cell volume = 30 ml) [10]. A Shimadzu LC-2 high-performance liquid chromatograph was also equipped with a synchronized accumulating radioisotope detector having five counting cells (cell volume = 1.1 ml) [11]. Gas chromatography (GC) was carried out under the following conditions: column, 1.5% OV-1 (2 m × 3 mm I.D.); injection port temperature, 220°C; column oven temperature, 200°C; carrier gas flow-rate, 50 ml/min (N_2). High-performance liquid chromatography (HPLC) operated under the following conditions; column, LiChrosorb C_{18} (4.6 mm × 15 cm, 5 μm); elution solvent, 0.01 *M* perchloric acid—methanol (9:1, v/v); flow-rate, 0.6 ml/min. SIM profiles were obtained with a Shimadzu LKB 9000B GC—MS instrument equipped with a multiple ion detector. Fluorometric assay was performed on a Hitachi MPF-2A fluorometer. Excitation and emission wavelengths were set to 380 nm and 500 nm, respectively.

Recovery of 5-HT from human plasma

To 200 μl of human plasma were added 100 nCi of 5-[^{14}C]HT and 5-HT was recovered by the following methods.

Method I. To the plasma sample were added 100 μl of 3% ascorbic acid, 2.5 ml of 2 *M* phosphate buffer (pH 10.5) and 2 ml of *n*-butanol. After shaking the sample for 20 min, the *n*-butanol layer was separated and washed with 0.5 ml of 0.05 *M* ammonium hydroxide. After addition of 2.5 ml of cyclohexane and shaking for 3 min, the upper layer was separated. To the obtained upper layer were added 200 μl of 0.05 *M* formic acid and the mixture was shaken for

5 min. After centrifugation, the aqueous layer was separated. This extraction was carried out once more with 200 μl of 0.05 *M* formic acid.

Method II. To the plasma sample were added 200 μl of 0.1 *M* zinc sulphate and 200 μl of 0.1 *M* barium hydroxide. After centrifugation, the supernatant was separated. To the residue were added 200 μl of 0.1 *M* zinc sulphate and 200 μl of 0.1 *M* barium hydroxide, and the supernatant was obtained after centrifugation.

Method III. To the supernatant described in Method II were added 200 μl of 0.5 *M* borate buffer (pH 10.0) and 5 ml of a mixed solution of *n*-butanol—diethyl ether (1:4, v/v). After shaking for 5 min, the organic layer was separated. This extraction was repeated twice.

Stability of 5-HT during recovery and derivatization processes

A portion of the recovered solutions (corresponding to about 1 nCi) obtained by Method I, II or III was injected into the radio-HPLC system. Each remaining solution was evaporated to dryness. After adding 100 μl of pentafluoropropionic anhydride (PFPA) to the residue, the mixture was heated at 60°C for 3 h. After evaporating the excess PFPA under a nitrogen stream, the residue was dissolved in 20 μl of ethyl acetate. A sample solution containing about 10 nCi of radioactivity was injected into the radio-GC system.

Derivatization conditions of 5-HT with PFPA

After adding 100 μl of PFPA to 200 nCi of 5-[^{14}C]HT, each solution was stood at room temperature, 60°C, 80°C, 100°C, 120°C, 140°C or 160°C. From each reaction mixture, 50 μl of solution were taken at 1, 2, 4 and 6 h after starting the reaction. Each solution was evaporated under a nitrogen stream. To the residue were added 20 μl of ethyl acetate and a portion of this solution (about 9 nCi) was injected into the radio-GC system.

Adsorption of 5-HT derivative on the surface of the GC connecting tube

The injection port was directly connected to a combustion tube by a brass tube (15 cm × 0.1 mm I.D.) to diminish adsorption of derivatized 5-HT on the column packing material. To 640 nCi of 5-[^{14}C]HT were added 100 μl of PFPA and the mixture was heated at 140°C for 2 h. After evaporating the excess PFPA, the residue was dissolved in 100 μl of ethyl acetate and a portion of this solution containing about 25 nCi was injected. $^{14}CO_2$ which was exhausted from the combustion tube was introduced into 6 ml of the mixed solution of methanol—ethanolamine (1:1, v/v). The radioactivity recovered in the ethanolamine was measured with a liquid scintillation counter.

Calibration curve for the GC—MS—SIM method

Appropriate amounts of 5-HT were added to 100 ng of 5-[^{2}H]HT in ratios from 0.1 to 2.0, and 100 μl of PFPA were added to the prepared mixture. After heating at 140°C for 2 h, the excess PFPA was evaporated under a nitrogen stream and the residue was dissolved in 20 μl of ethyl acetate. The multiple ion detector was focused on the ions of *m/z* 451 and 454, and 1 μl of each ethyl acetate solution was injected into the GC—MS system. The peak height ratio was calculated manually from the SIM profiles obtained.

Detection limit

To 5-HT in amounts of 0.1—100 ng were added 100 μl of PFPA and the mixture was heated at 140° C for 2 h. After evaporating the excess PFPA, the residue was dissolved in 200 μl of ethyl acetate and 1—5 μl of these solutions were injected into the GC—MS systems.

Determination of 5-HT in human plasma by GC—MS—SIM

After adding 100 ng of 5-[^{2}H] HT as an internal standard to 200 μl of human plasma, 5-HT was recovered from plasma by Method III. 5-HT was derivatized by adding 100 μl of PFPA and heating at 140° C for 2 h. The excess PFPA was evaporated under a nitrogen stream and the residue was dissolved in 20 μl of ethyl acetate; 2 μl of this solution were injected into the GC—MS system.

Determination of 5-HT in human plasma by fluorometric assay

To 1 ml of human plasma, which was the same sample used for the GC—MS—SIM assay, were added 0.5 ml of 5% disodium EDTA, 5 ml of water, 1 ml of 10% zinc sulphate and 0.5 ml of 1 *M* sodium hydroxide, then the mixture was shaken for 10 min. After centrifugation, 5 ml of the supernatant were added to a mixture of 1.5 ml of 0.1 *M* borate buffer (pH 12.0), 2.5 g of sodium chloride and 7 ml of *n*-butanol. After shaking for 10 min, the *n*-butanol layer was separated and washed with 5 ml of 0.1 *M* borate buffer. To 5 ml of the *n*-butanol layer obtained were added 7.0 ml of *n*-heptane and 1.5 ml of 0.05 *M* phosphate buffer (pH 7.0). After shaking for 5 min, the aqueous layer was separated. To 1 ml of the obtained aqueous solution were added 0.1 ml of 0.1 *M* ninhydrin and 0.1 ml of 0.15% ascorbic acid. The mixed solution was heated at 75° C for 30 min. After cooling to room temperature, fluorescence intensity was measured. The calibration curve was prepared by using a known amount of 5-HT instead of human plasma.

RESULTS AND DISCUSSION

To determine trace amounts of 5-HT in plasma with good accuracy using GC—MS—SIM, it is necessary to increase the recovery of 5-HT from plasma and the derivatization yield. Recovery methods for 5-HT from biological samples for GC analysis that have been used include adsorption with XAD-2 resin [12], deproteinization with zinc sulphate—barium hydroxide [13], or extraction with a polar organic solvent such as *n*-butanol [14]. In these reports, however, no detailed study on percentage recovery or derivatization yield was performed. In this present paper, fundamental investigations on recovery method and derivatization of 5-HT were carried out by use of a radioisotope tracer technique.

The recovery of 5-HT from plasma obtained by Method I, II or III was 63.9, 83.3 or 64.9%, respectively (Table I). To confirm the stability of 5-HT during the recovery procedure, a portion of the solution obtained from plasma by Method I, II or III was injected into the radio-HPLC system. It was apparent that decomposition of 5-HT did not occur during the recovery procedure, because a single peak corresponding to 5-HT appeared in each case. Radio gas chromatograms of the PFPA derivative of 5-[^{14}C] HT recovered from plasma by

TABLE I

RECOVERY OF 5-[^{14}C]HT AFTER PROCESSING FROM PLASMA

Method	Recovery (%)
I: Extraction using *n*-butanol following 0.5 *M* formic acid	63.9 ± 2.7*
II: Deproteinization using 0.1 *M* zinc sulphate and 0.1 *M* barium hydroxide	83.3 ± 1.7
III: Extraction using *n*-butanol—diethyl ether (1:4) after deproteinization	64.9 ± 2.5

*Mean ± S.D. (n = 3).

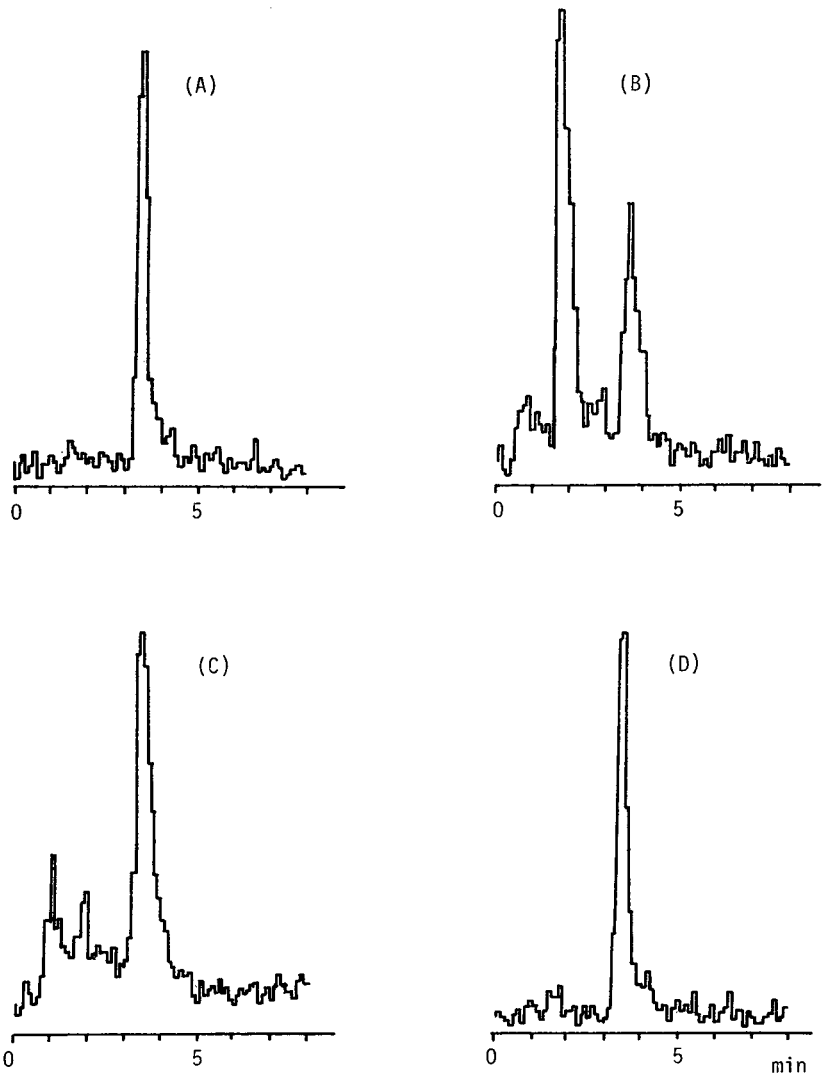


Fig. 1. Radio gas chromatograms of 5-[^{14}C]HT-tri-PFP: authentic (A), and recovered from plasma using Method I (B), Method II (C) and Method III (D).

Method I, II or III following reaction with PFPA according to the method of Beck et al. [9] (60°C, 3 h) are shown in Fig. 1. A single peak corresponding to 5-HT-tri-PFP appeared when Method III was used. On the other hand, peaks other than 5-HT-tri-PFP appeared when Methods I (about 65%) and II (about 40%) were used. When authentic 5-[^{14}C]HT was derivatized with PFPA in the presence of formic acid or zinc sulphate—barium hydroxide, similar peaks originating from the decomposed products appeared. It was apparent that the reagents used in the recovery procedure caused the decomposition of 5-HT in the derivatization reaction.

Equipping the radio-GC system with a synchronized accumulating radioisotope detector makes it possible to increase the detection efficiency without sacrificing resolution power and to obtain the radioactive intensity on the chromatogram in digital form. Thus the ratio of radioactive intensity on the chromatogram to injected radioactivity can be accurately estimated. We named this ratio "GC peak yield". The relationship between GC peak yield and derivatization conditions was examined, and the results obtained are shown in Fig. 2. The GC peak yield of 5-HT-tri-PFP reached a maximum at 140°C and then decreased at 160°C. At lower temperatures, the GC peak yield also increased when a longer reaction time was used. At higher temperatures, however, maximum GC peak yield was attained at 4 h. With the derivatization conditions reported by Beck et al. [9] (60°C, 3 h), the GC peak yield of 5-HT-tri-PFP was only 18%, while raising the reaction temperature to 140°C led to an increase of the GC peak yield to about 51% (reaction time 4 h). These derivatization conditions increased the GC peak yield more than three times in comparison with Beck's conditions. From these results it can be seen that the radio-GC system equipped with synchronized accumulating radioisotope detector is useful for estimating the GC peak yield and finding the best derivatization conditions.

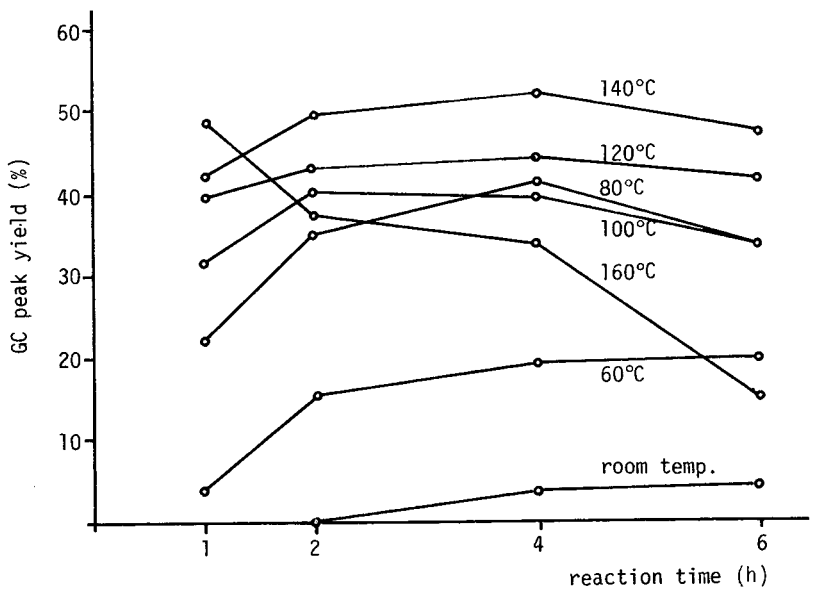


Fig. 2. Relationship between GC peak yield of 5-HT-tri-PFP and derivatization conditions.

To clarify whether the GC peak yield of 5-HT-tri-PFP was identical with the derivatization yield of 5-HT-tri-PFP, the following experiment was performed. An injection port was directly connected to a combustion tube by means of a brass tube, and the $^{14}CO_2$ that was exhausted from the combustion tube was absorbed into ethanolamine [15]. When the column oven temperature was set to 300°C, the recovery of the radioactivity was about 87%. Lowering the oven temperature to 200°C which was used for the assay of 5-HT-tri-PFP led to a decrease of the recovered radioactivity to 53%. It was apparent that the derivatization yield was about 87% or above under the conditions employed (140°C, 4 h), but an appreciable amount of this derivatized compound adsorbed onto the surface of the connecting metal tube. The derivatization of 5-HT for GC—MS—SIM analysis was carried out at 140°C for 2 h, because the GC peak yield reached an approximate equilibrium at 2 h when a temperature of 140°C was used. The calibration curve obtained by the GC—MS—SIM method showed good linearity between the peak height ratio and the molar ratio (r = 0.999). In preparing this calibration curve, the fragment ions m/z 451 and 454 were used as monitoring ions. There was some anxiety that some isotope effects might occur during this fragmentation process. However, the slope of the obtained calibration curve was close to 1. This means that no isotope effect occurred during the fragmentation process. Five picrograms of 5-HT were detected as the tri-PFP derivative with a signal-to-noise ratio of 2.5 (Fig. 3). When 200 µl of human plasma were used for the sample, the amount of derivatized 5-HT injection into the GC—MS system was estimated to be about a few nanograms. It was apparent that this method has an enough sensitivity to measure the 5-HT content in human plasma.

Quantitation of 5-HT in biological samples has mainly been performed by fluorometric assay. However, in this technique, 5-HT is not chromatographically separated and there is the possibility that compounds other than 5-HT affect the fluorescence intensity originating from 5-HT. In order to compare the analytical accuracy of the GC—MS—SIM method with that of the fluorometric assay, 5-HT was measured in a human plasma sample. Several fluorometric assays for 5-HT have been reported [16—18]. Of these, the

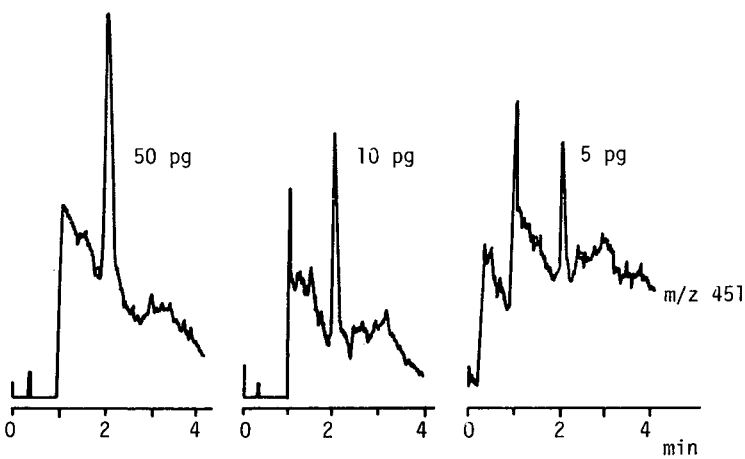


Fig. 3. Detection limit of 5-HT-tri-PFP by selected ion monitoring.

TABLE II

MEASUREMENT OF 5-HT IN HUMAN PLASMA BY GC—MS—SIM AND FLUOROMETRIC ASSAY

	GC—MS—SIM (ng/ml plasma)*	Fluorometric assay (ng/ml plasma)
1	307.8 ± 10.2	221.7
2	294.7 ± 6.7	191.2
3	281.2 ± 6.9	210.0
4	289.9 ± 4.7	169.1
5	301.6 ± 6.9	213.8
Mean ± S.D.	295.0 ± 9.2	201.2 ± 18.9
C.V. (%)	3.1	9.4

*Each value represents the mean ± S.D. of triplicate measurements.

ninhydrin method might be the most sensitive and has been used for clinical diagnosis. In this paper, the ninhydrin method was used.

The 5-HT level in human plasma was found to be 295.0 ± 9.2 ng/ml by the GC—MS—SIM method and 201.2 ± 18.9 ng/ml by the fluorometric assay (Table II), the former value being about 1.5 times higher than the latter. One of the reasons why the fluorometric assay showed a lower value might be due to the different extraction ratio of 5-HT from plasma and from an authentic sample solution which was used for the preparation of the calibration curve. A fixed amount of 5-[^{14}C]HT was added to the plasma sample or aqueous solution and 5-HT was extracted by the method used for the fluorometric assay. The recovery of radioactivity was 15.6 ± 0.1% for the aqueous solution and 13.3 ± 0.2% for the plasma sample. The addition of plasma lowered the recovery of radioactivity by about 15%. There is thus a possibility that lower values might be obtained when the calibration curve made 5-HT aqueous solution is used for measuring 5-HT in plasma. This phenomenon also suggests that a low extraction ratio is responsible for the low sensitivity in the fluorometric assay, because only 10% of radioactivity was recovered in the aqueous layer. The other reason why the fluorometric assay showed a lower assay value might be due to a quenching phenomenon which originates from biological compounds other than 5-HT. However, there is no evidence for this quenching phenomenon. A GC—MS—SIM method using 5-HT labelled with a stable isotope can automatically correct for the losses of 5-HT during the recovery and derivatization processes. The coefficients of variation were 3.1% for the GC—MS—SIM method and 9.4% for the fluorometric assay. This means that the GC—MS—SIM method has a better accuracy.

ACKNOWLEDGEMENTS

We are greatly indebted to Miss M. Akahane and Miss M. Shimokawa for skilled technical assistance.

REFERENCES

1 J. Matsumoto and M. Jouvet, C.R. Soc. Biol., 158 (1964) 2137.
2 M. Jouvet, Science, 163 (1969) 32.
3 R.M. Hardisty and R.S. Stacey, J. Physiol. (London), 130 (1955) 711.
4 J. Stratigos, A. Katsambas and P. Glanopoulou, Brit. J. Dermatol., 90 (1974) 451.
5 C.M.B. Pare, M. Sandler and R.C. Stacey, J. Neurol. Neurosurg. Psychiat., 23 (1960) 341.
6 F. Rosner, B.H. Ong, R.S. Paine and D. Mahanand, Lancet, i (1965) 1191.
7 J. Berman, M. Hulten and J. Lindsten, Lancet, i (1967) 730.
8 H.-Ch. Curtius, H. Farner and F. Rey, J. Chromatogr., 199 (1980) 171.
9 O. Beck, F.-A. Wiesel and G. Sedvall, J. Chromatogr., 134 (1977) 407.
10 S. Baba and Y. Kasuya, J. Chromatogr., 196 (1980) 144.
11 S. Baba, M. Horie and K. Watanabe, J. Chromatogr., 244 (1982) 57.
12 J. Segra, F. Artigas, E. Martinez and E. Gelpi, Biomed. Mass Spectrom., 3 (1976) 91.
13 F. Cattabeni, S.H. Koslow and E. Costa, Science, 178 (1972) 166.
14 I.L. Martin and G.B. Ansell, Biochem. Pharmacol., 22 (1973) 521.
15 S. Baba, T. Konishi and H. Ido, Yakugaku Zasshi, 93 (1973) 532.
16 S. Udenfriend, H. Weissbach and B.B. Brodie, Methods Biochem. Anal., 6 (1958) 95.
17 D.F. Bogdanski, A. Pletscher, B.B. Brodie and S. Udenfriend, J. Pharmacol. Exp. Ther., 117 (1956) 82.
18 S.H. Snyder, J. Axelrod and M. Zweig, Biochem. Pharmacol., 14 (1965) 831.

Journal of Chromatography, 307 (1984) 11—21
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2008

VOLATILE PRODUCTS FROM ACETYLCHOLINE AS MARKERS IN THE RAPID URINE TEST USING HEAD-SPACE GAS—LIQUID CHROMATOGRAPHY

T.J. DAVIES*,*

Microbiology Department, Monash University Medical School, Prahran 3181, Victoria (Australia)

and

N.J. HAYWARD

Bacteriology Laboratory, Alfred Hospital, Prahran 3181, Victoria (Australia)

(Received September 22nd, 1983)

SUMMARY

Head-space gas—liquid chromatographic analyses of cultures of all *Proteus* spp. showed that the production of trimethylamine from acetylcholine could be the marker for the detection in 3.5 h of significant numbers of *P. mirabilis*, *P. vulgaris*, *P. rettgeri* and *P. inconstans A* in the rapid test for *Escherichia* and *Klebsiella* spp. in urine specimens. Trimethylamine was not detected in cultures of five other urinary pathogens. Six of fifteen strains of *K. aerogenes* produced trimethylamine from acetylcholine but were distinguished from *Proteus* spp. by ethanol production from arabinose. Ethyl acetate was produced from acetylcholine by *P. mirabilis*, *P. vulgaris*, *P. rettgeri*, *P. inconstans A*, *P. inconstans B*, *E. coli*, *K. aerogenes* and *Streptococcus faecalis*.

INTRODUCTION

Gas—liquid chromatography (GLC) for the detection of bacterial growth is notable for its high information content. A single analysis can detect several volatile compounds and provide information both about the identity and the

*Present address: Department of Microbiology, University of Melbourne, Parkville 3052, Victoria, Australia.

0378-4347/84/$03.00

quantity of each compound. These features of GLC have been used to develop a test by head-space GLC (HS-GLC) for the rapid detection of significant numbers, viz. $\geqslant 10^5$ colony-forming units (CFU) per ml [1] of *Escherichia, Klebsiella, Citrobacter* and *Proteus* spp. in urine specimens [2].

The HS-GLC rapid urine test requires two cultures, one with limited and one with ample aeration, from each urine specimen. Limited aeration in unshaken cultures is required for the production from arabinose of ethanol, the volatile compound (marker) detected by HS-GLC that indicates *Escherichia, Klebsiella* and *Citrobacter* spp. Ample aeration in shaken cultures is required for the production from methionine of methyl mercaptan, the marker that indicates *Proteus* spp. The rapid test would be greatly simplified if there were markers for *Escherichia, Klebsiella, Citrobacter* and *Proteus* spp. that were produced in one urine culture, either unshaken or shaken. Materials, labour and total analysis times for the test would be halved.

Trimethylamine is found in unshaken cultures of urine specimens containing *Proteus* spp. [2]. The present investigation was undertaken to determine whether, in the HS-GLC rapid urine test, trimethylamine could be the marker for *Proteus* spp. and ethanol the marker for *Escherichia, Klebsiella* and *Citrobacter* spp. in a single unshaken urine culture.

Substrates were tested for trimethylamine production by *Proteus* spp. and one was selected for further investigation. Trimethylamine production from the substrate was assessed under the conditions of the HS-GLC rapid urine test and its specificity for *Proteus* spp. was determined. A trial of the HS-GLC rapid urine test was made with trimethylamine as the marker for *Proteus* spp. in a single unshaken culture of each urine specimen.

EXPERIMENTAL

Gas chromatography—mass spectrometry

Head-space samples were prepared by adding 2 ml of each liquid for analysis to 3 g potassium carbonate in a 9-ml glass vial which was immediately sealed with a rubber stopper and aluminium cap, shaken on a Vortex mixer to disperse the salt in the liquid and held in a water bath at 60° C for 5 min. While the vial remained in the water bath, 0.3 ml of the supernatant vapour was removed with a gas-tight syringe (Scientific Glass Engineering, No. 500-RN-GSG) which had been heated to 60° C, and immediately injected into the gas chromatograph.

The gas chromatograph was a Varian Aerograph Series 2400 with dual flame ionization detectors and two stainless-steel columns, 2 m × 3 mm, packed with 0.4% Carbowax 1500 on graphite 60—80 mesh. The oven temperature was 110° C and the injector and detector temperatures were 160° C. Nitrogen carrier gas flow-rate was 12 ml/min, hydrogen 30 ml/min and air 300 ml/min. The retention times (t_R) of trimethylamine, ethanol, ethyl acetate and *n*-propanol were 1.00, 0.75, 2.65 and 1.50 min, respectively.

Aqueous solutions of ethanol and trimethylamine hydrochloride were added to potassium carbonate in glass vials and analysed with each day's samples to check t_R for the identification of products from cultures. The concentrations of ethanol and trimethylamine in cultures necessary for a response of twice the

noise level due to volatile compounds in the culture medium were 0.22 m*M* and 0.10 m*M*, respectively.

The mass spectrometer was a 300-mm radius 60° magnetic deflection instrument coupled to the gas chromatograph by a Watson—Bieman frit [3]. Trimethylamine, ethanol, ethyl acetate and *n*-propanol were identified by comparing their mass spectra with a reference library file [4].

Bacteria, culture media and incubation conditions

The identities of 60 strains of *Proteus* spp. including 10 each of *P. mirabilis*, *P. vulgaris*, *P. morganii*, *P. rettgeri*, *P. inconstans A* and *P. inconstans B* and of 65 strains of other bacteria that may cause urinary tract infections including 10 *E. coli*, 15 *K. aerogenes*, 10 *Streptococcus faecalis*, 10 *Staphylococcus epidermidis*, 10 *S. aureus* and 10 *Pseudomonas aeruginosa* were confirmed by standard tests [5].

A basal yeast-extract peptone medium concentrate was prepared by dissolving 3.3 g Proteose peptone (Difco) and 1 g yeast extract (Oxoid) in 100 ml of 0.33 *M* sodium phosphate buffer pH 7.2, dispensing in sterile screw-capped bottles of 28 ml capacity, autoclaving at 115°C for 15 min and storing at 4°C. Aqueous enrichment solutions (10%) of acetylcholine chloride, choline chloride, phosphatidyl choline, betaine hydrochloride and L-arabinose were sterilized by membrane filtration and stored at 4°C. Within 24 h of use, enriched media were prepared by mixing 1 part of each appropriate enrichment solution with 3 parts of basal medium concentrate and making up the volume to 10 parts with sterile distilled water. The final concentrations of ingredients were the same as those in the medium for the HS-GLC rapid test [2]. Unenriched medium was prepared by mixing 3 parts of basal medium concentrate with 7 parts of sterile distilled water. Media were dispensed in 2.5-ml aliquots in sterile screw-capped bottles of 28 ml capacity.

Media were inoculated, usually with one drop (0.025 ml) of an overnight broth culture of the bacterium under test and incubated at 37°C. Three degrees of aeration were tested. Two degrees of limited aeration were provided in unshaken cultures by incubation either standing upright (minimum aeration) or sloping at an angle of 12° from the horizontal. Ample aeration was provided in cultures incubated standing in a shaker operating at 200 horizontal 20-mm oscillations per min.

Inocula of known numbers of bacteria were counted as CFU per ml by the method of Miles and Misra [6].

Specimens of urine and method of HS-GLC rapid test

Specimens of urine, stored at 4°C, were selected as containing $\geqslant 10^5$ CFU bacteria per ml on the basis of routine laboratory analysis results [2] available on the day after voiding. The urine specimens were then examined by the method of the HS-GLC rapid test and re-examined in parallel by routine laboratory analysis.

Excepting that arabinose acetylcholine medium replaced arabinose methionine medium, urine cultures for the HS-GLC rapid test were inoculated as described previously [2], and incubated standing unshaken for 3.5 h. HS-GLC analysis of urine cultures using a gas chromatograph with an automatic

head-space injector (Perkin-Elmer F45) was carried out as described previously [2]. The t_R values were shorter than in the gas chromatograph used for manual injections viz. trimethylamine 0.45 min, ethanol 0.325 min, ethyl acetate 1.125 min and *n*-propanol 0.60 min.

RESULTS

Substrates for trimethylamine production

Acetylcholine, choline, phosphatidyl choline and betaine were selected for trial on the basis of chemical structure.

In media enriched with acetylcholine chloride or choline chloride, incubated standing unshaken for 3.5 h, trimethylamine was detected by HS-GLC in large amounts in cultures of *P. mirabilis*, *P. vulgaris*, *P. rettgeri* and *P. inconstans A* and detected in only small amounts in uninoculated media incubated in parallel. Trimethylamine was not detected in cultures of *P. morganii* and *P. inconstans B* in larger amounts than in incubated uninoculated media. Analysis of uninoculated media enriched with phosphatidyl choline or betaine hydrochloride incubated standing unshaken for 3.5 h showed large amounts of trimethylamine.

The spontaneous decomposition of phosphatidyl choline and betaine made them unsuitable for use in the HS-GLC rapid urine test. Acetylcholine and choline appeared satisfactory and acetylcholine was selected for further investigation.

Media enriched with 0.1%, 0.5%, 1%, 2.5% and 5% acetylcholine chloride were inoculated with all species of *Proteus*, incubated standing unshaken for 3.5 h and analysed by HS-GLC. Medium enriched with 1% acetylcholine chloride gave the highest yields of trimethylamine from *P. mirabilis*, *P. vulgaris*, *P. rettgeri* and *P. inconstans A*. Even 5% acetylcholine chloride did not stimulate the production of trimethylamine by *P. morganii* and *P. inconstans B*. Medium containing 1% acetylcholine chloride was used in all subsequent tests.

Trimethylamine production under the conditions of the HS-GLC rapid urine test

Experiments were done to determine whether trimethylamine was produced by *Proteus* spp. from acetylcholine in the same medium incubated under the same conditions as used for the production from arabinose of ethanol, the marker for *Escherichia*, *Klebsiella* and *Citrobacter* spp.

In unshaken cultures, either standing or sloping, of three strains of *P. mirabilis* in acetylcholine medium incubated for 3.5 h the yields of trimethylamine were 20—30 times the yields in similar shaken cultures. Standing cultures (minimum aeration) gave the highest yields. Therefore, as with ethanol, limited aeration was best for the production of trimethylamine.

The period of incubation of cultures in the rapid urine test is chosen to be long enough for the detection of markers if there are significant numbers of bacteria in the urine specimen, viz. 10^5 CFU per ml urine [1], but not long enough for the detection of markers if there are fewer bacteria in the urine specimen. Acetylcholine media were inoculated with 10^4, 10^5 and 10^6 CFU *P. mirabilis* per ml medium, incubated standing unshaken and analysed by

HS-GLC at hourly intervals up to 6 h. Trimethylamine was detected in cultures with inocula of 10^6 CFU per ml after 3 h, with inocula of 10^5 CFU per ml between 3 and 4 h and with inocula of 10^4 CFU per ml after 4 h. Therefore, as with *Escherichia*, *Klebsiella* and *Citrobacter* spp., an incubation period of 3.5 h appeared to be suitable for the detection of the equivalent of significant numbers of *P. mirabilis* in urine specimens.

Specificity of trimethylamine production from acetylcholine

The distribution of the production of trimethylamine and/or other volatile compounds among *Proteus* spp. and among other species of bacteria that may cause urinary tract infections was determined.

Volatile compounds produced by all species of Proteus. Cultures of 10 strains of each species of *Proteus* in unenriched medium and in acetylcholine medium were incubated standing unshaken for 3.5 h and analysed by HS-GLC. Results from inoculated media were corrected by subtraction of trace amounts of ethanol and trimethylamine detected in uninoculated media incubated in parallel with cultures.

The results are shown in Table I. Trimethylamine and ethanol were produced by all strains of *P. mirabilis*, *P. vulgaris* and *P. rettgeri* in unenriched medium and in larger amounts in acetylcholine medium. Ethyl acetate was produced by all strains of the same three species in acetylcholine medium but not in unenriched medium. Trimethylamine, ethanol and ethyl acetate were produced similarly by *P. inconstans A* but in smaller amounts than by the other three species, an 8-times more sensitive setting of the gas chromatograph amplifier being required for their detection. These smaller amounts would be sufficient to detect *P. inconstans A* in urine specimens because the inocula gave concentrations of approximately 10^5 CFU per ml culture medium, the lowest limit of a significant count. The results showed that trimethylamine could be the primary marker for *P. mirabilis*, *P. vulgaris*, *P. rettgeri* and *P. inconstans A* with ethanol and ethyl acetate as secondary markers under the conditions of the HS-GLC rapid urine test.

Ethyl acetate was produced by all strains of *P. inconstans B* in amounts similar to *P. inconstans A* in acetylcholine medium and not in unenriched medium.

No volatile product was detected in cultures of all strains of *P. morganii*.

Volatile compounds produced by other bacteria that may cause urinary tract infections. Cultures of at least 10 strains of *E. coli*, *K. aerogenes*, *S. faecalis*, *S. epidermidis*, *S. aureus* and *P. aeruginosa* in arabinose medium and arabinose acetylcholine medium were incubated standing unshaken for 3.5 h and analysed by HS-GLC. Cultures of 10 strains of *P. mirabilis* were included for comparison. The results were corrected by subtraction of trace amounts of ethanol and trimethylamine in incubated uninoculated media.

The results are shown in Table II. The substrates for markers, acetylcholine and arabinose, were mutually compatible in one culture medium. In arabinose acetylcholine medium, arabinose did not interfere with the production of trimethylamine by all strains of *P. mirabilis*, and acetylcholine did not interfere with the production of ethanol by all strains of *K. aerogenes* and *E. coli*.

Trimethylamine was produced by 6 of the 15 strains of *K. aerogenes* but the

TABLE I

VOLATILE COMPOUNDS PRODUCED BY *PROTEUS* SPP. IN UNENRICHED MEDIUM AND ACETYLCHOLINE MEDIUM

Results of HS-GLC analysis after incubation standing unshaken for 3.5 h.

Proteus species (No. of strains)	Amplifier attenuation	Trimethylamine		Ethanol		Ethyl acetate	
		Unenriched medium	Acetylcholine medium	Unenriched medium	Acetylcholine medium	Unenriched medium	Acetylcholine medium
P. mirabilis (10)	× 8	28*	150	5	20	0	7.2
		12—30**	93—254	2—7	11—24		2.1—9
P. vulgaris (10)	× 8	8	73	3	10	0	2.6
		2.5—25	16—142	2—5	6—14		0.8—4.9
P. rettgeri (10)	× 8	2.5	16	0.8	2.3	0	0.5
		0.2—12	5—53	0.5—1.5	1.4—4.8		0.2—1.2
P. inconstans A (10)	× 1	3	13	0.7	2.1	0	0.5
		0.2—10	4.3—45	0.3—2	2.1—4.7		0.2—1.9
P. inconstans B (10)	× 1	0	0	0	0	0	0.8
							0.1—1.4
P. morganii (10)	× 1	0	0	0	0	0	0

*Median of peak areas in mm^2.
**Range of peak areas in mm^2.

TABLE II

VOLATILE COMPOUNDS PRODUCED BY URINARY PATHOGENS IN ARABINOSE MEDIUM (AM) AND ARABINOSE ACETYLCHOLINE MEDIUM (AAM)

Results of HS-GLC analysis after incubation standing unshaken for 3.5 h.

Bacterial species (No. of strains)	Amplifier attenuation	Trimethylamine		Ethanol		Ethyl acetate		*n*-Propanol	
		AM	AAM	AM	AAM	AM	AAM	AM	AAM
P. mirabilis (10)	× 8	6*	108	10	20	0	6	0	0
		2—12**	73—150	4—18	16—33		4.5—13		
K. aerogenes (6)	× 8	2	48	49	48	0	15	0	0
		0—3	5—173	9—155	9—152		3—79		
K. aerogenes (9)	× 8	0	0	100	101	0	32	0	0
				35—130	34—126		9—43		
S. faecalis (10)	× 1	0	0	23	15	0	1.8	0	0
				19—24	7—17		0.8—2.2		
E. coli (10)	× 8	0	0	90	83	0	24	7	4.5
				66—135	56—110		14—30	1—12	1—8
S. epidermidis (10)	× 1	0	0	0	0	0	0	0	0
S. aureus (10)	× 1	0	0	0	0	0	0	0	0
P. aeruginosa (10)	× 1	0	0	0	0	0	0	0	0

*Median of peak areas in mm^2.
**Range of peak areas in mm^2.

amount of ethanol relative to trimethylamine was larger than for *Proteus* spp. Trimethylamine-producing strains of *K. aerogenes*, with ethanol peak areas of size similar to trimethylamine peak areas, were readily distinguishable from *P. mirabilis* with small ethanol and large trimethylamine peak areas.

Trimethylamine was not produced by 9 of the 15 strains of *K. aerogenes*, nor by *S. faecalis*, *E. coli*, *S. epidermidis*, *S. aureus* or *P. aeruginosa*.

Ethanol was produced by all strains of *S. faecalis* in both arabinose medium and arabinose acetylcholine medium. The amounts were small relative to *E. coli* and *K. aerogenes*, requiring an 8-times more sensitive setting of the gas chromatograph amplifier for their detection.

Ethyl acetate was produced by all strains of *P. mirabilis*, *K. aerogenes* and *E. coli* and, in trace amounts, by *S. faecalis* in arabinose acetylcholine medium but not in arabinose medium.

n-Propanol was produced by all strains of *E. coli* in both arabinose medium and arabinose acetylcholine medium as previously reported [7].

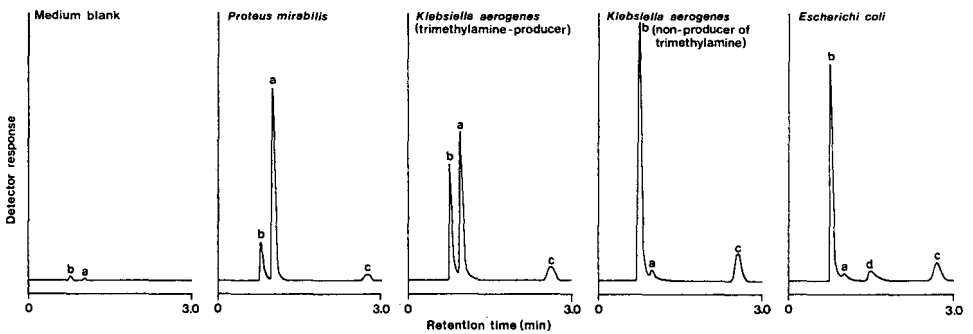


Fig. 1. Chromatograms of medium blank and of cultures of *P. mirabilis*, *K. aerogenes* (trimethylamine-producer), *K. aerogenes* (non-producer of trimethylamine) and *E. coli* in arabinose acetylcholine medium incubated standing unshaken for 3.5 h. Amplifier attenuation × 8, i.e. one-eighth the sensitivity in Fig. 2. Peaks: a = trimethylamine, b = ethanol, c = ethyl acetate, d = *n*-propanol.

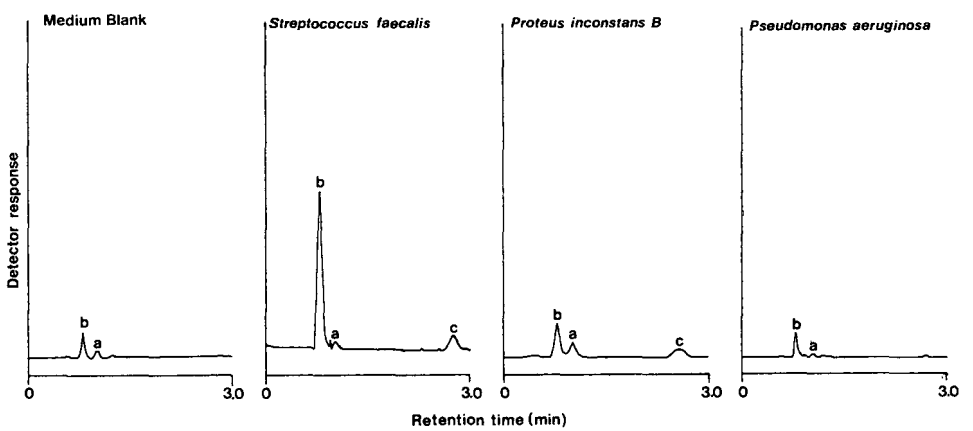


Fig. 2. Chromatograms of medium blank and of cultures of *S. faecalis*, *P. inconstans B* and *P. aeruginosa* in arabinose acetylcholine medium incubated standing unshaken for 3.5 h. Amplifier attenuation × 1, i.e. eight times the sensitivity in Fig. 1. Peaks: a = trimethylamine, b = ethanol, c = ethyl acetate.

It was possible to distinguish six different types of chromatogram from cultures in arabinose acetylcholine medium compared with incubated uninoculated medium (Medium blanks). The chromatograms illustrated in the figures, are characteristic of

(A) *P. mirabilis*, *P. vulgaris*, *P. rettgeri* and *P. inconstans A* (Fig. 1) with trimethylamine, ethanol and ethyl acetate peaks, the ethanol peak being only a fraction of the trimethylamine peak,

(B) *K. aerogenes*, trimethylamine-producer (Fig. 1) with trimethylamine, ethanol and ethyl acetate peaks, the ethanol and trimethylamine peaks being of similar size,

(C) *K. aerogenes*, non-producer of trimethylamine (Fig. 1) and *S. faecalis* (Fig. 2) with ethanol and ethyl acetate peaks,

(D) *E. coli* (Fig. 1) with ethanol, ethyl acetate and *n*-propanol peaks,

(E) *P. inconstans B* (Fig. 2) with an ethyl acetate peak and

(F) *P. morganii*, *S. epidermidis*, *S. aureus* and *P. aeruginosa* (Fig. 2) with no volatile products other than those in the medium blank.

Trial of HS-GLC rapid urine test using arabinose acetylcholine medium

Specimens of urine (35) were examined by routine laboratory analysis, which was regarded as the standard of correct diagnosis, and by the HS-GLC rapid urine test using a single culture in arabinose acetylcholine medium.

The results of HS-GLC analysis of urine cultures were corrected for volatile

TABLE III

TRIAL OF HS-GLC RAPID URINE TEST USING SINGLE CULTURES OF URINE SPECIMENS IN ARABINOSE ACETYLCHOLINE MEDIUM

Results of HS-GLC analysis after incubation standing unshaken for 3.5 h.

Routine laboratory analysis result*	No. of specimens	HS-GLC rapid test result				
		Trimethyl-amine	Ethanol	Ethyl acetate	*n*-Propanol	Chromatogram type
P. mirabilis	6	113** 23—254***	12 6—17	13 1.5—32	0	A
K. aerogenes	6	4.5 0.5—6	16 2.7—32	2.4 0.3—5.3	0	B
K. aerogenes	2	0	0.4, 4.6	0.1, 0.3	0	C
S. faecalis	4	0	1.0 0.5—2.5	0.5 0.3—4	0	C
E. coli	4	0	100 25—122	17 3—22	3.5 1—7	D
P. inconstans B	2	0	0	1.5, 12	0	E
S. epidermidis	2	0	0	0	0	F
P. aeruginosa	3	0	0	0	0	F
Nil	6	0	0	0	0	F

*Species detected in significant numbers viz. $\geq 10^5$ CFU per ml urine.
**Median of peak areas in mm^2.
***Range of peak areas in mm^2.

compounds in the culture medium or urine specimens [2]. All six types of chromatogram were represented in the results (Table III). *P. mirabilis*, *K. aerogenes* (producers and nonproducers of trimethylamine), *S. faecalis*, *E. coli* and *P. inconstans B* were detected in significant numbers in 24 urine specimens and the findings were confirmed the following day by the routine laboratory analysis result. *S. epidermidis* and *P. aeruginosa* showed no volatile products. Six of the urine specimens did not yield significant numbers of bacteria and markers were undetected in all six of them, confirming previous experience that the HS-GLC rapid urine test does not give false positive results [2].

DISCUSSION

The present investigation has shown that trimethylamine could be the marker for *P. mirabilis*, *P. vulgaris*, *P. rettgeri* and *P. inconstans A* and ethanol the marker for *Escherichia* and *Klebsiella* spp. in single unshaken cultures of urine specimens in arabinose acetylcholine medium in the HS-GLC rapid urine test. Materials, labour and total analysis times would be half those in the present test in which methyl mercaptan is the marker for *Proteus* spp. and two cultures, one unshaken and the other shaken, are required.

Two species of *Proteus*, *P. inconstans B* and *P. morganii* did not produce trimethylamine. However, *P. inconstans B* was detected both in pure cultures and in cultures of urine specimens in acetylcholine medium by the production of ethyl acetate, a marker not previously reported in the HS-GLC rapid test. In addition, *P. morganii* differs from other *Proteus* spp. in producing methyl mercaptan and its oxidation product dimethyl disulphide from methionine in cultures incubated with limited aeration [8]. It is probable that if methionine, in addition to arabinose and acetylcholine, were added to medium used for unshaken cultures of urine specimens in the HS-GLC rapid test, six volatile products would be detected and quantitated, viz. trimethylamine, ethanol, ethyl acetate, *n*-propanol, methyl mercaptan and dimethyl disulphide. In this way the high information content of a single HS-GLC analysis would detect and suggest the identity of *Escherichia*, *Klebsiella* and *Citrobacter* spp., all six *Proteus* spp. and *S. faecalis* in significant numbers in specimens of urine.

ACKNOWLEDGEMENTS

The thanks of one of us (T.J.D.) are due to Dr. W.J. Spicer, Director of the Bacteriology Laboratory, Alfred Hospital, for his generosity in enabling this investigation in his laboratory, to Mr. Reginald Butcher, Austin Hospital, for providing urine specimens, to Mr. W. Fock, C.S.I.R.O. Division of Chemical Physics, for mass spectral analyses, to the Alfred Hospital Whole-time Medical Specialists' Trust Fund for buying the Perkin-Elmer gas chromatograph with automatic head-space injector, and to the Alfred Hospital Medical Research Fund for a personal grant to one of us (N.J.H.).

REFERENCES

1 E.H. Kass, Trans. Assoc. Amer. Physicians, 69 (1956) 56.

2 N.J. Hayward, J. Chromatogr., 274 (1983) 27.
3 W. Stark, J.F. Smith and D.A. Forss, J. Dairy Res., 34 (1967) 123.
4 A. Cornu and R. Massot, Compilation of Mass Spectral Data, Heyden, London, 1966, 1st Suppl. 1967 and 2nd Suppl. 1971.
5 S.T. Cowan, Cowan and Steel's Manual for the Identification of Medical Bacteria, Cambridge University Press, London, 2nd ed., 1974.
6 A.A. Miles and S.S. Misra, J. Hyg., 38 (1938) 732.
7 N.J. Hayward and T.H. Jeavons, J. Clin. Microbiol., 6 (1977) 202.
8 N.J. Hayward, unpublished results.

Journal of Chromatography, 307 (1984) 23—28
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2022

STUDIES ON STEROIDS. CC. DETERMINATION OF 17-KETOSTEROID SULPHATES IN SERUM BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION USING PRE-COLUMN DERIVATIZATION

KAZUTAKE SHIMADA, MAKOTO TANAKA and TOSHIO NAMBARA*

Pharmaceutical Institute, Tohoku University, Sendai 980 (Japan)

(Received November 20th, 1983)

SUMMARY

A new sensitive method is described for the determination of 17-ketosteroid sulphates, particularly dehydroepiandrosterone sulphate, in human serum by high-performance liquid chromatography with electrochemical detection. The 17-ketosteroid sulphates in serum were extracted with acetonitrile and derivatized with *p*-nitrophenylhydrazine in trichloroacetic acid—benzene solution. The *p*-nitrophenylhydrazones were separated by high-performance liquid chromatography on a μBondapak C_{18} column using methanol—0.5% ammonium dihydrogen phosphate (8:3) as a mobile phase. The proposed method proved to be applicable to the quantitation of 17-ketosteroid sulphates with satisfactory sensitivity and reliability, providing a quantitation limit of 80 ng/ml and coefficient of variation of 4%. A good correlation was observed between the values obtained by the present method and radioimmunoassay for dehydroepiandrosterone sulphate in serum.

INTRODUCTION

The measurement of dehydroepiandrosterone sulphate, one of the principal adrenal secretory products in man, has proved to be valuable for assessing adrenocortical disorders [1, 2]. Numerous methods have been developed for the analysis of dehydroepiandrosterone sulphate in biological fluids, including gas chromatography [3, 4], gas chromatography—mass spectrometry [5], high-performance liquid chromatography (HPLC) [6, 7] and radioimmunoassay (RIA) [8—10]. In almost all cases, the conjugated steroids are determined indirectly after solvolysis and/or hydrolysis. However, the incomplete deconjugation and formation of artifacts may disturb the analytical results. In addition, deconjugation has a disadvantage that no information about the conjugated position can be obtained. Recently, Kawasaki et al. [11] reported a sensitive

0378-4347/84/$03.00

HPLC method for the determination of conjugated 17-ketosteroids in biological fluids using dansylhydrazine as a fluorescence labelling reagent.

HPLC with electrochemical detection (ED) is well recognized to be as sensitive as HPLC with fluorescence detection and extremely useful for the determination of trace components in biological fluids [12, 13]. A highly sensitive method has previously been developed for the quantitation of 17-ketosteroids in human serum after deconjugation, using *p*-nitrophenylhydrazine as a derivatization reagent for HPLC—ED [14]. This paper describes a simple and sensitive method for the determination of 17-ketosteroid sulphates in human serum by means of HPLC—ED.

EXPERIMENTAL

Instruments

The apparatus used for this work was a Waters Model ALC/GPC 202 high-performance liquid chromatograph (Waters Assoc., Milford, MA, U.S.A.) equipped with a Yanagimoto Model VMD 101 electrochemical detector (Yanagimoto Co., Kyoto, Japan). The applied potential was set at 0.8 V versus an Ag/AgCl reference electrode. A test sample was introduced by a Waters Model U6K sample loop injector with an effective volume of 2 ml. HPLC was carried out on a μBondapak C_{18} (5 μm) column (30.5 × 0.4 cm I.D.) (Waters Assoc.) using methanol—0.5% $NH_4H_2PO_4$ (pH 3.0, adjusted with phosphoric acid) (8:3, v/v) as a mobile phase at a flow-rate of 1 ml/min.

Materials

Androsterone, *p*-nitrophenylhydrazine and trichloroacetic acid were purchased from Tokyo Kasei Co. (Tokyo, Japan). Dehydroepiandrosterone, etiocholanolone and epiandrosterone were kindly donated by Teikoku Hormone Mfg. Co. (Tokyo, Japan). The 17-ketosteroid sulphates were prepared by the conventional method in these laboratoires. 2-Hydroxyestrone 3-methyl ether was prepared from estrone according to the method of Honma and Nambara [15].

Reagent solutions

p-Nitrophenylhydrazine solution. *p*-Nitrophenylhydrazine was purified by repeated recrystallization from methanol. Ten milligrams of *p*-nitrophenylhydrazine were dissolved in 0.2 ml of ethyl acetate. The reagent solution was freshly prepared prior to use.

Trichloroacetic acid—benzene solution. Thirty milligrams of trichloroacetic acid were dissolved in 10 ml of benzene.

Steroid stock solutions. Each solution was prepared by dissolving each 2 mg of dehydroepiandrosterone, androsterone, epiandrosterone and etiocholanolone sulphates in 10 ml of methanol and stored at −20° C until use.

Procedure

To human serum (0.1 ml) in a centrifuge tube was added acetonitrile (2 ml), and the whole was allowed to stand at room temperature for 5 min and then centrifuged at 1000 *g* for 5 min. The supernatant was transferred to another

tube. After addition of 2-hydroxyestrone 3-methyl ether (internal standard) (100 ng), the mixture was evaporated to dryness below 40°C. To the residue were added *p*-nitrophenylhydrazine solution (10 μl) and trichloroacetic acid—benzene solution (100 μl), successively. The solution was heated at 60°C for 20 min, and then evaporated to dryness under a stream of nitrogen gas. The residue was redissolved in methanol (200 μl) and an aliquot of the solution was applied to HPLC.

RESULTS AND DISCUSSION

In the preliminary report [14] we described a highly sensitive method for the determination of 17-ketosteroids in human serum using *p*-nitrophenylhydrazine as a derivatization reagent for HPLC—ED. The conventional method involving prior solvolysis and/or hydrolysis of the conjugated steroids is tedious and time-consuming. In addition, the information about the conjugated form would be lost by deconjugation. Therefore, we have attempted to develop a new method for the direct determination of 17-ketosteroid sulphates without solvolysis. Initially, a suitable procedure for derivatization was investigated using dehydroepiandrosterone sulphate as a model compound. Condensation of dehydroepiandrosterone sulphate with *p*-nitrophenylhydrazine proceeded quantitatively without fission of the sulphate bond, when these two were heated at 60°C for 20 min in 0.3% trichloroacetic acid—benzene solution. The favourable condition was found to be similar to that for free 17-ketosteroids described in the previous paper [14]. 2-Hydroxyestrone 3-methyl ether, an internal standard, was similarly transformed into the *p*-nitrophenylhydrazone. Among several columns tested, μBondapak C_{18} was most suitable for efficient separation of conjugated steroids and the internal standard. A typical hydrodynamic voltammogram is illustrated in Fig. 1. The current (peak height) at each applied potential was divided by the current at the most positive potential to obtain the relative current ratio. This value was plotted against the applied

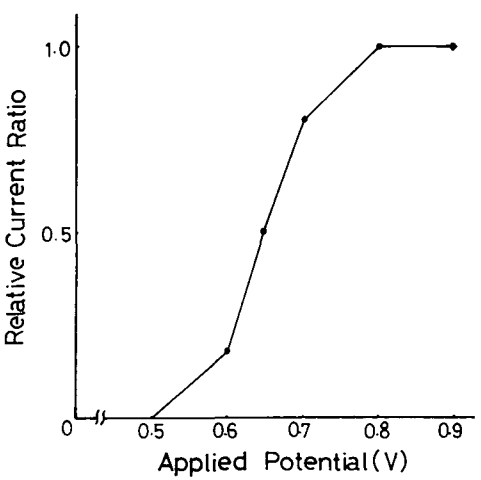


Fig. 1. A hydrodynamic voltammogram of the *p*-nitrophenylhydrazone formed from dehydroepiandrosterone sulphate.

potential. The detector gave a linear response up to +0.8 V versus an Ag/AgCl reference electrode. The *p*-nitrophenylhydrazones formed from dehydroepiandrosterone, epiandrosterone, etiocholanolone and androsterone sulphates and internal standard were satisfactorily resolved on a μBondapak C_{18} column (Fig. 2a). Each derivatized steroid showed a single peak of the theoretical shape. The detection limit of the *p*-nitrophenylhydrazones was 360 pg at 4 nA full scale (signal-to-noise ratio = 2).

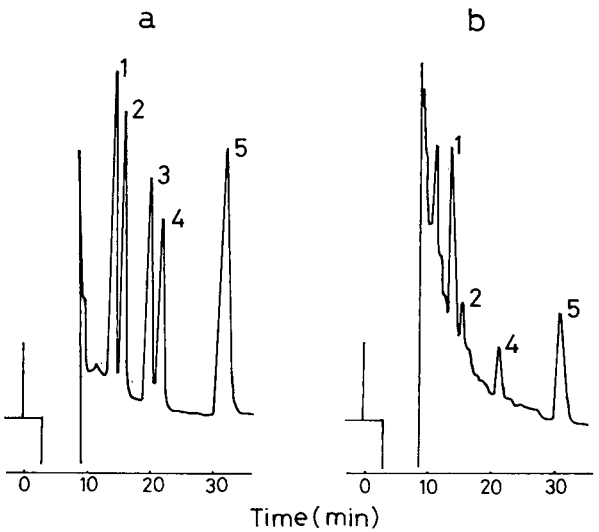


Fig. 2. High-performance liquid chromatograms obtained with (a) standard samples and (b) normal human serum sample. Peaks: 1 = dehydroepiandrosterone sulphate, 2 = epiandrosterone sulphate, 3 = etiocholanolone sulphate, 4 = androsterone sulphate, 5 = internal standard. Interference from unused reagent and endogenous polar substances due to saturation of the output of the detector was overcome by removing the connector for about 5 min after injection.

The next effort was focused on establishing a clean-up procedure for conjugated 17-ketosteroids in serum. After deproteinization of a serum sample with acetonitrile, 17-ketosteroids in the supernatant were derivatized with *p*-nitrophenylhydrazine in trichloroacetic acid—benzene solution. A typical chromatogram of 17-ketosteroid sulphates in human serum is illustrated in Fig. 2b. Three peaks corresponding to dehydroepiandrosterone, epiandrosterone and androsterone sulphates appeared on the chromatogram. No detectable amount of etiocholanolone sulphate, however, was observed with serum specimens analyzed in this study. The internal standard peak was not disturbed by coexisting substances in serum. This assay procedure was rather simple and suitable for the routine work.

A known amount of dehydroepiandrosterone sulphate was added to human serum and the overall recovery was estimated by the standard procedure. As listed in Table I, the spiked steroid was recovered at the rate of about 80% with a coefficient of variation (C.V.) of 4%. The calibration curve for each 17-ketosteroid sulphate was constructed by plotting the peak height ratio against the amount of 17-ketosteroid sulphate where a satisfactory linearity was observed

TABLE I

RECOVERY OF DEHYDROEPIANDROSTERONE SULPHATE ADDED TO NORMAL HUMAN SERUM

Added (μg/ml)	Expected (μg/ml)	Found (μg/ml)	Recovery ± S.D.* (%)
0	—	2.07	—
0.94	3.01	2.82	79.3 ± 3.3
1.88	3.95	3.59	80.9 ± 3.0

*n = 6.

in the range of 2.5–15 ng. When 0.1 ml of serum sample was used for the assay, the detection limit of dehydroepiandrosterone sulphate was approximately 8 μg/dl. The detection limit obtainable by the present method is comparable to that by the fluorescence HPLC method [11].

For the purpose of assessing the reliability of the proposed method, dehydroepiandrosterone sulphate levels in serum samples taken from fourteen healthy male volunteers (24—38 years' old) were determined by both HPLC and direct RIA without hydrolysis [16]. The values obtained by the two methods showed a good correlation (r = 0.952; n = 14), the regression equation being $Y = 0.98X + 0.011$ (Fig. 3).

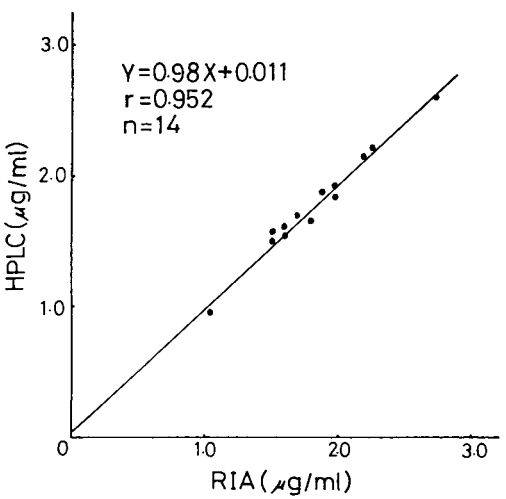


Fig. 3. Comparison of serum dehydroepiandrosterone sulphate levels determined by HPLC and RIA.

In conclusion, the newly developed HPLC method is satisfactory with respect to sensitivity, accuracy and precision. The assay procedure is simple and convenient, and therefore clinically applicable to the routine analysis of serum 17-ketosteroid sulphates.

ACKNOWLEDGEMENT

This work was supported by the Ministry of Education, Science and Culture of Japan.

REFERENCES

1 T. Yamaji and H. Ibayashi, J. Clin. Endocrinol., 29 (1969) 273.
2 S. Korth-Schutz, L.S. Levine, M.I. New and D.M. Chow, J. Clin. Endocrinol. Metab., 42 (1976) 1005.
3 D.Y. Wang, R.D. Bulbrook, B.S. Thomas and M. Friedman, J. Endocrinol., 42 (1968) 567.
4 J.C. Touchstone and M.F. Dobbins, J. Steroid Biochem., 6 (1975) 1389.
5 Y. Kawabe, K. Tsuneda, A. Kambegawa, R. Ohuchi and H. Mori, Folia Endocrinol. Jap., 55 (1979) 153.
6 F.A. Fitzpatrick, S. Siggia and J. Dingman, Sr., Anal. Chem., 44 (1972) 2211.
7 T. Kawasaki, M. Maeda and A. Tsuji, J. Chromatogr., 226 (1981) 1.
8 H. Sekihara, N. Ohsawa and H. Ibayashi, Steroids, 20 (1972) 813.
9 S. Cattaneo, G. Forti, G. Fiorelli, U. Barbieri and M. Serio, Clin. Endocrinol., 4 (1975) 505.
10 M.G. Metcalf, Steroids, 28 (1976) 311.
11 T. Kawasaki, M. Maeda and A. Tsuji, J. Chromatogr., 233 (1982) 61.
12 P.T. Kissinger, Anal. Chem., 49 (1977) 477A.
13 W.R. Heineman and P.T. Kissinger, Anal. Chem., 52 (1980) 138R.
14 K. Shimada, M. Tanaka and T. Nambara, Anal. Lett., 13 (B13) (1980) 1129.
15 S. Honma and T. Nambara, Chem. Pharm. Bull., 22 (1974) 687.
16 T. Nambara, K. Shimada, M. Numazawa, H. Ohta and Y. Sawada, J. Pharm. Dyn., 3 (1980) 407.

Journal of Chromatography, 307 (1984) 29—38
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2009

SEPARATION OF PEPTIDES ON A POLYSTYRENE RESIN COLUMN

TATSURU SASAGAWA*, LOWELL H. ERICSSON, DAVID C. TELLER, KOITI TITANI** and KENNETH A. WALSH*

Department of Biochemistry, University of Washington, Seattle, WA 98195 (U.S.A.)

(First received August 16th, 1983; revised manuscript received November 18th, 1983)

SUMMARY

A high-performance liquid chromatographic column of porous spherical polystyrene—divinylbenzene copolymer (Hamilton PRP-1) was found to be useful for reversed-phase resolution of a wide range of peptides. Recoveries and resolution were comparable with those from more widely used alkyl silica-based columns. Tests involving more than 40 peptides, ranging from 2 to 34 residues, and 3 proteins, indicated wide applicability of this column. The retention times of peptides of known composition are predicted. The stability of the resin at high pH permits protocols of separation involving successive chromatograms at widely different pH values, and offers a second dimension to the resolving power of a single column.

INTRODUCTION

In the past several years, reversed-phase high-performance liquid chromatography (HPLC) on alkyl-modified silica columns has become one of the most important techniques for separation of peptide mixtures. However, alkyl-modified silica lacks chemical stability outside the pH range of 1.5 to 8. Solvation of large denatured peptides may require more extreme pH values, particularly in the alkaline range. Neutral porous polystyrene—divinylbenzene copolymer columns, e.g. Amberlite XAD-2, have been known for years to be stable over a wide range of pH [1, 2]. Recently Lee and Kindsvater [3] introduced a 10-μm spherical preparation of this adsorbent, the Hamilton PRP-1 column, which is designed for HPLC. The column has been successfully used to separate nucleosides [3], other small molecules [4], and peptides [5],

*Present address: Toya Soda Co., Ltd., 2743-1 Hayakawa, Ayase-shi, Kanagawa-ken, Japan.
**Investigator of the Howard Hughes Medical Institute.

0378-4347/84/$03.00

although the latter study encompassed only peptides of six residues or less. We now show that complex mixtures of larger peptides can be separated on a porous polystyrene column (Hamilton PRP-1) at both high and low pH.

EXPERIMENTAL

Materials

Almost all peptides were obtained by tryptic degradation of sperm whale myoglobin, hen lysozyme (Sigma), or bovine brain calmodulin (a gift from Dr. Brad Olwin, University of Washington). Other oligopeptides were purchased from the indicated sources: Tyr-Gly-Gly-Phe-Met (Pierce); Gly-Phe (California Biomedical Research); Gly-Trp, Phe-Phe (Mann Research Labs.); Trp-Tyr-NH_2 (Vega Biochemicals). TPCK trypsin was purchased from Worthington. Sperm whale myoglobin was further purified before use [6]. Reagents were obtained as follows: acetonitrile (Burdick and Jackson Labs.); trifluoroacetic acid (Pierce).

The PRP-1 column was obtained from Hamilton (Reno, NV, U.S.A.).

Methods

Chromatography was carried out on a Hamilton PRP-1 column using a Varian Model 5000 liquid chromatograph. The column (150 × 4.1 mm) contains spherical, uniform, 10-μm particles of a polystyrene—divinylbenzene copolymer with a pore volume of 0.76 ml/g and a large surface area (415 m^2/g). Mobile phases comprised of linear gradients from 0.1% aqueous trifluoroacetic acid (pH 2) to acetonitrile containing 0.07% trifluoroacetic acid, or from 5 m*M* ammonium bicarbonate (adjusted to pH 8, 9.6 or 11 with ammonium hydroxide) to acetonitrile. These are referred to as pH 2, 8, 9.6, or 11 systems, respectively. The concentration of acetonitrile was increased linearly from 0 to 60% over 30 min (2%/min) at a flow-rate of 2 ml/min. Amino acid analyses were performed with a Dionex amino acid analyzer (Model D-500).

Retention times (t_{Ri}) of peptides were recorded and fitted [7] to the relationship $t_{Ri} = A \ln (1 + \Sigma D_j n_{ij}) + C$, where n_{ij} is the number of residues of amino acid j in peptide i, D_j is a retention constant for residue j, and A and C are constants. Non-linear multiple regression analyses were done on a PDP-12 computer with a floating-point processor to compute values for A and C and to obtain best fits for the D_j values in 46 peptides. Initial values of D_j were those for C_{18} columns [7]. Matrix inversion was performed using double-precision arithmetic.

RESULTS

Separation of tryptic peptides from myoglobin and lysozyme

Myoglobin was digested with trypsin (100:2, w/w, in 0.1 *M* NH_4HCO_3 for 6 h at 37°C), and an aliquot of the digest (30 nmol) was chromatographed (Fig. 1A) on a column of PRP-1 using the mobile-phase system at pH 2. Peak fractions were collected and subjected to amino acid analysis after hydrolysis to check purity and recovery. Unresolved mixtures (fractions 3, 5, 9 in Fig. 1A)

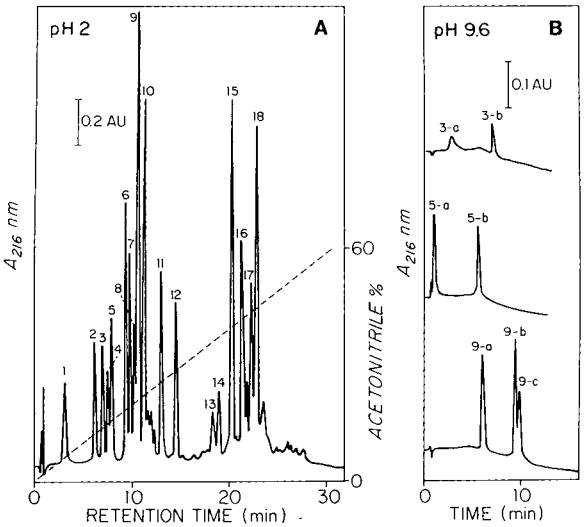


Fig. 1. (A) Chromatogram of 30 nmol of a tryptic digest of myoglobin on a column of PRP-1 in the system at pH 2. (B) Unresolved peaks 3, 5, and 9 were rechromatographed under the same conditions but using the pH 9.6 system. Purified peptides are identified in Table I.

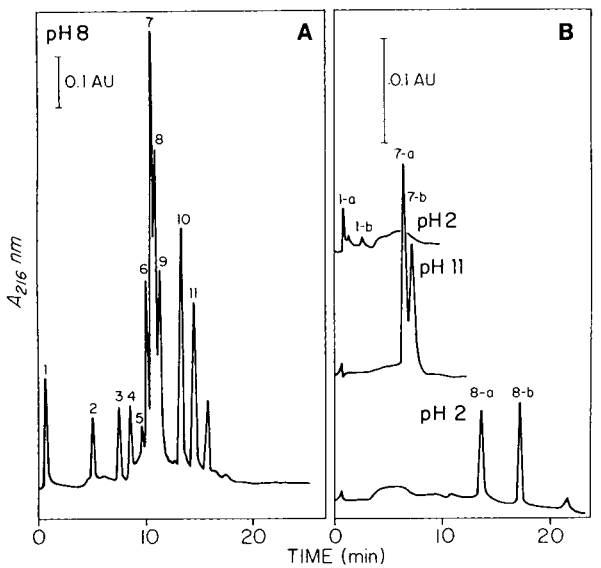


Fig. 2. (A) Chromatogram of 10 nmol of tryptic digest of reduced and S-carboxymethylated lysozyme on a PRP-1 column at pH 8 as in Fig. 1A. (B) Unresolved peaks were rechromatographed under the same conditions but using either the pH 2 or pH 11 system. Peaks: 1-a = NR, 1-b = GCR, 2 = TPGSR, 3 = CELAAMKR, 4 = HGLDNYR, 5 = KVFGR, 6 = FESNFNTQATNR, 7-a = WWCNDGR, 7-b = NTDGSTDYGILQINSR, 8-a = GTDVQAWIR, 8-b = NLCNIPESALLSSDITASVNCAK, 9 = GTDVQAWIR, 10 = GYSLGNWVCAAK, 11 = IVSDGDGMNAWVAWR.

TABLE I

AMINO ACID COMPOSITIONS OF TRYPTIC PEPTIDES PURIFIED FROM MYOGLOBIN

The peptides were purified as described in Fig. 1. In each case, the identity of the peptide was established by comparison of its amino acid composition with that (in parentheses) of a segment of myoglobin [8].

Amino acid	Peptide No. (Fig. 1)						
	1	2	3-a	3-b	4	5-a	5-b
Residues	146—147	48—50	141—145	46—47	57—63	51—56	43—45
Asp			1.0 (1)		1.0 (1)		1.0 (1)
Thr						1.0 (1)	
Ser					0.9 (1)		
Glu					1.1 (1)	2.0 (2)	
Pro							
Gly							
Ala			1.7 (2)		1.0 (1)	1.0 (1)	
Val							
Met						0.8 (1)	
Ile			0.8 (1)				
Leu		0.8 (1)			1.0 (1)		
Tyr	0.7 (1)						
Phe				0.7 (1)			0.9 (1)
His		0.9 (1)					
Lys	1.0 (1)	1.0 (1)	1.1 (1)	1.0 (1)	1.8 (2)	1.2 (1)	
Arg							1.0 (1)
Trp							
Length (residues)	2	3	5	2	7	6	3
Yield (%)	94	100	66	67	97	83	93

Amino acid	Peptide No. (Fig. 1)						
	6	7	8	9-a	9-b	9-c	10
Residues	35—42	97—102	32—34	148—153	79—96	78—96	119—133
Asp							2.8 (3)
Thr	0.9 (1)				1.0 (1)	1.0 (1)	
Ser	1.0 (1)				1.1 (1)	1.1 (1)	
Glu	2.0 (2)			2.0 (2)	3.3 (3)	2.9 (3)	1.3 (1)
Pro	1.0 (1)	1.1 (1)			1.0 (1)	1.1 (1)	0.9 (1)
Gly				2.0 (2)	1.2 (1)	1.3 (1)	3.0 (3)
Ala					3.0 (3)	3.0 (3)	3.0 (3)
Val							
Met							0.9 (1)
Ile		2.0 (2)					
Leu	1.0 (1)		1.0 (1)	1.0 (1)	2.0 (2)	2.0 (2)	
Tyr				0.8 (1)			
Phe			0.8 (1)				0.8 (1)
His	1.0 (1)	1.1 (1)			3.0 (3)	3.0 (3)	1.0 (1)
Lys	1.2 (1)	2.2 (2)	1.2 (1)		2.6 (3)	3.4 (4)	1.3 (1)
Arg							
Trp							
Length (residues)	8	6	3	6	18	19	15
Yield (%)	100	91	98	86	43	21	100

TABLE I (continued)

Amino acid	Peptide No. (Fig. 1)						
	11	12	13	14	15	16	18
Residues	17—31	134—139	64—78	64—77	1—16	103—118	1—31
Asp	1.8 (2)						2.0 (2)
Thr			1.7 (2)	2.0 (2)			
Ser					1.0 (1)	1.5 (2)	1.2 (1)
Glu	1.9 (2)	1.2 (1)			3.0 (3)	2.0 (2)	5.0 (5)
Pro							
Gly	2.0 (2)		2.0 (2)	2.0 (2)	1.1 (1)		3.0 (3)
Ala	2.3 (2)	1.1 (1)	2.2 (2)	2.1 (2)	1.2 (1)	1.0 (1)	3.0 (3)
Val	1.8 (2)		2.0 (2)	2.0 (2)	3.1 (3)	1.6 (1)	5.0 (5)
Met							
Ile	1.9 (2)		1.0 (1)	0.9 (1)		1.7 (3)	2.2 (2)
Leu	1.4 (1)	2.0 (2)	3.0 (3)	2.9 (3)	2.7 (3)	2.0 (2)	3.9 (4)
Tyr						0.8 (1)	
Phe		0.9 (1)				0.8 (1)	
His	0.9 (1)		1.1 (1)	1.2 (1)	1.0 (1)	1.8 (2)	2.1 (2)
Lys			2.4 (2)	1.5 (1)	1.5 (1)		2.0 (1)
Arg	0.9 (1)	1.0 (1)				0.8 (1)	1.0 (1)
Trp					ND (2)		ND (2)
Length (residues)	15	6	15	16	14	16	31
Yield (%)	81	98	33	36	42	74	18

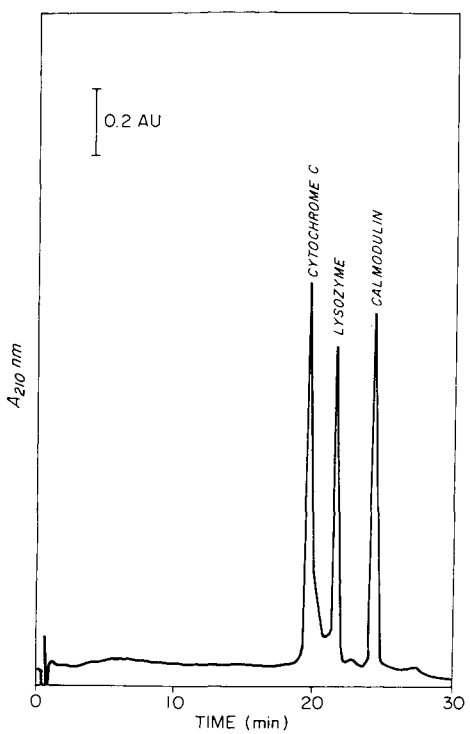


Fig. 3. Separation of a mixture of 1 nmol each of three proteins using the same protocol as in Fig. 1A.

were rechromatographed under the same conditions but using the mobile-phase system at pH 9.6. Twenty-one peptides were isolated in pure form. Ten of the 12 peptides shorter than 10 residues were recovered in better than 80% yield. Two of the longer peptides (Nos. 10 and 16 in Table I) were recovered in 100

TABLE II

RETENTION TIMES FOR VARIOUS PEPTIDES ON THE PRP-1 COLUMN

Retention times were measured on the Hamilton PRP-1 column using the gradient described in Methods, then compared with predicted retention times calculated from the amino acid compositions and computed D_j values from Table III.

No.	Sequence*	Retention time			
		pH 2		pH 8	
		Observed	Predicted	Observed	Predicted
1	YK	3.1	4.0	2.3	3.9
2	TPGSR	5.6	5.0	5.1	4.2
3	HLK	6.2	5.9	5.8	7.3
4	DIAAK	7.0	6.8	1.6	1.5
5	FK	7.0	7.1	6.5	7.6
6	ASEDLKK	7.5	6.9	1.3	0.8
7	TEAEMK	7.9	8.3	0.9	1.1
8	FDR	7.9	7.9	5.0	5.5
9	GF	8.7	9.1	8.6	8.2
10	DTDSEEEIR	9.1	8.6	0.4	0.2
11	SHPETLEK	9.3	9.6	5.0	4.7
12	EAFR	9.5	9.3	5.5	6.9
13	HKIPIK	9.7	9.6	10.7	9.8
14	HGLDNYR	10.0	9.3	8.5	7.1
15	LFK	10.2	11.1	11.0	9.9
16	GHHEAELKPLAQSHATK	10.5	10.3	9.7	10.4
17	ELGYQG	10.5	10.5	6.0	6.6
18	GW	10.5	10.4	10.5	9.4
19	FESNFNTQATNR	11.2	11.4	10.1	9.9
20	HPGNFGADAQGAMNK	11.2	11.0	9.2	9.0
21	WY-NH_2	11.2	11.2	13.8	13.8
22	CELAAMKR	11.8	11.3	6.5	5.8
23	ELGTVMR	11.8	13.4	8.8	10.2
24	DGNGYISAAELR	12.7	13.1	8.6	9.1
25	VEADVAGHGQDILIR	13.0	15.6	10.2	11.3
26	FF	13.5	12.6	11.7	11.1
27	NTDGSTDYGILQINSR	13.5	13.6	10.7	10.6
28	WWCNDGR	13.6	13.6	10.7	10.5
29	YGGFM	13.6	13.5	11.2	11.9
30	GTDVQAWIR	14.0	13.7	11.3	11.3
31	ALELFR	14.5	14.8	12.9	11.1
32	VFDKDGNGYISAAELR	14.6	15.6	11.0	11.8
33	Ac-ADQLTEEQIAEFK	14.9	14.9	7.2	7.2
34	EAFSLFDKDGDGTITTK	15.0	15.9	10.3	10.6
35	GYSLGNWVCAAK	15.8	15.1	13.3	12.8
36	HVMTNLGEK*LTDEEVDEMIR	16.3	16.3	9.2	9.2
37	NLCNIPESALLSSDITASVNC	17.2	17.9	11.0	11.8
38	NKALELFRKDIAAKYKELGYQG	17.4	17.2	17.7	13.8
39	IVSDGDGMNAWVAWR	17.6	18.3	14.5	15.7
40	EADIDGDGQVNYEEFVQM	18.2	16.6	9 0	7.5
41	HGVTVLTALGAILKK	18.3	17.4	—	15.0
42	EADIDGDGQVNYEEFVQMMTAK	18.3	17.6	11.0	10.0
43	HGVTVLTALGAILKKK	19.0	18.0	—	15.0
44	VLSEGEWQLVLHVWAK	20.2	20.2	17.2	17.5
45	YLEFISEAIIHVLHSR	21.3	19.7	19.7	17.6
46	SLGQNPTEAELQDMINEVDADGNGTIDFPEFLTM	22.0	22.6	11.6	13.7
47	VLSEGEWQLVLHVWAKVEADVAGHQDILIR	22.7	23.2	17.8	19.5

Single letter abbreviations for amino acid residues are listed in Table III. In addition, the following abbreviations are used: C, S-carboxymethyl cysteine; K, trimethyllysine; Ac-, N-acetyl; -NH_2, carboxyl-terminal amide.

and 74% yield, respectively. The other longer peptides (14 to 29 residues) were each products of incomplete cleavage, but the sum of yields within each redundant set indicated overall recoveries ranging from 60% (residues 1—16, the sum of yields of peptides 15 and 18) to 99% (residues 17—31, peptides 11 and 8).

Similar experiments with 10 nmol of S-carboxymethyl lysozyme (Fig. 2) used the pH 8 system for the first chromatography and either the pH 2 system or the pH 11 system to resolve mixtures. Although fractions 7 and 8 each contained two unresolved peptides, other peptides ranging in length from 2 residues (fraction 1-a in Fig. 2) to 23 residues (fraction 8-b) were resolved in narrow symmetrical peaks with no indication of distortion at the higher molecular weights.

Separation of a mixture of proteins

A mixture of horse cytochrome c, hen lysozyme, and bovine brain calmodulin (1 nmol each) was separated on a PRP-1 column (Fig. 3) using the system at pH 2. Recoveries were estimated to be 82, 87, and 89% respectively for cytochrome c (11,702 daltons), lysozyme (14,314 daltons), and calmodulin (16,705 daltons).

TABLE III

COMPUTED RETENTION CONSTANTS (D_j) OF AMINO ACID RESIDUES IN PEPTIDES

Amino acid		C_{18} Silica* pH 2	Polystyrene PRP-1		No. of residues considered
			pH 2	pH 8	
Tryptophan	(W)	2.34	1.79	2.48	12
Phenylalanine	(F)	1.71	1.37	1.89	21
Isoleucine	(I)	1.38	1.06	1.28	26
Leucine	(L)	1.34	1.23	1.09	39
Tyrosine	(Y)	1.23	0.77	0.67	12
Methionine	(M)	0.85	0.97	1.31	13
Proline	(P)	0.48	0.55	−0.32	8
Valine	(V)	0.38	0.73	0.85	27
Threonine	(T)	0.12	0.21	−0.01	23
Histidine	(H)	0.34	−0.12	0.66	16
Alanine	(A)	0.13	0.15	−0.03	45
Glutamine	(Q)	0.36	−0.39	−0.22	18
Glutamic acid	(E)	0.27	0.17	−0.55	41
Glycine	(G)	0.22	0.04	0.27	43
Serine	(S)	0.18	−0.16	0.60	21
Arginine	(R)	0.26	−0.21	0.32	18
Aspartic acid	(D)	0.10	−0.08	−1.04	35
Asparagine	(N)	−0.45	−0.17	−0.25	22
Lysine	(K)	0.05	−0.50	0.04	25
Carboxymethylcysteine	(CMC)	1.57	0.09	−0.86	6
Trimethyllysine	(TML)	−1.38	−2.42	−1.69	1
Acetyl-	(Ac-)	0.81	0.69	0.71	1
-Amide	(-NH_2)	−0.56	−0.42	2.92	1

*On a Waters μBondapak C_{18} column (Sasagawa et al. [7]).

Retention behavior of peptides

Retention times (t_{Ri}) were measured in both the pH 2 and the pH 8 systems for 47 peptides of known structure (Table II). Computed best fit individual residue retention constants (D_j) are listed in Table III. The predicted retention time of each peptide was then calculated, using the computed D_j values, and compared with the observed t_{Ri} in Table II. The correlation of observed and predicted retention times at pH 2 is 0.98 (Fig. 4A). The mean percent deviation of retention times is only 4.9%. The analogous values at pH 8 (Fig. 4B) are 0.96 and 11.6%, respectively.

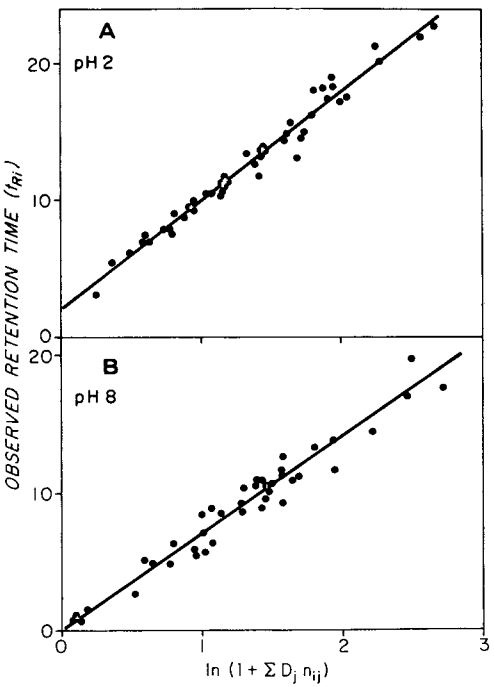


Fig. 4. Relationship between the amino acid composition of a peptide and its retention time on the PRP-1 column at pH 2 (A) and pH 8 (B). The observed retention times were plotted against ln (1 + $\Sigma D_j n_{ij}$), where D_j is the computed retention constant of amino acid j (Table III) and n_{ij} is the number of residues of amino acid j in peptide i.

DISCUSSION

The present experiments with the polystyrene-based PRP-1 column indicate that both the resolving power and the recovery of peptides are comparable with those of the more commonly used silica-based reversed-phase columns. Whereas elution of cationic peptides from the silica-based columns is occasionally perturbed by negative charges on exposed silica surfaces, polystyrene-based columns should resolve peptides exclusively by hydrophobic interactions. Previous studies with very small peptides had suggested that good resolution on the PRP-1 column was possible [5], and the present studies demonstrate resolution of complex mixtures at pH values between 2 and 11

(Figs. 1, 2) with peptides ranging from 2 to 34 residues (Table II). Similar conditions separated three small proteins (Fig. 3) although it is probable that such chromatography will generally result in denaturation and loss of biological activity.

Recoveries of peptides were generally better than 80%. Of the 21 peptides documented in Table I, 9 were recovered in better than 90% yield in a single chromatographic step. Five, which required a second purification step (Fig. 1B), were obtained in 66—93% yield. Those which were recovered from the myoglobin digest in less than 50% yield were each the result of incomplete enzymatic digestion either at residues 77—79 (Lys-Lys-Lys) or at residues 16—17 (Lys-Val), where nearby acidic residues apparently retard cleavage.

The principal advantage of the polystyrene-based column is its utility over a wide range of pH. The same column is used at pH 2, 8, 9.6, and 11 in Figs. 1 and 2. With silica-based columns, the higher values would not be possible. Inclusion of the alkaline range facilitates resolution of a mixed fraction from initial chromatography by rechromatography at a very different pH. Yang et al. [9] recognized the value of such rechromatography but were restricted on their silica-based column to pH 6.0 and 2.15. Without such restrictions on the PRP-1 column, one can optimize a first separation at one pH, then rechromatograph an unresolved fraction at a widely disparate elution pH. For example, peptides 9-b and 9-c, which differ only by an extra amino-terminal lysine in 9-c, co-elute at pH 2 (Fig. 1A) but separate at pH 9.6 (Fig. 1B) where the hydrophobic character of the lysyl residue is not countered by its positive charge. Conversely, by changing the pH from 8 to 2 two co-eluting peptides, 8-a and 8-b, separate because three more carboxylates are neutralized in 8-b

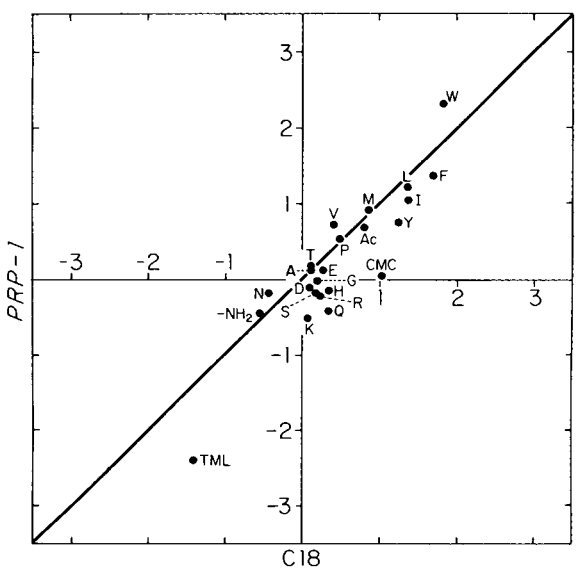


Fig. 5. Correlation of the specific retention constants (D_j) at pH 2 for each amino acid residue (see code in Table III) on the polystyrene (PRP-1) column and on the alkyl silica (C_{18}) column. Points falling on the diagonal line have identical retention constants in the two systems.

than in 8-a (Fig. 2). We have recently reported other two-step purification schemes involving the analogous principle, but with successive chromatograms interrupted by chemical modification of the hydrophobicity of a specific amino acid residue [10, 11]. Other selective procedures and newer matrices should facilitate even further the isolation of peptides from complex mixtures.

Previous studies with silica-based columns [7] indicated that the retention behavior of a few peptides can serve as the basis for predicting the mobility of others on the same column. Table II presents such retention data for 47 peptides on the PRP-1 column and Table III lists individual retention constants for each amino acid residue, as computed from these data. The retention constants for the polystyrene column differ significantly from the corresponding values for an octadecyl silica column (Fig. 5). This allows for further resolution of difficult mixtures by successive combinations of columns.

ACKNOWLEDGEMENTS

The authors are grateful to Roger D. Wade for the amino acid analyses, to Andrew Karplus for help with the computer programming and to Dr. Dan Lee for the gift of a Hamilton PRP-1 column. This work is supported in part by NIH grants GM 15731 (KAW) and GM 13401 (DCT).

A Fortan IV program for calculating predicted retention time from amino acid composition is available on request.

REFERENCES

1 E.P. Kroeff and D.J. Pietrzyk, Anal. Chem., 50 (1978) 502.
2 D.J. Pietrzyk, E.P. Kroeff and T.D. Rotsch, Anal. Chem., 50 (1978) 489.
3 D.P. Lee and J.H. Kindsvater, Anal. Chem., 52 (1980) 2425.
4 D.P. Lee, J. Chromatogr. Sci., 20 (1982) 203.
5 Z. Iskandarani and D.J. Pietrzyk, Anal. Chem., 53 (1981) 489.
6 K.D. Hapner, R.A. Bradshaw, C.R. Hartzell and F.R.N. Gurd, J. Biol. Chem., 243 (1968) 683.
7 T. Sasagawa, T. Okuyama and D.C. Teller, J. Chromatogr., 240 (1982) 329.
8 A.B. Edmundson, Nature (London), 205 (1965) 883.
9 C.Y. Yang, E. Pauly, H. Kratzin and N. Hilschmann, Hoppe-Seyler's Z. Physiol. Chem., 362 (1981) 1131.
10 T. Sasagawa, K. Titani and K.A. Walsh, Anal. Biochem., 128 (1983) 371.
11 T. Sasagawa, K. Titani and K.A. Walsh, Anal. Biochem., 124 (1983) 224.

Journal of Chromatography, 307 (1984) 39—51
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1998

HIGH-PERFORMANCE SIZE EXCLUSION CHROMATOGRAPHY AS A RAPID METHOD FOR THE SEPARATION OF STEROID HORMONE RECEPTORS

RONALD D. WIEHLE, GLEN E. HOFMANN*, AXEL FUCHS and JAMES L. WITTLIFF*

Department of Biochemistry, and the James Graham Brown Cancer Center, University of Louisville, Health Sciences Center, Louisville, KY 40292 (U.S.A.)

(First received June 8th, 1983; revised manuscript received November 16th, 1983)

SUMMARY

High-performance size exclusion chromatography (HPSEC) on TSK 3000SW molecular sieve columns was used to separate estrogen, progestin, and androgen receptors from several target tissues within 50 min on the basis of size and shape (Stokes radius). Moreover, this system provided for the detection of heterogeneity of receptor species (isoforms) in a manner superior to that observed with sucrose density gradient centrifugation. HPSEC separated various estrogen receptor isoforms having Stokes radii of > 61 Å, ~ 48 Å and 29—32 Å. Agents such as potassium chloride and sodium molybdate which alter the distribution of estrogen receptor species on sucrose density gradient centrifugation, promote similar alterations in receptor profiles when HPSEC was employed. Our investigations suggest the use of [^{125}I]iodoestradiol-17β and HPSEC allows the sequential analysis of estrogen receptor species providing new insights into receptor composition and structure.

It is concluded that HPSEC has a broad application in the field of steroid hormone receptors. This method should be useful in studies ranging from measurements of molecular and kinetic properties to their mode of cellular interaction and regulation.

INTRODUCTION

Intracellular proteins termed receptors exist for the steroid hormones. These constituents appear to be a prerequiste for eliciting a biological response by a target organ [1—3]. Current methodologies for the analyses of these proteins (gel chromatography, isoelectric focusing, ion-exchange chromatography, sucrose density gradient centrifugation) are laborious and time-consuming considering the highly labile nature of these macromolecules [3]. For example, the

*Present address: Department of Obstetrics and Gynecology, University of Cincinnati, Cincinnati, OH 45267, U.S.A.

0378-4347/84/$03.00

variety of molecular forms of the estrogen receptor has been attributed to such diverse causes as subunit dissociation, enzymatic processing, and proteolysis. Recently we described a rapid and efficient procedure for resolution of these forms on the basis their surface charge using either high-performance chromatofocusing [4] or high-performance ion-exchange chromatography [5].

In this study we explored rigid, macroporous size exclusion columns for protein separation in a high-performance size exclusion chromatographic (HPSEC) system similar to those described by Regnier and Gooding [6] as a means of separating various species (isoforms) of a given class of steroid hormone receptors on the basis of size (i.e. Stokes radius). The speed and efficiency of this technology make it ideal for investigating molecular properties of steroid hormone receptors. These data on size and shape may be compared with those obtained from gel chromatography and sucrose density gradient centrifugation to determine the interrelationships of the receptor isoforms.

It was of particular interest to determine if HPSEC was as useful for the characterization of estrogen receptors as sucrose density gradient centrifugation. The latter procedure is utilized currently as a clinical method of determining the molecular species of estrogen receptors in human breast cancer [7, 8]. To determine if our earlier proposal regarding molecular heterogeneity is viable [9, 10] the influence of a number of conditions such as ionic strength, buffer type, pH and protease inhibition were evaluated. Also the clinical utility of the HPSEC method has been examined using radioactively labeled ligands for estrogen, progestin, and androgen receptors in a variety of tissues.

In human breast carcinoma, the concentration [11] and properties [9, 12] of estrogen receptors are valuable biochemical criteria for selecting hormonal therapies by the clinician. Our data clearly indicate that the use of size exclusion columns in high-performance liquid chromatography (HPLC) provides rapid separation of steroid hormone receptors which may be applied as a clinical method. This may be particularly significant with metastatic breast carcinoma where biopsies usually are smaller. In this case, we suggest that ^{125}I-labeled estradiol-17β be used as the ligand due to its high-specific radioactivity [10, 13].

MATERIALS AND METHODS

Reagents and chemicals

The ligands used for receptor studies, [16α-^{125}I]iodoestradiol-17β, [2,4,6,7-^{3}H]estradiol-17β, [17α-*methyl*-^{3}H]methyltrienolone (^{3}H-R1881), [11β-*methoxy*-^{3}H]moxestrol (^{3}H-R2858), [17α-*methyl*-^{3}H]promegestrone (^{3}H-R5020), and several of their unlabeled counterparts were obtained from the New England Nuclear Corporation. Purity of labeled steroids was checked by thin-layer chromatography using two solvent systems; only those with purities of $> 95\%$ were utilized. Other unlabeled ligands such as diethylstilbestrol were purchased from Sigma.

The purified proteins used as markers, namely horse cytochrome *c*, chicken ovalbumin, whale myoglobin and hemoglobin, catalase, and thyroglobulin of human origin were obtained from Sigma.

Animals and tissues

Lactating mammary glands of Sprague-Dawley rats were obtained from animals bred in our vivarium. Calf uterus was obtained from animals at the time of sacrifice in a local slaughterhouse. All human tissues were obtained through the aegis of pathologists at local hospitals where tumor pathology was confirmed. Specimens were either frozen in pathology or brought to the laboratory on ice and frozen in liquid nitrogen. Usually human breast carcinomas were used in the form of a powder consisting of pooled specimens pulverized in liquid nitrogen and stored at −86° C.

Preparation of cytosol and ligand-binding reactions

Fresh tissue was minced at 0—4° C whereas frozen tissue was sliced into thin sections with a scalpel blade before mincing. Pulverized tissue was mixed directly with buffer prior to homogenization. Homogenization was carried out at 0—4° C utilizing a Brinkman Polytron [3, 4]. One of the following homogenization buffers was used: TEGM [10 m*M* Tris—HCl, pH 7.4 at 4° C, containing 1.5 m*M* EDTA, 10 m*M* monothioglycerol, 10% (v/v) glycerol]; PEGM [25 m*M* Na_2HPO_4/NaH_2PO_4, pH 7.4 at 4° C, containing 1.5 m*M* EDTA, 10 m*M* monothioglycerol, 10% (v/v) glycerol]; TEGMM [10 m*M* Tris—HCl, pH 7.4 at 4° C, containing 1.5 m*M* EDTA, 10 m*M* monothioglycerol, 10% (v/v) glycerol, 20 m*M* sodium molybdate]. Homogenates were centrifuged at 105,000 *g* for 30 min to sediment nuclei, organelles, and cellular debris. The infranates were separated from the lipid-rich layer and used immediately in binding studies. The cytosolic protein concentration was determined by the method of Waddell [14] using bovine serum albumin as a standard. When cytosols prepared in the presence of molybdate were assayed, the Waddell method was modified since molybdate interferes with this procedure. In this case, the dual-beam spectrophotometer was used and initially balanced using molybdate-containing buffers diluted to the same extent as the unknowns (1:100). Protein standards were prepared in molybdate-containing buffer, read at the appropriate wavelengths and the standard curve constructed. These curves were linear although the parameters of the line were different from the non-molybdate-containing standard curves.

All ligand binding reactions were carried out at 0—4° C. Cytosols were incubated with a saturating concentration (4—5 n*M*) of one of the labeled ligands either in the presence or absence of a 200- to 250-fold excess of unlabeled steroid or competitor to estimate non-specific (low-affinity, high-capacity) binding [3]. Certain incubations were performed in 400 m*M* potassium chloride to achieve a high ionic strength. The reactions were terminated after various times by adding the reaction mixture to pellets of dextran-coated charcoal which removed unbound ligand from solution [4]. A low-speed centrifugation step precipitated the charcoal.

Sucrose density gradient centrifugation

Linear gradients of 10—35% (w/w) sucrose were made by hand layering solutions of different sucrose concentrations into $\frac{7}{16}'' \times 2\frac{3}{8}''$ Beckman cellulose nitrate centrifuge tubes. The original sucrose solutions were made using various buffers: TEK (10 m*M* Tris—HCl, pH 7.4 at 4° C, containing 1.5 m*M*

EDTA and 400 m*M* KCl); TE (10 m*M* Tris—HCl, pH 7.4, containing 1.5 m*M* EDTA) or PE (25 m*M* Na_2HPO_4/NaH_2PO_4, pH 7.4, containing 1.5 m*M* EDTA).

The binding reactions were applied to gradients as a narrow band of 200 μl. Marker proteins such as catalase may be applied at this time in a small volume (20—50 μl) to the gradient. The gradients were centrifuged at 60 000 rpm (335 000 *g*) for 16.5 h at 0—4°C ($\omega^2 t = 2.25 \times 10^{12}$). The position of the heme-containing proteins in the fractionated gradients was visualized by taking advantage of their ability to react and form a colored species after the additions of 1% benzidine in glacial acetic acid and 3% hydrogen peroxide. Other protein markers were identified spectrophotometrically.

Calculation of specific binding capacity

Gradient or chromatography fractions containing tritium-labeled steroid were counted in a Beckman LS-9000 scintillation counter. [^{125}I] Iodoestradiol was counted in a Beckman 4000 gamma counter giving 65% counting efficiency. Specific-binding capacity was expressed as fmol/mg cytosol protein using the difference betweeen the accumulated total binding and the binding in the presence of excess unlabeled ligand.

High-performance size exclusion chromatography

All chromatography was performed in a cold room (0—5°C) utilizing the Spherogel TSK-3000SW size exclusion column (7.5 × 700 mm) with a Beckman Model 322 HPLC system equipped with an in-line Hitachi Model 100-40 spectrophotometer. The chromatographic column comprised two separable units, a short (7.5 × 100 mm) TSK 3000SW guard column and, immediately downstream, the longer (7.5 × 600 mm) TSK 3000SW size exclusion column. Reactions were applied in 20—250 μl volumes using a Hamilton syringe and the Model 210 sample injection valve. Essentially no difference in resolution was noted when injection volumes of 20—250 μl and protein concentrations of 2—12 mg/ml were used. An additional aliquot was taken at this time to estimate specific binding capacity and recovery. The elution buffers were either $TEGK_{100}$ [10 m*M* Tris—HCl, pH 7.4 at 4°C, containing 1.5 m*M* EDTA, 10% (v/v) glycerol, 100 m*M* KCl], $TEGK_{400}$ [10 m*M* Tris—HCl, pH 7.4 at 4°C, 1.5 m*M* EDTA, 10% (v/v) glycerol, 400 m*M* KCl] or $PEGK_{100}$ [25 m*M* NaH_2PO_4/Na_2HPO_4 buffer, pH 7.4 at 4°C, containing 1.5 m*M* EDTA, 10% (v/v) glycerol, 100 m*M* KCl]. The $PEGK_{100}$ and $TEGK_{100}$ buffers gave similar results on the HPSEC when used for incubation and elution. All buffers were filtered with a 0.45-μm filter (Millipore). Elution was carried out at a flow-rate of 0.7 ml/min. Column effluent was collected as either 0.5- or 1-min fractions. Following a day of chromatography, the entire column was washed overnight with filtered, distilled, deionized water. The entire column was washed weekly with a filtered solution of 15% dimethyl sulphoxide in methanol whereas the TSK 3000SW guard column was washed periodically with a solution of 6 *M* urea. The chromatographic system was stored in filtered, distilled, deionized water.

RESULTS AND DISCUSSION

Comparison of HPSEC and sucrose density gradient centrifugation

Our early experiments compared the size exclusion properties of estrogen receptors in a variety of hormone target tissues. The lactating mammary gland of the rat is known to contain predominantly a single form of the estrogen receptor which sediments at 8—9 S using buffers containing Tris [15]. An example of these receptors in cytosol of mammary gland from a rat, 15 days post partum, is shown in Fig. 1A. These receptors were extracted in PEGM buffer as described in Materials and methods.

When an identical sample of cytosol was applied to a TSK-3000SW column and eluted with $PEGK_{100}$ buffer, the profile shown in Fig. 1B was observed. The specific estrogen binding capacity was distributed primarily as a high-molecular-weight species eluting just after the void volume of the column seemingly analogous to the 8—9 S form observed with the sucrose gradient method. Unbound [^{125}I]iodoestradiol-17β eluted at the total volume of the column, i.e. at approximately 41 min. It has been our experience that the amount of unbound ligand remaining after dextran-coated charcoal treatment

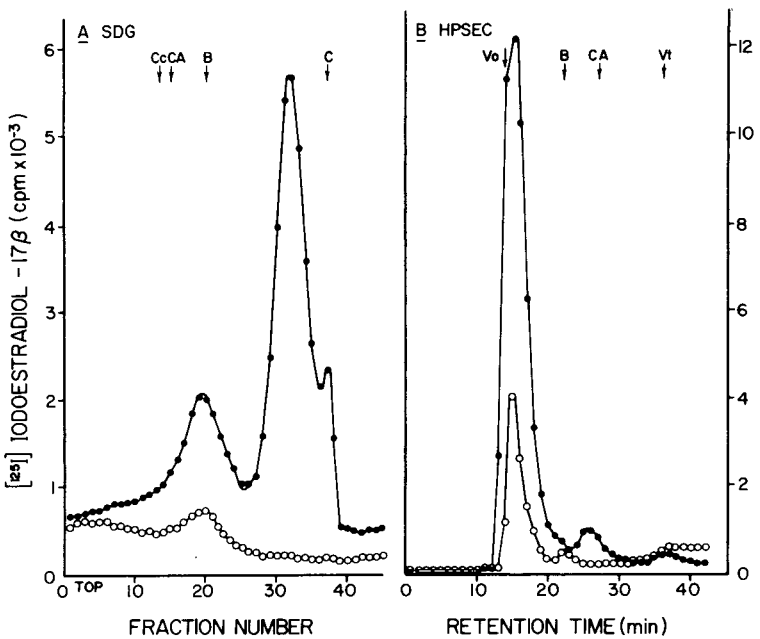


Fig. 1. Comparison of HPSEC and sucrose gradient centrifugation (SDG) for the separation of estrogen receptors from rat lactating mammary gland. Mammary glands of rats lactating 15 days were used as a tissue source of cytosol. Incubation was performed as described in Materials and methods using PEGM buffer and 4 n*M* [16α-^{125}I]iodoestradiol-17β as ligand in the presence (○) or absence (●) of a 250-fold excess of diethylstilbestrol. A portion of each reaction was used for analysis either by sucrose density gradient centrifugation (A) or by HPSEC (B). (A) A 200-μl aliquot was loaded onto a linear gradient of sucrose (10—35%) in PE buffer and centrifuged as described in Materials and methods. (B) A 25-μl aliquot was applied to the TSK 3000SW column and the receptors were eluted with $PEGK_{100}$ buffer as described in Materials and methods. Cc = cytochrome *c*; CA = carbonic anhydrase; B = bovine serum albumin; C = catalase; Vo = void volume as determined using blue dextran; Vt = total volume as determined using ^3H_2O.

and eluting in this position is highly variable and, in certain cases, seems to leach slowly from the column representing a potential problem in terms of interpretation of data. The high non-specific binding peak present at the void volume (Fig. 1B) of the column is a finding representative of cytosol from lactating mammary gland which is not observed when separated on sucrose gradients. This non-specifically associated ligand may represent an artifact in which the components were preferentially preserved by the HPSEC method in distinction to the sucrose gradient procedure.

A similar comparison of separation profiles by sucrose gradient centrifugation and HPSEC was conducted using estrogen receptors from human breast carcinomas [10]. Earlier we reported that breast tumors exhibited a wide variety of estrogen receptor profiles including those that contain both the high- (8—9 S) and low- (4—5 S) molecular-weight forms, only one or the other species or neither [3, 9, 10]. HPSEC revealed three estrogen binding components including a species of large size which eluted from the column near the void volume [10].

In certain experiments in this study using human breast tumors, there was good agreement on the distribution of receptor isoforms using HPSEC and sucrose density gradient centrifugation with the exception of the high-molecular-weight species. The recovery of specific estrogen binding capacity usually was comparable (75—98%) for the two methods although this was highly dependent upon the characteristics of the individual columns.

HPSEC separation of estrogen receptors in uterus

To demonstrate the utility of HPSEC methodology in the analysis of steroid receptors, we examined these proteins in a wide variety of estrogen responsive tissues. Using calf and human uteri, estrogen receptors were extracted in PEGM

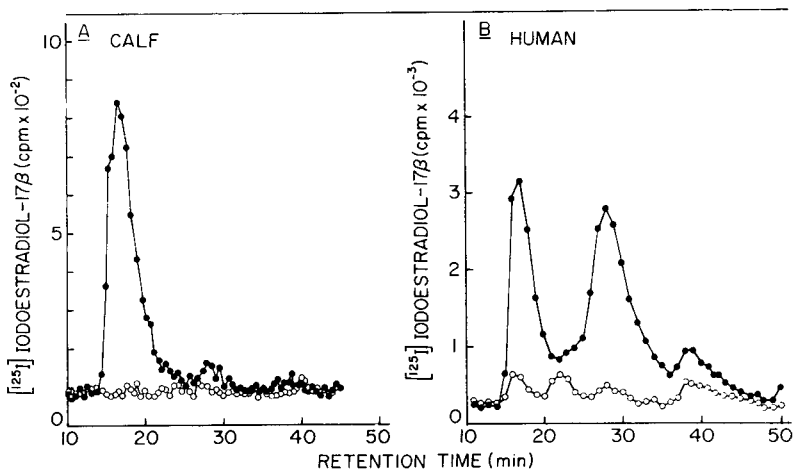


Fig. 2. HPSEC of estrogen receptors from uterus. Calf uterus (A) or human uterus (B) was used as a tissue source of cytosolic estrogen receptors. Cytosol was prepared and incubation performed as described in Materials and methods using PEGM buffer and 5 n*M* [16α-^{125}I]-iodoestradiol-17β as ligand in the absence (•) or presence (○) of a 250-fold excess of diethylstilbestrol. A 200-μl aliquot of each reaction was applied to the TSK 3000SW column. The column was eluted using PEGK$_{100}$ buffer at a flow-rate of 0.7 ml/min.

buffer and separated on TSK 3000SW columns (Fig. 2). As shown, cytosol from calf uterus (Fig. 2A) exhibited a single species which had a Stokes radius of > 61 Å. Human uterus consistently exhibited both a high-molecular-weight form (> 61 Å) and a species with Stokes radius of 29—32 Å (Fig. 2B). We have observed isoforms of the estrogen receptor in human endometrial carcinoma and leiomyomata also [16].

HPSEC separation of receptors for progestins and androgens

The size exclusion properties of progestin receptors in myometrium, ovary and breast carcinoma were estimated using TSK 3000SW columns (Fig. 3). Surprisingly the profiles contained a single receptor species of high molecular weight. Using marker proteins, the estimated size of this binding component was > 61 Å (Stokes radius) similar to that observed for the estrogen receptor. Lower-molecular-weight species were observed rarely, perhaps due to the rapid separation afforded by the HPSEC.

A human ovarian follicular cyst was used as a source of androgen receptor for separation by HPSEC (Fig. 4). The synthetic androgenic ligand, R1881, was used to detect these receptors. Again a high-molcular-weight species (Stokes radius > 61 Å) of the receptor was demonstrated. Unlike most tissues examined, this preparation exhibited a large peak of unbound ligand (fractions 38—42).

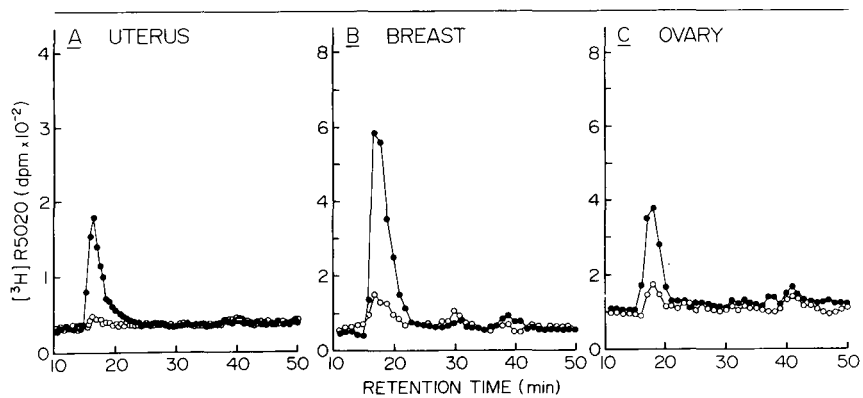


Fig. 3. HPSEC of progestin receptors from various human tissues. Human myometrium (A), human breast carcinoma (B), and human ovary (C) were used as tissue sources of the cytosolic progestin receptors. Cytosols were prepared and the reactions were performed as described in Materials and methods using PEGM buffer and 4 n*M* ^{3}H-R5020, a synthetic progestin, as ligand in the presence (○) or absence (●) of a 200-fold excess of unlabeled R5020. A 250-μl aliquot of reach reaction was applied to a TSK 3000SW column and the receptors were eluted with $PEGK_{100}$ buffer at a flow-rate of 0.7 ml/min.

Influence of potassium chloride and sodium molybdate on separation properties

As discussed earlier, estrogen receptors prepared in low-ionic-strength buffers containing monothioglycerol separated primarily as 8—9 S and 4—5 S components on linear sucrose gradients subjected to centrifugation (Figs. 1A and 5A). In a fashion similar to that which we reported earlier for lactating mammary gland [15], the 8—9 S component in cytosol of human breast

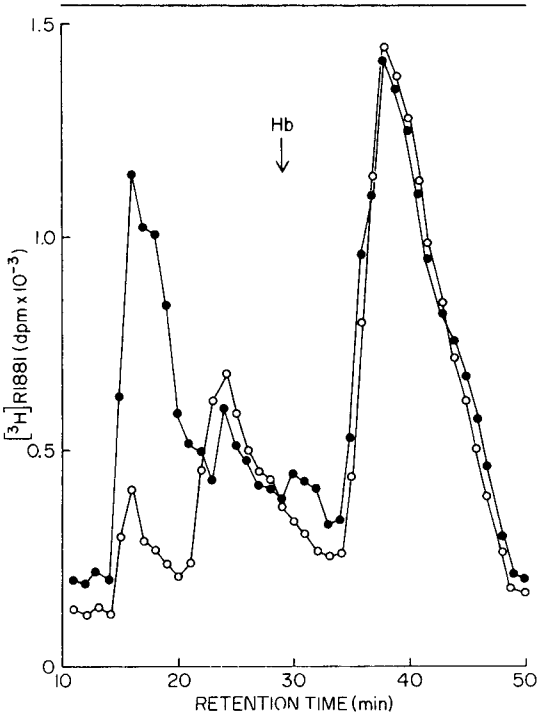


Fig. 4. HPSEC of androgen receptors from human ovarian follicular cyst. A human ovarian follicular cyst was used as a tissue source of the cytosolic androgen receptors. Cytosols were prepared and the reactions were performed as described in Materials and methods using PEGM buffer and ^{3}H-R1881 as ligand in the presence (○) or absence (●) of a 200-fold excess of unlabeled R1881. A 250-μl aliquot of each was applied to a TSK 3000SW column and the receptors were eluted with $PEGK_{100}$ buffer at a flow-rate of 0.7 ml/min. Hb = hemoglobin.

cancer appeared to dissociate into 4—5 S forms in the presence of 400 m*M* potassium chloride (Fig. 5B). However if 20 m*M* sodium molybdate was added to the homogenizing buffer and the receptors separated on linear sucrose gradients, an increased quantity of the 8—9 S species of the estrogen receptor was observed (Fig. 5C). These findings are consistent with the results of a number of investigators using a variety of receptors [17—20].

A problem of considerable magnitude in discerning the interrelationships of estrogen receptor species has been the long separation times required by the sucrose gradient centrifugation which favor both ligand and subunit dissociation and/or receptor degradation. HPSEC was employed to assess the relative importance of these problems since it may be an alternative method. Fig. 6A illustrates the separation of estrogen receptor species in human breast cancer on HPSEC. These profiles were obtained using the same cytosols as those utilized in the sucrose gradient profiles illustrated in Fig. 5. Each HPSEC separation was accomplished within 1 h while centrifugation required 16 h.

Fig. 6A is a representative profile of estrogen receptors in human breast cancer extracted in TEGM buffer and separated by HPSEC in the presence of $TEGK_{100}$ buffer. It is our experience that this is the minimal ionic strength (100 m*M*) required to maintain a linear relationship in the elution sequence

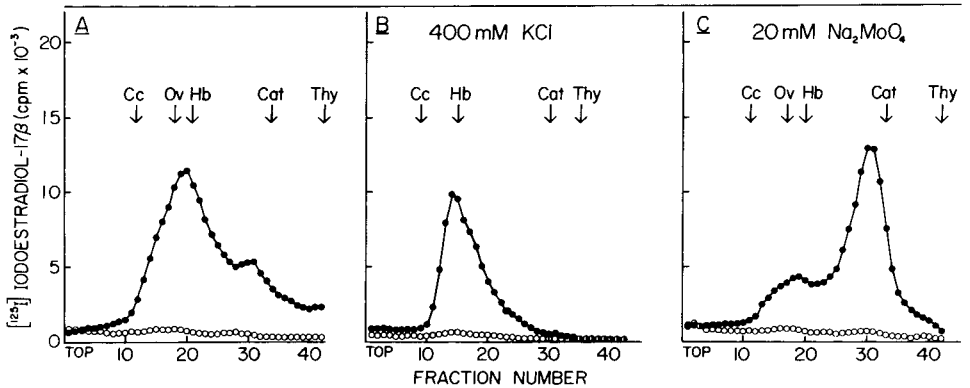


Fig. 5. Influence of potassium chloride and molybdate on the sedimentation properties of estrogen receptors in human breast carcinoma by sucrose density gradient centrifugation. Cytosol preparation and reaction conditions are described in Materials and methods. Human hemoglobin was added to each gradient as an internal standard. The marker proteins cytochrome *c* (Cc), ovalbumin (Ov), hemoglobin (Hb), catalase (Cat), and thyroglobulin (Thy) were separated in an additional gradient. Reactions were performed using 4 n*M* [^{125}I]-iodoestradiol-17β in the presence (○) and absence (●) of a 200-fold excess of diethylsilbestrol. (A) The reaction medium consisted of TEGM buffer whereas centrifugation was performed in sucrose (10—35%) gradients made with TE buffer. A receptor preparation containing 0.8 mg of protein was applied. (B) The initial reaction medium was TEGM buffer but sufficient potassium chloride was added after 1 h incubation to bring the final potassium chloride concentration to 400 m*M*; centrifugation was performed in sucrose (10—35%) gradients made with TEK buffer. Cytosol containing 0.5 mg of protein was applied. (C) The reaction medium consisted of TEGM buffer whereas centrifugation was performed in sucrose (10—35%, w/w) gradients made with TE buffer. The preparation applied to the gradient contained 0.7 mg of protein.

of marker proteins employed in this study. The elution positions of these proteins are indicated in Fig. 6. The sedimentation positions of the same markers on sucrose gradients are indicated in Fig. 5. A comparison of the high-molecular-weight (size) species in Fig. 5A (fraction 31) and Fig. 6A (approximately 18 min) relative to the position of the catalase marker demonstrates the difference observed with these methods. Using HPSEC, the high-molecular-weight species appeared to elute with a Stokes radius of > 61 Å whereas on sucrose gradients the largest isoform sedimented more slowly than catalase (51 Å). This seeming discrepancy is usually resolved by the assumption that the molecule is highly asymmetric and in the shape of a prolate ellipsoid of relatively high frictional ratio (1.65) [21].

The concentration of estrogen receptors determined from profiles generated by the respective separation systems as demonstrated in Figs. 5A and 6A were calculated. The sucrose gradient procedure gave 122 fmol/mg cytosol protein whereas HPSEC gave a level of estrogen receptor equivalent to 99 fmol/mg cytosol protein. The sucrose gradient method revealed that 65% of total specific binding was represented by the 4—5 S species and 20% was represented by the 8—9 S species. On HPSEC, a molecular species indistinguishable from the 4 S species seen on sucrose gradients (data not shown) and the high-molecular-weight species constituted 66% and 18% of the total specific binding, respectively.

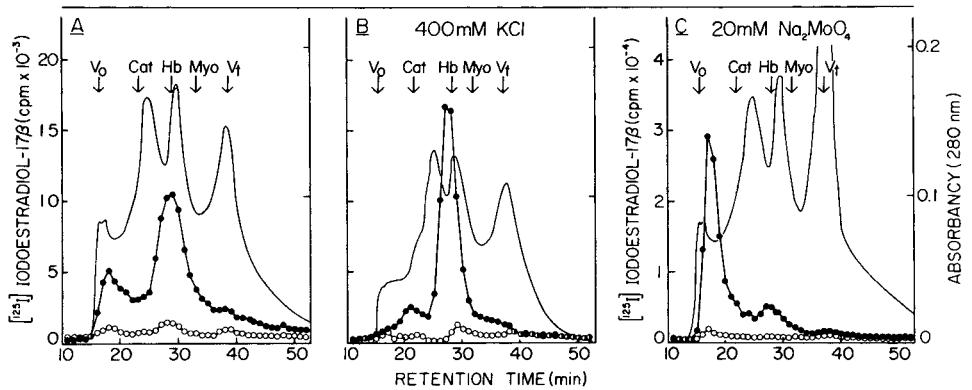


Fig. 6. Influence of potassium chloride and molybdate on the size exclusion properties of estrogen receptors in human breast carcinoma by HPSEC. Cytosol preparation and reaction conditions are described in Materials and methods. In each case, the reactions were performed using 4 n*M* [16α-^{125}I]iodoestradiol-17β as ligand in the presence (○) or absence (●) of a 200-fold excess of diethylstilbestrol. A 100-μl aliquot of each reaction was applied to the column which was eluted at a flow-rate of 0.7 ml/min using $TEGK_{100}$ or $TEGK_{400}$. The marker proteins were chromatographed individually and as a group in separate runs and their retention times were determined from their absorption peaks at 280 nm. The cytosols and reactions were identical to those described in Fig. 5. (A) The reaction medium consisted of TEGM buffer whereas the chromatographic elution buffer was $TEGK_{100}$. A total of 0.8 mg of cytosol protein was applied. (B) The initial reaction medium was TEGM buffer, but sufficient potassium chloride was added after 1 h of incubation to bring the final potassium chloride concentration to 400 n*M*. The chromatographic elution buffer was $TEGK_{400}$ and the quantity of cytosol protein applied was 0.5 mg. (C) The reaction medium consisted of TEGMM buffer whereas the chromatographic elution buffer was $TEGK_{100}$. A total of 0.7 mg of cytosol protein was applied. The trace of eluted materials absorbing at 280 nm is given. Identification of peaks as in Fig. 5. V_0 = void volume as determined using blue dextran; V_t = total volume as determined using ^3H_2O.

Treatment of the cytosol with 400 m*M* potassium chloride resulted in the appearance of a 29—32 Å form on HPSEC which dominated the profile (Fig. 6B). There was a small quantity of a component retained at 22 min which appeared to have a Stokes radius of ~ 48 Å. The sucrose gradient method gave (Fig. 5B) a level of 120 fmol/mg cytosol protein while HPSEC estimated a level of 121 fmol/mg cytosol protein. The gradient technique showed that 88% of the specific estrogen receptor binding was in the 4—5 S form and only 5% was in the 8—9 S form, whereas HPSEC exhibited 85% in the 29—32 Å form and 12% in the high-molecular-weight region.

When 20 m*M* sodium molybdate was added to the homogenizing buffer (TEGM), virtually all of the specific binding was exhibited by a component eluting after the void volume in the HPSEC experiments (Fig. 6C). These data suggest a component with a Stokes radius of > 61 Å. Only a small quantity of the 29—32 Å species remained. A specific estrogen receptor concentration of 150 fmol/mg cytosol protein was estimated by centrifugation whereas HPSEC gave 187 fmol/mg cytosol protein. The higher recovery of receptor by HPSEC relative to that from sucrose gradients may be attributed to the longer incubation time with the stabilizing reagent, sodium molybdate.

The higher level of binding attained in the presence of molybdate was consistent with the stabilizing effects that ion has shown on other receptors [17—20,

22]. Using the sucrose gradient centrifugation, 57% of the specific binding was attributable to the 8—9 S species and 35% to the 4—5 S species, whereas, utilizing HPSEC, 74% of the specific binding corresponds to the high-molecular-weight species and 23% to the 28—32 Å moiety.

Whereas the highly comparable data given in Figs. 5 and 6 are representative of many samples, we have observed several cases where gradients and HPSEC exhibited different profiles. Fig. 7 is a sample of another human breast carcinoma treated in the same manner as that of the sample shown in Fig. 5, i.e. sedimented in the presence of low salt (panel A), adjusted to 400 m*M* potassium chloride and sedimented in high salt (Fig. 5B), or homogenized in molybdate-containing buffers and sedimented on low salt gradients (Fig. 5C). The effects of these treatments are given in Fig. 7 and are qualitatively similar to those illustrated in Fig. 5. The corresponding HPSEC profiles given in Fig. 8 are, however, not nearly as comparable. In general, the profile in Fig. 8A exhibited much less of the 29—32 Å species than would be expected if this species corresponded to the 4—5 S moiety. Fig. 8B demonstrates relatively more of the 48 Å species separating at 22 min than was apparent in the sample shown in Fig. 6B. The high-molecular-weight species (> 61 Å) seen in Fig. 8C appears proportional to that observed on the gradient.

The results of these experiments indicated a common occurrence, namely the tendency for this chromatographic system either to preserve the high-molecular-weight form or precursor, or to induce the aggregation of the

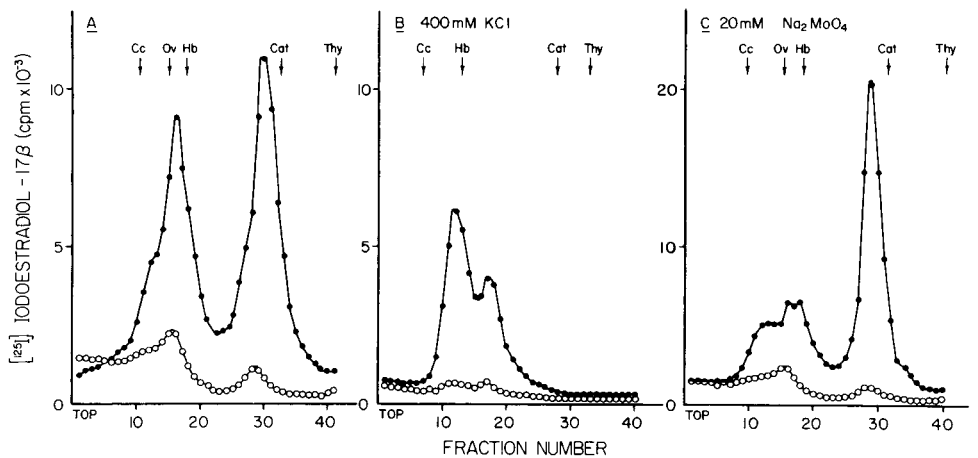


Fig. 7. Influence of potassium chloride and molybdate on the sedimentation properties of estrogen receptor in human breast carcinoma by sucrose density gradient centrifugation. Conditions for cytosol preparation and receptor separation were identical to those given in the legend of Fig. 5. (A) The reaction medium consisted of TEGM buffer whereas centrifugation was performed in sucrose (10—35%) gradients made with TE buffer. A receptor preparation containing 0.8 mg of protein was applied. (B) The initial reaction medium was TEGM buffer but sufficient potassium chloride was added after 1 h incubation to bring the final potassium chloride concentration to 400 m*M*; centrifugation was performed in sucrose (10—35%) gradients made with TEK buffer. Cytosol containing 0.5 mg of protein was applied. (C) The reaction medium consisted of TEGMM buffer whereas centrifugation was performed in sucrose (10—35%, w/w) gradients made with TE buffer. The preparation applied to the column contained 0.8 mg of protein. Identification of peaks as in Fig. 5.

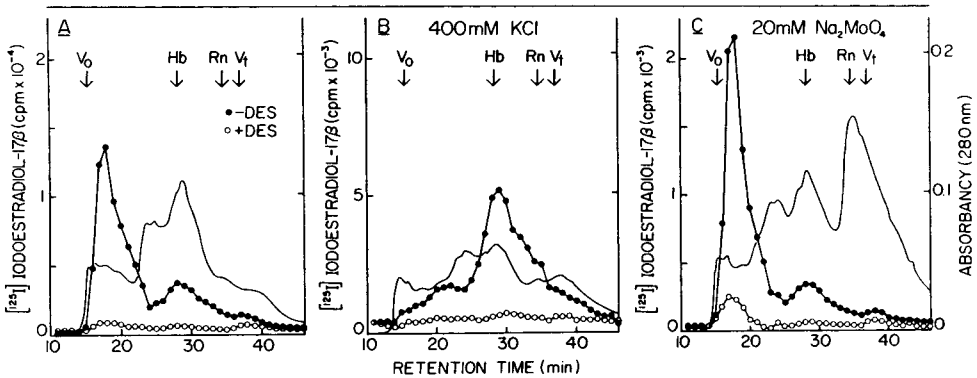


Fig. 8. Influence of potassium chloride and molybdate on the size exclusion properties of estrogen receptors in human breast carcinoma by high-performance liquid chromatography. Conditions for cytosol preparation and receptor separation were identical to those given in the legend of Fig. 6. (A) The reaction medium consisted of TEGM buffer whereas the chromatographic elution buffer was $TEGK_{100}$. A total of 0.8 mg of cytosol protein was applied. (B) The initial reaction medium was TEGM buffer, but sufficient protassium chloride was added after 1 h of incubation to bring the final potassium chloride concentration to 400 n*M*. The chromatographic elution buffer was $TEGK_{400}$ and the quantity of cytosol protein applied was 0.5 mg. (C) The reaction medium consisted of TEGMM buffer whereas the chromatographic elution buffer was $TEGK_{100}$. In this case 0.8 mg of cytosol protein was applied. The trace of eluted materials absorbing at 280 nm is given. V_0 = void volume as determined using blue dextran; V_t = total volume as determined using ^3H_2O; Hb = hemoglobin; Rn = ribonuclease; DES = diethylstilbestrol.

receptor. Since molybdate is considered to be a potential inhibitor of protease activity as well as of phosphatase action [22, 23], our finding of a high-molecular-weight species supports the concept of a processing step in the steroid hormone receptor cascade. In summary, HPSEC separates the various species of receptors for the sex-steroid hormones on the basis of size and shape. Furthermore, the speed and efficiency of HPSEC in combination with [^{125}I]-iodoestradiol-17β of high-specific radioactivity should permit sequential analyses of estrogen receptor isoforms prepared by a variety of methods.

ACKNOWLEDGEMENTS

The authors are indebted to their colleagues, Drs. Greg Kuhns, Stewart E. Wolfson and George Barrows, who provided tissue specimens for this study. Their appreciation is also expressed to Sandra Wiehle, Judith Siegel, Derich Wittliff, Neil Clements, Jr. and Jay Eckman for assistance in the collection and preparation of tissues. Dr. P.A. Wheeler Feldhoff is acknowledged for assistance in the preparation of certain cytosols. We acknowledge the expert help of Debbie Schwab and Ellen Ford with the manuscript preparation. Also, we wish to thank the Medical Illustration and Photography Unit for their superb workmanship.

This work was supported in part by USPHS grants CA-19657 and CB-23876 from the National Cancer Institute and by funds from the Marie Overbey

Memorial grant from the American Cancer Society and from the Phi Beta Psi Sorority. Ronald D. Wiehle is a university fellow; Glen E. Hofmann is a summer research fellow; Axel Fuchs is a research fellow of the Deutsche Forschungsgemeinschaft and of the Cancer Center.

REFERENCES

1 E.V. Jensen, G.E. Block, S. Smith, K. Kyser and E.R. DeSombre, Natl. Cancer Inst. Monogr., 34 (1971) 55.
2 B.W. O'Malley, W.T. Schrader and T.C. Spelsberg, in B.W. O'Malley and A.R. Means (Editors), Receptors for Reproductive Hormones, Advances in Experimental Medicine and Biology, Plenum Press, New York, 1973, p. 174.
3 J.L. Wittliff, Methods Cancer Res., 9 (1975) 293.
4 T.W. Hutchens, R.D. Wiehle, N.A. Shahabi and J.L. Wittliff, J. Chromatogr., 266 (1983) 115.
5 R.D. Wiehle and J.L. Wittliff, III International Symposium on HPLC of Proteins, Peptides, and Polynucleotides, in press.
6 F.E.Regnier and K.M. Gooding, Anal. Biochem., 103 (1980) 1.
7 J.L. Wittliff, R. Hilf, W.F. Brooks, E.D. Savlov, T.C. Hall and R.A. Orlando, Cancer Res., 32 (1972) 1983.
8 J.L. Wittliff and E.D. Savlov, in W.L. McGuire, P.P. Carbone and E.P. Vollmer (Editors), Estrogen Receptors in Human Breast Cancer, Raven Press, New York, 1975, p. 73.
9 T.E. Kute, P. Heidemann and J.L. Wittliff, Cancer Res., 38 (1978) 4307.
10 J.L. Wittliff, P.W. Feldhoff, A. Fuchs and R.D. Wiehle, in R.J. Soto, A.F. DeNicola and J.A. Blaquier (Editors), Pathophysiology of Endocrine Diseases and Mechanisms of Hormone Action, Alan R. Liss, New York, 1981, p. 375.
11 E.R. DeSombre, P.P. Carbone, E.V. Jensen, W.L. McGuire, S.A. Wells, J.L. Wittliff and M.B. Lipsett, N. Engl. J. Med., 301 (1979) 1011.
12 J.L. Wittliff, Cancer, 46 (1980) 2953.
13 R.B. Hochberg, Science, 205 (1979) 1138.
14 W.J. Waddell, J. Lab. Clin. Med., 48 (1956) 311.
15 D.G. Gardner and J.L. Wittliff, Biochemistry, 12 (1973) 3090.
16 G. Daxenbichler, H.J. Grill, W. Geir, J.L. Wittliff and O. Dapunt, in J.L. Wittliff and O. Dapunt (Editors), Steroid Receptors and Hormone-dependent Neoplasia, Masson Publishing USA, New York, 1980, p. 59.
17 H. Nishigori and D. Toft, Biochemistry, 19 (1980) 77.
18 G. Shyamala and L. Leonard, J. Biol. Chem., 255 (1980) 6028.
19 L.K. Miller, F.B. Tuazon, E.-M. Niu and M.R. Sherman, Endocrinology, 108 (1981) 1369.
20 T.W. Hutchens, F.S. Markland and E.F. Hawkins, Biochem. Biophys. Res. Commun., 103 (1981) 60.
21 G.A. Puca, E. Nola, V. Sica and F. Bresciani, Biochemistry, 10 (1971) 3769.
22 K.L. Leach, M.K. Dehmer, N.D. Hammond, J.J. Sando and W.B. Pratt, J. Biol. Chem., 254 (1979) 11884.
23 K. Paigen, J. Biol. Chem., 233 (1958) 388.

Journal of Chromatography, 307 (1984) 53—64
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2000

ION-PAIR CHROMATOGRAPHY OF SELECTED NUCLEOSIDES, BASES AND OTHER LOW-MOLECULAR-WEIGHT ULTRAVIOLET ABSORBING COMPOUNDS

PAMELA A. PERRONE and PHYLLIS R. BROWN*

University of Rhode Island, Department of Chemistry, Kingston, RI 02281 (U.S.A.)

(First received August 23rd, 1983; revised manuscript received November 15th, 1983)

SUMMARY

Chromatographic conditions used in the profiling of nucleosides and bases in physiological fluids by ion-pair chromatography were invstigated. The parameters examined included type, concentration and size of counter-ion, pH of the mobile phase, temperature, percent organic modifier in the mobile phase and the alkyl chain length of the stationary phase. The compounds studied were creatinine, hypoxanthine, orotidine, L-tyrosine, uracil, uric acid, uridine monophosphate and xanthine. It was found that the quaternary amines were better pairing agents than the sulfonic acids for varying the retention of these compounds and improving the resolution of the chromatogram over the first several minutes. The optimum set of conditions for retention was a mobile phase of 1 m*M* tetrabutylammonium phosphate in 20 m*M* phosphate buffer, adjusted to pH 5.7, at a temperature 40°C and with a C_8 stationary phase. Under these conditions the first segment of the chromatogram of plasma samples was simplified because the retention time of uric acid was increased; thus, it is now possible to determine the compounds present in the first part of the chromatogram which are normally masked by the uric acid peak. Conditions are also presented for the improved separation of hypoxanthine, xanthine and uridine. Finally, evidence is given to support the ion-exchange mechanism for retention of the solutes.

INTRODUCTION

In earlier research conducted in our laboratory on the high-performance liquid chromatographic (HPLC) profiling of the nucleosides, bases and amino acids in serum and/or plasma, noticeable differences were detected between the profiles of normal and diseased individuals [1, 2]. These differences were both qualitative and quantitative in nature. For example, breast cancer patients could be differentiated from the normal subjects and patients with benign fibrocystic changes by the presence of 1-methylinosine and N-2-methyl-

0378-4347/84/$03.00

guanosine [1]; adenosine and elevated levels of several other nucleosides were determined in the plasma of acute lymphocytic leukemics [2]. In a chemometric approach to classifying the chromatographic profiles of acute and chronic leukemic and normal patients by multivariant linear analysis [3] and pattern recognition techniques [4], it was further demonstrated that the earliest time segment was the most important in discriminating between the diseased and normal groups. This time interval is composed of weakly retained compounds (i.e. L-tyrosine and uric acid) or ionic, polar or other compounds which are not retained under reversed-phase conditions. In another area of research in which the authors have been active, the nucleoside and base profiling of Australian marsupials for taxonomical and phylogenetic purposes [5], this quantitative difference in the beginning portion of the chromatogram again surfaced as an important piece of data in determining the individual groups.

Ion-pair chromatography (IPC) is a technique which by modification of the partitioning process permits the simultaneous separation of ionic as well as non-ionic compounds [6, 7]. By the addition of a pairing agent to the mobile phase, ionic sample components combine with the counter-ion of the pairing agent to form neutral ion-pairs which are retained by the chromatographic system. Theoretically neutral sample components are unaffected by the pairing agent. It is believed that if IPC can be applied to the chromatographic systems utilized above, the components of the earliest time segment may be separated and further identified, providing a better understanding of the biochemistry and physiology involved.

In this report, results are presented on the reversed-phase, ion-pair investigation of a series of standards, some of which are known to be present in the beginning segment of the chromatograms. The variables of IPC examined include the type of pairing agent (i.e. chain length), concentration of the pairing agent, pH of the mobile phase, percent organic modifier, hydrophobic chain length of stationary phase and temperature.

EXPERIMENTAL

Instrumentation

A Series 3B liquid chromatograph (Perkin-Elmer, Norwalk, CT, U.S.A.) equipped with a Rheodyne Model 7125 injector, LC-75 spectrophotometric detector, LC-15 fixed-wavelength (254 nm) detector and LC-100 column oven was used. A dual-channel Omniscribe recorder (Houston Instruments, Austin, TX, U.S.A.) was used to record the 280- and 254-nm detector signals. Retention times and peak areas were obtained in earlier work using the Sigma 10 Data Station (Perkin-Elmer) and later with the Model 3600 Chromatographics Intelligent Terminal (Perkin-Elmer).

Columns and chromatographic conditions

An analytical C_{18} (Perkin-Elmer) chemically bonded, 10-μm column (25 cm $\times$ 4.6 mm) was used. A pre-column (5 cm $\times$ 4.6 mm) tap-packed with pellicular, 30—38 μm, reversed-phase material (Whatman, Clifton, NJ, U.S.A.) was used to guard and prolong the life of the main column. The dead time of

the system was determined by the injection of 5 μl of a 3 *M* potassium chloride solution with double-distilled, deionized water as the mobile phase. For ten replicate injections an average value of 2.17 min with a standard deviation of zero was measured for the dead time. In order to study the effect of stationary-phase chain length, a Partisil PXS 10/25 C_8 column (25 cm × 4.6 mm, Whatman) was used for one data set.

The mobile phase in all of the experiments was comprised of 0.02 *M* potassium dihydrogen phosphate, HPLC grade (Fisher Scientific, Fair Lawn, NJ, U.S.A.), plus some concentration of pairing agent, prepared by dissolving the anhydrous powder in double-distilled, deionized water and adjusting the pH either with phosphoric acid or potassium hydroxide. Prior to use, the eluent was filtered by passage through 0.45 μm filters (Millipore, Bedford, MA, U.S.A.) and helium-degassed. Column equilibration with the desired mobile phase occurred overnight at low flow-rates. All analyses were performed at a flow-rate of 1.5 ml/min and unless specified, at ambient temperature.

Chemicals

Pairing agents were purchased from various vendors. 1-Hexanesulfonic acid, 1-octanesulfonic acid and tetrabutylammonium phosphate were obtained from Eastman-Kodak (Rochester, NY, U.S.A.); tetramethylammonium hydroxide and tetraethylammonium hydroxide were from Aldrich (Milwaukee, WI, U.S.A.); 1-dodecanesulfonate was bought from Altex, a division of Beckman Instruments (Berkeley, CA, U.S.A.) and 1-heptanesulfonic acid from Fisher Scientific. All standard reference compounds were purchased from Sigma (St. Louis, MO, U.S.A.). Stock solutions were prepared in double-distilled, deionized water.

TABLE I

COMPOUNDS INVESTIGATED, THEIR ABBREVIATIONS AND THEIR ACID DISSOCIATION (p*K*) CONSTANTS*

Compound	p*K*
Creatinine (Cre)	3.6
Hypoxanthine (Hyp)	8.9, 12.1
Orotidine (Ort)	—
L-Tyrosine (Tyr)	2.20, 9.11, 10.13
Uracil (Ura)	9.5, >13
Uric acid (UA)**	5.57, 10.3
Uridine (Urd)	9.2, 12.5
Uridine monophosphate (UMP)	1, 6, 9.5
Xanthine (Xan)	7.5, 11.1

*Values from H.A. Sober (Editor), CRC Handbook of Biochemistry, The Chemical Rubber Co., Cleveland, OH, 2nd ed. 1970.

**Values from D.S. Newcombe, Inherited Biochemical Disorders and Uric Acid Metabolism, University Park Press, Baltimore, MD, 1975.

RESULTS

Since the identity of the constituents of the early portion of the chromatogram are unknown, we have selected for this investigation compounds encompassing negatively charged, neutral and positively charged species which are thought to be present in blood fluids. The standard compounds selected include creatinine (Cre), hypoxanthine (Hyp), orotidine (Ort), L-tyrosine (Tyr), uracil (Ura), uric acid (UA), uridine (Urd), uridine monophosphate (UMP) and xanthine (Xan). Table I lists these compounds and their acid dissociation constants; Fig. 1 depicts their structures. UA, Tyr and Cre are known to be constitutents in the early time segment of the chromatogram; Ura and Ort are components which might be present. Hyp, Urd and Xan were included

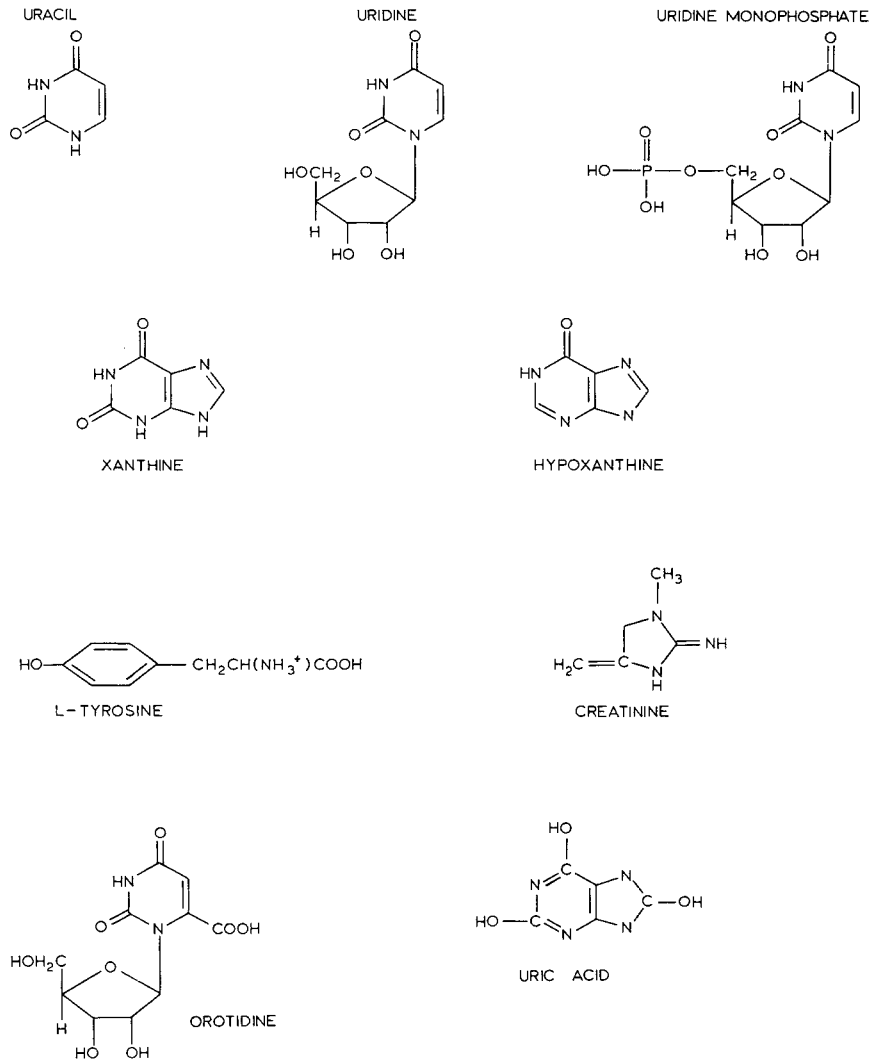


Fig. 1. Structures of the compounds investigated.

because of their importance in clinical manifestations and the inability to resolve readily all three of these components using the present chromatographic conditions. UMP was chosen as a model for any nucleotides which might exist in the physiological fluids.

Generally for negatively charged entities, quarternary amines are used as the pairing agent, while for positively charged components, alkyl sulfonates are employed. Because of the uncertainty as to the chemical composition of the early time segment, both classes of pairing agents have been investigated. Table II lists the pairing agents, their molecular formulae, and the abbreviations we shall use.

TABLE II

ION-PAIR REAGENTS INVESTIGATED

Quaternary amines	
Tetramethylammonium hydroxide pentahydrate (TMA)	$(CH_3)_4NOH\ 5H_2O$
Tetraethylammonium hydroxide (TEA)	$(C_2H_5)_4NOH$
Tetrabutylammonium phosphate (TBA)	$(C_4H_9)_4NH_2PO_4$
Sulfonates	
1-Hexanesulfonic acid, sodium salt (HxSA)	$CH_3(CH_2)_5SO_3Na$
1-Heptanesulfonic acid, sodium salt (HpSA)	$CH_3(CH_2)_6SO_3Na$
1-Octanesulfonic acid, sodium salt (OSA)	$CH_3(CH_2)_7SO_3Na$
1-Dodecanesulfonate, sodium salt (DDS)	$CH_3(CH_2)_{11}SO_3Na$

Effect of the pairing agent

In order to determine the effects of the variables in IPC, we have selected a mobile phase of 0.02 *M* phosphate buffer adjusted to pH 5.7 as our reference point. In an investigation of the pairing agent, there are three important parameters: the type of pairing agent itself (acidic or basic), its chain length, and its concentration. Each of the pairing agents listed in Table II was investigated over a concentration range of several orders of magnitude, typically 0.01 to 10 m*M*. Experimentally, the acidic pairing agent, tetrabutylammonium phosphate (TBA), produced the most significant changes (Fig. 2). The chain length of the alkyl portion of the pairing agent was found to be very critical. For example, the retention times are virtually unaffected by the addition of the quaternary amines, tetramethylammonium hydroxide pentahydrate (TMA) and tetraethylammonium hydroxide (TEA); however, large shifts occur when TBA is used for UMP, Ort and UA which have negative charges at pH 5.7 (Table I). Cre, on the other hand, which has a slight positive charge, is repelled by TBA leading to a decrease in retention. It is interesting to note that neutral species also decrease in retention at the higher concentrations of TBA. For the majority of standard compounds in this study, the sulfonates are not particularly useful. With the sulfonates the retention is longest with 1-heptanesulfonic acid sodium salt (HpSA) and decreases with acids of chain length to either side of it. Ort and UMP have retention times in the presence of the sulfonates shorter than the dead time (2.17 min) of the system. This observation indicates ionic repulsion within the column. Since these compounds have negative charges under the conditions employed, this

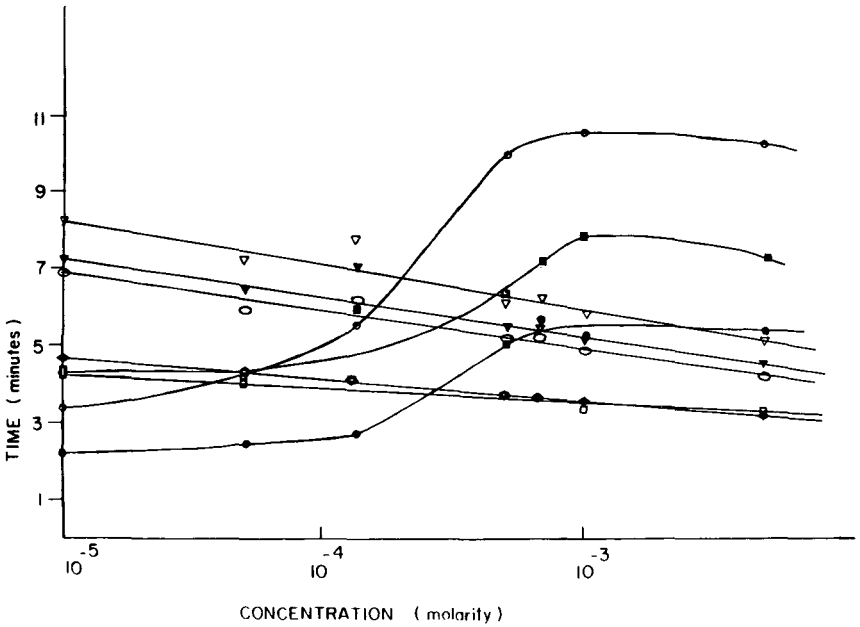


Fig. 2. Effect of concentration of TBA on retention time. Detection, 254 nm; column, analytical C_{18}; guard column, Co-Pell ODS; program, isocratic; eluent, 0.02 *M* monobasic phosphate buffer + pairing agent, adjusted to pH 5.7; flow-rate, 1.5 ml/min; temperature, ambient. Symbols: (♦) creatinine; (▾) hypoxanthine; (●) orotidine; (◇) L-tyrosine; (□) uracil; (■) uric acid; (⊂) uridine; (○) uridine monophosphate; (▽) xanthine.

type of evidence has been used in support of the ion-exchange mechanism to explain the retention behavior in ion-pairing [8—10]. In this hypothesis the ion-pair reagent coats the stationary phase forming a dynamic ion-exchanger. As a result, the stationary phase now has a charge associated with it; components of similar charge to the stationary phase are repelled by the stationary phase and hence elute from the column faster. The observed decrease in retention of the neutral species can also be explained by this coating action, which would cover some of the sites normally available for partitioning of these compounds [11]. Further evidence for alteration of the stationary phase is seen by the very rapid decrease in retention of all components with the increasing concentration of the longest alkyl chain pairing agent, dodecanesulfonic acid. From an exprimental standpoint, the large volume of mobile phase (typically several hundred milliliters) necessary before equilibration was obtained and the large volume of water necessary to remove the pairing agent and restore the column to its previous conditions, also supports the dynamic ion-exchange mechanism.

Brown et al. [12] reported a reversed-phase IPC separation of Hyp, Xan and UA using heptanesulfonic acid; however, Urd was not investigated. Similarly, Popovich [13] separated Ura and Xan with a mobile phase containing HpSA, but did not examine the possibility of interferences from Urd or Hyp. During our investigation of the sulfonic acids, improved resolution of Hyp, Xan and Urd was noted. With a mobile phase of 0.02 *M* phosphate buffer, pH 5.7, these three compounds were not resolved; however, upon the addition of either

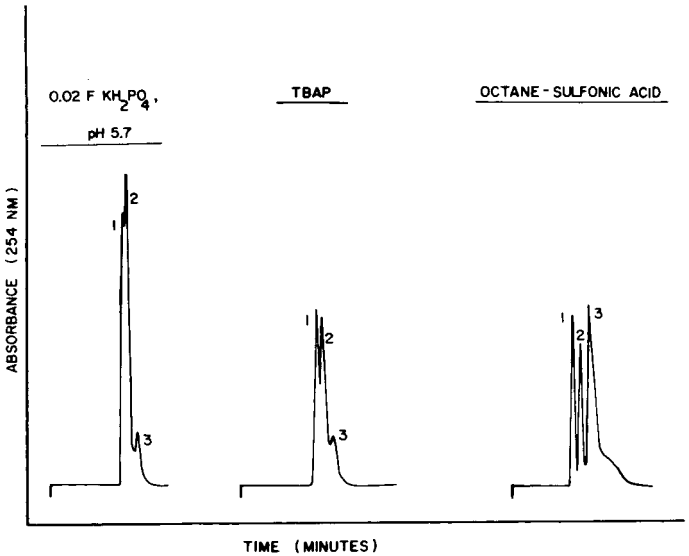


Fig. 3. Separation of uridine (1), hypoxanthine (2) and xanthine (3) with different mobile phases adjusted to pH 5.7: 0.02 *M* monobasic phosphate buffer (left), 0.02 *M* monobasic phosphate buffer + 0.001 *M* TBA (middle), and 0.02 *M* monobasic phosphate buffer + 0.05 m*M* OSA (right). Remaining chromatographic conditions are as in Fig. 2.

hexane-, heptane- or octanesulfonic acid, baseline resolution was achieved (Fig. 3).

Although the retention times of Hyp, Xan and Urd either do not change or change by less than 0.5 min, baseline resolution is obtained. Of the three pairing agents, the heptane- and octanesulfonic acids reduced the peak tailing of xanthine. Since lower concentrations of octanesulfonic acid are necessary to improve the separation as compared to the other sulfonic acids, it is the preferred pairing agent. This improved separation does not occur with the quarternary amines. It is difficult to understand why improved resolution of Hyp, Urd and Xan is obtained with the sulfonic acids. Hyp and Xan are purines differing by a carbonyl group at the 2-position (Fig. 1), while Urd is a dioxopyrimidine with a ribose group at the 9-position. Xan has a lower acid dissociation constant than Hyp and Urd; however, at the pH of the mobile phase, 5.7, this is not a significant factor. It is possible that these compounds are undergoing lactam—lactim tautomerism and that the lactim has some affinity for the pairing agent [14]. Another explanation though, may be that the pairing agent is disrupting the base stacking between the Hyp, Xan and Urd [14]. Because of the high concentrations of these compounds in the standard solutions, it is possible that heterogeneous or homogeneous base stacking occurs.

Effect of pH

Since it was determined that a 1 m*M* solution of TBA in 0.02 *M* phosphate buffer, pH 5.7, produced the most significant changes in retention times, in all subsequent discussions, this mobile phase will be used as the basis for comparison. Table III summarizes the changes in retention times with the mobile phase from pH 5.7 to 3.0, pH values higher than 7 were not investigated

TABLE III

EFFECT OF pH OF THE MOBILE PHASE ON RETENTION TIME (min)

Values listed are the average of three injections.

Compound	pH	
	3.0	5.7
Creatinine	1.92	3.44
Hypoxanthine	6.20	5.12
Orotidine	14.43	5.26
L-Tyrosine	5.00	4.38
Uracil	4.09	3.47
Uric acid	6.16	7.76
Uridine	6.74	4.78
Uridine monophosphate	22.40	10.48
Xanthine	7.24	5.72

because of silica-based column limitations. Cre has a pK of 3.6; UA has a pK value for the 9-position of 5.57. The decreased retention of Cre at pH 3 is thought to be caused by charge repulsion between Cre and TBA. However, at a mobile-phase pH of 5.7, Cre is a neutral molecule, therefore this charge repulsion is no longer a factor and increased retention is observed. The effect of pH on UA is difficult to understand. UA has a greater negative-charge density at pH 3 than at pH 5.7; hence ion-pairing should occur more efficiently at pH 3 and increased retention should result. This is not observed. At pH 5.7 UA is predominately non-ionic and it is thought that the hydrophobic interactions of reversed-phase chromatography (RPC) may dominate. A marked decrease in retention is observed for Ort and UMP from pH 5.7 to pH 3. For UMP at a pH of 3 the phosphate moiety undergoes primary dissociation; at pH 5.7 secondary dissociation of this phosphate group occurs yielding a more negatively charged molecule which should exhibit greater affinity for the cationic TBA. Thus the retention time should increase; however, this was not observed. Gelijkens and De Leenheer [15] have also reported a decrease in the capacity factor of UMP with increasing pH of the mobile phase. The retention times of Hyp, Tyr, Ura, Urd and Xan are slightly longer with a mobile phase of pH 5.7. This trend has been reported in the literature for these compounds for the pH range 2.2—6.3 [16]; however, we do not have an explanation for this change in retention.

Effect of temperature

The effect of temperature on retention time is plotted in Fig. 4. For Ort, UA and UMP, the compounds which ion-pair with the TBA, a maximum in retention is observed at 40°C followed by a decrease with further increase in temperature. For the remaining six compounds a slow decrease is observed over the entire temperature range investigated which is typical behavior for a reversed-phase system. Although Cre and Urd were not included in Fig. 4, the trends observed are similar to those for Hyp, Tyr, Ura and Xan. Therefore, it is evident that control of temperature may aid in specific ion-pair separations.

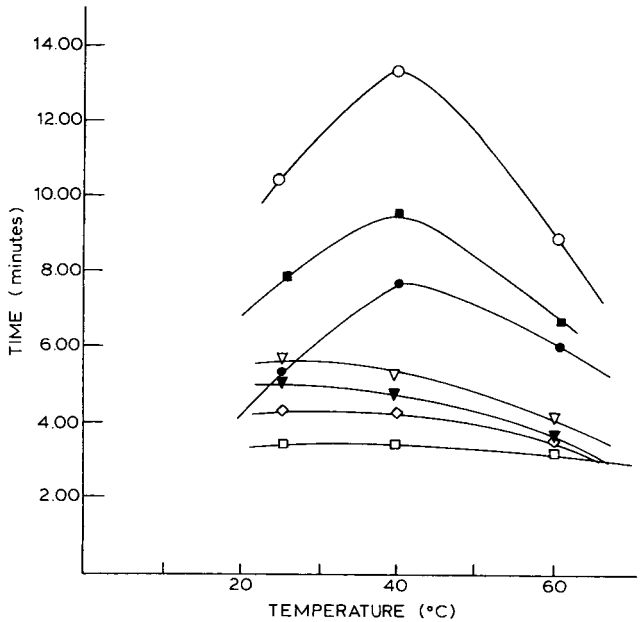


Fig. 4. Effect of temperature on retention time (min). Eluent, 0.001 *M* TBA in 0.02 *M* monobasic phosphate buffer adjusted to pH 5.7. Remaining chromatographic conditions and symbols are as in Fig. 2.

Effect of percent organic modifier

Generally, the addition of an organic modifier, such as methanol or acetonitrile, to a reversed-phase system decreases the retention of the solutes. In order to determine the effects of an organic modifier on ion-pairing, a mobile phase of 0.001 *M* TBA in 0.02 *M* potassium phosphate, pH 5.7, in which there was 5% methanol, was investigated. The retention times of solutes using a mobile phase with the methanol were compared to the retention times obtained without methanol (Table IV). It was observed that the retention times

TABLE IV

EFFECT OF AN ORGANIC MODIFIER IN THE MOBILE PHASE ON RETENTION TIME (min)

Values are the average of three injections.

Compound	No methanol	5% Methanol
Creatinine	3.44	3.88
Hypoxanthine	5.12	4.54
Orotidine	5.26	5.03
L-Tyrosine	4.38	3.94
Uracil	3.47	3.38
Uric acid	7.76	5.56
Uridine	4.78	4.17
Uridine monophosphate	10.48	7.61
Xanthine	5.72	4.98

of all the compounds evaluated decreased in the presence of methanol. This effect was more dramatic with the compounds UA and UMP, which ion-pair to the greatest extent. This effect has been noted in the literature for a variety of compounds [11, 17—20] and has been attributed to a decrease of the surface concentration of the counter-ion because of the competition by the cosolvent.

Effect of the stationary phase

The alkyl chain length of the stationary phase can be an important parameter in IPC optimization. Retention data were obtained for a C_8 and a C_{18} column (Table V). Retention for the neutral compounds is not significantly different between the two columns; however, the retention of Ort, UA and UMP is much longer with the C_8 versus the C_{18} column. This observation is difficult to interpret. The retention behavior of the compounds examined suggests an ion-exchange mechanism. Thus, adsorption of the counter-ion onto the stationary phase prior to ion-pair formation is postulated. This adsorption can occur at two sites, either onto the hydrophobic portion or any unreacted silanol groups present. If the adsorption were on the hydrophobic portion, one would expect to see little difference between the C_8 and C_{18} columns. However, if the adsorption is at the unreacted silanols more counter-ion could be adsorbed on the C_8 column since the C_8 chains are less bulky than the C_{18} chains; hence, increased retention would be expected. This line of reasoning though, must be applied cautiously. Two columns from two different manufacturers were used in this investigation. Therefore, the extent of capping of the unreacted silanols and the percent carbon loading of the C_8 or C_{18} onto the silica backbone may vary, which may contribute to the large change in retention times.

TABLE V

EFFECT OF THE STATIONARY PHASE ON RETENTION TIME (min)

Values are the average of three injections.

Compound	Column	
	C_8	C_{18}
Creatinine	3.38	3.44
Hypoxanthine	5.82	5.12
Orotidine	10.92	5.26
L-Tyrosine	4.94	4.38
Uracil	3.92	3.47
Uric acid	11.25	7.76
Uridine	6.15	4.78
Uridine monophosphate	21.09	10.48
Xanthine	6.63	5.72

Application

The optimum conditions for improving the separation of the early eluting compounds in plasma chromatograms are achieved by a reversed-phase system with a mobile phase of 1 m*M* TBA and 20 m*M* phosphate buffer, adjusted to

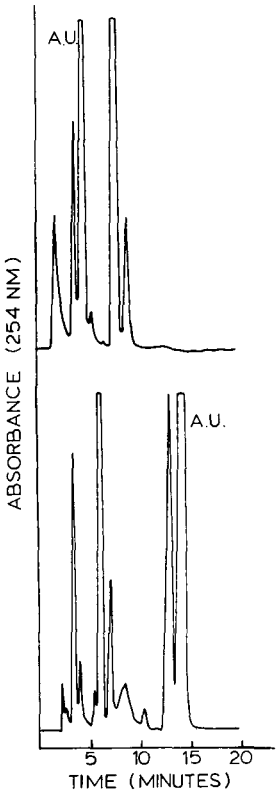


Fig. 5. A 40-μl sample of plasma from a normal individual chromatographed using a mobile phase, 0.02 *M* monobasic phosphate buffer, pH 5.7, without (top) and with (bottom) a pairing agent, 0.001 *M* TBA. Remaining chromatographic conditions are as in Fig. 2.

pH 5.7. The profiles of the plasma of a normal individual under chromatographic conditions with and without the pairing agent are shown in Fig. 5. Two changes are evident. First, the retention of uric acid and some other component not yet identified, increased. The removal of uric acid, which is present in such large amounts, greatly simplified the early part of the chromatogram. Second, decreased retention of the neutral compounds, Hyp, Urd and Xan, was observed. Both these observations were anticipated from the study on the standards. No other components were shifted; therefore, the components still present in this segment must be polar rather than ionic.

ACKNOWLEDGEMENT

We gratefully acknowledge the Perkin-Elmer Corporation for the research fellowship of P.A.P. and for use of instrumentation.

REFERENCES

1 A.M. Krstulovic, R.A. Hartwick and P.R. Brown, Clin. Chim. Acta, 97 (1979) 159.
2 M. Zakaria, P.R. Brown, M.P. Farnes and B.E. Barker, Clin. Chim. Acta, 126 (1982) 69.

3 H.A. Scoble, M. Zakaria, P.R. Brown and H.F. Martin, Comp. Biomed. Res., 16 (1983) 300.
4 H.A. Scoble, J.L. Fasching and P.R. Brown, Anal. Chim. Acta, 150 (1983) 171.
5 P.A. Perrone, P.R. Brown and J.D. Sallis, Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy, March, 1983, Abstract No. 469.
6 S. Eksborg and G. Schill, Anal. Chem., 45 (1973) 2092.
7 L.R. Snyder and J.J. Kirkland, Introduction to Modern Liquid Chromatography, Wiley, New York, 2nd ed., 1979.
8 P.T. Kissinger, Anal. Chem., 49 (1977) 883.
9 B.A. Bidlingmeyer, S.N. Deming, W.P. Price, Jr., B. Sachok and M.M. Petrusek, J. Chromatogr., 186 (1979) 419.
10 J.H. Knox and R.A. Hartwick, J. Chromatogr., 204 (1981) 3.
11 C.T. Hung and R.B. Taylor, J. Chromatogr., 202 (1980) 333.
12 N.D. Brown, J.A. Kintzios and S.E. Koetitz, J. Chromatogr., 177 (1979) 170.
13 D.J. Popovich, Selective Detection in Liquid Chromatography, Tracor Chromatography, Appl. 79-10.
14 P.R. Brown and E. Grushka, Anal. Chem., 52 (1980) 1210.
15 C.F. Gelijkens and A.P. de Leenheer, J. Chromatogr., 194 (1980) 305.
16 M. Zakaria, P.R. Brown and E. Grushka, Anal. Chem., 55 (1983) 547.
17 C.P. Terweij-Groen, S. Heemstra and J.C. Kraak, J. Chromatogr., 161 (1978) 69.
18 A. Tilly-Melin, Y. Askemark, K.G. Wahlund and G. Schill, Anal. Chem., 51 (1979) 976.
19 R.S. Deelder, H.A.J. Linssen, A.P. Konijnendijk and J.L.M. van de Venne, J. Chromatogr., 185 (1979) 241.
20 S.O. Jansson, I. Andersson and B.A. Persson, J. Chromatogr., 203 (1981) 93.

Journal of Chromatography, 307 (1984) 65—79
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2002

A QUANTITATIVE DENSITOMETRIC METHOD FOR THE RAPID SEPARATION AND QUANTITATION OF THE MAJOR TISSUE AND LIPOPROTEIN LIPIDS BY HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHY

I. SAMPLE PREPARATION, CHROMATOGRAPHY, AND DENSITOMETRY

GERD SCHMITZ and GERD ASSMANN*

Zentrallaboratorium der Medizinischen Einrichtungen der Westfälischen Wilhelms-Universität, Domagkstrasse 3, and Institut für Arteriosklerosеforschung an der Universität Münster, 4400 Münster/Westf. (F.R.G.)

and

DAVID E. BOWYER

Department of Pathology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QP (U.K.)

(First received March 14th, 1983; revised manuscript received November 25th, 1983)

SUMMARY

A rapid method for the separation and quantitation of the major lipids of tissues and lipoproteins by automated high-performance thin-layer chromatography is presented. Solvent systems for one-dimensional separation of neutral lipids, of cholesteryl esters, and of phospholipids are described. Separated lipids are measured following treatment with methanolic sulphuric acid containing manganese chloride and scanned in fluorescence or absorption mode. Absolute quantitation is obtained by the use of an internal standard and by references to standards for each lipid run on the same plates as samples. The method described here is particularly suitable for the rapid quantitation of small amounts of lipid (0.01—0.02 nmol per sample), for example in tissue culture studies; 100 μg of fibroblast or macrophage protein are sufficient for complete lipid analysis. The coefficients of variation due to the sample preparation, application to the plates and densitometry are in the range 7.2—9.1%.

The method was compared with enzymatic determinations for cholesterol and gave correlation coefficients of 0.95 for total cholesterol and 0.91 for unesterified cholesterol. Phospholipid estimation was compared with large-plate thin-layer chromatography and

0378-4347/84/$03.00

phosphorus analysis and gave correlation coefficients of 0.90 for phosphatidylcholine and 0.89 for sphingomyelin.

INTRODUCTION

There is a need in many studies of tissue and lipoprotein lipids for rapid separation and subsequent estimation of small amounts of material. For example, in studies of cells in culture, where the concentration of most lipids is of the order of femtomoles (10^{-15}) per cell, unless very sensitive methods of analysis are available, large cell populations must be used. This may complicate experimental conditions. Similarly, in studies of influx and efflux of lipoprotein lipids in cells, there is a need for sensitive methods for analysing the individual lipids of both cells and lipoproteins.

Various methods have been described including enzymatic fluorimetric methods [1], gas–liquid chromatography (GLC) [2–6], high-performance liquid chromatography (HPLC) [7–9], thin-layer chromatography (TLC) with subsequent microchemical assay [10, 11], Iatroscan [12], and high-performance thin-layer chromatography (HPTLC) followed by quantitative densitometry on the plate [13–15].

TLC offers relatively simple separation systems, which principally allow inexpensive analysis of multiple samples at the same time with sufficient sensitivity. However, in situ quantification of the separated compounds on a TLC plate as done by scanning densitometry presents various difficulties and in the past has often had a poor reputation. Thus, satisfactory quantification is dependent upon consistent chromatographic separations, the homogeneity of impregnation of the plates with detection reagent and uniform background staining. In addition, the densitometric quantification of a spot depends not only on the mass of material in the spot, but also upon the area which the spot occupies, and the signal per unit mass is also related to the absorption and/or fluorescence spectra of the material and these may be different for unknowns and standards.

Due to the recent availability of HPTLC plates [16, 17], automated sample application systems working in the nanolitre range and improved separation systems (Camag linear development chamber), certain disadvantages (e.g. poor reproducibility, irregular spot shape, etc.) of TLC are minimized. The high performance of the plates ensures excellent and reproducible separation of the lipids. The total mass of material added to the HPTLC plates can be kept very low, thus reducing the dependance of the integrated signals on the spot geometry. The method is fast and the cost of expendable materials is small, giving a low cost per analysis.

We have, therefore, extensively re-investigated the technique of quantitative HPTLC and included the experiences of various published procedures [13–15] to elaborate a method which avoids most of the difficulties enumerated above and gives fast, accurate, and reproducible analysis of the major tissue and lipoprotein lipids combined with fully automated data calculation.

MATERIALS AND METHODS

Thin-layer plates, organic solvents, and lipid standards

High-performance thin-layer plates, 10 × 20 cm from Merck (Silica Gel 60, Cat. No. 5641) were used for the separation of the lipids. If a documentation by ultraviolet (UV)-sensitive films (see below) was required, plates with fluorescence indicator F 254 (Silica Gel 60 F 254, Cat. No. 5642) were used. All chromatography plates were prewashed before chromatography in a solvent with the same composition as that used for the separation. Before sample application the plates were activated in an oven for 1 h at 110° C. Drying the plates in this way markedly improved the separation. Plates were stored and handled carefully in order to avoid touching the surface and to prevent contact with dust.

Diethyl ether, acetic acid methyl ester and *n*-propanol were laboratory grade. Chloroform, methanol, *n*-hexane, and *n*-heptane were LiChrosolv quality (Merck). Satisfactory chromatography may also be achieved using freshly redistilled laboratory grade reagents. Pure lipid standards were purchased from Sigma, Munich (cholesterol No. CH 8253, triolein No. T 7502, cholesteryl formate No. C 9398, cholesteryl stearate No. C 9503, cholesteryl oleate No. C 9253, cholesteryl linoleate No. C 9003, phosphatidylethanolamine No. P 4513, phosphatidylcholine No. P 6267, sphingomyelin No. S 7004).

Sample application

For all applications of lipids to the thin-layer plates, the Camag Nanomat (Camag, Muttenz, Switzerland) was used with disposable 0.5-μl Microcap pipettes or the 100-nl and 200-nl platinum capillary. Spots were applied at a constant distance apart, 0.5 cm or 1.0 cm.

Chromatographic separation

The separation of the neutral lipids was performed as a one-step procedure with 4.5 ml of *n*-hexane—*n*-heptane—diethyl ether—acetic acid (63:18.5:18.5:1, v/v) in the Camag HPTLC linear developing chamber (Cat. No. 28520) at room temperature for 10 min.

For cholesteryl ester separation, one-step chromatography on 10 × 20 cm HPTLC plates in chloroform—*n*-heptane (40:60, v/v) was used. The separation was done in the linear developing chamber with 4.5 ml of solvent at 4° C for 15 min. Chilled solvents gave an improved separation.

Phospholipid separation was done with a one-step procedure as previously described [18], using 10 × 20 cm HPTLC plates. The solvent was acetic acid methyl ester—*n*-propanol—chloroform—methanol—43 mmol/l potassium chloride in distilled water (25:25:25:10:9, v/v). This separation was done with 4.5 ml of solvent in the linear developing chamber at room temperature for 20 min.

Detection of separated lipids

Following chromatography, spots were detected using the manganese chloride—sulphuric acid reagent containing 3.2 g of manganese chloride, 480 ml of methanol, 32 ml of concentrated sulphuric acid and 480 ml of deionized

water [19]. Following chromatography, the plates were dried for 10 min in an oven at 110°C and then immediately immersed in a tank filled with reagent for 20 sec. The plates were then placed horizontally on a paper towel to remove the excess reagent from the back. They were then placed in an oven on a PTFE block to ensure even heating at 110°C for precisely 30 min.

Documentation of separated lipids on the chromatography plate

The charred plates can be easily documented on UV-sensitive films (Technifax DZO blue) using a Technifax apparatus (Technifax, A. Leistenschneider, Düsseldorf) as follows: the back of the thin-layer plate is carefully cleaned and the plate layed on the UV-light source. A sheet of film is then placed on the coated side of the HPTLC plate and exposed for 2 min. The film is then developed in ammonia vapour for 1 min.

TLC scanning and quantitation

Quantitation of the lipids on the HPTLC plates was performed with a Shimadzu CS 910 TLC scanner (Shimadzu Seisakusho, Japan) working semi-automatically or with the fully automated Camag TLC scanner. Both instruments were interfaced with a Spectra Physics Basic integrator SP 4100 equipped with a Kerr minifile 4100 D for data storage (Spectra Physics, Darmstadt, F.R.G.).

Plates were scanned either by fluorescence excitation or by absorption. For fluorescence scanning, the emitted fluorescence light was scanned in the reflection mode. For absorption scanning in preliminary experiments either reflected or transmitted light was scanned. However, the reflection mode is far superior, because it is not dependent on slight differences in thickness and dispersion of particles of the gel and, therefore, was used in all subsequent experiments with absorption scanning.

The light sources used for fluorescence scanning were a xenon lamp (Shimadzu) or a mercury lamp (Camag). The excitation wavelengths selected by the monochromator depended on the lamp used as follows: 370 nm for the xenon lamp and 366 nm for the mercury lamp. The light sources used for absorption scanning were a xenon lamp (Shimadzu) or a deuterium lamp (Camag).

Slit conditions were selected according to the spot size. The slit height should always cover the whole spot diameter (0.4—0.6 cm). The slit width was kept constant at 0.1 mm for the Shimadzu scanner or 0.2 mm for the Camag TLC scanner. A scanning speed of 60—80 cm/min was used and gave good peak resolution.

The densitometric signals of all tracks (2—3 scans per track) were integrated in the SP 4100 Basic integrator and stored on the floppy disc. For the data manipulation and standard correction, a Basic computing program has been elaborated and is described elsewhere [20].

Internal standard correction

In this method, an internal standard was used to correct for inevitable slight losses of material during extraction and chromatography. Various internal standards were used.

Cholesteryl formate. This was obtained from Sigma, Munich (Cat. No. C 9398) and made up as a stock solution containing 10 mg/dl (24.1 nmol/l) in chloroform. The standard was added according to the lipid content of the samples being analysed, so that the mass of standard was approximately equal to the mass of the major lipid in the sample (for example, for cells such as cultured fibroblasts, about 50 μg internal standard per mg protein).

[^{14}C] Cholesteryl oleate and [^{14}C] phosphatidylcholine. These were obtained from NEN Chemicals, Munich: cholesteryl [1-^{14}C] oleate (Cat. No. NEC 638; 50—60 mCi/mmol) and L-α-[*dipalmitoyl*-1-^{14}C] phosphatidylcholine (Cat. No. NEC 682; 60—100 mCi/mmol). The volume added to each sample was sufficient to give about 5500 dpm/μl of the application volume. Radioactivity was measured directly on the plate using a Berthold TLC linear analyser Type LB 2832 (Laboratorium Prof. Dr. Berthold, Wildbad, F.R.G.) at gain 3, voltage 550 V and scanning time 180—900 sec.

Lipid extraction

Cells from tissue cultures. Medium was removed from the dishes and the cells were washed three times with 2 ml of Dulbecco's modified Eagle medium (DMEM, Flow No. 10-331-24) and once with 2 ml of Dulbecco's phosphate-buffered saline (PBS, Flow No. 18-610-54). The cells were harvested with a rubber policeman in 500 μl of PBS and transferred to a siliconized conical glass tube. The cells were sonicated with a Branson sonifier (Branson Scientific) three times for 20 sec at 30 W, in tubes chilled in ice water. Defined volumes (0.3—0.7 ml) of the sonicated samples containing 100—400 μg cell protein were delipidated by a modification of the Folch procedure [17] with 5 volumes of chloroform—methanol (2:1, v/v) containing the appropriate internal standard. The two phases were separated by centrifugation at 1100 *g* for 10 min in a laboratory centrifuge. The lower phase was transferred to a conical glass tube and the upper phase was reextracted for 1 h, with repeated vortexing, with an equal volume of chloroform—methanol—normal saline (86:14:1, v/v) to ensure a good recovery of lipids [10]. The two phases were again separated by centrifugation and the first and the second lower phases (about 3 ml + 2 ml) were mixed and evaporated at 40°C under vacuum. To avoid oxidation, butylated hydroxytoluene (Fluka, Heidelberg, F.R.G.) at a concentration of 0.005% was present in all organic solvents [21]. The dried samples were stored in a desiccator overnight and redissolved in 50 μl of chloroform—methanol (2:1, v/v) as solvent.

Medium. A defined volume of medium was subjected to ultracentrifugation at d = 1.21 g/ml potassium bromide for 48 h at 220,000 *g* using a 50.3 Ti rotor in a Beckman ultracentrifuge. The 1.21 g/ml potassium bromide supernatant was dialysed against 1 mmol/l ammonium bicarbonate, 0.01 mmol/l EDTA, pH 8.6 for 24 h. Samples were then dialysed for at least 1 h against distilled water and lyophilized. The samples were then redissolved in 1 ml of normal saline with vortexing. Protein was determined in an aliquot and 700—1000 μl containing 250—500 μg protein were delipidated after addition of an appropriate amount of internal standard as described for cells.

Lipoprotein fractions. Appropriate volumes of lipoprotein fractions obtained after ultracentrifugation were analysed for protein and 10—30 μl of lipoprotein

fractions were delipidated as described for cells with 5 volumes of chloroform—methanol (2:1, v/v) after addition of 500 μl of normal saline and internal standard (1 μg internal standard per 1—10 μg of protein depending on the lipoprotein fraction).

Other methods

Protein was determined according to the method of Lowry et al. [22]. Cholesterol and triglycerides were measured using enzymatic methods. The analysis for total cholesterol was performed with cholesterol oxidase—para-aminophenazon (CHOD—PAP) reagent (Boehringer Mannheim, Cat. No. 148 393) using a Cobas-Bio centrifugal analyser (Hoffmann-La Roche, Zürich, Switzerland). Free cholesterol was measured using the CHOD—PAP method without cholesteryl ester hydrolase (Boehringer Mannheim, Cat. No. 310 328). Esterified cholesterol was calculated by difference. Triglycerides were measured using an enzymatic method (Boehringer Mannheim, Cat. No. 475 429). Phospholipids were also measured following separation on standard TLC plates using the solvent system described above and analysis for phosphurus [23].

Mouse peritoneal macrophages were prepared as described by Ho et al. [24]; the preparation of acetyl-LDL (low-density lipoproteins) was performed according to the description of Brown et al. [4] and Goldstein et al. [25].

RESULTS

Chromatographic separation

The separation of the neutral lipids is shown in Fig. 1. It was demonstrated by co-chromatography of pure lipids that the separated spots of cholesterol, triglycerides and cholesteryl esters and the internal standard were not contaminated by other lipids such as diglycerides as may occur in certain solvent systems.

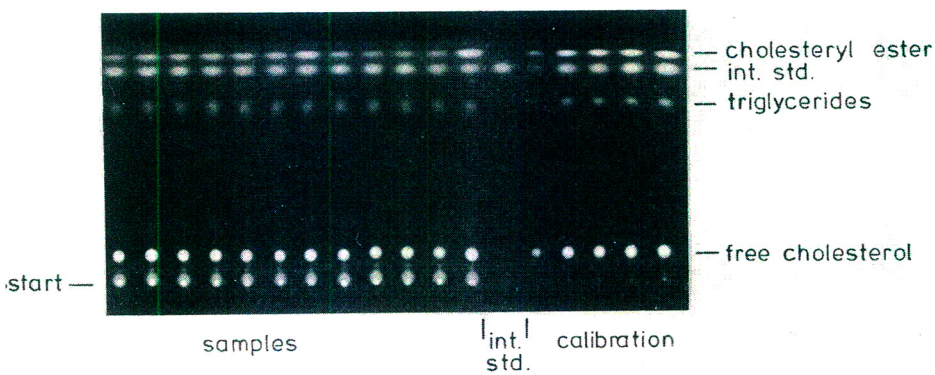


Fig. 1. Separation of the neutral lipids on HPTLC plates in *n*-hexane—*n*-heptane—diethyl ether—acetic acid (63:18.5:18.5:1, v/v). The external standards consisting of cholesterol, triolein, cholesteryl formate (internal standard), and cholesteryl linoleate for calculating the calibration curves are applied on the first five tracks (cholesterol 0.008—0.155 nmol; triolein 0.018—0.36 nmol; cholesteryl formate and cholesteryl linoleate 0.013—0.26 nmol). Track 6 contains the internal standard in the concentration as added to the samples (0.104 nmol/spot). The samples are applied on the remaining tracks. For documentation purposes, the HPTLC plates have been overloaded with the samples.

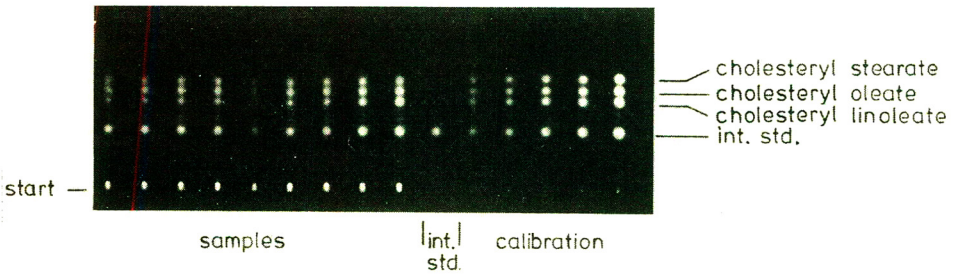


Fig. 2. Separation of the cholesteryl esters (cholesteryl stearate, cholesteryl oleate, cholesteryl linoleate, and the internal standard cholesteryl formate) on HPTLC plates in *n*-heptane—chloroform (60:40, v/v). The external standards are applied in masses between 0.039 nmol and 0.26 nmol. The concentration of the internal standard applied on track 5 is 0.104 nmol per spot. For documentation purposes, the HPTLC plates have been overloaded.

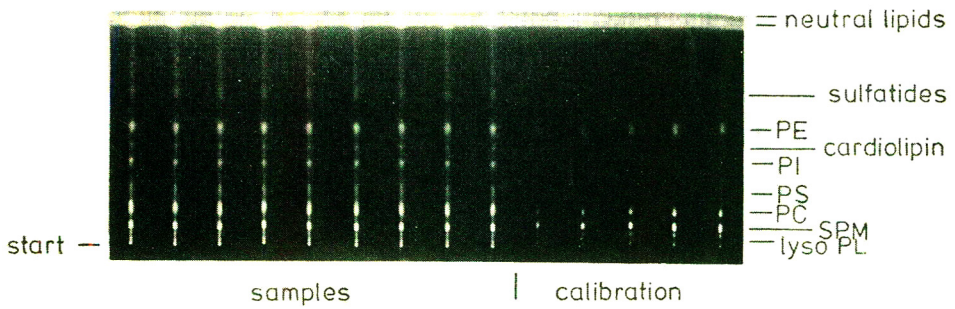


Fig. 3. Separation of phospholipids on HPTLC plates in *n*-propanol—acetic acid methyl ester—chloroform—methanol—43 mmol/l potassium chloride in distilled water (25:25:25:10:9, v/v). This chromatography system separates lysophospholipids (lyso PL), sphingomyelin (SPM), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI), cardiolipin, phosphatidylethanolamine (PE), and sulphatides. The external standard consists of SPM, PC, and PE (0.039—0.39 nmol). For documentation purposes, the HPTLC plates have been overloaded.

The separation of cholesteryl esters is shown in Fig. 2. The solvent system separates the cholesteryl esters according to the degree of unsaturation of the fatty acids, thus permitting resolution of the major saturated esters, palmitate and stearate, from the major monoene, oleate and from the major diene, linoleate.

The separation of phospholipids is shown in Fig. 3. The solvent system separates lysophosphatidylcholine, sphingomyelin, phosphatidylcholine, phosphatidylserine, phosphatidylinositol, cardiolipin, phosphatidylethanolamine and sulphatides.

On each plate the external standards are applied in the expected concentration range of the samples (0.01—0.5 nmol per spot).

Fluorescence-absorption measurements

Characteristics of the fluorescent lipid derivatives. Using the described staining procedure, the in situ fluorescence characteristics of all fluorescent

lipid derivatives are very similar, all showing an excitation maximum around 366 nm and an emission maximum around 410 nm (Fig. 4).

When the absorption spectrum of the lipid derivatives was determined, the absorption maxima for all compounds were found at 366 nm. This is virtually identical with the maxima for fluorescence excitation.

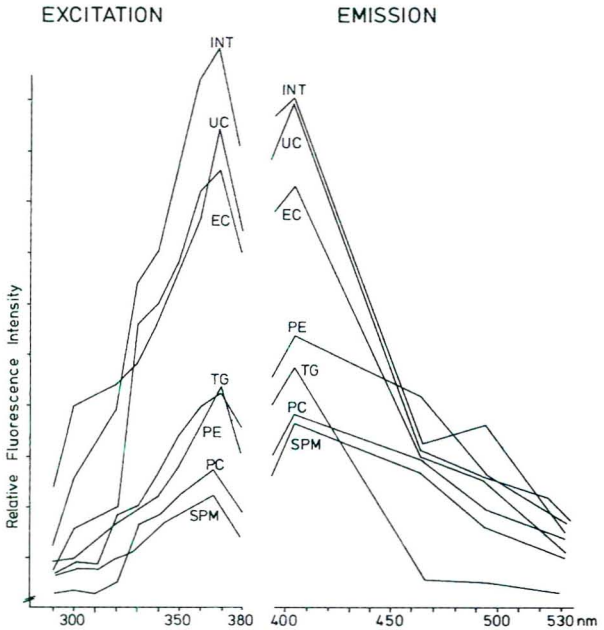


Fig. 4. In situ excitation and emission spectra of the quantitated lipids on the HPTLC plates immersed in the detection reagent for 20 sec and then heated at 110°C (see Methods) for 20 min. INT = cholesteryl formate; UC = unesterified cholesterol; EC = esterified cholesterol; TG = triglycerides; PE = phosphatidyl ethanolamine; PC = phosphatidylcholine; SPM = sphingomyelin. The HPTLC plates were measured at identical settings of the instrument.

Effect of heating time on the fluorescence intensity. Heating of the HPTLC plate after treatment with the detection reagent induces the formation of fluorescent derivatives. This process is time-dependent with the maximum effect reached after about 40 min with heating at 110°C (Fig. 5A). Longer heating time ashes the derivatives and the fluorescence ultimately decreases to zero. On the other hand, absorption (Fig. 5B) increases until it reaches a maximum at 60 min.

Comparison of calibration curves measured by fluorescence and absorption. The calibration curves for the major neutral lipids and phospholipids are given in Fig. 6 and the regression equations are shown in Table I. For all compounds whose separation is demonstrated in Figs. 1—3, there is a linear relationship between the logarithm of the integrated signal and the logarithm of the corresponding mass of compound over a wide range. At concentrations exceeding 0.3 nmol either fluorescence quenching or absorption saturation causes non-linearity of the calibration curves.

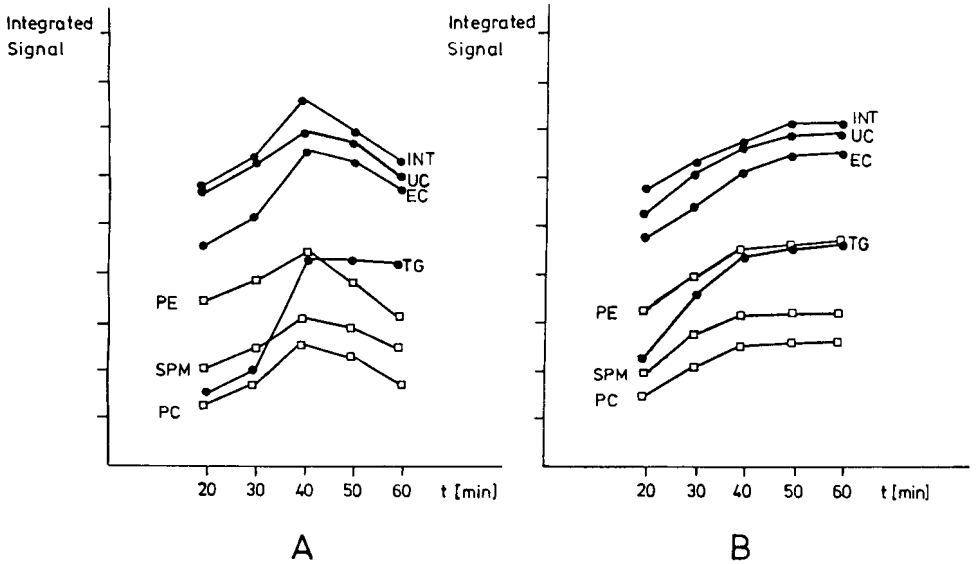


Fig. 5. In situ fluorescence emission and absorption after different heating times at 110°C. Twenty tracks with identical concentrations of a lipid standard mixture were applied on an HPTLC plate, chromatographed, and immersed in the detection reagent as described. Before heating of the plate at 110°C, the HPTLC plate was cut into five pieces, each with four tracks on it. Then the glass pieces were heated for either 20, 30, 40, 50, or 60 min at 110°C, and either fluorescence (panel A) or absorption (panel B) was measured at identical settings of the instrument. Integrated signals for fluorescence and absorption were set identical for the internal standard (cholesteryl formate). The data represent mean values of each heating time. (•), neutral lipid separation; (□), phospholipid separation. Abbreviations as in Fig. 4.

Within the cholesteryl ester class, the degree of unsaturation of the fatty acid does not significantly affect the shape of the calibration curve. Cholesteryl linoleate, oleate, stearate and palmitate exhibit practically the same response for each concentration applied and reveal identical calibration curves (not shown). However, in both the fluorescence and absorption measurement, cholesteryl formate (used as an internal standard) showed a slightly higher signal response per mass as compared to the other cholesteryl esters tested (Fig. 6, upper plots).

For triglycerides containing unsaturated fatty acids, signal intensity is slightly higher than that of the saturated homologues (not shown). Since most of the tissue and lipoprotein triglycerides contain oleic acid, triolein was used as a standard.

For phosphatidylcholine and sphingomyelin substituted with different fatty acids, the degree of unsaturation did not significantly affect the calibration curves (not shown).

Standards separated on different plates yielded almost identical shapes of calibration curves, although the absolute value of the integrated signal varied depending on the plate and the sensitivity setting of the scanner. It is possible, therefore, to calibrate each plate from a single standard. Thus, as an example the 0.1-μg standard can be applied to the plate and the sensitivity for the scanner is set to give 50,000 units of integrated signal before measuring the whole plate.

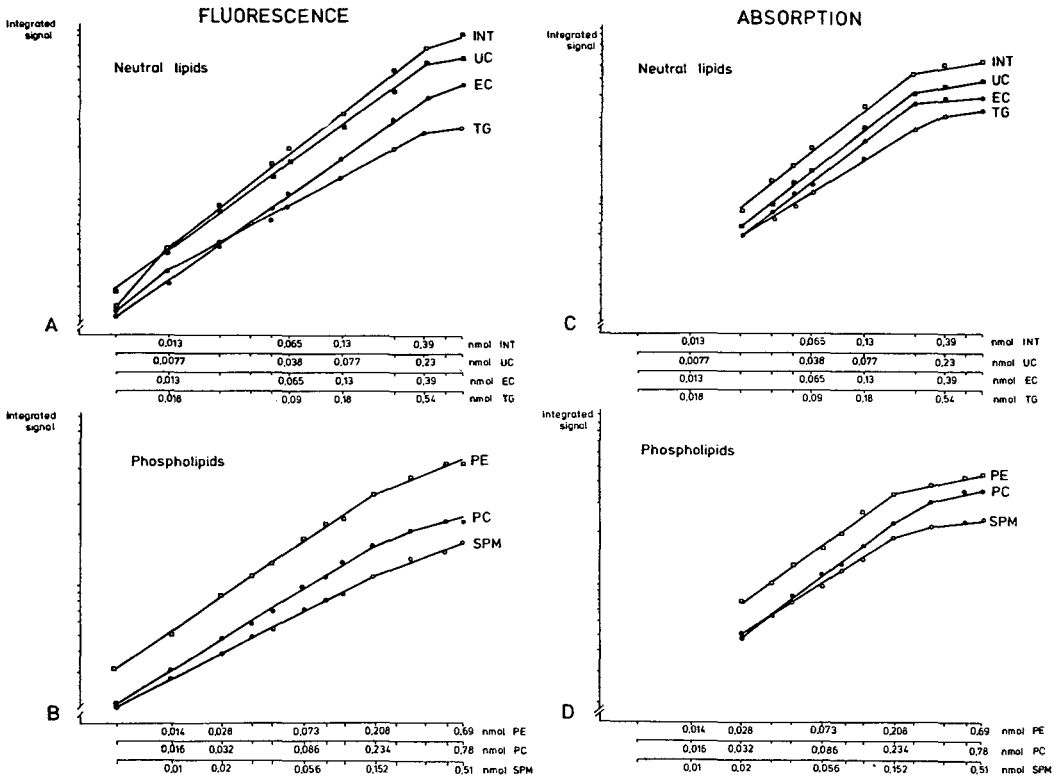


Fig. 6. Calibration curves of the lipids usually quantitated, separated on an HPTLC plate, immersed in the detection reagent for 20 sec and heated for 40 min at 110°C. The plates were measured either in the fluorescence (panels A and B) or absorption (panels C and D) mode. Panels A and C: □ = cholesteryl formate, ■ = unesterified cholesterol, ● = esterified cholesterol, ○ = triglycerides. Panels B and D: □ = phosphatidylethanolamine, ● = phosphatidylcholine, ○ = sphingomyelin. Abbreviations as in Fig. 4.

TABLE I

REGRESSION EQUATIONS AND CORRELATION COEFFICIENTS FOR CALIBRATION CURVES OF MAJOR NEUTRAL LIPIDS AND PHOSPHOLIPIDS

Compound*	Fluorescence		Absorption	
	Regression equation	Correlation coefficient	Regression equation	Correlation coefficient
INT	$Y = 0.73X + 5.12$	0.998	$Y = 0.75X + 5.08$	0.995
UC	$Y = 0.50X + 4.89$	0.987	$Y = 0.53X + 4.94$	0.981
EC	$Y = 0.61X + 4.75$	0.991	$Y = 0.71X + 4.76$	0.996
TG	$Y = 0.61X + 4.47$	0.974	$Y = 0.74X + 4.57$	0.989
PE	$Y = 0.61X + 4.73$	0.971	$Y = 0.70X + 4.83$	0.994
PC	$Y = 0.63X + 4.39$	0.988	$Y = 0.71X + 4.65$	0.977
SPM	$Y = 0.55X + 4.35$	0.991	$Y = 0.61X + 4.56$	0.973

*For abbreviations see legend to Fig. 4.

Internal standard correction

The internal standard technique is used to correct for inevitable slight losses of material during the extraction, sample application and chromatography. Various internal standards have been tested:

Cholesteryl formate. This standard was well separated from all naturally occurring lipids (Figs. 1 and 2). Replicate analyses of a mass of 0.1 μg applied as a series of spots to one HPTLC plate showed that the coefficient of variation (C.V.) was $< 0.9\%$ for repeated measurement of one track and $< 5.4\%$ for all tracks on one HPTLC plate.

[^{14}C] Cholesteryl oleate and [^{14}C] phosphatidylcholine. The added radioactive material was of sufficiently high specific activity not to increase the mass of the naturally occurring lipids. In preliminary experiments, the C.V. for these standards was determined using two different amounts of radioactivity (1100 dpm per spot, and 5500 dpm per spot) and counting for different times between 180 and 900 sec. It was demonstrated that the addition of 5500 dpm per sample and a counting time above 200 sec gave a C.V. of 5.8—6.0% which is in the range of that obtainable with the cholesteryl formate mass standard.

Effect of different scanning directions

With both scanners it is possible to scan separated spots either along the length of a single track (track-by-track), or across tracks (cross-track). The C.V. for track-by-track measurement (0.7—1.0%) was significantly better than for cross-track (1.2—4.0%). Hence, in subsequent experiments, track-by-track scanning was always used.

Reproducibility

When the lipids of a single sample were scanned repeatedly by the track-by-track method (see above), the C.V. for all substances was $< 1\%$. When a single lipid extract was chromatographed on twelve separate tracks on one plate, the C.V. for all lipids was between 2.1 and 5.0%. If the single lipid extract was chromatographed on separate plates, however, the C.V. lay between 4.7 and 7.6%.

In order to test the precision for the complete method of extraction and analysis, five aliquots of a single serum sample were delipidated and analysed. The C.V. was between 7.2 and 9.1%.

Correlation of HPTLC method with other analytical techniques

The HPTLC method described here was compared with conventional enzymatic analyses for cholesterol and triglyceride and with large-plate TLC and phosphorus analysis for phospholipids. The following results were obtained. HPTLC versus CHOD—PAP method: for total cholesterol $r = 0.95$, for unesterified cholesterol $r = 0.91$. HPTLC versus enzymatic triglyceride method: for triglycerides $r = 0.92$. HPTLC versus TLC phosphorus method: for phosphatidylcholine $r = 0.90$, for phosphatidylserine $r = 0.91$, for sphingomyelin $r = 0.89$.

Application to cultured cells

As an example of tissue culture lipid analysis (shown in Fig. 7), mouse peritoneal macrophages were preincubated for 24 h at 37° C with human acetyl-

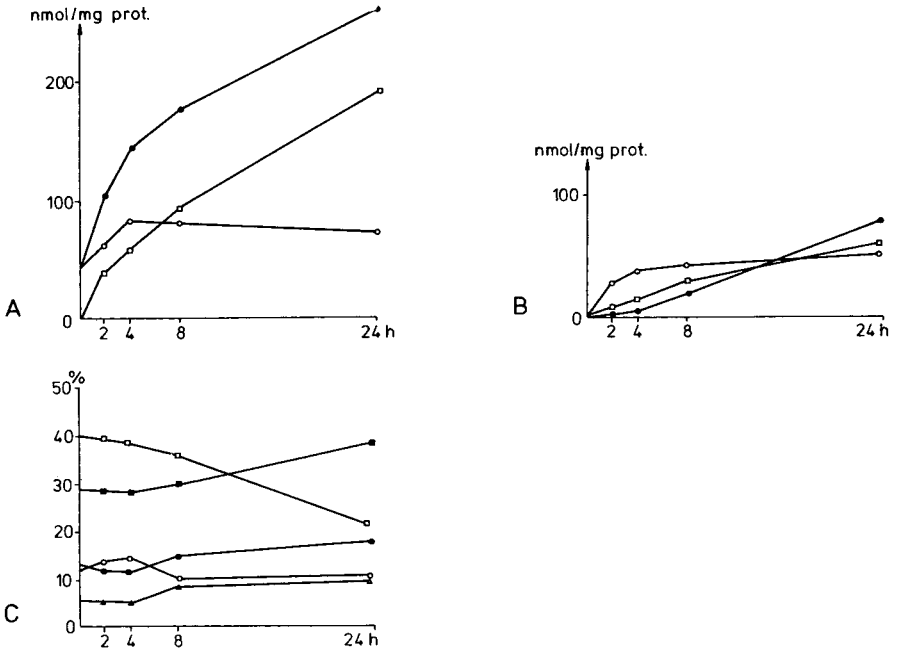


Fig. 7. Effect of incubation time on the lipid content of mouse peritoneal macrophages incubated with acetyl-LDL. Mouse peritoneal macrophage monolayers (60-mm dishes) were washed with DMEM and incubated at 37°C with 3 ml of DMEM containing 35 μg/ml acetyl-LDL for the indicated time. After incubation, two dishes were washed and harvested, and their lipid content measured. Panel A: • = total cholesterol, ○ = unesterified cholesterol, □ = esterified cholesterol. Panel B: • = cholesteryl oleate, □ = cholesteryl stearate, ○ = cholesteryl linoleate. Panel C: ■ = PC, □ = SPM, • = PE, ○ = PS, △ = PI.

LDL (35 μg protein per ml) to enrich cells with cholesterol [24, 25]. Total cholesterol increased in the cells during the incubation period due to a massive accumulation of esterified cholesterol (Fig. 7A). During the first 4 h of incubation, influx of cholesteryl linoleate was responsible for the increase in cellular cholesteryl esters (Fig. 7B). During further incubation the content of cholesteryl linoleate remained nearly constant. Whereas only small amounts of cholesteryl oleate were detectable in the first 4 h, this cholesteryl ester moiety increased and became predominant at the end of the incubation period. Free cholesterol increased in the cells in the first 4 h and did not change much in the following 20 h of incubation. In comparing the relative distribution of the phospholipids (Fig. 7C), it is evident that there was a relative increase in phosphatidylcholine and a decrease in sphingomyelin. Phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol remained nearly constant.

DISCUSSION

A method is presented for the rapid separation and estimation of major tissue and lipoprotein lipids. Separation and detection is uncomplicated and reliable, densitometry and subsequent calculation are fully automated [20]. Besides the classical solvent systems for neutral and polar lipids [26—28], we

have tested numerous published solvents with various internal standards on HPTLC plates.

For the separation of neutral lipids, due to the use of an internal standard, it was necessary to find a one-dimensional chromatography system which revealed uncontaminated spots and a good resolution of the internal standard from the naturally occurring lipids. The described system represents a suitable compromise for both these problems.

For the one-step differentiation between cholesteryl linoleate (90% of the unhydrolysed cholesteryl esters from extracellular origin) and cholesteryl oleate (most of the cholesteryl esters synthesized within the cell), the one-step solvent system used revealed optimal results. However, in complex mixtures cholesteryl palmitate can partially overlap with cholesteryl stearate.

For the phospholipid separation, the solvent system published by Vitiello and Zanetta [18] gave by far the best resolution, accuracy and precision of all one-step separations tested for cellular phospholipids. This method is sufficiently sensitive to monitor the major cellular phospholipids. Splitting of the sphingomyelin spot according to differences in the fatty acid composition did not occur using the Camag HPTLC linear chamber. The individual lysophospholipids do not contaminate the major phospholipid spots.

In our experience, one-dimensional separation of neutral lipids and phospholipids on the same plate [14] can cause contamination of the separated spots by other lipids (e.g. cholesterol contaminated by diglycerides). In addition, there are calibration problems due to large differences in concentration between individual lipid classes (for example, when cholesteryl esters are on the upper limit of the calibration curve, the phospholipids may only reach the lower limit of the calibration curve). We conclude, therefore, that the quantitative analysis of neutral and phospholipids in natural mixtures using one-dimensional HPTLC should be done on separate plates to reduce overlapping of lipid compounds.

The use of either the mass or radioactive internal standard technique improves the precision of the method if absolute values of individual lipids must be compared from different samples on different tracks and different HPTLC plates. However, this standardization is not necessary for the comparison of relative mass distribution of lipids between individual tracks as described by Touchstone et al. [15]. The advantage of a radioactive internal standard is that the ratio of standard to sample can vary over a much wider range than a mass standard without affecting the accuracy and precision of the method. A mass standard has to be present in approximately the same amount as the lipids of the sample. The advantage of a mass internal standard, however, is that it does not require a radiochromatogram scanner and can be measured with the scanning densitometer.

Chromatography was compared between ordinary tanks and the linear development system. Using the linear development system we found more stable conditions, better linearity and paralellism of sample tracks, and very low reagent consumption due to the application of a defined amount of solvent (4.5 ml per plate). For the detection of the separated lipids, we immersed the total HPTLC plate in the liquid detection reagent. We have tested various detection systems, including the ammonia vapour technique [21] which is very

time-consuming, and various spraying reagents [15, 26, 29, 30]. The manganese chloride—methanolic sulphuric acid reagent had the highest sensitivity both in reflectance and transmission mode and plates scanned after immersion revealed a much better C.V. when compared to spraying which leads to inevitable inhomogeneities of the spots.

As shown in Fig. 4, the in situ fluorescence and absorption characteristics of the various lipids are practically identical, all showing absorption and excitation maxima around 366 nm and emission maxima around 410 nm. It has been suggested [31] that fluorescence measurement is more sensitive than absorption. This is correct for measurements of the natural fluorescence of a compound, but the fluorescence of derivatives formed by reaction with a detection reagent lead to non-specific background fluroscence and hence to reduced sensitivity. We have compared the transmission and reflectance scanning mode and found a lower signal-to-noise ratio for the reflectance mode compared to the transmission mode. The transmission measurement is very sensitive to differences in layer thickness, whereas in reflectance the signal-to-noise ratio is basically determined by the surface quality of the layer [13]. In addition, we found in our studies that the transmission mode was ten times less sensitive than the fluorescence emission mode and had a smaller linear portion in the calibration curves.

Comparison of fluorescence and absorption measurements (Fig. 6 and Table I) indicates that the absorption curves have a slightly higher slope, but the useful range of the absorption measurement is more limited. The coefficients of variation for absorption and fluorescence measurements are comparable. The minimum amount detectable by the described fluorescence method is ten times lower compared with data from the literature [14]; 100—150 μg of cell protein were sufficient to obtain a complete lipid analysis.

The method described here allows the rapid separation and quantification of the major tissue and lipoprotein lipids with a high degree of sensitivity, precision, and accuracy. In particular, the method is suitable for the quantification of lipids in tissue culture studies. The idea of these studies was to give refinements and extensions (especially for cholesteryl esters) to information already published on the use of quantitative HPTLC for microlipid analysis. A computer program written in BASIC, using linear regression of logarithmically transferred data for the reduction and calculation of the data produced by this method, is described elsewhere [20].

NOTE TO READERS

The BASIC program [20] is available from the authors free of charge when a 5.25 inch standard discette is sent, together with a prepaid envelope, to our laboratory.

REFERENCES

1 J.G. Heider and R.L. Boyett, J. Lipid Res., 19 (1978) 514.
2 M.S.J. Dallas, L.J. Morris and B.W. Nichols, in O. Mikes (Editor), Laboratory Handbook of Chromatographic and Allied Methods, Ellis Horwood, Chichester, 1979, Ch. 17, p. 527.

3 C.C. Sweeley and D.E. Vance, in G.V. Marinetti (Editor), Lipid Chromatographic Analysis, Vol. I, Marcel Dekker, New York, 1967, p. 465.
4 M.S. Brown, J.L. Goldstein, M. Krieger, Y.K. Ho and R.G.W. Anderson, J. Cell Biol., 82 (1979) 597.
5 M.S. Brown, J.R. Faust and J.L. Goldstein, J. Clin. Invest., 55 (1975) 783.
6 A.M. Fogelman, J. Seager, P.A. Edwards and G. Popjak, J. Biol. Chem., 252 (1977) 644.
7 L.R. Snyder and J.J. Kirkland, Introduction to Modern Liquid Chromatography, Wiley, New York, 2nd ed., 1979.
8 G.L. Hawk, Biological/Biomedical Applications of Liquid Chromatography, Vols. I and II, Marcel Dekker, New York, 1979.
9 J.W. Duncan, P.H. Culbreth and C.A. Burtis, J. Chromatogr., 162 (1979) 281.
10 D.E. Bowyer and J.P. King, J. Chromatogr., 143 (1977) 473.
11 P.S. Gentner and A. Haasemann, Fette Seifen Anstrichm., 81 (1959) 357.
12 D.M. Bradley, C.R. Richards and N.S.T. Thomas, Clin. Chim. Acta, 92 (1979) 293.
13 W. Bertsch, S. Hara, R.E. Kaiser and A. Zlatkis, Instrumental HPTLC, Hüthig Verlag, Heidelberg, Basel, New York, 1980.
14 I.R. Kupke and S. Zeugner, J. Chromatogr., 146 (1978) 261.
15 J.C. Touchstone, M.F. Dobbins, C.Z. Hirsch, A.R. Baldino and D. Kritchevsky, Clin. Chem., 24 (1978) 1496.
16 J. Ripphahn and H. Halpaap, J. Chromatogr., 112 (1975) 81.
17 H. Halpaap and K.-F. Krebs, J. Chromatogr., 142 (1977) 823.
18 F. Vitiello and J.P. Zanetta, J. Chromatogr., 166 (1978) 637.
19 P. Halpaap, Merck-Kontakte, 1 (1978) 32.
20 G. Schmitz, H.-U. Jabs and G. Assmann, Clin. Chem., 29 (1983) 1435.
21 R. Segura and A.M. Gotto, Jr., J. Chromatogr., 99 (1974) 643.
22 O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, J. Biol. Chem., 193 (1951) 265.
23 D.R. Bartlett, J. Biol. Chem., 234 (1959) 466.
24 Y.K. Ho, M.S. Brown and J.L. Goldstein, J. Lipid Res., 21 (1980) 391.
25 J.L. Goldstein, Y.K. Ho, S.K. Basu and M.S. Brown, Proc. Nat. Acad. Sci. U.S., 76 (1979) 333.
26 D.C. Malins and H.K. Mangold, J. Amer. Oil Chem. Soc., 37 (1960) 576.
27 H. Wagner, L. Hörhammer and P. Wolff, Biochem. Z., 334 (1961) 175.
28 E. Stahl (Editor), Dünnschichtchromatographie, Springer Verlag, Berlin, Heidelberg, New York, 2nd ed., 1967.
29 H.P. Martin, Biochim. Biophys. Acta, 25 (1957) 408.
30 R.F. Witter, G.V. Marinetti and A. Morrison, Arch. Biochem. Biophys., 68 (1957) 15.
31 W. Funk, Vom Wasser, 54 (1980) 208.

Journal of Chromatography, 307 (1984) 81—89
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2003

A QUANTITATIVE DENSITOMETRIC METHOD FOR THE RAPID SEPARATION AND QUANTITATION OF THE MAJOR LIPIDS OF TISSUES AND LIPOPROTEINS BY HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHY

II*. REDUCTION OF THE DENSITOMETRIC DATA

GERD SCHMITZ and MARLIES LENCZYK

Zentrallaboratorium der Medizinischen Einrichtungen der Westfälischen Wilhelms-Universität, und Institut für Arteriosklerosеforschung an der Universität Münster, Domagkstrasse 3, 4400 Münster/Westfalen (F.R.G.)

DAVID ORD

Spectra Physics, Darmstadt (F.R.G.)

DAVID E. BOWYER

Department of Pathology, University of Cambridge, Tennis Court Road, Cambridge, CB 21QP (U.K.)

and

GERD ASSMANN*

Zentrallaboratorium der Medizinischen Einrichtungen der Westfälischen Wilhelms-Universität, und Institut für Arteriosklerosеforschung an der Universität Münster, Domagkstrasse 3, 4400 Münster/Westfalen (F.R.G.)

(First received March 14th, 1983; revised manuscript received November 25th, 1983)

SUMMARY

A BASIC program is described for acquisition of data and data reduction for an automated densitometric system for quantitation of lipids separated by high-performance thin-layer chromatography. The program allows calculation of mass of samples from log/log calibration curves computed from standards. The calculated masses are reported as nmol/volume or

*For part I, see ref. 1.

0378-4347/84/$03.00

nmol/mg protein. The program contains a flexible dialog system which permits its use for a variety of applications in addition to the system described for quantitation of lipids.

INTRODUCTION

The reduction of data from quantitative densitometry of high-performance thin-layer chromatography (HPTLC) can be an extremely laborious process which can detract from the many other advantages of this technique. There are two main reasons: (1) Due to improvements in the TLC plates, a large number of samples can be loaded on to each plate and the good resolution may produce a large number of components to be measured. (2) For quantitative analysis it is usually necessary to run a standard curve on each plate and it is desirable to scan each standard and sample more than once. Thus a large amount of data is generated from each analysis.

This paper describes a computer program for the reduction of data from scanning TLC systems. The program is written in BASIC for the Spectra Physics SP 4100 computing integrator which is interfaced to the TLC scanner. It was written primarily for handling quantitative analysis of lipids which we have described elsewhere [1], but it is flexibly organized so that it can be used for other analyses [2].

MATERIALS AND METHODS

The SP 4100 (Spectra Physics, Darmstadt, F.R.G.), a computing integrator programmable in BASIC, was used for data manipulation in conjunction with Minifile 4100 D (Spectra Physics), a floppy disc with 81 Kbyte memory for the automated data manipulation, reporting and storage of results.

HPTLC plates were analysed with either the semiautomated Shimadzu CS 910 TLC scanner (Shimadzu, Kyoto, Japan) or the fully automated Camag TLC scanner (Camag, Muttenz, Switzerland). When used with the Camag scanner a modified Camag interface was used to control the bidirectional

```
**************************************************************************

DECIDE WHICH PROGRAM TO RUN !

"SCAN"      FOR STORING REPORTS ON DISC
OR
"CALC"      FOR RESTORING REPORTS FROM DISC
            AND POST RUN CALCULATIONS

ENTER NAME OF PROGRAM !

PROGRAM: SCAN

ENTER NAME OF SCANNER ( CAMAG OR SHIMADZU )
SCANNER: CAMAG
**************************************************************************

"SCANNING" WILL BE TRANSFERRED TO SP4100
AND START AUTOMATICALLY
**************************************************************************
```

Fig. 1. The printout demonstrates the dialog for the program selection (SCAN or CALC) and for the scanner used (Camag or Shimadzu).

scanning, the SP 4100 integrator and the Minifile. The program is structured in modules as described in Fig. 1. As can be seen, the two major subprograms are "SCAN" for storing scanning data on disc and "CALC", which recalls data from disc and permits a variety of subsequent data reductions. The "BOOT MOD" module is the fundamental program which is present in all subprograms and contains the necessary disc operating software. It manages and calls up the necessary subprograms.

DESCRIPTION OF PROGRAM

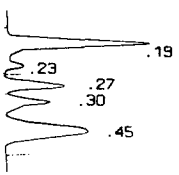
Data acquisition program ("SCAN")

The SCAN program starts by setting up the scan parameters (Fig. 2). The number of scans/track and the scanning parameters must be defined at the

```
**************************************************************************

SELECT BLOC OF FILES TO STORE REPORTS ON DISC !

SELECTED BLOC No. : 2

ENTER NUMBER OF TRACKS !
NUMBER OF TRACKS : 14

ENTER NUMBER OF SCANS/TRACK
SCANS/TRACK : 3


SCANNING PARAMETERS

ATTENUATION          (AT) : 64
CHART SPEED          (CS) : 4
PEAK PARAMETER       (PW) : 3
                     (PT) : 500
NUMBER OF TRACKS          : 14
NUMBER OF SCANS/TRACK     : 3

R E A D Y   T O   S C A N
**************************************************************************
```

Fig. 2. The printout demonstrates the dialog for the scanning parameters in the SCAN program (number of scans, tracks, etc.) and asks for the file where the data shall be stored (BLOC-No.). The variable names of the scanning parameters, e.g. (F 7), (F 8), are given in parentheses.

```
INJECT  TIME    10:25:57
```




```
TRACK 1
SCAN No.  1                SCANS/TRACK  1
  1     0.19          23451       02
  2     0.23           1923       03
  3     0.27           8812       02
  4     0.30          10080       02
  5     0.45          28287       03

SAVED ON DISC FILE No. 201
```

Fig. 3. The printout demonstrates the report format of the scanned track. Each scan is reported in terms of retention time, peak area and the code number for baseline correction (01-65).

```
**************************************************************************

ALL TRACKS HAVE BEEN SCANNED AND THE REPORTS
ARE STORED ON DISC IN BLOC No. 2
**************************************************************************

R E T E N T I O N   T I M E S

SCAN No.          PEAK RETENTION TIMES
-------------------------------------
# 1          0.19    0.23    0.27    0.30    0.45
# 2          0.18    0.23    0.26    0.29    0.45
# 3          0.19    0.24    0.27    0.31    0.46
  |
  |
# 41         0.18    0.23    0.28    0.31    0.46
# 42         0.19    0.24    0.27    0.30    0.45
**************************************************************************

FOR NEW MEASUREMENT OR CALCULATION
DEPRESS THE KEYS "SHIFT" AND "SLOPE"
"BOOT MOD" WILL THEN BE TRANSFERRED TO SP4100
ENTER "RUN" WHEN TRANSFER IS FINISHED
**************************************************************************
```

Fig. 4. The printout demonstrates the list of all peak retention times encountered during scanning. Up to six retention times per scan can be listed. This list allows selection of the peak of interest for further calculation according to the retention times.

beginning of the program. Up to three are possible; the number of tracks is dependent on the plate used and the spot size. The program prints a listing of all scan parameters and responds "Ready to scan".

The analog signal from the densitometer is integrated by the SP 4100 using a 50-Hz data sampling rate, permitting high resolution of signal from noise for the very fast peaks produced by the scanner. Each scan is recorded on printer plotter. Peaks are automatically baseline-corrected and reported in terms of retention times and the area under the peak (Fig. 3). At the end of each scan, the data are automatically stored in files on discette, defined by its track-number and number of scans for each track.

```
**************************************************************************

DATE OF MEASUREMENT : "12.02.82
DATE OF EXPERIMENT  : "10.02.82
EXPERIMENT          : PERI/PREINCUBATION

SELECT BLOC OF FILES TO RESTORE REPORTS FROM DISC !
SELECTED BLOC No. :2

ENTER NUMBER OF TRACKS !
TRACKS : 9

ENTER NUMBER OF SCANS/TRACK !
SCANS/TRACK : 3
**************************************************************************

      PEAK IDENTIFICATION

NUMBER OF PEAKS : 3

RETENTIONTIME     : .51
MOLECULAR WEIGHT  : 647.1
NAME              : CHOL'ESTER

RETENTIONTIME     : .57
MOLECULAR WEIGHT  :
NAME              : INT. STD.

RETENTIONTIME     : 1.57
MOLECULAR WEIGHT  : 386.6
NAME              : CHOL

PEAK IDENTIFICATION WINDOW :
**************************************************************************
```

Fig. 5. The printout demonstrates the dialog of the CALC program to define the experiment (up to 35 characters are available), to address the relevant peaks as demonstrated in Fig. 4 and to name the components by means of the substance and molecular weight.

```
**************************************************************************
     E X T E R N A L   S T A N D A R D S

NUMBER OF EXTERNAL STANDARDS : 5
EXT. STD. CONC. LEVEL DATA

CHOL'ESTER
LEVEL 1 : 1
LEVEL 2 : .8
LEVEL 3 : .5
LEVEL 4 : .3
LEVEL 5 : .2

INT. STD.
LEVEL 1 : 1
LEVEL 2 : .8
LEVEL 3 : .5
LEVEL 4 : .3
LEVEL 5 : .2

CHOL
LEVEL 1 : 1
LEVEL 2 : .8
LEVEL 3 : .5
LEVEL 4 : .3
LEVEL 5 : .2
**************************************************************************
     I N T E R N A L   S T A N D A R D S

NUMBER OF INTERNAL STANDARDS : 2
PEAK No. OF INT. STD. : 2
INT. STD. CONC. LEVEL DATA

INT. STD.
LEVEL 1 : .30
LEVEL 2 : .60
**************************************************************************
```

Fig. 6. The printout demonstrates the dialog for the applied masses of the external standard calibration curves and the internal standards.

```
**************************************************************************
     S A M P L E   D A T A

SPECIFY THE KIND OF INPUTS YOU NEED !
ENTER "YES" OR "NO"
REDISSOLVED SAMPLE VOLUME : YES
APPLICATED SAMPLE VOLUME  : YES
ALIQUOT VOLUME            : YES
DISH VOLUME               : YES
ALIQUOT PROTEIN           : YES
INT. STD. AMOUNT          : YES


NUMBER OF SAMPLES : 2

SAMPLE No. 1
SAMPLE NAME: TEST A
REDISSOLVED SAMPLE VOLUME : 60
APPLICATED SAMPLE VOLUME  : 1
ALIQUOT VOLUME            : 1000
DISH VOLUME               : 500
ALIQUOT PROTEIN           : 70.4
INT. STD. AMOUNT          : .3

SAMPLE No. 2
SAMPLE NAME: TEST B
REDISSOLVED SAMPLE VOLUME :
APPLICATED SAMPLE VOLUME  :
ALIQOUT VOLUME            :
DISH VOLUME               :
ALIQUOT PROTEIN           : 70.38
INT. STD. AMOUNT          :
**************************************************************************
     E N D   O F   D I A L O G

"EXT.CALC" WILL BE TRANSFERRED TO SP4100
AND START AUTOMATICALLY
**************************************************************************
```

Fig. 7. The printout demonstrates the dialog for the sample data as indicated (e.g. volumes, protein, etc.). There is a major "YES" or "NO" decision to select only the inputs which are necessary. The program asks in the following only for the YES input parameters. For the first sample all variables must be entered. For the following samples the default value for any input is the last valid input that was made.

When all scans are done, the program lists the retention times of all components encountered when scanning (Fig. 4). This enables the user to identify each component and to give it a name by which it is identified in subsequent calculations.

Calculation program ("CALC")

Definition of the calculation program ("DIALOG"). The calculation program starts with the subprogram DIALOG (Figs. 5—7). This is necessary to: (1) define the experiment (Fig. 5); (2) address the relevant sample peaks (Fig. 5); (3) address the external and internal standards (Fig. 6); (4) label the sample and enter sample variables (Fig. 7).

Calibration curve calculation ("EXT. CALC"). At the end of the dialog the

```
**********************************************************************

EXTERNAL STANDARD AREA

TRACK 1         SCAN 1          SCAN 2          SCAN 3          REJECT ?
CHOL'ESTER      71780           73889           73890
INT. STD.       93737           96009           96021
CHOL            92049           94616           94598


TRACK 2         SCAN 1          SCAN 2          SCAN 3          REJECT ?
CHOL'ESTER      157089          68398           69853             1
INT. STD.       0               85905           86214             1
CHOL            78755           79830           80798


TRACK 3         SCAN 1          SCAN 2          SCAN 3          REJECT ?
CHOL'ESTER      58411           57440           57517
INT. STD.       69055           71042           71321
CHOL            73450           70005           70180


TRACK 4         SCAN 1          SCAN 2          SCAN 3          REJECT ?
CHOL'ESTER      45192           44847           45447
INT. STD.       54291           55042           55937
CHOL            54943           54460           54987


TRACK 5         SCAN 1          SCAN 2          SCAN 3          REJECT ?
CHOL'ESTER      37758           34910           34832
INT. STD.       43503           42952           43327
CHOL            45315           45184           45084
**********************************************************************


    MEAN STANDARD DEVIATION OF AREAS


CHOL'ESTER          1.88%
INT. STD.           1.1 %
CHOL                1.28%
**********************************************************************

    C A L I B R A T I O N   C U R V E S

  Y=mX+b FOR Y=LOG(AREA)  AND  X=LOG(CONC)

                m  "SLOPE"          b  "INTERCEPT"      CORRELATION
CHOL'ESTER       0.445                4.879               0.989
INT. STD.        0.483                4.984               0.996
CHOL             0.435                4.965               0.987
**********************************************************************
END OF EXTERNAL STANDARD CALCULATIONS
THE NEXT SUBPROGRAM WILL BE TRANSFERRED
TO SP4100 AND START AUTOMATICALLY
**********************************************************************
```

Fig. 8. This printout provides the opportunity to reject a maximum of two scanned areas for a substance in a track. At the end of each line the system asks for this rejection of areas. Input can either be nothing (then the program calculates the mean area and the standard deviation for all areas of this line), or the numbers of the areas to be rejected. If more than one area is to be rejected, the numbers must be separated by a point. The program will then calculate a mean area and standard deviation of the remaining areas. The system then prints the mean standard deviations of areas for each substance and the coefficients of the calibration curve.

calibration curve subprogram (EXT. CALC) is automatically transferred from the disc storage to the SP 4100. The peak areas of the external standard tracks identified by their retention times are transferred from disc storage into the RAM of the computing integrator. The transferred data are then printed for all tracks, scans and substances as can be seen from Fig. 8. This listing provides the opportunity for the user to control the values of the areas and if necessary to reject a maximum of two scanned areas for a substance in a track. From the peak areas the mean value and the relative standard deviation for each substance are calculated and printed out (Fig. 8). From these values log/log calibration curves are calculated and slope (m), intercept (b) and correlation coefficient of the function $\log y = m \log x + b$ are printed as described in Fig. 8.

Internal standard calculation ("INT. CALC"). At the end of the EXT. CALC the internal standard subprogram is automatically transferred from disc storage to the SP 4100. The peak areas of the internal standard are transferred and reported as described for EXT. CALC. The concentration of the internal standard is calculated from the mean of the internal standard integral = INT. STD. CALC and compared to the actual internal standard concentration (see Fig. 9). This comparison offers the opportunity to the user to carry out an "intercept correction of internal standard calibration curve".

```
**************************************************************************

INTERNAL STANDARD AREA

TRACK 6          SCAN 1          SCAN 2          SCAN 3          REJECT ?
INT. STD.        59637           53360           54012             1

TRACK 7          SCAN 1          SCAN 2          SCAN 3          REJECT ?
INT. STD.        88717           78664           0                 1.3
**************************************************************************

     MEAN STANDARD DEVIATION OF AREAS

INT. STD.          0.86%
**************************************************************************

     I N T E R N A L   S T A N D A R D S

                    APPLICATED          CALCULATED          DIFF. %
                    CONCENTRATION       CONCENTRATION
INT. STD. No. 1        0.3                 0.298              -.75
INT. STD. No. 2        0.6                 0.657             9.44

INTERCEPT CORRECTION OF INTERNAL STANDARD CALIBRATION CURVE

ACCORDING TO INT. STD. No. 1

                    APPLICATED          CALCULATED          DIFF %
                    CONCENTRATION       CONCENTRATION
INT. STD. No. 1        0.3                 0.3                0
INT. STD. No. 2        0.6                 0.662             10.28

INTERCEPT CORRECTION OF INTERNAL STANDARD CALIBRATION CURVE

ACCORDING TO INT. STD. No.
**************************************************************************
"SPL.CALC" WILL BE TRANSFERRED TO SP4100
AND START AUTOMATICALLY
**************************************************************************
```

Fig. 9. This printout demonstrates the rejection sequence similar to the one of the external standards. The program compares the calculated concentrations of internal standard in the internal standard tracks to the applied concentrations and offers the opportunity to correct the internal standard calibration curve according to one of the used internal standards. Input is either the number of internal standard, then the intercept correction is carried out, or 0, then the original calibration curve is reestablished, or nothing, then the program will continue.

```
**************************************************************************

SPECIFY FORMAT OF SUMMARY REPORT !

"MOL/PROTEIN"  FORMATS THE RESULTS AS FOLLOWS:
               "nmol/mg PROTEIN     %       nmol/DISH"

"MG/DL"        FORMATS THE RESULTS AS FOLLOWS:
               "mg/dl    %                  nmol/dl"

REPORT FORMAT : MOL/PROTEIN
**************************************************************************
```

Fig. 10. This printout illustrates the output for one of the two available summary reports (MOL/PROTEIN or MG/DL).

```
**************************************************************************

        S U M M A R Y   R E P O R T
        ---------------------------

DATE OF MEASUREMENT: 08.03.82
DATE OF EXPERIMENT : 08.03.82
EXPERIMENT         : PWS


                    mg/dl          %              nmol/dl
SERUM A
TOTAL           117.03275
PC              100.52451      85.89          128.87758
SPM             16.50824       14.11          21.164417

                    mg/dl          %              nmol/dl
SERUM B
TOTAL           106.17328
PC              88.157841      83.03          113.02287
SPM             18.015437      16.97          23.096714

**************************************************************************

FOR PRINTING ANOTHER REPORT, ENTER "RUN"
FOR NEW MEASUREMENT OR CALCULATIONS
DEPRESS THE KEYS "SHIFT" AND "SLOPE"
"BOOT MOD" WILL THEN BE TRANSFERRED TO SP4100
ENTER "RUN" WHEN TRANSFER IS FINISHED
**************************************************************************

**************************************************************************

        S U M M A R Y   R E P O R T
        ---------------------------

DATE OF MEASUREMENT: 12.02.82
DATE OF EXPERIMENT : 12.02.82
EXPERIMENT         : PERI/PREINCUBATION


                nmol/mg PROTEIN     %          nmol/DISH
TEST A
TOTAL             57.840295
CHOL'ESTER        2.9240131         5.06       1.0292526
CHOL              54.916282        94.94       19.330531

                nmol/mg PROTEIN     %          nmol/DISH
TEST B
TOTAL             58.487073
CHOL'ESTER        1.1855367         2.03       0.4349734
CHOL              57.301536        97.97       21.023934

**************************************************************************

FOR PRINTING ANOTHER REPORT, ENTER "RUN"
FOR NEW MEASUREMENT OR CALCULATIONS
DEPRESS THE KEYS "SHIFT" AND "SLOPE"
"BOOT MOD" WILL THEN BE TRANSFERRED TO SP4100
ENTER "RUN" WHEN TRANSFER IS FINISHED
**************************************************************************
```

Fig. 11. This figure gives an example of both of the available summary reports (MOL/PROTEIN or MG/DL).

Sample calculation sequence ("SPL. CALC"). At the end of INT. CALC the sample calculation subprogram is automatically transferred from the disc storage to SP 4100. The peak areas of the samples are transferred as described for the EXT. CALC program. For the mean areas and the compound calibration curves the mass of compound in μg/ml applied volume is calculated. For each sample a correction factor resulting from the comparison of the calculated samples internal standard concentration and the INT. STD. CALC is computed. At the end of SPL. CALC one of two alternative summary reports can be selected (Fig. 10).

One of the summary reports offers the results as nmol/mg protein, percentage mass of total mass, nmol/dish or nmol/vol. The other formats are according mg/dl, percentage submass of total mass/dl, nmol/dl. An example for each report format is given in Fig. 11.

DISCUSSION

Automatic computation of data from an HPTLC scanner requires the calculation of an appropriate regression equation relating the integrated fluorescence or absorption signal to the mass of material. In the BASIC program described here a linear regression between the log of the integrated signal and the log of the mass is used. This technique gives the best fit for most of the HPTLC calibration curves [3, 4]. As with any computed regression equation, it is not important that values for unknowns are calculated by interpolation of the curve and that any data which lie beyond the range of the appropriate calibration curves are detected and can then be rejected. As is shown in Fig. 8, the calculated correlation coefficient (r) may be used as an indicator of the quality of the calibration curve. Thus the user has the opportunity to reject curves of poor quality where, for example, r is less than 0.96. The program allows the use of an internal standard so that the analysis is independent of the exact volume of sample applied to the plate.

The use of a BASIC programmable computing integrator with a log/log linear curve fitting enables the large quantities of data to be reduced with a high degree of precision and accuracy and reported in an acceptable form, in vastly improved turn-around time.

NOTE TO READERS

The BASIC program is available from the authors free of charge when a 5.25 inch standard discette is sent, together with a prepaid envelope, to our laboratory.

REFERENCES

1 G. Schmitz, G. Assmann and D.E. Bowyer, J. Chromatogr., 307 (1984) 65.
2 G. Schmitz, H.-U. Jabs and G. Assmann, Clin. Chem., 29 (1983) 1435.
3 R.E. Kaiser, in W. Bertsch, S. Hara, R.E. Kaiser and A. Zlatkis (Editors), Instrumental HPTLC, Hüttig-Verlag, Heidelberg, Basel, New York, 1980, p. 165.
4 D.T. Downing and A.M. Stranieri, J. Chromatogr., 192 (1980) 208.

Journal of Chromatography, 307 (1984) 91—102
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2025

ANALYSIS OF THE GLOBINS FROM FAST HUMAN HAEMOGLOBINS BY ISOELECTROFOCUSING ON POLYACRYLAMIDE GEL RODS

MASSIMO CASTAGNOLA*, PAOLO CARADONNA, LOREDANA CASSIANO, CLAUDIA DEGEN, FRANCESCA LORENZIN, DIANA ROSSETTI and MARIA LETIZIA SALVI

Istituto di Chimica, ed Istituto di Patologia Medica, Facoltà di Medicina e Chirurgia, Università Cattolica del Sacro Cuore (e C.N.R., Centro di Studio per la Chimica dei Recettori e delle Molecole Biologicamente Attive), Largo Francesco Vito 1, 00168 Rome (Italy)

(Received July 5th, 1983)

SUMMARY

The globins from all fast haemoglobin (Hb) components obtainable by Bio-Rex 70 cation-exchange chromatography were examined by isoelectrofocusing on polyacrylamide gel rods with 8.0 mol/l urea. From this analysis HbA_{1a_1} and HbA_{1a_2} seem to be very heterogeneous components. HbA_{1b} is separable into two components, one of which is varied in both the β chains. Between HbA_{1b_2} and the well-known HbA_{1c} components two chromatographic peaks are separated, one with a noticeable percentage of glucosylated β chain and one that probably contains HbF. HbA_{1c} has both β chains glucosylated, while HbA_{1x} seems to be a β monoglucosylated Hb form. Finally, the early part of the HbA_0 peak has a large amount of glucosylation on both α and β chains.

INTRODUCTION

We recently showed, by cation-exchange chromatography, that normal human haemoglobin can be separated into a number of fast components, more than previously known [1, 2]. One of these components, HbA_{1c}, is structurally defined as glucosylated on the α-NH_2 of the terminal valines of the β chains [3]. Some components (HbA_{1a1}, HbA_{1a2}, HbA_{1x}) are awaiting confirmation of their identity [1, 2, 4]. Others (HbA_{1b} species) are still structurally unknown [5].

In this present study we describe the globin analysis of all these components, also concerning the early part of HbA_0 peak, where glucosylation on ϵ-NH_2 of lateral lysines is postulated [1, 6]. For the analytical separation of globin chains we improved the isoelectrofocusing by using polyacrylamide gel rods in 8.0 mol/l urea.

0378-4347/84/$03.00

Some authors [7—9] performed similar globin separations by isoelectrofocusing on slab gels, with or without non-ionic detergents, choosing to screen a large number of samples. The aim of our study, however, was to check a reduced number of samples with a large separation between globin chains, thus we preferred to use the higher separation properties of rod isoelectrofocusing. In fact, in comparison to slab isoelectrofocusing [9], the rod method allows the simultaneous characterization of modifications on α and β globin chains.

MATERIAL AND METHODS

Reagents

All common reagents were analytical grade from Merck (Darmstadt, F.R.G.) and from Carlo Erba (Milan, Italy). Bio-Rex 70 (200—400 mesh, Na^+) and AG 501-X8 (D), (20—50 mesh) mixed-bed resin were from Bio-Rad Labs. (Richmond, CA, U.S.A.). YM 10 membranes were from Amicon (Lexington, MA, U.S.A.). Ampholine 6—8 and 7—9 were from LKB (Bromma, Sweden). Fibrous cellulose powder CF12 was from Whatman (Maidstone, Kent, U.K.). N,N,N'N'-tetramethyl-1,2-diaminomethane (TEMED) was from BDH (Poole, U.K.) and Coomassie Brillant Blue R-250 (C.I. 42,660) was from Serva (Heidelberg, F.R.G.).

Preparation of haemoglobin samples and chromatographic separation of fast components

The haemolysis of Hb samples and their chromatography on Bio-Rex 70 were performed on the basis of the method previously described [1]. In order to obtain a quantity of small components sufficient for the subsequent isoelectrofocusing, the sample load was 25 ml of hemolysate and column dimensions were 7 cm^2 × 60 cm. The volume of elution buffer was proportionally increased as shown in the results. Elution with 0.10 mol/l sodium chloride in 0.05 mol/l potassium phosphate buffer (pH 6.55 ± 0.02) was increased with respect to the previous method to provide better resolution in the zone of the HbA_{1b} components (see Fig. 1). Each chromatographic component was pooled and concentrated, under nitrogen pressure, by Amicon YM 10 membranes.

Separation of globin from haemoglobin components

The globin from each Hb chromatographic component was obtained on the basis of the acid acetone method of Rossi-Fanelli et al. [10]. The dried globin samples were kept at —20°C.

Isoelectrofocusing in polyacrylamide gel rods with 8.0 mol/l urea

Before the gel preparation 10.0 mol/l urea was filtered on fibrous cellulose powder CF12 and subsequently eluted on a small column of AG 501-X8 (D) mixed-bed resin. Then 2.425 g of acrylamide and 0.075 g of N,N-methylene-bis-acrylamide were dissolved in 30 ml of 10.0 mol/l urea solution; 250 mg of AG 501-X8 (D) resin were added to this solution and left under mild stirring for 30 min. After lint filtration 1.6 ml of Ampholine 6—8 and 1.6 ml of Ampholine 7—9, 5.0 ml of glycerol, 10.0 ml of 10.0 mol/l urea and distilled

water were added up to a total volume of 49.5 ml. This solution, after stirring, was degassed under vacuum for 15 min.

Then 50 μl of TEMED and 0.5 ml of ammonium persulphate (freshly prepared) at a concentration of 18 mg/ml (20°C) were added; in the various preparations this persulphate concentration was slightly changed depending on room temperature. Immediately, 2.0-ml aliquots of this solution were poured into tubes (bottom capped) 5 mm (diameter) × 120 mm and 100 μl of distilled water were gently layered on the surface. The gels were left to polymerize overnight.

Isoelectrofocusing was performed in a vertical jacketed chamber (Minivolt, Rome, Italy). The gel surface was washed once with a small volume of 10.0 mol/l urea and the top of the tube was filled with a solution obtained by mixing 12 ml of 10.0 mol/l urea, 2 ml of glycerol, 0.5 ml of Ampholine 6—8 and 0.5 ml of Ampholine 7—9. The bottom chamber was filled with 0.01 mol/l ethanolamine and the upper chamber with 0.01 mol/l glutamic acid. Prefocusing was performed in the precooled chamber at a constant current of 1 mA/gel (LKB 2197 power supply) (top, anode; bottom, cathode) for 20 min. The protective upper solution was then discharged and 20 μl of the sample were layered on the surface of the gel.

The samples were prepared by dissolving 2.0 mg/ml of each globin in a solution obtained by mixing 0.8 ml of 10.0 mol/l urea, 20 μl of β-mercaptoethanol, 40 μl of Ampholine 6—8, 40 μl of Ampholine 7—9 and 100 μl of glycerol.

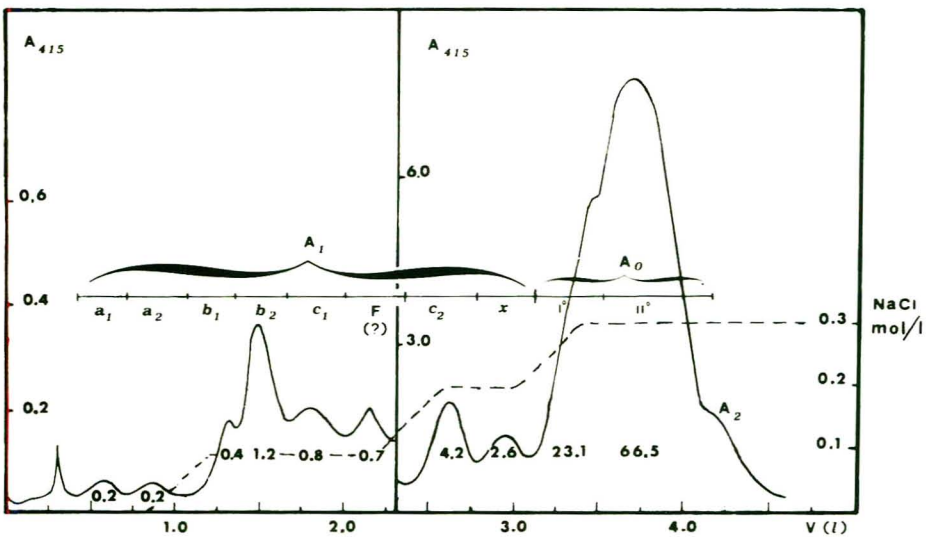


Fig. 1. Bio-Rex 70 cation-exchange chromatography of a normal Hb sample. The elution buffer was a potassium phosphate solution 0.05 mol/l, pH 6.55 ± 0.02 [1]. The dashed line represents the progressive increase of sodium chloride concentration in the buffer. The central line shows how the division of the pools is performed. The name assigned to each pool is also reported; the separation of two new peaks between HbA_{1b_2} and $HbA_{1c(2)}$ caused some problems in name assignation. Thus it was preferred, on the basis of the following experiments, to call the first peak HbA_{1c_1} and the second HbF, and transform the HbA_{1c} denomination to HbA_{1c_2}. The percentage of each pool (component) is reported on the peak base.

Isoelectrofocusing was performed for 210 min (10°C) at a constant current of 2 mA/gel with a maximal cut-off of 500 V. At the end of the run the gels were removed from the rods by rimming with a water jet. A small piece of gel was cut obliquely, at the bottom of the gel, to determine the gel orientation. The gels were kept for 1 h in fixing solution (17.25 g of 5-sulphosalicylic acid and 57.5 g of trichloroacetic acid dissolved in 150 ml of methanol and 350 ml of distilled water). Then they were stained overnight with Coomassie Brillant Blue R-250 (115 mg in 100 ml of destaining solution) and finally destained in a solution of 250 ml of ethanol, 80 ml of acetic acid and 670 ml of distilled water.

Before the fixing step, one of the gels was cut into slices of 2 mm and the pH of each slice was measured after shaking in 2.0 ml of distilled water until pH stabilization. Each stained gel was scanned by a DD2 Kipp and Zonen densitometer with automated integrator.

RESULTS

In Fig. 1 the pattern obtained using Bio-Rex 70 chromatography of Hb normal samples is shown. The pattern is slightly different than that previously reported [1]: after the HbA_{1b2} component two peaks are now obtained. This is due to longer use of the plateau with 0.10 mol/l sodium chloride in potas-

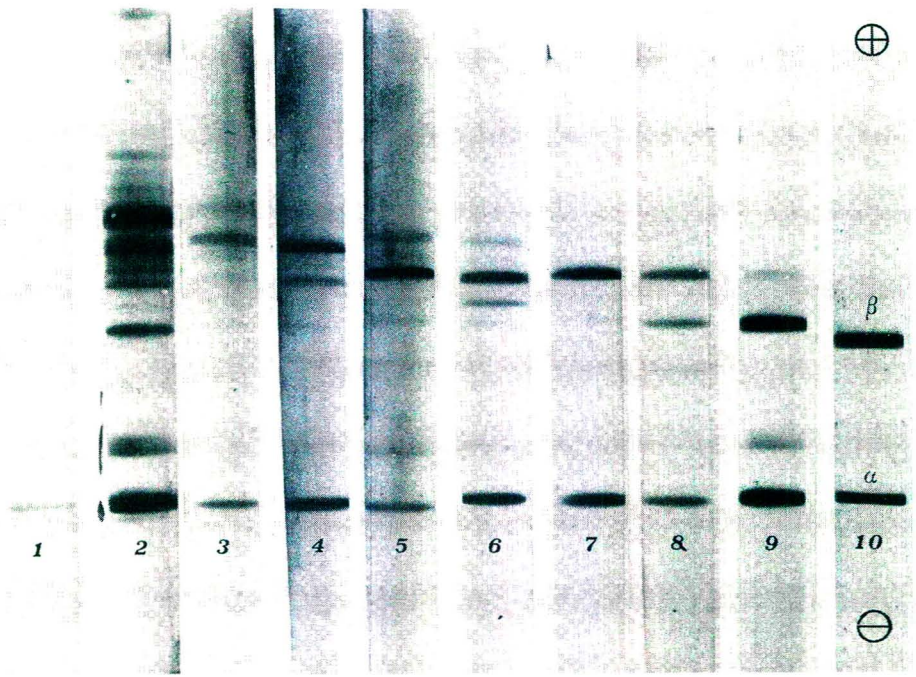


Fig. 2. Isoelectrofocusing rod gels of the globins obtained from the chromatographic pools. The numbers from 1 to 8 represent, in sequence, globin patterns from HbA_1: 1 = a_1; 2 = a_2; 3 = b_1; 4 = b_2; 5 = c_1; 6 = HbF; 7 = c_2; 8 = x. The numbers 9 and 10 are the globin patterns from HbA_0 pools: 9 = 0I°; 10 = 0II°.

sium phosphate buffer. This makes it difficult to assign a name to these components. It is possible to attribute these components to either HbA_{1b} or HbA_{1c} species. On the basis of the subsequent isoelectrofocusing we assign the first to HbA_{1c} species and suppose that in the second HbF is present. Thus, HbA_{1c} is divided into two components, named HbA_{1c1} and HbA_{1c2}, with the F component in between. In any case the nomenclature for the Hb components at this point in time seems to be inadequate and a revision is advisable.

In Fig. 1 the choice of the pools collected is indicated; the HbA_0 peak is divided into two pools, in line with both the thiobarbituric acid colorimetric test [1, 11] and the in vitro incubation experiments with labelled glucose [1]. HbA_{1b} is divided into two components and HbA_{1x} is a component which seems to be monoglucosylated [1]. At the bottom of each peak the percentage of the pool with respect to the total Hb sample is shown. The values of the A_{1b1} and A_{0I° pools do not provide accurate percentages since these peaks are poorly separated from nearby components. The final percentage (HbA_{0II° pool) also includes the HbA_2 component, although it is not considered for pool collection.
sidered for pool collection.

The collected pools are concentrated on Amicon YM 10 and the usual globin separation by the acetone–HCl method [10] is then performed. The yield of globin for each pool is very good except for HbA_{1a2}, where it is very low, and for HbA_{1a1} and HbA_{1x} components, which provide abnormally high yield.

The globins obtained are then examined by isoelectrofocusing on polyacrylamide gel rods with 8.0 mol/l urea solutions. The results are presented in Fig. 2 and in the following. Fig. 2 is a photo of the gels, and Figs. 3–5 are the densitometric scans from the gels, obtained by a scanning densitometer with integrator. In the scans, using as a reference globin from the HbA_{0II° pool, we prefer to report the sequence of components in the opposite order to that of the chromatographic elution (i.e. starting with HbA_{0II° and finishing with HbA_{1a1}). The bottom scale represents the distance (mm) from the top (anode) of the gels. Thus, each peak can be identified simply by its position.

The upper scale represents the pH measured along the gel. The cut-slice method used affords a pH value somewhat different from that obtained by Francina et al. [9] by direct measurement. Furthermore, we do not apply any correction for the presence of urea. In fact, the aim of our separation is principally to obtain a good separation simultaneously for α and β chain modifications and not to determine exact pI_{app} (apparent isoelectric point) values.

The use of Ampholine of two different ranges (6–8 and 7–9) causes an overlap in the middle and is not equivalent to the use of a unique range from 6 to 9. This middle Ampholine excess reflects an amplification in the middle pH zone which has a broader variation than the extreme ones, with the best separation in the zone between 6.75 and 7.50 pH units. Thus, the upper pH scale is not linearly related to the distance from the top of the gel.

The peak height represents the band intensity as measured by the scanning densitometer and the relative percentage of each peak is reported as measured

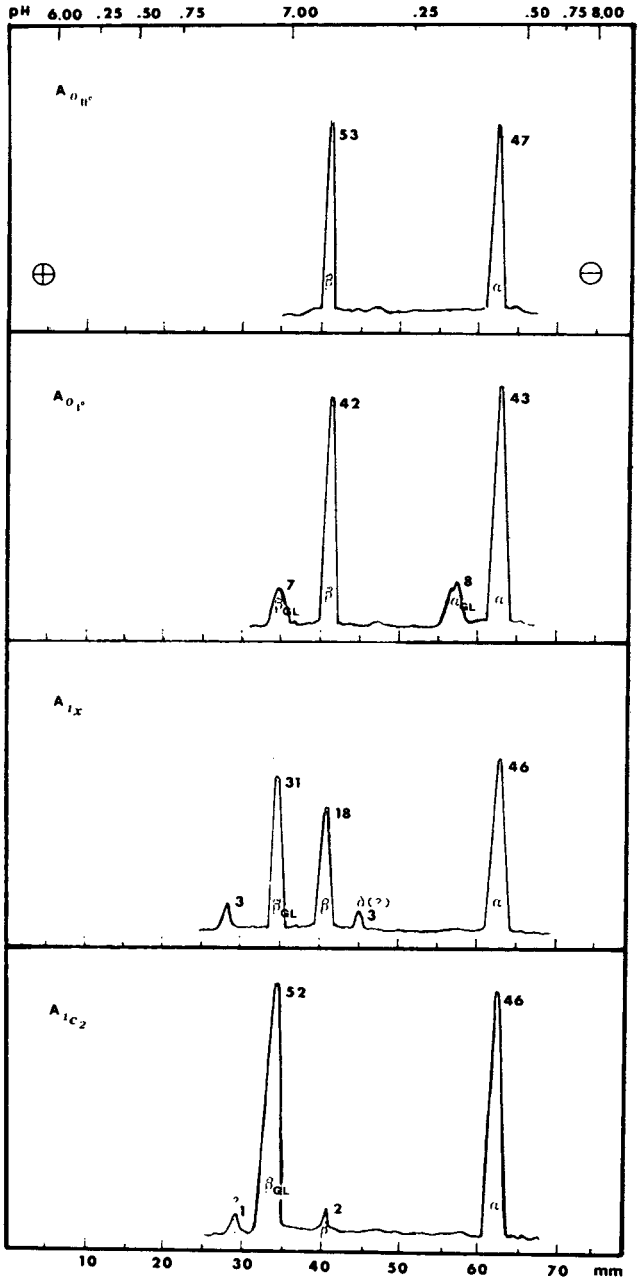


Fig. 3. Scanning of the 10, 9, 8, 7 gels from Fig. 2 by densitometer and integrator. The bottom scale represents the distance of each band from the top of the gel (+ = anodic electrode). The total gel measures 95 mm, thus only the significant zone is reported. The upper scale represents the pH measured by the cut-slice method. The pH scale is not linearly related to the distance owing to the use of Ampholine of two different ranges, with an overlap in the middle zone. The percentage of each peak is reported with respect to the total as considered within each globin sample. α and β subscripts are reported only when it is possible to assign, with some certainty, also on the basis of previous results [1, 11], the band identity. Thus α_{gl} and β_{gl} are for α and β globins glucosylated on the α-NH_2 of terminal valines or on unidentified ϵ-NH_2 of lateral lysines.

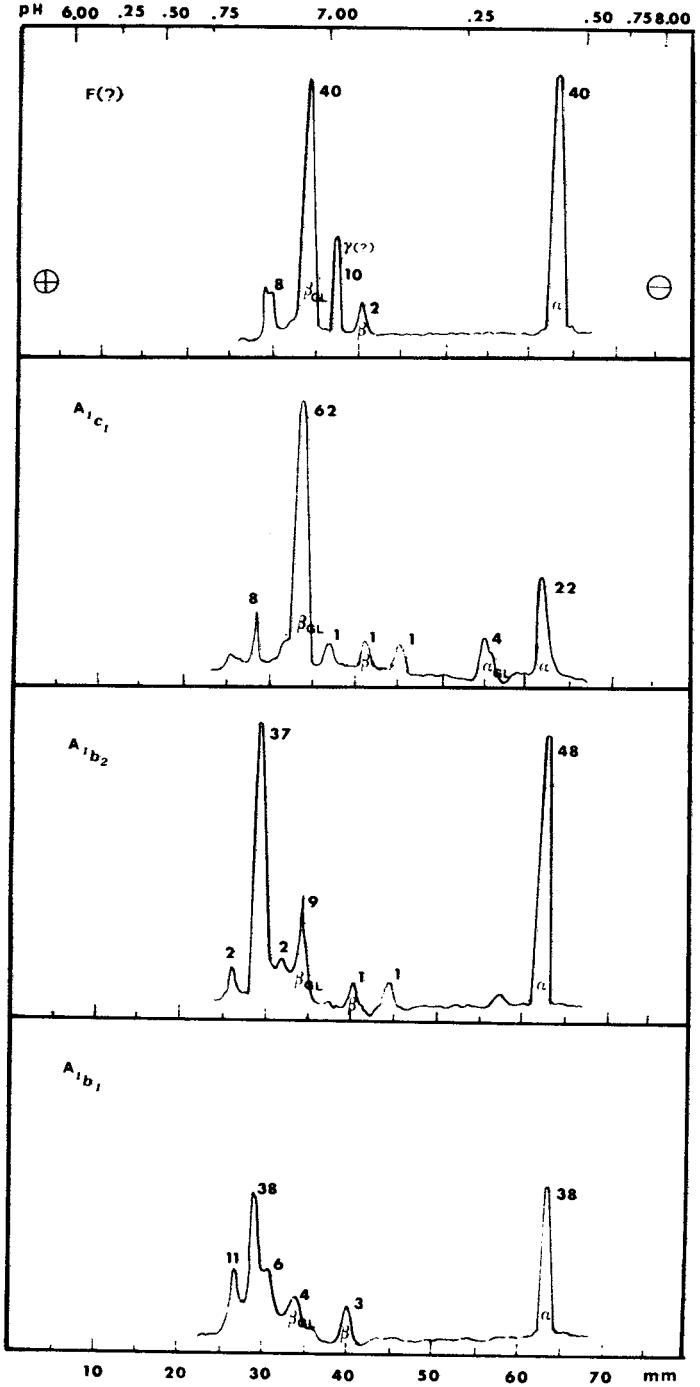


Fig. 4. Scanning of the 6, 5, 4, 3 gels from Fig. 2. The specifications are as in Fig. 3.

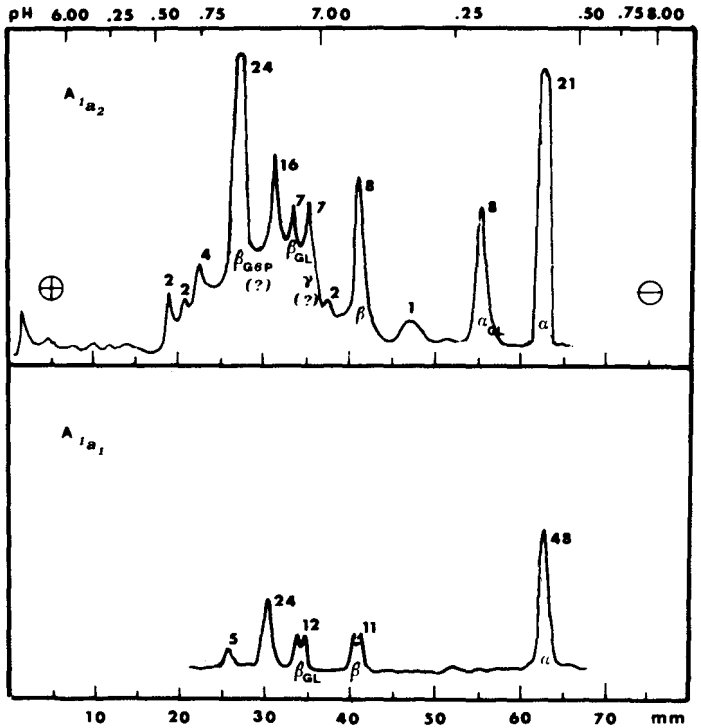


Fig. 5. Scanning of the 2, 1 gels from Fig. 2. The specifications are as in Fig. 3. β_{G_6P} is a β globin chain with a glucose-6-phosphate molecule linked to the α-NH_2 of the terminal valine.

by the integrator. The intensity values are consistent only within the same globin sample; from one scan to another the relative intensity reflects only approximately the variations between the gels because of slight adjustments made to the amplitude of the absorbance scale of the densitometer to avoid too high values in highly stained gels and too low values in poorly stained gels.

The differences in staining among the gels is not due to different quantities of total protein in the samples applied (always 40 μg for each sample) but rather to the possible presence in the sample of some protein contaminants (non-haemoglobin proteins: NHP) which have a pI so different from globin species that they are excluded from the gel ranges. We believe that NHP is definitely present in the globin from the HbA_{1a1} component and, perhaps, in the globin from the HbA_{1x} component. This supposition is confirmed, in the globin separation of these components, by a yield higher than expected.

The position of normal α and β globin chains from the HbA_{0II^0} pool is slightly different from those of the other pools, unfortunately, because this gel is from a different focusing run than the others. This is the best comparison that we can provide, due to various gel losses during the rimming from the tubes. For this reason it is advisable to refer the exact position of normal α and β globin chains to the major bands of the globin from the HbA_{0II^0} pool.

Finally, it must be noted that, until now, no great differences in the globin patterns among the pools collected from normoglycemic and diabetic samples are present.

DISCUSSION

We prefer, as mentioned above, to start our discussion with the $HbA_{0II^{\circ}}$ pool in the opposite order to the elution of the components.

HbA_0 is normally assumed to be the pure HbA form. Shapiro et al. [6] reported that about 10% of HbA_0 is glycosylated primarily at the ϵ-amino groups of the lysines. Their experiments allowed also the prevalent sites of glycosylation to be determined, but were partly disproved by the observations of Trueb et al. [12], for the presence of labelled contaminants in the radioactive glucose used in the experiments.

Our recent experiments [1, 11] have confirmed some of their observations and allow the separation, by Bio-Rex 70 chromatography, of a new component, named HbA_{1x}, which was postulated to be monoglucosylated by the thiobarbituric acid colorimetric test (TBA test), by in vitro incubation with labelled glucose and by a significant increase in diabetic samples compared to normoglycemic ones. For the same reasons further glucosylation seems to be present in the early part of the HbA_0 peak.

Thus we attempted to find an improved method for the analysis of modifications on both globin chains, for a control of the sites and the percentage of the glucosylation process. Isoelectrofocusing of globin in polyacrylamide gel rods with 8.0 mol/l urea solutions proved to be a very sensitive method for our purposes.

Application of the method to the two pools collected from the HbA_0 peak provides verification that the $HbA_{0II^{\circ}}$ pool, freed from its early ($HbA_{0I^{\circ}}$) and late (HbA_2) parts seems to be completely pure, showing only two bands (in the same percentage) which obviously represent the α and β normal chains. The pI_{app} is 7.05 for β chain and 7.45 for α chain, with some uncertainty resulting from the method of measurement and the presence of urea.

Both α and β chains of the globin from the $HbA_{0I^{\circ}}$ pool present an appreciable percentage of modified chain with a position exactly expected for a monoglucosylated chain, as also shown from the position of the β globin chain from HbA_{1c2}. The percentages obtained by peak integration indicate that about 14% of the β chain (7% with respect to the total) and 16% of the α chain (8% with respect to the total) are modified. These values account for about 60% of the total monoglucosylation on Hb from this pool. Since the pool percentage is about 23% of the total Hb, this glucosylated Hb should account for about 13% of the total Hb, half for α and half for β glucosylation. These results are obtained in a normal sample and previous results on diabetics seem to reveal an increase in these percentages. The data described above agree very well with those obtained by the TBA test on the $HbA_{0I^{\circ}}$ pool [11].

The glucosylation affects probably the ϵ-NH_2 of the lateral lysines, because it involves groups which keep, even after glucosylation, a positive charge at the pH of the cation-exchange chromatography (pK_a of the lysines = 10). Thus it is very difficult to separate these glucosylation products by chromatography at the pH normally in use. Combining the results of the globin analysis with the results obtained in previous experiments [11] we can confirm that HbA_{1x} is monoglucosylated on the β chain.

The percentages of the peaks of the β and glucosylated β (β_{gl}) chains show contamination by the HbA_{1c2} component. These results confirm the value

of 1.2 mol of glucose per mole Hb tetramer previously obtained [1, 11].

Obviously, from these data, it is impossible to determine the glucosylation site. If this component represents the monoglucosylation product in order to obtain HbA_{1c2}, it accounts for a lower total percentage than expected. In fact for 4% of the diglucosylated form (HbA_{1c2}), statistically 20% of the monoglucosylated form (HbA_{1x}) is expected. This fact could be explained assuming a splitting constant, $K_{2,4}$, for the monoglucosylated form much greater than of HbA_0 and HbA_{1c2}. This assumption should be still more consistent if the glucosylation on HbA_{1x} component is on a different site than the α-NH_2 of the valine of the β chain.

The two slight β bands, of the same percentage, could account for a second Hb component in this chromatographic zone; while that with a pI_{app} lower than that of β_{gl} could derive from a modification similar of that observed for HbA_{1b} components; that with a pI_{app} higher than β should be the δ chain.

No glucosylation on the α chain appears in this component. Finally, we believe that some NHP is also present in this component because of too high a yield of globin (by the acetone method) and a low staining of the bands on the gel. HbA_{1c} is known to be the most abundant fast component of Hb. Holmquist and Schroeder [13] postulated that it is a normal Hb ($\alpha_2\beta_2$) with a blocked N-terminal residue in one of the β chains. Bookchin and Gallop [14] subsequently proposed that both β chains are blocked by N-terminal hexose. Finally, Koenig et al. [15] showed that glucose is the blocking group and after attachment by aldimine linkage it undergoes an Amadori rearrangement which transforms it into a more stable ketoaminic group.

Our results are consistent with this proposal and clearly confirm that HbA_{1c} is glucosylated on both β chains, because of the presence in the globin pattern of only β_{gl} chain, except for a trace amount of normal β chain. The fact that two new peaks appear before this component, during the chromatographic elution, obliges us to introduce a slight variation into the nomenclature of Hb fast components; thus we change the name HbA_{1c} to HbA_{1c2}.

In usual cation-exchange chromatography of Hb it is known that HbF is not easily separable from the HbA_{1c} peak [16]. In the globin analysis of the HbA_{1c2} peak, obtained by the chromatographic conditions described, no peak near the β_{gl} chain is present which justifies the existence of a γ chain. In the globin analysis of the minor peak, eluted before the HbA_{1c2} peak, we find two globin bands with a pI_{app} strictly related to that of the β_{gl} chain (6.87; 6.92 β_{gl}; 7.02). The band with a pI_{app} of 7.02 seems to correspond to the γ chain, also from a preliminary comparison against globin samples from pure HbF preparations (data not reported). Thus we ascribe the chromatographic peak to the HbF component, although a noticeable quantity of HbA_{1c2} is still present in this peak and some uncertainty exists as to the exact position of the γ globin chain.

The globin from the HbA_{1c1} chromatographic peak reveals a very high presence of β_{gl} chain to which an equivalent amount of α chain does not correspond. Moreover, the α chain has an appreciable quantity of glucosylation and some other chains are present in the β position, the most abundant of which is the β chain related to the position of the β globin from HbA_{1b} species. This could derive more from chromatographic overlapping than from a complex Hb component. It is difficult to explain these results and we are performing

further experiments to clarify the globin patterns obtained from this Hb peak and the following ones. In any case, an appreciable quantity of $Hb(\beta_{gl})_4$ should be present in the HbA_{1c1} peak. It is known that HbH has a β_4 composition, thus also the β_{gl} chain could exist in a stable tetrameric form; it could have a stability greater than β_4 and, perhaps, replace the latter, by dissociation equilibrium, with a little disappearance of HbA_{1c2}. In fact no genetic excess of β_{gl} can be supposed. Because of the high percentage of β_{gl} chain we prefer to name this peak HbA_{1c1}, assigning it to HbA_{1c} rather than to HbA_{1b} species.

In the HbA_{1b2} chromatographic component the most abundant chain has a pI_{app} more acidic than β_{gl} (6.88 pH units), as expected from its chromatographic properties. A large percentage of β_{gl} chain is still present in this component, while no appreciable quantity of β chain is present. These results disprove the finding of Krishnamoorthy et al. [5], who, on the basis of electrophoretic data, postulated this Hb component as an asymmetrical hybrid, i.e. with a β normal and a β modified chain. It is possible that neither their chromatographic separation on Hb nor electrophoretic and chromatographic globin separations are able to distinguish the slight differences among the Hb components and between β_{gl} and β chains. If the β_{gl} chain is not due to contamination from a subsequent chromatographic peak, the asymmetrical hybrid must be considered at most, as $\alpha_2\beta_{gl}\beta_{1b}$.

In this case it is also possible that, at the pH of our chromatography, the dissociation equilibrium of Hb always favours glucosylated components instead of the normal ones. On the other hand, the HbA_{1b1} component is probably a Hb modified on both β chains, i.e. $\alpha_2(\beta_{1b})_2$.

In the examination of globin patterns of the two small HbA_{1a2} and HbA_{1a1} components, the first fact to discuss is the large differences in band staining. The HbA_{1a2} component is very intense in all the bands because of the great difficulty in exactly weighing its absolute amount after globin separation by the acetone method (0.2 mg); its yield in globin is very low, thus some contaminants should be present in this peak, but they do not precipitate during the globin separation. Thus the slight overload in the gel is due principally to difficulties in the globin weighing. The HbA_{1a1} component, on the other hand, has very faint bands, even though the acetone precipitate is very abundant; thus, in this peak, a large quantity of NHP, excluded from the gel by their pI, must be present.

The HbA_{1a2} globin pattern is surprisingly complex. This confirms that the rod isoelectrofocusing method has a high resolving power, but leads to the impossibility of being able to interpret the results exactly. It is possible to recognize both α and β modifications, with high percentages of α_{gl} chain, β_{gl} chain and β_{1b} chain; but the most abundant of the β chains is a band with a pI_{app} of 6.81 which corresponds well to a β_{G6P} (glucose-6-phosphate) chain, as expected for this Hb component [2]. HbA_{1a1} also has a complex pattern, but its faint bands do not allow us to verify if a $\beta_{F1,6DP}$ (fructose-1,6-diphosphate) chain is present. In any case, the presence of high heterogeneity in these chromatographic peaks, as previously postulated by McDonald et al. [4], is widely demonstrated.

CONCLUSIONS

Globin analysis of the peaks from Bio-Rex 70 chromatography of normal

Hb leads to a complex picture. The chromatographic peaks are often highly heterogeneous, either because of the presence of many components in the same peak or because of partial overlapping among the peaks. Thus, β_{gl} globin is present, either in high proportions or in trace amounts, in almost all the globin samples examined, while its characteristic peaks should be HbA_{0I^c}, HbA_{1x}, HbA_{1c2}, HbA_{1c1}. The same fact could be observed for the β_{1b} chain which, obviously present in high percentage in the HbA_{1b1} and HbA_{1b2} components, is still present, in trace amounts, from the HbA_{1a2} even to the HbA_{1x} component.

The chromatographic separation is more related to β chain modifications, while α modifications seem to affect only slightly the chromatographic properties of Hb. In fact, the globin analysis shows a continuous increase of the pI_{app} of the β chain in relation to the progressive elution.

Glucosylation on the α chain provides an earlier elution of the Hb within the peak itself, and not net peak separation. This is clear for the HbA_{0I^c} pool but can be postulated also for the HbA_{1a2} and HbA_{1c1} components. These characteristics agree well with the properties of the NH_2 of the terminal valine of the β chain, which seems to be the NH_2 group more prone to carbonyl attachment, either because its pK_a ($\sim$7) is very different from that of the NH_2 of lateral lysines ($\sim$10), or because of its external position which is different from that of the NH_2 of the terminal valine of the α chain.

The relatively easy use of rod electrofocusing makes this method a powerful tool for structural Hb analysis. The higher resolution of the rod compared with that of slab gels must be ascribed more to the higher Ampholine concentration in the rod gel, which allows a stable pH gradient, than to the greater dimension of the rod gels. Application of this method could be extended to many other Hb analysis problems, but, obviously, for some Hb variants, the Ampholine ranges should be varied to obtain maximum separation power.

REFERENCES

1 M. Castagnola, P. Caradonna, M.L. Salvi and D. Rossetti, J. Chromatogr., 272 (1983) 51.
2 M.J. McDonald, R. Shapiro, M. Bleichman, J. Solway and H.F. Bunn, J. Biol. Chem., 253 (1978) 2327.
3 H.F. Bunn, D.N. Haney, K.H. Gabbay and P.M. Gallop, Biochem. Biophys. Res. Commun., 67 (1975) 103.
4 M.J. McDonald, M. Bleichman, H.F. Bunn and R.W. Noble, J. Biol. Chem., 254 (1979) 702.
5 R. Krishnamoorthy, G. Gacon and D. Labie, FEBS Lett., 77 (1977) 99.
6 R. Shapiro, M.J. McManus, C. Zalut and H.F. Bunn, J. Biol. Chem., 255 (1980) 1406.
7 K. Altland, M. Kaempfer and H. Granda, Hum. Genet., 53 (1979) 97.
8 K. Valkonen, E. Gianazza and P.G. Righetti, Clin. Chim. Acta, 107 (1980) 223.
9 A. Francina, C. Baudonnet, E. Dorleac, F. Galacteros and J. Delaunay, Hemoglobin, 6 (1982) 481.
10 A. Rossi-Fanelli, E. Antonini and A. Caputo, Biochim. Biophys. Acta, 30 (1958) 608.
11 M. Castagnola, P. Caradonna, R. De Cristofaro, P.P. Sanna, D. Rossetti and M.L. Salvi, J. Res. Lab. Med., in press.
12 B. Trueb, C.G. Holenstein, R.W. Fisher and K.H. Winterhalter, J. Biol. Chem., 255 (1980) 6717.
13 W.R. Holmquist and W.A. Schroeder, Biochim. Biophys. Acta, 82 (1964) 639.
14 R.M. Bookchin and P.M. Gallop, Biochem. Biophys. Res. Commun., 32 (1968) 86.
15 R.J. Koenig, S.H. Blobstein and A. Cerami, J. Biol. Chem., 252 (1977) 2992.
16 W.A. Schroeder and T.H.J. Huisman, in M.K. Schwartz (Editor), Clinical and Biochemical Analysis, Vol. IX, Marcel Dekker, New York, 1980, pp. 39—40.

Journal of Chromatography, 307 (1984) 103—110
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2010

DETERMINATION OF GLYCOSYLATED HAEMOGLOBIN BY ISOELECTRIC FOCUSING IN NON-LINEAR pH GRADIENTS

GIANFRANCO COSSU, MARIO MANCA, M. GAVINA PIRASTRU and RENZA BULLITA

Laboratory of Immunohematology, A. Segni Hospital, Ozieri, Sassari (Italy)

ADRIANA BIANCHI BOSISIO

Clinical Chemistry Laboratory, S. Carlo Borromeo Hospital, Milan (Italy)

and

PIER GIORGIO RIGHETTI*

Department of Biomedical Sciences and Technologies and Chair of Biochemistry, Faculty of Pharmaceutical Sciences, University of Milano, Via Celoria 2, Milan 20133 (Italy)

(First received August 17th, 1983; revised manuscript received November 17th, 1983)

SUMMARY

A new isoelectric focusing technique for the separation and quantitation of glycosylated haemoglobin (HbA_{1c}) is described. By using an equimolar mixture of two separators (0.2 *M* β-alanine + 0.2 *M* 6-aminocaproic acid) a 2-pH unit Ampholine range (pH 6—8) is transformed in a shallow, 0.6-pH unit span (pH 6.7—7.3). This brings about an increment of resolution between HbA and HbA_{1c} by a factor of about three, thus allowing proper densitometric evaluation of the trichloroacetic acid-fixed MetHb bands by conventional gel scanners. Excellent agreement is found among microchromatography, isoelectric focusing followed by densitometry in situ, and isoelectric focusing followed by band excision, elution and spectrophotometric determination. The present method also allows full resolution between HbA_{1c} and fetal haemoglobins (F and F_{ac} bands).

INTRODUCTION

Haemoglobin A_{1c} is the major component of the glycosylated haemoglobin pool [1]. It derives from HbA molecules through a post-translational modifica-

0378-4347/84/$03.00

tion: the $-NH_2$ group of $\beta^{1\,Valine}$, in fact, reacts with glucose residues to form an aldimine adduct which subsequently undergoes an Amadori rearrangement to form a more stable ketoamine linkage [2]. The rate of these non-enzymatic reactions is primarily affected by the glucose concentration in the medium [3]. Determination of the percentage of HbA_{1c} in a haemoglobin sample is thus important in some pathological alterations of the glycosidic metabolic pathways. In red cells of patients with overt diabetes mellitus there is a two- to threefold increase in percentage of HbA_{1c} (from 4—7% to 10—18% of total Hb) [4]. It has therefore been proposed that the percentage of HbA_{1c} could be used in assessing the degree of diabetes, by providing an integrated measurement of blood glucose according to the red cell life span.

HbA_{1c} can be separated from HbA by isoelectric focusing (IEF) in commercial pH gradients [5—7] but accurate quantitative determinations of HbA_{1c} under these conditions can be obtained only by high-resolution microdensitometry [8—11]. Improved separations are necessary for the densitometric evaluation of HbA_{1c} by conventional scanners. They can be obtained by the use of separators [12, 13] or by resorting to home-made, narrow pH cuts [14]. However, home-made, shallow pH gradients (< 1 pH unit) [14] and some separators (such as the dipeptide His-Gly of ref. 12) will never be available in most routine clinical laboratories, thus severely limiting the use of these methodologies.

In an extensive screening of umbilical cord blood for β-thalassemias in northern Sardinia, we have recently described the generation of very flat pH gradients, easily obtainable from commercial Ampholine pH 6—8 spans by the addition of an equimolar mixture of two amphoteric substances, β-alanine and 6-aminocaproic acid [15]. Such gradients were most satisfactory for the separation and unambiguous quantitation of the three major components of cord blood: fetal, adult and acetylated fetal Hbs.

We report here how the same type of gradient can be used, in adult blood, for resolution and densitometric evaluation of HbA_{1c} in diabetic screening.

MATERIALS AND METHODS

Acrylamide, N,N′-methylene bisacrylamide, ammonium persulphate and N,N,N,N′-tetramethylethylenediamine (TEMED) were from Bio-Rad, Richmond, CA, U.S.A. β-Alanine (β-Ala) and 6-aminocaproic acid (6-ACA) were from BDH, Poole, U.K. Ampholine, pH 6—8, pH 7—9 and pH 3.5—10 ranges, were from LKB Produkter, Bromma, Sweden, and Silane A-174 (organosilane ester) was from Union Carbide Silicones, Sisterville, WV, U.S.A.

Sample collection

Adult blood, from normal and diabetic patients, was washed three to four times in saline (10 ml/ml blood). Red blood cells (RBC) were lysed in distilled water containing 0.05% potassium cyanide (5 vols. lysis solution per 1 vol. packed RBC) and, if not immediately analysed, stored as such at −80°C. Prior to IEF, CCl_4 is added to the lysate (0.5 vol. of CCl_4 per vol. of packed RBC) to precipitate the ghosts. After centrifugation for 10 min at 10,000 *g*, the sample is diluted to about 1% content and analysed by IEF.

IEF fractionation

For routine IEF analysis, we have found a 0.5 mm gel thickness to be most suitable, since it does not require any particular skill from technical staff. The slab can be cast vertically in a cassette utilizing a U-gasket cut out from a 0.5-mm-thick rubber sheet, or horizontally utilizing the ready-made LKB glass plates. When only a few samples have to be analysed, we use square 12.5 × 12.5 cm glass plates, which accommodate 14—15 samples; otherwise, the 25-cm-long glass slab can be used, which allows analysis of 28—30 samples. For densitometric analysis, we prefer to fix the polyacrylamide gel covalently to the glass plate via a silane bridge [16], so that the gel can be dried directly onto the glass plate without peeling off. For this purpose, the glass is dipped for 30 sec in 0.2% Silane A-174 in anhydrous acetone and then left to dry in the air. As a cassette cover, we use a Lucite slab (8—10 cm thick, to prevent warping) modified to accommodate a pocket-forming device. For this purpose, a strip of Dymo label (200—250 μm thin) is glued 2.5 cm from the long edge of the Lucite cover and then short strips are removed with a surgical blade so as to form 5 × 3 mm^2 pockets, at 2-mm intervals. The sample is usually applied at the cathodic side, in a volume of about 3—5 μl (corresponding to about 20—30 μg total protein load). The gel contains 6% T and 4% C (for definition see ref. 17), 2% Ampholine pH 6—8 and 0.2% Ampholine pH 3.5—10. Anodic and cathodic solutions are 1 *M* orthophosphoric acid and 1 *M* sodium hydroxide, respectively. Conditions for a typical run (in a 12.5 × 12.5 cm gel slab) are as follows: 15 min at 400 V, then the sample is loaded and the run is continued for 90 min at 15 W (with a limiting voltage of 1500 V, which is reached after 20 min from sample application). The run is performed on an LKB 2117 Multiphor cell with a constant-wattage power supply (LKB 2103) and with the thermostat at 4°C.

Shallow pH gradients

For a proper resolution of HbA_{1c} from HbA, non-linear pH gradients, which have a much shallower slope around pH 7, have to be utilized. For this purpose, we have gelled plates, as above, containing a mixture of 0.2 *M* β-Ala and 0.2 *M* 6-ACA, as previously suggested [15], and the following amounts of carrier ampholytes (in a final gel volume of 10 ml): 0.5 ml of pH 6—8, 0.12 ml of pH 7—9 and 0.05 ml of pH 3.5—10 ranges. In this latter case, since the migration to equilibrium position is slower, the gels are run, after sample application, for somewhat longer periods (120 min). In non-linear pH gradients, it is imperative to apply the sample at the cathode, since anodic application would result in very long focusing periods.

Densitometry

At the end of the IEF run, the gels are dipped in 20% trichloroacetic acid (TCA), as previously suggested [18], whereby all Hbs are fixed and converted to the ferric form (MetHb). After washing the gel in distilled water to remove excess TCA, the polyacrylamide layer is desiccated with a hair-dryer and the sample tracks are read and integrated at 465 nm with a Cliniscan densitometer (Helena, Beaumont, TX, U.S.A.). For comparing the present method with the microchromatography of Trivelli et al. [19], at the end of the IEF run, prior

TABLE I

COMPARISON BETWEEN CHROMATOGRAPHIC AND ISOELECTRIC FOCUSING DETERMINATION OF HbA_{1c}

The figures reported are percentile values of HbA_{1c}, taking as 100% the sum of the HbA and HbA_{1c} peaks.

Sample No.	Microchromatography	IEF (densitometry)	IEF (elution)
1	15.68	16.40	17.02
2	12.50	13.31	13.42
3	6.67	7.02	7.93
4	11.23	12.04	12.91
5	14.33	15.29	15.06
6	8.04	8.61	9.00
7	21.41	22.87	23.02
8	19.04	18.43	19.56
9	6.03	6.60	7.05
10	13.81	14.69	14.13
11	11.82	12.67	12.92
12	14.07	14.76	15.20
13	18.23	17.22	18.40
14	12.17	13.09	13.63
15	15.10	16.29	15.61
16	13.56	14.67	15.03
17	20.23	21.86	20.78
18	13.64	14.43	14.39
19	5.71	6.80	6.53
20	16.04	15.36	16.41
	a	c	
		b	

[a] $r = 0.988$, $P < 0.001$.
[b] $r = 0.995$, $P < 0.001$.
[c] $r = 0.990$, $P < 0.001$.

to TCA fixation, the focused Hb zones are removed with a surgical blade, eluted overnight in 1.5 ml (HbA_{1c}) and 4.5 ml (HbA) of 0.05% potassium cyanide and read in a spectrophotometer at 415 nm. These data, compared with the IEF scan values and the eluate values from the Bio-Rex 70 column, are tabulated in Table I.

Microchromatography

For comparison, the same samples were also analysed by microchromatography, as modified after Schnek and Schroeder [20]. We have used the Combi-Test Kit for HbA_{1c} from Boehringer Mannheim (F.R.G.). The columns were thermostatted at 23°C and the eluates read at 415 nm.

RESULTS

Fig. 1 (left) shows the separation of adult blood lysates in conventional Ampholine gradients encompassing a 2-pH unit span (pH 6—8). It can be seen

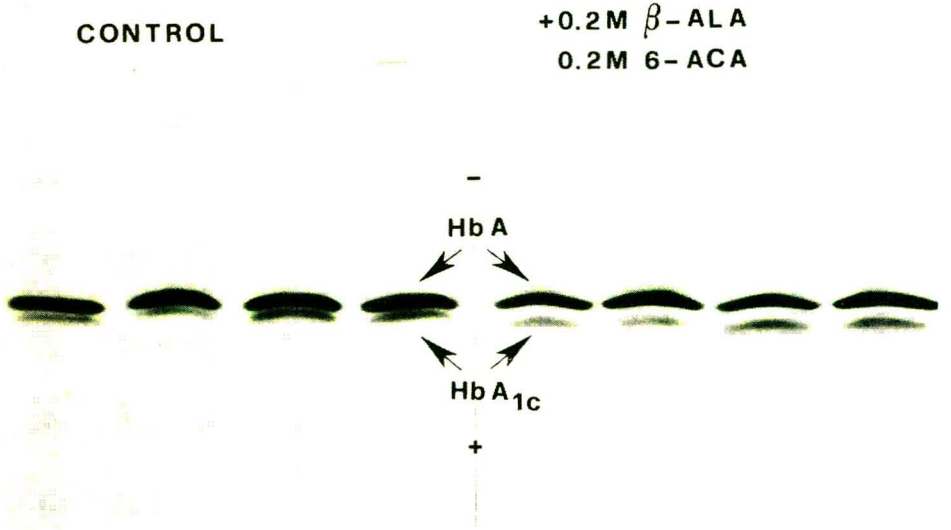


Fig. 1. Separation of normal human adult haemoglobin (HbA) from its glycosylated derivative (HbA_{1c}) by isolelectric focusing in polyacrylamide gel slabs. Left: control gel containing only 2% Ampholine pH 6—8, Right: the same gel with an equimolar (0.2 *M*) mixture of the two separators β-alanine and 6-aminocaproic acid. Experimental conditions are as described in Materials and methods.

that the glycosylated component (HbA_{1c}) is barely separated below the main HbA band: such a resolution is not generally enough for quantitation by the common scanners usually available in clinical laboratories. However, when the IEF run is repeated in a gel containing a mixture of separators (0.2 *M* β-Ala and 0.2 *M* 6-ACA), excellent resolution is obtained between HbA and its glycosylated form (Fig. 1, right). As shown in the densitometric scan of Fig. 2, in the latter case the tracing returns to the baseline before the onset of the second peak, thus allowing for meaningful integration; in the absence of separators, the HbA_{1c} peak is seen by the densitometer only as a shoulder of the main HbA zone.

What is happening in the presence of this mixture of "separators" is shown in Fig. 3: the 2-pH unit span of Ampholine pH 6—8 is converted into essentially a 0.6-pH unit interval (pH 6.7—7.3) which is centered on the isoelectric point of HbA (pI approximately 7.0). β-Ala flattens the portion of the pH gradient below HbA, while 6-ACA alters the pH course on the alkaline side of HbA. The combined action of the two "separators" produces a pH span which is optimal for separating not only HbA from HbA_{1c}, but also the fetal components, such as HbF and HbF_{ac} (the pI position of these four zones is shown by arrows in Fig. 3).

The reliability of this method is shown in Table I: in a double-blind experiment, twenty normal and diabetic samples were analysed by microchromatography, IEF followed by densitometry in situ and IEF followed by elution of excised bands and spectrophotometric quantitation. Excellent agreement was found among the three methods, as shown by the *r* and *P* values reported at the bottom of Table I, although there appears to be a trend, in both IEF

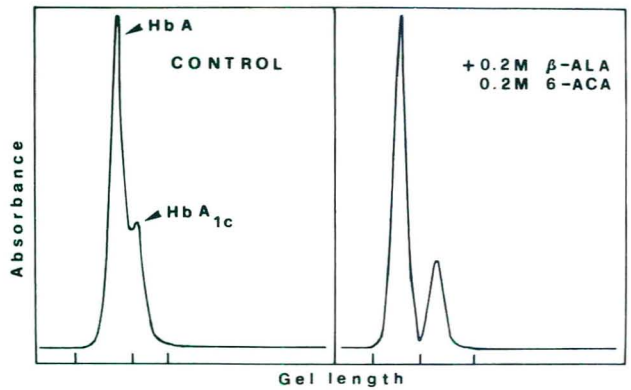


Fig. 2. Densitometric evaluation of the IEF-separated HbA and HbA_{1c} bands. Scans of TCA-precipitated, brown MetHb bands were performed at 465 nm with a Cliniscan densitometer. Left: scan of the Hb zones obtained by IEF in the control gel of Fig. 1. Right: scan of the Hb bands obtained by IEF in the presence of 0.2 *M* β-alanine and 0.2 *M* 6-amino caproic acid, as shown in Fig. 1 (right). The marks along the abscissa represent the integration intervals for each peak in the densitometric scan.

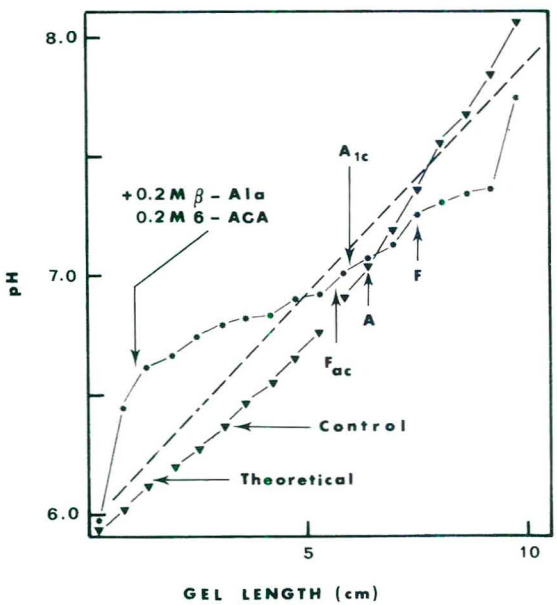


Fig. 3. Evaluation of the pH course in Ampholine gels. (- - -), Theoretical pH gradient in a gel containing only pH 6—8 ampholytes, assuming a linear pH course. (∗—∗), pH gradient obtained in a gel containing 2% Ampholine pH 6—8 and a mixture of 0.2 *M* β-alanine + 0.2 *M* 6-aminocaproic acid. (▾—▾), experimental pH gradient obtained with 2% Ampholine pH 6—8 in the absence of separators. In these last two cases, at the end of IEF, gel segments at 5-mm intervals were cut along the separation track, eluted with 300 μl of 10 m*M* potassium chloride and read in a pH meter. The arrows indicate the pI position, in the pH gradient with separators, of haemoglobins F, A, A_{1c} and F_{ac}.

analyses, to give somewhat higher amounts (5—10% higher). Four samples (Nos. 3, 6, 9 and 19) were correctly evaluated as normal.

DISCUSSION

In addition to the microchromatographic methods reported [19, 20], several other automatic techniques have been described for the separation and quantitation of HbA_{1c}. High-performance liquid chromatography [21, 22] has been adopted, but requires rather expensive equipment and still seems too sophisticated for non-specialized laboratories. Medium-pressure liquid chromatography [23] is also limited by cost factors in that it requires an expensive amino-acid analyser. Low-pressure liquid chromatography [14] uses simpler equipment and is cheaper to perform than the previous systems, but still has not found widespread application. The major drawback of all these chromatographic methods for assaying HbA_{1c}, is that this peak is eluted together with HbF [24]. Therefore when an abnormally elevated HbA_{1c} peak is found, the percentage of HbF has to be checked out by independent methods in order to rule out possible contamination of the HbA_{1c} peak. This problem is fully solved in our present technique: not only are HbA_{1c} and HbF widely separated (they focus on opposite sides as compared with the HbA zone) but the glycosylated Hb is also resolved from the acetylated HbF band (see Fig. 3 of the present report and Fig. 1B of ref. 15).

While several reports on IEF separation have been published [7—13] they do not seem to have found widespread application, possibly because of rather limited separation [7—10] or difficulties in synthesizing the right separator [12] or narrow pH range carrier ampholytes [14]. Even in what appears to be the simplest and most reliable method [9], the authors have, in a subsequent paper [25], resorted to band excision, overnight elution and spectrophotometric quantitation, thus rendering the technique no longer attractive for routine separations. Jeppsson et al. [25] have used 0.33 *M* β-Ala as the sole separator in their IEF gels: since β-Ala has a pI of 6.9, just below the pI of HbA_{1c} (6.95) and of HbA (pI 7.0), it is quite possible that, as the separation proceeds and the cathodic drift becomes more pronounced [26], the two protein zones roll down from the β-Ala plateau and fall in a pH region where the steep incline compresses the separation. In our present case, since we have made sure that the pH gradient is flattened both below and above the pI of the components of interest, the protein zones can drift along the wide plateau formed by the mixture of the two separators without hampering the separation obtained. The method is fast, accurate and requires only simple equipment. Moreover, several gels can be polymerized simultaneously and stored in the refrigerator before use for periods of at least two months.

We should like to emphasize that excellent separations of HbA_{1c} are obtained also in the recently invented technique of immobilized pH gradients [27, 28] but the gel casting technique is more complex and the focusing times, at present, are rather long (8—10 h).

ACKNOWLEDGEMENT

Supported in part by grants from the Ministero della Pubblica Istruzione (Roma).

REFERENCES

1 W.R. Holmquist and W.A. Schroeder, Biochemistry, 5 (1966) 2489—2503.
2 H.F. Bunn, D.N. Haney, K.H. Gabbay and P.M. Gallop, Biochem. Biophys. Res. Commun., 67 (1975) 103—109.
3 R. Flückiger and K.H. Winterhalter, FEBS Lett., 71 (1976) 356—360.
4 H.F. Bunn, K.H. Gabbay and P.M. Gallop, Science, 200 (1978) 27—33.
5 J.W. Drysdale, P.G. Righetti and H.F. Bunn, Biochim. Biophys. Acta, 229 (1971) 42—51.
6 P. Basset, Y. Beuzard, M.C. Garel and J. Rosa, Blood, 51 (1978) 971—980.
7 R. Schoos, S. Schoos-Barbette and C. Lambotte, Clin. Chim. Acta, 86 (1978) 61—65.
8 K.M. Spicer, R.C. Allen and M.G. Buse, Diabetes, 27 (1978) 384—388.
9 J.O. Jeppsson, B. Franzén and K.O. Nilsson, Sci. Tools, 25 (1978) 69—75.
10 K.M. Spicer, R.C. Allen, D. Hallett and M.G. Buse, J. Clin. Invest., 64 (1979) 40—46.
11 H.B. Mortensen, J. Chromatogr., 182 (1980) 325—333.
12 L. Beccaria, G. Chiumello, E. Gianazza, B. Luppis and P.G. Righetti, Amer. J. Hematol., 4 (1978) 367—374.
13 M.D. Fitzgerald and M.N. Cauchi, Amer. J. Hematol., 9 (1980) 311—316.
14 P. Basset, F. Braconnier and J. Rosa, J. Chromatogr., 227 (1980) 267—304.
15 G. Cossu, M. Manca, M.G. Pirastru, R. Bullitta, A. Bianchi-Bosisio, E. Gianazza and P.G. Righetti, Amer. J. Hematol., 13 (1982) 149—157.
16 A. Bianchi-Bosisio, C. Loeherlein, R.S. Snyder and P.G. Righetti, J. Chromatogr., 189 (180) 317—330.
17 S. Hjertén, Arch. Biochem. Biophys. Suppl. 1 (1962) 147—151.
18 F. Galacteros, K. Kleman, J. Caburi-Martin, Y. Beuzard, J. Rosa and B. Lubin, Blood, 56 (1980) 1068—1071.
19 L.A. Trivelli, H.M. Ranney and H. Lai, N. Engl. J. Med., 284 (1971) 353—357.
20 A.G. Schnek and W.A. Schroeder, J. Amer. Chem. Soc., 83 (1961) 1472—1478.
21 P.S. Dunn, R.A. Cole and J.S. Soeldner, Metabolism, 28 (1979) 777—783.
22 C.A. Gruber and M.D. Kolts, Clin. Chem., 25 (1979) 1970—1972.
23 H. Wajcman, B. Dastugue and D. Labie, Clin. Chim. Acta, 92 (1979) 33—38.
24 T.K. Mayer and Z.R. Freedman, Clin. Chim. Acta, 127 (1983) 147—184.
25 J.O. Jeppson, B. Franzén and A.B. Gaal, in B.J. Radola (Editor), Electrophoresis '79, de Gruyter, Berlin, 1980, pp. 655—661.
26 P.G. Righetti and J.W. Drysdale, Ann. N.Y. Acad. Sci., 209 (1973) 163—186.
27 B. Bjellqvist, K. Ek, P.G. Righetti, E. Gianazza, A. Görg, W. Postel and R. Westermeier, J. Biochem. Biophys. Methods, 6 (1982) 317—339.
28 E. Gianazza, P.G. Righetti, B. Bjellqvist, K. Ek, A. Görg and R. Westermeier, Prot. Biol. Fluids, 30 (1983) 603—606.

Journal of Chromatography, 307 (1984) 111—119
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2024

DETERMINATION OF UROCHLORALIC ACID, THE GLUCURONIC ACID CONJUGATE OF TRICHLOROETHANOL, BY GAS CHROMATOGRAPHY WITH ELECTRON-CAPTURE DETECTION AND ITS APPLICATION TO URINE, PLASMA AND LIVER

MIKIKO IKEDA*, HIROKO HATTORI, YOKO KOYAMA (TSUDA) and SHINJI OHMORI

Faculty of Pharmaceutical Sciences, Okayama University, Tsushima-Naka-1, Okayama 700 (Japan)

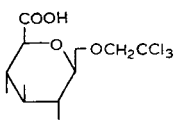
(Received October 12th, 1983)

SUMMARY

Gas chromatography with electron-capture detection was used to quantify the glucuronic acid conjugate of trichloroethanol, urochloralic acid. The conjugate was extracted into ethyl acetate—ethanol (19:1, v/v) under acidic conditions, and analyzed as its trifluoroacetylated methyl ester or as its acetylated methyl ester. As little as 0.2 μg of the conjugate was quantified, and the method was applicable to urine, plasma and liver. The synthesis of urochloralic acid from trichloroethanol by rat liver microsomal fraction was demonstrated in the presence of uridine diphosphoglucuronic acid as coenzyme.

INTRODUCTION

We have studied the metabolism of trichloroethylene in vitro using rat liver [1]. It is well known that trichloroethylene is oxidized to its epoxide, which is transformed to chloral hydrate and successively reduced to trichloroethanol (TCE). TCE has been reported to be conjugated with glucuronate in vivo to form urochloralic acid (UCA). Human and animals excrete UCA along with



TCE when they have inhaled trichloroethylene vapour [1—4], or have been administered chloral hydrate [4, 5], triclofos [6], or TCE [4, 7]. However, biochemical studies on the glucuronide formation have not until now been reported. We desired to investigate the formation of UCA in vitro, but found no sensitive method for its determination.

0378-4347/84/$03.00

A number of methods for the determination of UCA have been published [8—12]. In our preliminary experiments, colorimetry of UCA [8, 9] could not be applied to the biochemical studies in vitro because of its low sensitivity and low specificity. With the gas chromatographic methods [10—12] the amount of UCA is calculated as the difference between free TCE and total TCE determined after hydrolysis of UCA by acid or β-glucuronidase. The indirect method for the assay of UCA has been widely applied to serum and urine samples, but this method is more useful if UCA is present in much larger, or about equal, amounts compared to free TCE in the samples. However, when determining a relatively small amount of UCA as, for example, in our in vitro experiment on the conjugation of TCE, the indirect method is inadequate for accurate determination.

Here we describe a direct and sensitive method for the assay of UCA using gas chromatography with electron-capture detection (ECD). The method is applicable to urine, plasma and liver homogenates. Several features of the conjugation of TCE in vitro are also presented here.

MATERIALS AND METHODS

Reagents and instruments

All chemicals were of analytical grade, and the organic solvents for extraction were of especially fine grade; these were purchased from Wako Pure Chemicals (Osaka, Japan). Uridine diphosphoglucuronic acid (UDPGA) was obtained from Yamasa Shoyu (Chiba, Japan), and uridine diphospho-N-acetylglucosamine (UDPAG) and ATP from Sigma (St. Louis, MO, U.S.A.). Authentic methyl ester of acetyl-UCA was synthesized in this laboratory according to the method of Seto and Schultze [13]; authentic UCA was kindly supplied by the Central Institute of Chugai Pharmaceutical Co. (Tokyo, Japan).

Organic solvents were evaporated under reduced pressure by a Speed Vac Concentrator (Savant, U.S.A.).

Extraction of UCA from biological samples

An aliquot (0.5 ml) of plasma, urine, or liver homogenate prepared as described below was added to *n*-hexane—benzene (1:1, v/v, 1.5 ml), stirred vigorously, and centrifuged at 1000 *g* for 10 min. After removal of the organic layer by aspiration, the same procedure was repeated again. After acidification of the aqueous phase with 6 *M* hydrochloric acid (0.1 ml), UCA was extracted twice with ethyl acetate—ethanol (19:1, v/v) (1.5 and 1 ml). The organic solvents were combined, dried over anhydrous sodium sulphate, and evaporated to dryness under reduced pressure.

Rat liver was homogenized in 9 vols. of 1.15% potassium chloride with a Potter-Elvejhem PTFE pestle homogenizer. The homogenate was centrifuged at 700 *g* for 10 min. The supernatant fluid was used for recovery tests. When the formation of UCA in vitro was studied using rat liver microsomal fraction, the incubation mixtures described below were treated in the same manner as described above.

Derivatization of UCA

The residue containing UCA was dissolved in 1 ml of ethyl acetate and 0.1 ml of methanol, into which diazomethane was introduced according to the microscale procedure of Schlenk and Gellerman [14]. After standing at room temperature for about 30 min, the solvent was evaporated to dryness under reduced pressure. To the dried residue, ethyl acetate (175 μl) and trifluoroacetic anhydride (TFAA, 25 μl) were added in turn. For acetylation, pyridine (150 μl) and acetic anhydride (50 μl) were added to the residue. Both acetylating reaction mixtures were allowed to stand at room temperature overnight. In the case of trifluoroacetylation, an aliquot (1 μl) of the reaction mixture was injected into the gas chromatograph after appropriate dilution with ethyl acetate. In the case of the acetylation, excess reagents were evaporated under a nitrogen stream at about 40°C, and the residue was dissolved in ethyl acetate (0.2 ml). An aliquot (1 μl) was subjected to gas chromatography after appropriate dilution with ethyl acetate.

Gas chromatography

The gas chromatographic analysis was carried out on a Shimadzu Model GC-4CM gas chromatograph equipped with an electron-capture detector (^{63}Ni), which was operated at a pulse-rate of 10 kHz and a temperature of 230°C for the methyl ester of trifluoroacetyl-UCA and 270°C for that of acetyl-UCA. The glass column, 2 m × 3 mm I.D., was packed with 1.5% Silicone OV-17 on Shimalite W (AW DMCS), 80—100 mesh. The carrier gas was nitrogen at a flow-rate of 40 ml/min. The electrometer setting was continuously kept at range 10^2, attenuation 8. The injection temperature was 230°C and the column oven temperature 180°C for trifluoroacetyl-UCA, and 270°C and 250°C for acetyl-UCA. The peak height was used for quantification.

Preparation of enzyme source

Livers of male Wistar strain albino rats (200—300 g body weight) were used as enzyme source. Rats were killed by decapitation, and the microsomal fraction was prepared by the method of Schöllhammer et al. [15]. Briefly, liver homogenates were centrifuged at 9000 *g* for 20 min, and the supernatant fluid decanted was centrifuged at 105,000 *g* for 60 min. The pellet thus obtained was suspended in an aliquot of 1.15% potassium chloride, and recentrifuged at 105,000 *g* for 60 min. After repeating this washing procedure, the pellet was suspended in a volume of 1.15% potassium chloride equivalent to the original weight of wet liver; this is referred to as the "washed microsomal fraction".

Incubation

The incubation system for conjugation was constituted according to the method of Schöllhammer et al. [15]. The incubation mixture consisted of 0.1 ml of the washed microsomal fraction, 2.5 m*M* magnesium chloride, 0.5 m*M* TCE, 0.5 m*M* UDPGA and 37.5 m*M* Tris—HCl buffer (pH 7.4) in a total volume of 0.5 ml. The mixture in a 10-ml test tube was incubated at 37°C for 60 min with shaking.

UCA formed in the incubation mixture was extracted, reacted with diazomethane and acetylated as described above.

RESULTS

Reaction conditions for derivative formation

Typical gas chromatograms are shown in Fig. 1a, indicating that symmetrical sharp peaks appeared for both trifluoroacetylation and acetylation.

Trifluoroacetylation. To establish optimum reaction conditions for trifluoroacetylation of methyl urochloralate, the reaction temperature and time, and the amount of TFAA were investigated. UCA (2 mg) was previously reacted with diazomethane as described under Materials and methods, and aliquots corresponding to 5 and 10 μg of UCA were reacted with TFAA under various conditions. As shown in Fig. 1b, the maximum peak height was obtained after reaction at room temperature for about 8 h, and remained constant up to 46 h. However, reaction at 60°C for 6 h resulted in the disappearance of the peak. It required 20—30 μl of TFAA to acetylate the methyl ester quantitatively.

Acetylation. We examined the acetylation conditions of methyl uro-

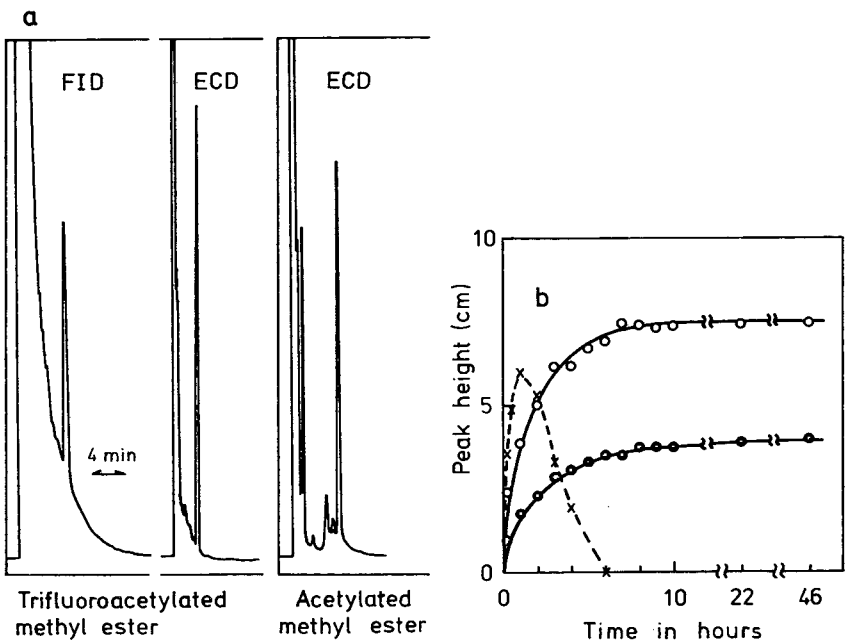


Fig. 1. Typical gas chromatograms obtained from authentic UCA, and reaction conditions for trifluoroacetylation. (a) UCA (10 μg) was reacted with diazomethane and TFAA or acetic anhydride as described under Materials and methods. In the case of the trifluoroacetylation, a 5-μl aliquot of the reaction mixture (0.2 ml) was subjected to gas chromatography with flame-ionization detection (GC—FID), and a 1-μl aliquot after 2000 times dilution with ethyl acetate to GC—ECD. Analytical conditions for GC—FID were as follows: injection and detector temperature 200°C, column temperature 160°C, flow-rate of nitrogen 50 ml/min, attenuation 8, sensitivity 10^3. In the case of acetylation, a 1-μl aliquot of the reaction mixture was injected for GC—ECD after 200 times dilution with ethyl acetate. Analytical conditions were the same as described under Materials and methods. (b) Methyl urochloralate [corresponding to 5 (•) and 10 μg (○) of UCA] was reacted with TFAA (30 μl) in ethyl acetate (0.17 ml) at 25°C (——) and 60°C (- - -) for varius time intervals. In the latter case methyl urochloralate corresponding to 10 μg of UCA was tested. A 5-μl aliquot was subjected to GC—FID.

chloralate with acetic anhydride, by comparison with authentic methyl ester of acetyl-UCA. The best yield was obtained when 25—50 μl of acetic anhydride were added to the reaction mixture (0.2 ml in a total volume with pyridine), followed by standing at room temperature overnight.

Standard and calibration curves

The present assay consists of extraction (Step 1), methylation (Step 2) and acetylation (Step 3). Three experiments were carried out: Step 3 only; Steps 2 and 3; and all the steps. Three curves were prepared as shown in Fig. 2. Accordingly, the difference between standard curves I and II gives the yield of methylation (92%), which proved to fall 40% below 1 μg of UCA in 0.2 ml of the reaction mixture (see inset to Fig. 2). The difference between standard curve II and the calibration curve gives the extraction ratio (89%).

These results were obtained for trifluoroacetylation; the acetylation method gave a similar result (data not shown).

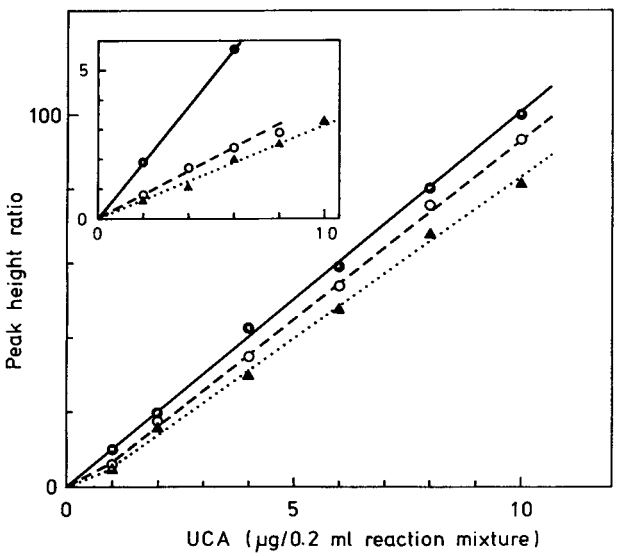


Fig. 2. Standard and calibration curves of the methyl ester of trifluoroacetyl-UCA. Standard curve I (•): UCA (1 mg) was dissolved in ethyl acetate (about 1 ml), reacted with diazomethane at room temperature for 30 min, and excess reagent and the solvent were evaporated. The residue was dissolved in ethyl acetate (1.0 ml), from which standard solutions (corresponding to 100 μg and 10 μg UCA per ml ethyl acetate) were prepared. An aliquot (corresponding to 0.2—10 μg of UCA) of the standard solutions was subjected to trifluoroacetylation, and to gas chromatography as described under Materials and methods. Standard curve II (○): The UCA standard solutions in ethyl acetate (100 and 10 μg/ml) were prepared, and an aliquot (corresponding to 0.2—10 μg of UCA) of the standard solutions was subjected to methylation and trifluoroacetylation, and to gas chromatography as described under Materials and methods. Calibration curve (▲): An aqueous solution (0.5 ml) containing 0.2—10 μg of UCA was prepared, extracted, and derivatized as described under Materials and methods. A 1-μl aliquot of the reaction mixture was injected into the gas chromatograph after appropriate dilution with ethyl acetate. The peak height obtained from 10 μg of UCA in standard curve I was multiplied by a dilution factor (2000), and is represented as 100 in the figure.

Recovery tests

In order to apply the present procedure to the assay of UCA in urine, plasma and liver homogenates, typical gas chromatograms were taken after trifluoroacetylation or acetylation (see Fig. 3), and then recovery tests were carried out. As shown in Fig. 3a, the trifluoroacetylation method was applicable to plasma and liver. However, trifluoroacetylation was found to be unsuitable for UCA determination in urine, since a large peak behind the methyl ester of trifluoroacetyl-UCA interfered (data not shown). Therefore, the acetylation method had to be applied to urine, and the separation of the peaks was satisfactory (Fig. 3b). For the assay of UCA in liver homogenates the acetylation method was also applicable, and was time-saving (compare the chromatograms in Fig. 3a and b). Acetylation could be applied to plasma, but a chromatogram is not shown.

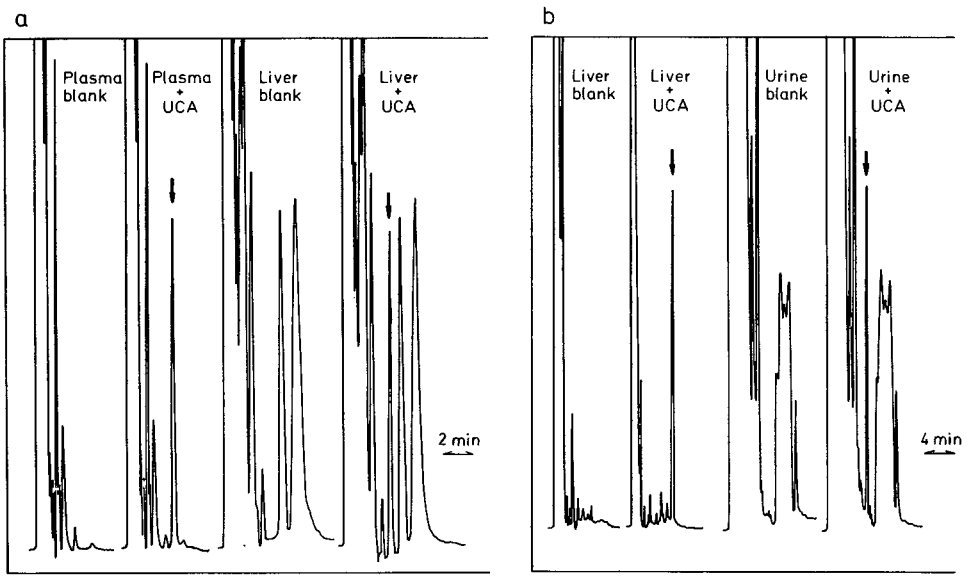


Fig. 3. Typical gas chromatograms obtained from biological samples with and without addition of UCA. After addition of UCA (1 μg) or no addition to biological samples (plasma, liver and urine), they were treated as described under Materials and methods. In the case of trifluoroacetylation (a), a 1-μl aliquot of the reaction mixture was injected into the gas chromatograph after 200 times dilution with ethyl acetate. In the case of acetylation (b), a 1-μl aliquot was subjected to gas chromatography after 20 times dilution. The arrows in the figure indicate the peaks of the derivatives.

Varying amounts of UCA were added to each sample, and the amount of UCA was measured as the methyl ester of trifluoroacetyl-UCA for plasma and liver homogenates, and that of acetyl-UCA for urine. The results are summarized in Table I, indicating that good recoveries were obtained in all cases.

UCA formation by rat liver microsomal fraction

In order to clarify the overall profile of the in vitro formation of UCA, experiments using rat liver microsomal fraction as the enzyme source were per-

TABLE I

RECOVERY TESTS

Biological samples (0.5 ml) with and without addition of UCA were treated in the same manner as described in Materials and methods. Each value represents the mean of duplicate determinations.

Specimen	UCA added (μg)	UCA determined (μg)	Recovery	
			%	Mean ± S.D.
Urine	0	0		97.3 ± 6.9
	1	0.870	87.0	
	2	1.96	98.0	
	3	2.99	99.7	
	5	5.30	106.0	
	10	9.60	96.0	
Plasma	0	0		84.1 ± 4.3
	1	0.876	87.6	
	2	1.73	86.5	
	3	2.42	80.7	
	5	3.91	78.2	
	10	8.74	87.4	
Liver homogenate	0	0		97.4 ± 4.5
	1	0.994	99.4	
	2	1.91	95.5	
	3	2.87	95.7	
	5	4.61	92.2	
	10	10.4	104.0	

formed, and the amount of UCA was determined by the present procedure (acetylation method). The results were as follows.

Effect of incubation time, pH and amount of enzyme. Fig. 4a shows the effect of incubation time on the formation of UCA, revealing that the conjugation proceeds even after 120 min. In this experiment, about 4.5% of the amount of TCE added had been conjugated to UCA after 120 min of incubation. Fig. 4b depicts the effect of pH on the formation of UCA, indicating that the pH optimum is around 7. The dependency of the amount of microsomal fraction on the formation of UCA is shown in Fig. 4c. About 12 nmol of UCA were formed in a test tube that contained about 5 mg of microsomal protein.

Requirements for cofactors. Cofactor requirements for the conjugation were examined. The omission of UDPGA from the complete system described under Materials and methods caused no formation of UCA, while omission of magnesium chloride decreased the formation of UCA from 5.06 nmol/mg protein in the complete system to 2.69 nmol/mg protein. The addition of ATP and UDPAG to the complete system increased the formation of UCA to 17.5 nmol/mg protein; this finding will be discussed later.

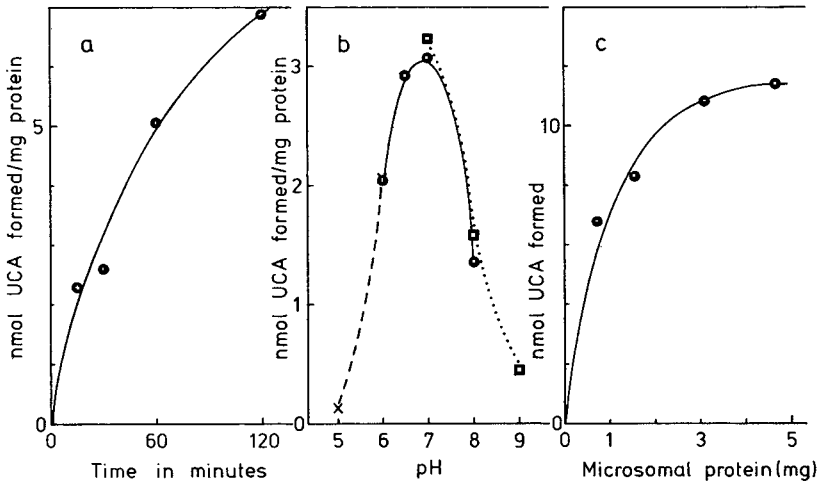


Fig. 4. Effect of incubation time, pH and amount of enzyme source on the formation of UCA. (a) TCE (0.5 m*M*) was incubated with microsomal protein corresponding to 100 mg of wet weight liver in the system described under Incubation at 37°C for 15, 30, 60 or 120 min. (b) Incubation was conducted at 37°C for 60 min as described above, except that acetate (×), potassium phosphate buffer (•) or Tris—HCl (▪) was used to examine the effect of pH on the formation of UCA. (c) TCE (0.5 m*M*) was incubated with various amounts of microsomal fraction from 0.05 to 0.3 ml (about 5 mg of protein) at 37°C for 60 min as described above.

DISCUSSION

As described in Introduction, UCA has been determined as TCE after hydrolysis. Attempts to apply the methods so far published to an in vitro study of the conjugation of TCE with glucuronate were unsuccessful. When UCA was synthesized from TCE by rat liver microsomal fraction, a much smaller amount of UCA than TCE was formed. In this case we had first to remove a large excess of free TCE through repeated extraction with *n*-hexane, but the TCE could not be removed completely. Therefore the exact amount of UCA could not be determined from the difference between total TCE after hydrolysis and remaining TCE by gas chromatography. However, evaporation of the incubation mixture under reduced pressure resulted in complete removal of free TCE, and UCA could be determined as TCE after hydrolysis. By this method, it is not proved whether only glucuronide was determined or other conjugates. For this reason we desired to determine UCA itself.

At first we tried to determine UCA as its trimethylsilylated methyl ester as reported by Verweij and Kientz [16]. That is, UCA was reacted with diazomethane, followed by treatment of the dried residue containing methyl urochloralate with pyridine (0.15 ml), trimethylsilane (50 μl) and hexamethyldisilazane (50 μl) at room temperature for 30 min. The derivative was subjected to gas chromatography with flame-ionization detection. The reproducibility of derivative formation was found to be unsatisfactory.

We thus established two derivatization procedures applicable to biological samples, i.e. trifluoroacetylation and acetylation of the hydroxyl groups of the glucuronic acid moiety. When both methods were compared, the ECD response

of the methyl ester of acetyl-UCA was about one-tenth that of trifluoroacetyl-UCA. However, in practice, the sensitivity of the two methods was almost the same, since at least 100 times dilution of the reaction mixture was necessary to avoid tailing due to TFAA. On the other hand, the acetylated derivative could be analyzed without such interference even after five times dilution of the reaction mixture. In the case of liver homogenates, several peaks appeared behind the acetylated derivative (see Fig. 3); and the trifluoroacetylation method was not applicable to urine as described in Results. As a whole, the acetylation method seems to be more advantageous than the trifluoroacetylation method for analysis of UCA.

UDP-glucuronyltransferase (EC 2.4.1.17), which is responsible for the formation of UCA from TCE, is predominantly located in the liver endoplasmic reticulum [17]. The latency of the enzyme activity in the microsomes has been reported by several investigators [18—24] who studied the conjugation of *p*-nitrophenol, phenolphthalein or bilirubin with glucuronate in vitro. They previously treated the microsomes with perturbants or several reagents to activate the enzyme activity. Similar induction phenomena were observed for TCE as described in the present paper. The addition of ATP and UDPAG, which are supposed to be allosteric effectors for the liver enzyme, resulted in about a four-fold increase in the conjugation of TCE. The concentration of UDPAG in this experiment was 1.24 m*M*, while that in liver was reported to be 0.32 m*M* [25]. Magnesium ion was also shown to stimulate the conjugation of TCE.

REFERENCES

1 T.C. Butler, J. Pharmacol. Exp. Ther., 97 (1949) 84.
2 B. Souček and D. Vlachová, Brit. J. Ind. Med., 17 (1960) 60.
3 J.W. Daniel, Biochem. Pharmacol., 12 (1963) 795.
4 G. Kimmerle and A. Eben, Arch. Toxicol., 30 (1973) 115.
5 E.M. Sellers, M. Lang, J. Koch-Weser, E. LeBlanc and H. Kalant, Clin. Pharmacol. Ther., 13 (1972) 37.
6 E.M. Sellers, M. Lang-Sellers and J. Koch-Weser, J. Clin. Pharmacol., 18 (1978) 457.
7 E.W. Garret and H.J. Lambert, J. Pharm. Sci., 62 (1973) 550.
8 B.E. Cabana and P.K. Gessner, Anal. Chem., 39 (1967) 1449.
9 M. Ogata, Y. Takatsuka and K. Tomokuni, Brit. J. Ind. Med., 27 (1970) 378.
10 D.D. Breimer, H.C.J. Ketelaars and J.M. van Rossum, J. Chromatogr., 88 (1974) 55.
11 R. Helbolsheimer and L. Funk, Arch. Toxicol., 32 (1974) 209.
12 M. Ogata and T. Saeki, Int. Arch. Arbeitsmed., 33 (1974) 49.
13 T.A. Seto and M.O. Schultze, J. Amer. Chem. Soc., 78 (1956) 1616.
14 H. Schlenk and J.L. Gellerman, Anal. Chem., 32 (1960) 1412.
15 I. Schöllhammer, D.S. Poll and M.H. Bickel, Enzyme, 20 (1975) 269.
16 A. Verweij and C.E. Kientz, J. Chromatogr., 226 (1981) 165.
17 C.B. Kasper and D. Henton, in W.B. Jakoby (Editor), Enzymatic Basis of Detoxication, Vol. 2, Academic Press, New York, 1980, pp. 4—27.
18 A.B. Graham and G.C. Wood, Biochim. Biophys. Acta, 276 (1972) 392.
19 A.B. Graham and G.C. Wood, Biochim. Biophys. Acta, 311 (1973) 45.
20 A. Winsnes, Biochim. Biophys. Acta, 191 (1969) 279.
21 G.W. Lucier, O.S. McDaniel and H.B. Matthews, Arch. Biochem. Biophys., 145 (1971) 520.
22 A. Winsnes, Biochim. Biophys. Acta, 284 (1972) 394.
23 A. Winsnes, Biochim. Biophys. Acta, 289 (1972) 88.
24 N. Nemoto and H.V. Gelboin, Biochem. Pharmacol., 25 (1976) 1221.
25 C. Berry, A. Stellon and T. Hallinan, Biochem. Soc. Trans., 2 (1974) 937.

Journal of Chromatography, 307 (1984) 121—127
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2007

DIRECT ENANTIOMERIC RESOLUTION OF MEPHENYTOIN AND ITS N-DEMETHYLATED METABOLITE IN PLASMA AND BLOOD USING CHIRAL CAPILLARY GAS CHROMATOGRAPHY

P.J. WEDLUND, B.J. SWEETMAN, C.B. McALLISTER, R.A. BRANCH and G.R. WILKINSON*

Department of Pharmacology, Vanderbilt University, Nashville, TN 37232 (U.S.A.)

(First received August 4th, 1983; revised manuscript received November 18th, 1983)

SUMMARY

A gas chromatographic method was developed for the determination of the *R*- and *S*-enantiomers of the anticonvulsant, mephenytoin, and its N-demethylated metabolite, 5-phenyl-5-ethylhydantoin (PEH), in plasma and blood. Direct enantiomeric separation of mephenytoin and its internal standard was obtained using a chiral capillary column (Chirasil-Val®) followed by nitrogen specific detection. However, resolution of the enantiomers of PEH and its internal standard required propylation at the 3-position of the hydantoin ring prior to analysis. Similar linear and reproducible standard curves were obtained from both plasma and blood over the concentration range 50 ng/ml to 5 μg/ml, and above 100 ng/ml the reproducibility was less than 8% (coefficient of variation).

Pronounced stereoselective differences in the plasma concentration—time curves for both mephenytoin and PEH were observed in a normal subject who received a single oral dose of 300 mg racemic mephenytoin. The peak plasma level of *S*-mephenytoin was only one-fifth that of the *R*-enantiomer and its elimination half-life was less than 3 h compared to over 70 h for *R*-mephenytoin. Similarly, *S*-PEH levels were barely detectable whereas concentrations of *R*-metabolite steadily increased over 4—6 days before slowly declining.

INTRODUCTION

Molecular interactions in biological systems frequently exhibit stereoselectivity of which drug action is a particular example. Numerous examples exist where optical enantiomers exhibit qualitatively and quantitatively different pharmacological activities as a result of differences in receptor interactions and/or drug disposition within the body. For example, the metabolism of the anticonvulsant, mephenytoin, has been shown to be stereoselective in both the dog [1] and in the majority of humans [2]. In the latter case, the hydroxyla-

0378-4347/84/$03.00

tion of the *S*-(+) enantiomer appears to be almost stereospecific [2]. The degree of stereoselectivity in drug disposition processes may be assessed by separately studying each enantiomer in vivo; however, this cumbersome approach neglects the possibility that one of the isomers may have an effect on the other. The latter is an important consideration since the majority of chiral drugs are clinically evaluated and marketed as racemic mixtures. Accordingly, stereoselective drug disposition is best and most directly studied by an analytical technique which resolves the two enantiomers following administration of the racemate.

The use of a pseudoracemate consisting of differentially labelled enantiomers based on either radioactive [2] or heavy elements [3] has been successfully used, but this elegant approach often has significant limitations and is not too well suited to the usual therapeutic situation, where unlabelled drug is administered. For example, the earlier studies with mephenytoin [2] were based on urinary excretion profiles using radiolabelled enantiomers, the specific activities of which were too low to permit measurements of the circulating plasma concentrations of the compounds of interest. Gas—liquid chromatography has been successfully applied to the enantiomeric resolution problem using two approaches. The first is dependent on derivatization with a pure enantiomer of an optically active reagent and separation of the resulting diastereoisomers on a non-chiral stationary phase [4—8]. Alternatively, direct separation of the enantiomers on a chiral column is possible [9—14]. The second approach is obviously more desirable because of its analytical simplicity, and the recently developed chiral phases based on polysiloxanes with covalently bound amino acid or peptide groups [15—17] potentially offer several advantages over more established methods [9—14]. Accordingly, the suitability of this type of phase, specifically Chirasil-Val®, to separate the enantiomers of mephenytoin and its demethylated metabolite was investigated, and an analytical procedure was developed to routinely determine these compounds in biological fluids, including blood and plasma.

EXPERIMENTAL

Chemicals

The purified enantiomers of mephenytoin (3-methyl-5-phenyl-5-ethylhydantoin) and 5-phenyl-5-ethylhydantoin (PEH) were kindly supplied by Dr. Adrian Küpfer (University of Bern, Bern, Switzerland). Racemic mephenytoin was a gift from Sandoz Pharmaceuticals (Hanover, NJ, U.S.A.). Propiophenone, butyrophenone, isobutyrophenone, 5-phenyl-5-methylhydantoin and hydantoin were obtained from Aldrich (Milwaukee, WI, U.S.A.). Iodomethane, iodoethane, 1-iodopropane, 2-iodopropane, 1-iodobutane and 2-iodo-2-methylbutane were purchased from Eastman Kodak (Rochester, NY, U.S.A.). Glass-distilled methanol and dichloroethane were obtained from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). All chemicals and reagents were used without further purification.

Synthesis

The syntheses of racemic PEH, 5-phenyl-5-propylhydantoin and 5-phenyl-5-

isopropylhydantoin were carried out according to the methods of Henze and Isbell [18] utilizing propiophenone, butyrophenone and isobutyrophenone, respectively, as starting material. Alkylation of the hydantoins at the 3-position was achieved by dissolving the hydantoin in 0.1 *M* sodium hydroxide in methanol—iodoalkane (4:1) and stirring the mixture at 50°C for 24 h, except for the reaction with iodomethane which was carried out at room temperature. Alkylation was stopped by adding 4 parts 0.1 *M* hydrochloric acid and extracting the final product into 12 parts dichloroethane. The organic layer was then washed twice with an equal volume of aqueous 0.1 *M* sodium hydroxide to remove any unreacted hydantoin. The dichloroethane layer was then evaporated to dryness at room temperature under nitrogen and the resulting residue was utilized without further purification.

Possible internal standard for mephenytoin and its N-demethylated metabolite, PEH, were examined by synthesizing the methyl through butyl series of alkylated derivatives of 5-phenyl-5-methyl-, 5-phenyl-5-ethyl-, 5-phenyl-5-propyl-, and 5-phenyl-5-isopropylhydantoins, respectively. Solutions of the selected compounds, 3-methyl-5-phenyl-5-isopropylhydantoin (3MIPPH) and 5-phenyl-5-propylhydantoin (PPH), were prepared in ethyl acetate to provide concentrations of 8 and 17 μg/ml, respectively.

Analytical procedure

Internal standard solutions (0.1 ml each) were added to 1 ml of plasma or blood which was then acidified with 1 ml of 0.01 *M* acetic acid and extracted with 6 ml of dichloromethane by reciprocal shaking. Following centrifugation, the supernatant was removed by aspiration and the organic phase transferred to a 5-ml Reacti-Vial™ (Pierce Chemical, Rockford, IL, U.S.A.) and evaporated to dryness under nitrogen at room temperature. The residue was dissolved in 1 ml of 4:1 solution of 0.1 *M* sodium hydroxide in methanol—iodopropane and then incubated in a water bath at 50°C for 18 h. Derivatization was stopped by addition of 1 ml of 0.1 *M* hydrochloric acid and 3 ml of dichloroethane were added to each vial. Following extraction as described previously, the organic phase was transferred to a 1-ml Reacti-Vial with a PTFE-lined septum, evaporated to dryness under nitrogen at room temperature, and the residue dissolved in 10 μl of ethyl acetate. A 0.5—1.0 μl aliquot of the resulting solution was chromatographed on a Varian 2100 gas chromatograph modified for capillary use and with a thermionic nitrogen—phosphorus specific detector. The column was a 25 m × 0.25 mm I.D. Chirasil-Val glass capillary (Applied Science, Deerfield, IL, U.S.A.) with the following conditions: injector temperature, 225°C; oven temperature 165°C, detector temperature, 300°C; helium flow-rate, 1.2 ml/min with a 20:1 split ratio; make-up, 2.5 ml/min; hydrogen flow-rate, 3.5 ml/min; and air flow-rate, 175 ml/min.

RESULTS AND DISCUSSION

Preliminary studies showed that separation of hydantoin enantiomers on the Chirasil-Val column was only possible if there was a 3-alkyl group located on the hydantoin ring; in the absence of such a group or when the 1,3-dialkyl

derivatives were chromatographed, enantiomeric resolution was lost. Accordingly, a mild and selective procedure was developed for converting the N-demethylated metabolite, PEH, and its internal standard, PPH, to their 3-propyl derivatives. Since mephenytoin was unaffected by this procedure, it was considered important to use a separate internal standard with a similar characteristic. Of the various hydantoin derivatives which were synthesized and examined, most were either poorly resolved or had retention times that overlapped with the other compounds of interest. Only 3MIPPH was found to have suitable chromatographic properties.

Under the described conditions, excellent chromatographic separations were obtained between the different compounds of interest and their enantiomers (Fig. 1) and no interfering peaks were observed from either plasma or blood. The *S*-enantiomers eluted earlier than the corresponding *R*-enantiomers with almost baseline separation between the two, and the respective retention times (*S*/*R*) were: mephenytoin, 19.2/19.9 min; 3MIPPH, 23.5/25.6 min; PEH, 28.8/30.1; and PPH, 40.3/42.7 min. Standard curves for the mephenytoin and PEH exhibited good linearity between peak height ratio of the enantiomer to the appropriate internal standard and plasma concentrations over the range 50 ng/ml to 5 μg/ml (Fig. 2). The curves were reproducible in that the coefficient of variation of the slopes was less than 5% for the mephenytoin enantiomers and 13% for those of PEH (n = 8), and the lowest measurable concentration was about 50 ng/ml. Analysis of blood gave identical results to those with plasma; however, repetitive analyses quickly contaminated the injection port leading to a decrease in peak resolution. Intraday variability in the assay was less than 4.5% for mephenytoin (n = 6—8) over the concentration range 50 ng/ml to 1.0 μg/ml and below 8% for PEH. However, poorer reproducibility (< 19%) was observed with PEH at concentrations below 100 ng/ml. A similar situation was apparent with the interday reproducibility; the

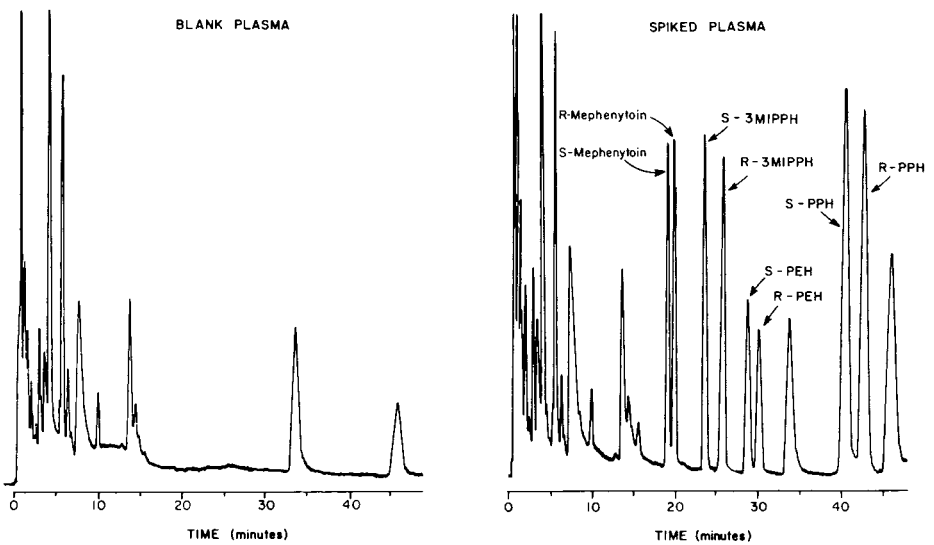


Fig. 1. Separation of the *R*- and *S*-enantiomers of mephenytoin, PEH and their internal standards following extraction from plasma.

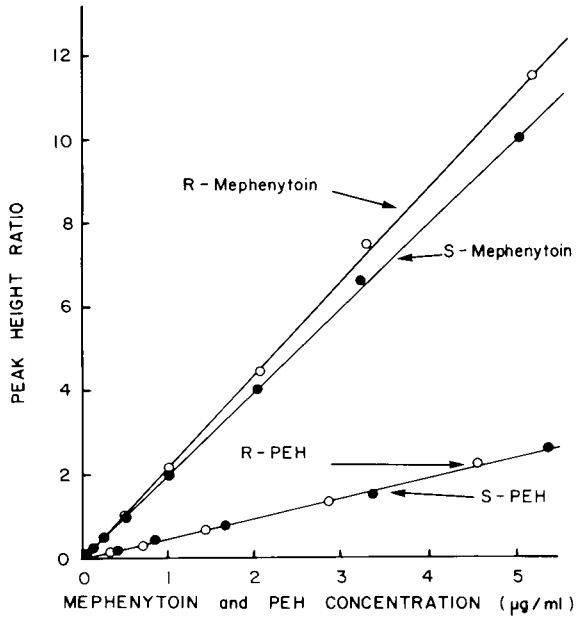


Fig. 2. Standard curves for the *R*- and *S*-enantiomers of mephenytoin and PEH extracted from plasma.

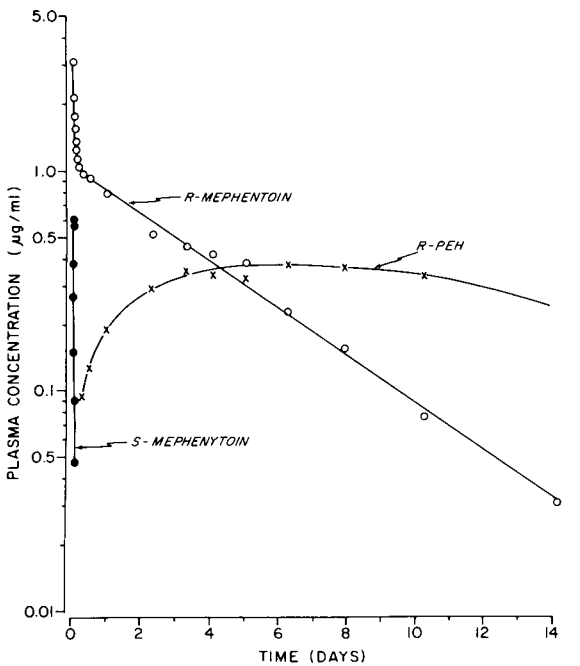


Fig. 3. Plasma concentration—time curves of the *R*- and *S*-enantiomers of mephenytoin and its N-demethylated metabolite (PEH) in a normal subject after receiving a 300-mg oral dose of racemic mephenytoin.

coefficient of variation (n = 8) being less than 8% for both mephenytoin and PEH over the above range except for the lowest concentrations of PEH where only 20% reproducibility was obtainable. The interday reproducibility studies were performed over a 6—8 month period, but no trends were apparent to suggest that either mephenytoin or PEH were unstable in frozen plasma during this time.

In order to examine the clinical applicability of the developed procedure to determine the circulating plasma concentrations of mephenytoin and PEH enantiomers, a normal subject was given a 300-mg oral dose of racemic mephenytoin. This was followed by serial blood sampling over the next two weeks, and the plasma concentrations of the enantiomers were determined (Fig. 3). Pronounced stereoselective differences were apparent in the plasma concentration—time curves for both mephenytoin and PEH. For example, the peak concentration of *R*-mephenytoin was five-fold greater than that of the *S*-enantiomer and the rates of elimination were widely disparate; the half-life of *S*-mephenytoin being less than 3 h compared to over 70 h for the *R*-isomer. Similarly, large enantiomeric differences were present with the N-demethylated metabolite; *S*-PEH concentrations were barely measurable and rapidly fell below the detection limit of the assay whereas *R*-PEH levels steadily increased over 4—6 days to exceed these of *R*-mephenytoin and then declined very slowly. These findings are consistent with earlier studies based on urinary excretion profiles [2] and support the conclusion that the metabolism of *S*-mephenytoin is very rapid and involves 4-hydroxylation followed by conjugation. In contrast, the *R*-enantiomer is mainly N-demethylated at a much slower rate to the pharmacologically active metabolite, *R*-PEH. Since this metabolite is not further metabolized and its renal clearance is small, *R*-PEH accumulates in the plasma and is only slowly removed from the body. Such differences in drug disposition and their clinical consequences clearly demonstrate the need for analytical methodology capable of separating and quantifying the two stereoisomers and their metabolites. The described direct separation procedure using a chiral gas chromatographic column will, therefore, allow investigation of the determinants of mephenytoin's stereoselective metabolism and clinical effectiveness.

Chirasil-Val is synthesized by coupling L-valine, *tert.*-butylamide to a copolymer of dimethylsiloxane and carboxyalkylmethylsiloxane units [15]. It was originally developed for analyzing the optical purity of amino acids derived from synthetic peptides [15, 16]. However, preliminary studies [17] have shown that it is also applicable to the enantiomeric resolution of certain sympathomimetic amines and catecholamines, glycols and α-hydroxycarboxylic acids [17]. The mechanism of separation of enantiomers in the gas phase probably involves formation of hydrogen-bonded diastereomeric association complexes with the stationary phase. The degree of enantiomeric separation appears to be dependent on the sequence and spatial relationship between hydrogen-donating amino groups and hydrogen-accepting carbonyl groups around the chiral center [17]. The resolution of the enantiomers of mephenytoin on this phase was excellent, but as indicated with PEH, the degree of alkylation of the molecule was critical. The ease and simplicity of this direct analytical approach to the important area of stereoselective drug disposi-

tion would suggest that further studies should be pursued to characterize the necessary structural requirements for enantiomeric resolution of other types of compounds using Chirasil-Val or other chiral stationary phases.

ACKNOWLEDGEMENTS

This study was supported by USPHS grants GM 31304 and RR-05424.

REFERENCES

1 A. Küpfer and J. Bircher, J. Pharmacol. Exp. Ther., 209 (1979) 190.
2 A. Küpfer, R.K. Roberts, S. Schenker and R.A. Branch, J. Pharmacol. Exp. Ther., 218 (1981) 193.
3 T.A. Baillie, Pharmacol. Rev., 33 (1981) 81.
4 B. Halpern and J.W. Westley, Biochem. Biophys. Res. Commun., 19 (1965) 361.
5 E. Gil-Av, R. Charles-Sigler, G. Fischer and D. Nurok, J. Gas Chromatogr., 4 (1966) 51.
6 B.L. Karger, R.L. Stern, W. Keane, B. Halpern and J.W. Westley, Anal. Chem., 39 (1967) 228.
7 C.J.W. Brooks, M.T. Gilbert and J.D. Gilbert, Anal. Chem., 45 (1973) 896.
8 W.A. Kõnig, W. Rahn and J. Eyem, J. Chromatogr., 133 (1977) 141.
9 B. Feibush and E. Gil-Av, J. Gas Chromatogr., 5 (1967) 257.
10 B. Feibush and E. Gil-Av, Tetrahedron, 26 (1970) 1361.
11 W. Koenig, W. Parr, H. Lichtenstein, E. Bayer and J. Orõ, J. Chromatogr. Sci., 8 (1970) 183.
12 W. Parr, C. Yang, E. Bayer and E. Gil-Av, J. Chromatogr. Sci., 8 (1970) 591.
13 W. Parr, J. Pleterski, C. Yang and E. Bayer, J. Chromatogr. Sci., 9 (1971) 141.
14 J.A. Corbin, J.E. Rhoad and L.B. Rogers, Anal. Chem., 43 (1971) 327.
15 H. Frank, G.J. Nicholson and E. Bayer, Angew. Chem. Int. Ed. Engl., 17 (1978) 363.
16 H. Frank, G.J. Nicholson and E. Bayer, J. Chromatogr., 146 (1978) 197.
17 I. Abe, K. Izumi, S. Kuramoto and S. Musha, J. High Resolut. Chromatogr. Chromatogr. Commun., 4 (1981) 549.
18 H.R. Henze and A.F. Isbell, J. Amer. Chem. Soc., 76 (1954) 4152.

Journal of Chromatography, 307 (1984) 129—135
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2001

GAS—LIQUID CHROMATOGRAPHIC ASSAY OF AMINOGLUTETHIMIDE AND A HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR ITS ACETYL METABOLITE IN BIOLOGICAL FLUIDS

A.M. ADAM and H.J. ROGERS*

Department of Clinical Pharmacology, Guy's Hospital Medical School, London Bridge, London SE1 9RT (U.K.)

(First received May 18th, 1983; revised manuscript received November 4th, 1983)

SUMMARY

A rapid, sensitive and selective gas—liquid chromatographic assay for aminoglutethimide is described. The same extraction procedure may be employed prior to a high-performance liquid chromatographic assay for acetamidoglutethimide which is also detailed. Both assays are suitable for the study of the pharmacokinetics of aminoglutethimide and acetamidoglutethimide in biological fluids in man.

INTRODUCTION

Aminoglutethimide [3-(4-aminophenyl)-3-ethylpiperidine-2,6-dione] was initially developed as an anticonvulsant in the 1950s but was later withdrawn from clinical use after reports of adrenal insufficiency [1]. It was subsequently shown to suppress adrenal steroid synthesis by inhibiting the enzymic conversion of cholesterol to pregnenolone [2]. It has therefore been used in the treatment of adrenocortical tumours, Cushing's syndrome (in combination with metyrapone [3]), and to perform "medical adrenalectomy" in patients with metastatic breast cancer [4, 5]. In post-menopausal women the major oestrogen source is the aromatisation of androstenedione to oestrone in fat, muscle and liver. Aminoglutethimide also blocks this enzyme: an additional and perhaps more important action [6] since this is the rate-limiting step in oestrogen production.

A spectrophotometric method for estimation of aminoglutethimide in biological fluids has been previously described [7]. These authors, however, found cross-reactions with Ehrlich's aldehyde reagent by a number of endogenous and exogenous compounds to restrict the applicability of this assay es-

0378-4347/84/$03.00

pecially for aminoglutethimide concentrations of less than 1 μg/ml. The concentrations of aminoglutethimide expected in saliva after a single dose of aminoglutethimide are thus too low to be measured by this method [8]. A high-performance liquid chromatographic (HPLC) assay has been briefly described utilising an isocratic acetonitrile—water (containing 0.05% perchloric acid) system [9] but the authors experienced difficulty in measuring plasma levels with this system and the isocratic system was replaced with a methanol gradient [10]. Under these conditions the retention times were very long: 15 min for aminoglutethimide and 17 min for acetamidoglutethimide [11]. No internal standard was used to correct for variable extraction of these compounds from biological fluids.

In the present study a gas—liquid chromatographic (GLC) method for aminoglutethimide was developed. Acetamidoglutethimide gave an asymmetric peak shape on this system. A simple and rapid HPLC assay was therefore developed for the acetyl metabolite. Although the extraction process was carried out separately for the two assays, this could easily be simplified to a single extraction process since the two internal standards used do not interfere with either of the chromatographic techniques.

MATERIALS AND METHODS

Chemicals and reagents

Aminoglutethimide was a gift of Ciba-Geigy (Basle, Switzerland). Acetamidoglutethimide was kindly donated by Dr. P.J. Nicholls (Welsh National School of Pharmacy, Cardiff, U.K.) and Dr. M. Jarman (Ludwig Institute, Sutton, U.K.). Heptabarbitone was obtained from Ciba-Geigy (Horsham, U.K.) and primidone from ICI (Macclesfield, U.K.). All other reagents and solvents were AnalaR grade from BDH (Poole, U.K.), and were used without further purification.

Apparatus

The gas chromatograph used was a Pye Unicam Series 104 model fitted with a nitrogen-selective detector. The glass column (1.5 m × 0.4 mm I.D.) was packed with 2% CDMS (cyclohexanedimethanol succinate on Chromosorb W, 80—100 mesh, acid-washed, dichloromethylsalisilane treated).

For the HPLC assay an Altex Model 110A solvent pump was used with a fixed-wavelength (254 nm) detector (Applied Chromatography Systems, Luton, U.K.). The column was a stainless-steel tube (15 cm × 4.6 mm I.D.) packed with reversed-phase Magnusphere C_{18}, 7-μm particle size (Magnus Scientific, Sandbach, U.K.). Injections were made via a Rheodyne type 7125 valve and 20-μl injection loop.

Extraction procedure for aminoglutethimide

Plasma and saliva. To 0.5 ml of plasma or saliva in a centrifuge tube 15 μl of an aqueous solution of primidone (100 μg/ml) were added as internal standard followed by 1 ml of 0.1 *M* citrate buffer, pH 4 (0.1 *M* citric acid—0.2 *M* disodium hydrogen phosphate 62:38 and 5 ml of dichloromethane. After stoppering, the tube was shaken on a Rolamix (Luckhams, Burgess Hill, U.K.)

for 10 min. The tubes were then centrifuged at 800 *g* for 15 min and the dichloromethane transferred to a clean, dry conical tube and evaporated to dryness in a stream of air at 35°C. The contents of the conical tube were reconstituted in 20 μl of methanol. After mixing, 1—2 μl of this solution were injected directly into the column of the gas chromatograph. The peak height ratio of aminoglutethimide to primidone was used to construct a standard curve and to estimate unknown aminoglutethimide concentrations by interpolation.

Urine. For urine, the same procedure was used except that 15 μl of 1 mg/ml primidone in methanol were used as internal standard.

The operating conditions for the chromatograph were: column temperature 240°C, detector temperature 350°C. Gas flow-rates were: carrier gas (nitrogen) 100 ml/min, hydrogen 65 ml/min and air 300 ml/min.

Calibration procedure

Blank plasma or saliva was spiked with an aqueous solution of aminoglutethimide over the range 1—10 μg/ml and carried through the whole procedure. For urine, blank urine was spiked with aminoglutethimide in methanol over the range 10—300 μg/ml.

Extraction procedure and calibration for acetamidoglutethimide

The same extraction method was used. The internal standard was 100 μl of 100 μg/ml heptabarbitone in water. Calibration curves were prepared over the concentration range 500 ng/ml—4 μg/ml for plasma or saliva and 5—100 μg/ml for urine. The amount of internal standard used for the urine assay was 100 μl of 2 mg/ml heptabarbitone in methanol.

Chromatography

The mobile phase was prepared from citrate buffer made by adding 0.1 *M* citric acid to 0.2 *M* disodium hydrogen phosphate in the ratio of 76:24. The pH of this mixture was then adjusted to 3.4 by addition of 1 *M* hydrochloric acid or 1 *M* sodium hydroxide as required. This buffer was mixed with methanol in the ratio 500:280. The mobile phase flow-rate was 1.2 ml/min (about 10 MPa).

RESULTS AND DISCUSSION

Typical chromatograms are shown in Figs. 1 and 2. The retention times were: aminoglutethimide 2.8 min and primidone 4.9 min on the GLC system; acetamidoglutethimide 3.5 min and heptabarbitone 5.6 min on the HPLC system. No interfering peaks due to endogenous substances were observed.

Aminoglutethimide

The inter-day coefficients of variation (C.V.) are shown in Table I. The within-assay coefficients of variation for the aminoglutethimide are shown in Table II.

The calibration curve was linear between 0.25 and 300 μg/ml (the mean correlation coefficient for assays shown in Table I was 0.996, S.D. 0.003) and the minimum level of detection of aminoglutethimide was 100 ng/ml. The fol-

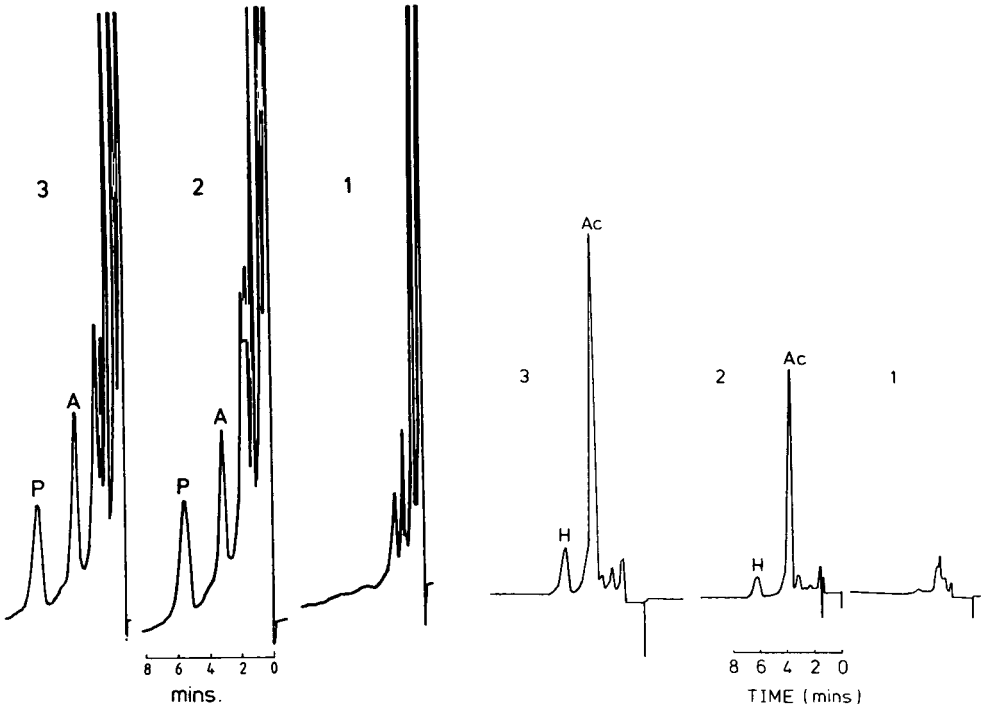


Fig. 1. Representative gas chromatograms of human plasma. 1, Blank plasma; 2, plasma containing 2 μg/ml aminoglutethimide (A), and the internal standard, primidone (P); and 3, plasma from a patient 6 h after oral administration of 500 mg of aminoglutethimide. In each chromatogram the injection artifact is shown on the right-hand side of the trace.

Fig. 2. Representative high-performance liquid chromatograms of blank human plasma. 1, Blank plasma; 2, plasma containing 2 μg/ml acetyl aminoglutethimide (Ac) and the internal standard, heptabarbitone (H); and 3, plasma from a patient 6 h after oral administration of 500 mg of aminoglutethimide.

TABLE I

INTER-DAY REPRODUCIBILITY OF THE AMINOGLUTETHIMIDE ASSAY

	Spiked aminoglutethimide concentration (μg/ml)	Mean peak height ratio (n = 6)	S.D.	C.V. (%)
Plasma	1	0.51	0.05	9.8
	3	1.49	0.01	0.7
	4	2.05	0.09	4.4
	6	3.10	0.07	2.3
	8	4.13	0.14	3.4
	10	5.17	0.17	3.3
Saliva	1	0.60	0.06	10.0
	2	1.09	0.17	15.6
	4	2.33	0.08	3.4
	6	3.58	0.12	3.4
	8	4.69	0.13	2.8
Urine	20	1.22	0.19	15.6
	100	5.6	0.52	9.3
	200	11.74	0.30	2.6
	300	17.2	0.41	2.4

TABLE II

WITHIN-ASSAY COEFFICIENT OF VARIATION (AMINOGLUTETHIMIDE ASSAY)

	Spiked aminoglutethimide concentration (μg/ml)	Mean (n = 6) (μg/ml)	S.D.	C.V. (%)
Plasma	2	1.98	0.05	2.5
	5	5.04	0.14	2.8
	10	9.98	0.25	2.5
Saliva	2	2.0	0.02	1.0
	4	4.05	0.09	2.2
	8	7.99	0.03	0.4
Urine	10	10.2	0.24	2.4
	50	49.9	0.19	0.4
	200	199.2	1.70	0.9

lowing drugs were found not to interfere with this assay: glutethimide (an impurity in the tablet), phenytoin, paracetamol, aspirin, dextropropoxyphene, diazepam and temazepam.

Acetamidoglutethimide

Tables III and IV give comparable data on assay variability over several months of investigation for acetamidoglutethimide.

The calibration curve was linear between 50 ng/ml and 80 μg/ml. The mean correlation coefficient for the data in Table III was 0.998, S.D. 0.004. The minimum level of detection was 10 ng/ml. None of the following drugs which our patients were taking was found to interfere with this assay: methyldopa, aspirin, paracetamol, nitrazepam, temazepam, dextropropoxyphene. Glute-

TABLE III

INTER-DAY REPRODUCIBILITY OF THE ACETAMIDOGLUTETHIMIDE ASSAY

	Spiked aminoglutethimide concentration (μg/ml)	Mean peak height ratio (n = 6)	S.D.	C.V. (%)
Plasma	0.5	0.95	0.10	11
	1	1.90	0.16	8.4
	2	3.90	0.31	7.9
	3	5.75	0.45	7.8
	4	7.8	0.22	2.8
Saliva	0.5	0.96	0.03	3.1
	1	1.73	0.16	9.2
	2	3.67	0.22	6.0
	3	5.62	0.10	1.8
	4	7.58	0.14	1.8
Urine	5	0.36	0.06	16.7
	10	0.82	0.03	3.7
	20	1.71	0.05	2.9
	30	2.38	0.07	2.9
	50	3.97	0.08	2.0
	80	6.18	0.09	1.5

TABLE IV

WITHIN-ASSAY COEFFICIENT OF VARIATION (ACETAMIDOGLUTETHIMIDE ASSAY)

	Spiked aminoglutethimide concentration (μg/ml)	Mean (n = 7) (μg/ml)	S.D.	C.V. (%)
Plasma	0.5	0.52	0.01	1.9
	1	1.00	0.01	1.0
	3	3.01	0.02	0.7
Saliva	1	0.99	0.02	2.0
	2	2.00	0.02	1.0
	4	4.00	0.02	0.5
Urine	10	10.0	0.29	2.9
	50	49.6	2.50	5.0
	80	80.5	2.63	3.3

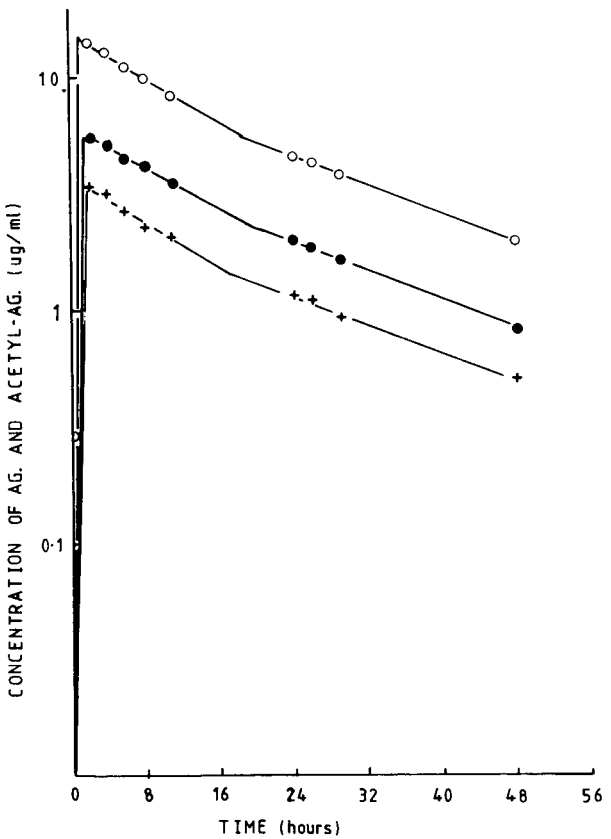


Fig. 3. Concentration—time curves for aminoglutethimide in plasma (○) and acetylaminoglutethimide in plasma (●) and saliva (+) following oral administration of 500 mg of aminoglutethimide to a patient with carcinoma of the breast.

thimide, which may be an impurity in the aminogluthethimide tablet also did not interfere with the assay.

Aminoglutethimide and acetamidoglutethimide

These two assays were used to study the pharmacokinetics of aminoglutethimide and acetamidoglutethimide in patients and volunteers and were both found to be satisfactory (Fig. 3). The salivary pharmacokinetics of acetamidoglutethimide could also be studied. Aminoglutethimide was not detected in saliva despite adequate assay sensitivity and a reported plasma protein binding for aminoglutethimide of 21—25% [8] confirmed in this laboratory to be 31—34%. This subject excreted 8.4% of the dose as aminoglutethimide in urine in 48 h.

Chromatographic assays have been developed to quantify aminoglutethimide and its acetyl metabolite in biological fluids. Adequate sensitivity and reproducibility of calibration data have been shown and these assays are suitable for the conduct of pharmacokinetic studies in normal subjects or patients undergoing treatment for breast cancer with aminogluthetimide.

ACKNOWLEDGEMENTS

This work was supported by a W.H.O. research fellowship and is part of the work for a post-graduate degree of the University of Nairobi. We are grateful to Dr. R.D. Rubens for allowing us to study his patients.

REFERENCES

1 A.M. Camacho, A.J. Brough, R. Cash and R.S. Wilroy, J. Paediat., 68 (1966) 852.
2 R. Cash, A.J. Brough, M.N.P. Cohen and P.S. Satoh, J. Clin. Endocrinol. Metab., 27 (1967) 1239.
3 D.F. Child, C.W. Burke, D.M. Burley, L.H. Rees and T.R. Fraser, Acta Endocrinol., 82 (1976) 300.
4 R.J. Santen, A. Lipton and J. Kendall, J. Amer. Med. Ass., 230 (1974) 1661.
5 R.J. Santen, T.J. Worgul, A. Lipton, H. Harvey, A. Boucher, E. Samojlik and S.A. Wells, Ann. Int. Med., 96 (1982) 94.
6 H.A. Salhanick, Cancer Res. Suppl., 42 (1982) 3315S.
7 F.T. Murray, S. Santner, E.A. Samojlik and R.J. Santen, J. Clin. Pharm., 19 (1979) 704.
8 T.A. Thompson, J.D. Vermeulen, W.E. Wagner and A.R. Le Sher, J. Pharm. Sci., 70 (1981) 1040.
9 M.H. Baker, A.B. Foster, S.J. Harland and M. Jarman, Brit. J. Pharmacol., 74 (1981) 243.
10 R.C. Coombes, A.B. Foster, S.J. Harland, M. Jarman and E.C. Nice, Brit. J. Cancer, 46 (1982) 340.
11 R.C. Coombes, in F.J.A. Paesi (Editor), Aminoglutethimide (Orimeten): Mechanism of Action and Clinical Results in Breast Cancer, Ciba-Geigy, Basle, 1982, p. 130.

Journal of Chromatography, 307 (1984) 137—144
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2004

MICRODOSAGE DE L'ISONIAZIDE ET DE L'ACETYLISONIAZIDE PLASMATIQUES PAR CHROMATOGRAPHIE LIQUIDE À HAUTE PERFORMANCE

C. LACROIX*, G. LAINE et J.P. GOULLE

Laboratoire de Biochimie, Centre Hospitalier Général, 55 bis, rue Gustave Flaubert, B.P. 24, 76083 Le Havre Cedex (France)

et

J. NOUVEAU

Service de Pneumologie, Hôpital Calmette, 76083 Le Havre Cedex (France)

(Reçu le 20 juin 1983; manuscrit modifié reçu le 15 novembre 1983)

SUMMARY

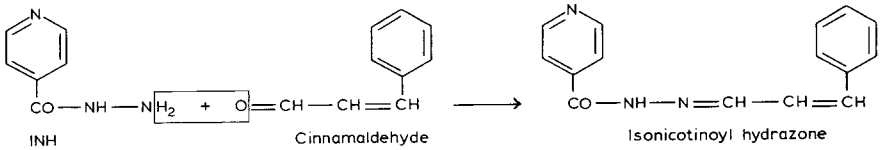
Determination of isoniazid and acetylisoniazid in plasma or serum by high-performance liquid chromatography

A method for the analysis of iosoniazid and acetylisoniazid in plasma or serum has been developed. This report describes a simple, quick and economically optimized reversed-phase high-performance liquid chromatographic assay using a small sample size without solvent extraction step. Isoniazid was condensed with cinnamic aldehyde after trichloroacetic acid deproteinisation. The method correlated well with a fluorimetric procedure. Precision, sensibility and accuracy were good. Common antituberculous drugs did not interfere.

INTRODUCTION

Largement utilisé depuis de nombreuses années dans la chimiothérapie antituberculeuse, l'isoniazide (INH) a fait la preuve de son efficacité et acquis ainsi une place prépondérante. Sa dégradation hépatique, génétiquement déterminée, donne naissance à différents métabolites dont le plus important est représenté par l'acétylisoniazide (Ac-INH). La connaissance de leurs taux plasmatiques permet d'ajuster la posologie quotidienne en fonction de la vitesse d'inactiva-

0378-4347/84/$03.00



tion du sujet [1], diminuant ainsi le risque d'effets secondaires de types neuropsychiques, hépatiques, ou l'instauration d'un traitement inefficace.

Diverses méthodes de dosage ont été proposées: microbiologique utilisant différentes souches de *Mycobacterium* [2, 3], spectrophotométrique [4—7], spectrofluorométrique [8, 9], par chromatographie en phase gazeuse [10], par chromatographie liquide à haute performance (HPLC) [11—13].

La technique proposée s'attache au dosage HPLC de l'INH après formation d'un hydrazone par condensation à l'aldehyde cinnamique (AC) [6, 7] sans extraction préalable.

MATÉRIEL ET MÉTHODE

Appareillage

L'appareil utilisé est un chromatographe liquide à haute performance Du Pont de Nemours (Orsay, France). Ce système comprend une pompe 870 équipée d'une vanne à boucle de 20 µl (Rhéodyne 71-25; Berkeley, CA, É.U.), un four thermostaté à 50°C, un spectrophotomètre UV à longueur d'onde variable (réglé à 330 nm) raccordé à un registreur Omniscribe (1 mV; Houston Instruments). La colonne est une Zorbax CN Du Pont de Nemours (6 µm; 250 × 4.6 mm I.D.).

Prélèvements étalonnage

L'INH et l'Ac-INH sont fournies gracieusement par le laboratoire Roche (Neuilly-sur-Seine, France). Les prélèvements sanguins (3 ml) sont recueillis dans des tubes de verre héparinés (Vacutainers; 5 ml), immédiatement centrifugés à 1000 *g* pendant 10 min. La fraction plasmatique est congelée à —20°C jusqu'au dosage. L'étalonnage est réalisé en surchargeant des plasmas par des solutions d'INH fraîchement préparées à différentes concentrations.

Réactifs

Aldehyde cinnamique, méthanol qualité HPLC (Carlo Erba, St. Cloud, France). Acide trichloracétique, acide acétique, acétate de sodium ($3H_2O$), acide chlorhydrique (tous de chez E. Merck, Darmstadt, R.F.A.). Ces produits sont utilisés sans autres purifications.

Méthode

Dosage de l'INH. Le plasma (50 µl) est déprotéinisé par 50 µl d'acide trichloracétique à 10% dans des microtubes à usage pédiatrique et centrifugé pendant 5 min à 10,000 *g* (microtubes, microcentrifugeuse; Beckman, Gagny, France). Prélever 50 µl de surnageant, ajouter 5 µl d'AC (10 g/l de méthanol). Mélanger. Après un délai de 2 min injecter 20 µl dans le chromatographe. La phase mobile est constituée d'un mélange méthanol—eau—acide acétique (400:590:10)

contenant 3 mmol/l d'acétate de sodium trihydraté. Avant l'usage, la phase mobile est passée sur filtre Millipore (0.45 µm) et dégazée par un courant d'hélium. Le débit est de 2 ml/min soit une pression d'environ 14 MPa; la colonne est thermostatée à 50°C. La détection se fait à 330 nm et la quantification est basée sur la hauteur des pics.

Dosage de l'Ac-INH. L'Ac-INH est hydrolysé à chaud en milieu acide en INH. La valeur mesurée représente donc la somme de l'INH déterminé auparavant et de l'Ac-INH. Pour ceci, ajouter 5 µl d'acide chlorhydrique 6 mol/l à 50 µl de surnageant trichloracétique et chauffer 1 h à 80°C. Après refroidissement, mélanger 5 µl d'AC et opérer comme précédemment. La gamme d'étalonnage et son blanc sont traités de la même façon. La concentration de l'Ac-INH correspond donc à:

[Ac-INH] = [INH (après hydrolyse)] − [INH (avant hydrolyse)]

Pour exprimer cette valeur en Ac-INH vrai, il suffit de la multiplier par 1.3 (1.3 mg d'Ac-INH = 1 mg d'INH).

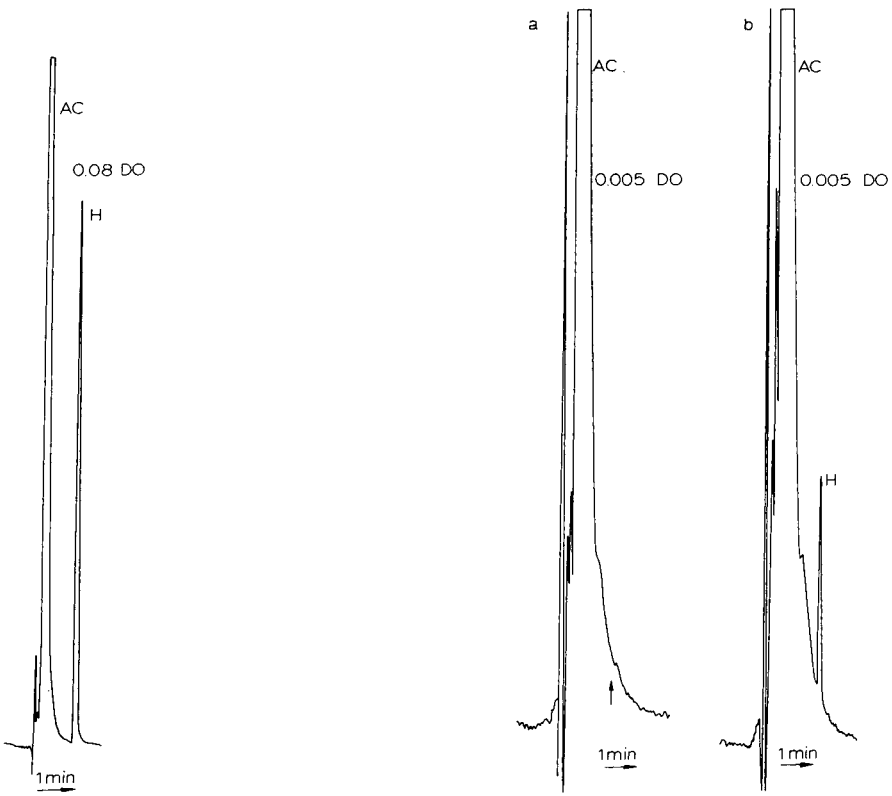


Fig. 1. Chromatogramme obtenu après injection d'un plasma contenant 5 µg/ml. H = isonicotinyl hydrazone de l'aldehyde cinnamique; AC = aldehyde cinnamique.

Fig. 2. Chromatogrammes de (a) blanc plasma à 0.005 densité optique pleine échelle; et (b) plasma contenant 0.1 µg/ml à 0.005 densité optique pleine échelle.

RÉSULTATS

La Fig. 1 représente un tracé obtenu avec un plasma surchargé d'INH (5 μg/ml). Le temps de rétention dans les conditions indiquées est de 3 min. La linéarité a été évaluée à deux sensibilités différentes:

(1) 0.08 densité optique (DO) pleine échelle pour des concentrations de 0.5, 1, 2.5, 5 et 10 μg/ml atteintes en thérapeutique.

(2) 0.005 DO pleine échelle pour des valeurs de 0.025, 0.05 et 0.1 μg/ml pour une étude plus fine de la cinétique du produit (Fig. 2).

La répétabilité a été évaluée à six concentrations différentes et figure dans le Tableau I. L'exactitude a été vérifiée à l'aide de plasmas surchargés de 0.1 à 10 μg/ml (Tableau II). L'équation de la droite de régression linéaire des ajouts

TABLEAU I

ÉTUDE DE LA RÉPÉTABILITÉ

Dix déterminations par niveau.

	Concentration (μg/ml)	Écart-type (μg/ml)	Coefficient de variation (%)
INH	0.025	0.0013	5
	0.05	0.0015	3.1
	0.1	0.0032	3.2
	0.5	0.002	3.5
	2	0.05	2.4
	4	0.11	2.9
AC—INH	0.125	0.015	2.4
	0.25	0.010	1.3
	0.5	0.05	2.4
	1	0.027	1.3
	2	0.13	3.3
	4	0.071	1.0

TABLEAU II

EXACTITUDE DE LA MÉTHODE

Cinq déterminations par niveau.

Concentration théorique des plasmas surchargés (μg/ml)	Moyenne des concentrations mesurées (μg/ml)
0.1	0.095
0.5	0.50
1	0.97
2.5	2.40
5	4.91
7.5	7.68
10	9.90

mesurés (X) par rapport à la valeur théorique (Y) est la suivante:

$Y = 1.00X + 0.03$; $r = 0.999$

La limite de détection est de 17 ng/ml pour l'INH et de 50 ng/ml pour l'Ac-INH.

DISCUSSION

Corrélation avec une technique fluorimétrique

La méthode proposée a fait l'objet d'une comparaison avec la méthode fluorimétrique (Fig. 3) de Bourdon et al. [9]. La droite de régression est de la forme:

$Y = 1.07X + 0.13$; $r = 0.992$

Toutefois, le test t sur couples appariés indique une différence significative ($P < 0.001$) entre les deux techniques. En effet, la défécation trichloroacétique libère l'INH des hydrazones labiles formées avec l'acide pyruvique et l'acide α-cétoglutarique [14] dont les activités antibactériennes ont été démontrées [15, 16]. La méthode fluorimétrique employée comporte une défécation du plasma par l'acétate de cadmium à pH 8. Ce procédé n'entraîne pas la libération de l'INH de ses hydrazones labiles mais sous-estime la concentration d'INH active. Dans le Tableau III figurent les résultats obtenus par les deux méthodes.

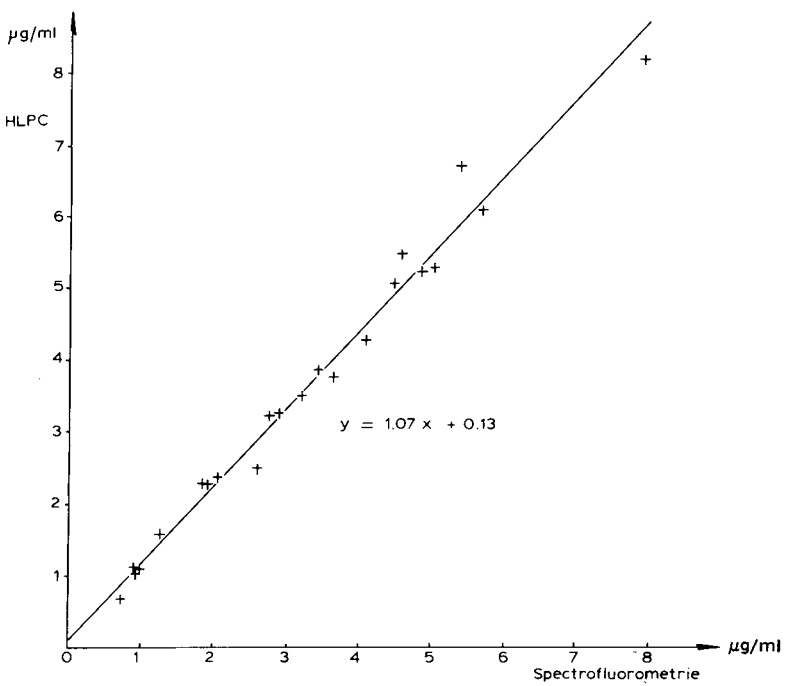


Fig. 3. Comparaison HPLC et spectrofluorimétrie.

TABLEAU III

VALEUR DE L'INH LABILE APRÈS DÉFÉCATION ACIDE

	INH (μg/ml)											
Défécation trichloracétique HPLC	2.30	1	3.25	3.85	1.10	0.7	8.20	3.80	4.3	5.30	1.05	1.55
Défécation cadmique pH 8 fluorimétrie	1.90	0.9	2.75	3.45	0.95	0.7	7.95	3.65	4.1	5.05	0.90	1.25
"INH labile"	0.40	0.10	0.50	0.40	0.15	0	0.25	0.15	0.20	0.25	0.15	0.30

Concentration en aldehyde cinnamique

Le volume d'aldehyde cinnamique a été fixé volontairement à 5 μl afin de ne pas diminuer la sensibilité de la technique par une dilution supplémentaire. Nous avons recherché la réponse optimale en comparant les hauteurs de pic d'hydrazone formée à partir de solutions d'INH (5 μg/ml), en fonction de concentrations différentes d'AC. La réponse ne varie plus au-delà d'une valeur de 10 g/l d'AC dans le méthanol (Fig. 4).

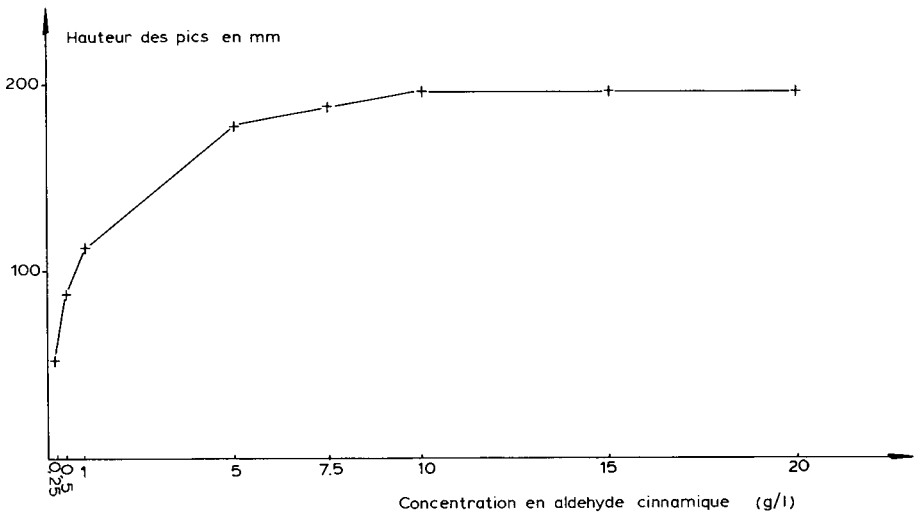


Fig. 4. Evolution de la hauteur des pics d'hydrazone déterminée avec une solution à 5 μg/ml d'INH et des concentrations croissantes d'aldehyde cinnamique.

Temps de contact aldehyde—INH

La formation du produit de condensation est très rapide au pH du défécat trichloracétique. Elle n'évolue plus à partir d'une minute. Nous avons fixé le temps de réaction à 2 min avant l'injection dans un souci de reproductibilité.

Conservation des prélèvements

Cette étude a été réalisée sur deux pools de plasma (1.70 et 4.45 μg/ml). Après fractionnement, chaque échantillon a été conservé de trois façons: à 4° C, à 4° C après défécation trichloroacétique et congelés à —20° C. Des dosages

ont été effectués chaque jour pendant une semaine. Les meilleures conditions de conservation sont obtenues par une congélation à —20° C en accord avec les travaux de Ellard et al. [17].

Les méthodes décrites en HPLC sont fondées sur l'extraction de l'INH et de l'Ac-INH par un solvant organique ou un dosage direct après défécation et lecture à 254 ou 265 nm. Toutefois, le rendement de cette extraction est faible, compte tenu de la polarité de la molécule et nécessite une prise d'essai importante (0.5 à 1 ml) avec une limite de détection insuffisante. Un dosage direct après simple défécation du plasma implique une sensibilité de détection élevée et le risque non négligeable d'interférences. La formation préalable d'une hydrazone procure plusieurs avantages: une limite de détection exceptionnelle, la possibilité d'une prise d'essai réduite et un tracé sans pics parasites par une lecture à 330 nm. La détermination de l'Ac-INH après action du nitrite de sodium, de sulfamate d'ammonium et hydrolyse par acide chlorhydrique [17] fait apparaître deux problèmes: un élargissement notable du pic sur le chromatogramme et la présence de blancs plasmatiques élevés et très variables. C'est

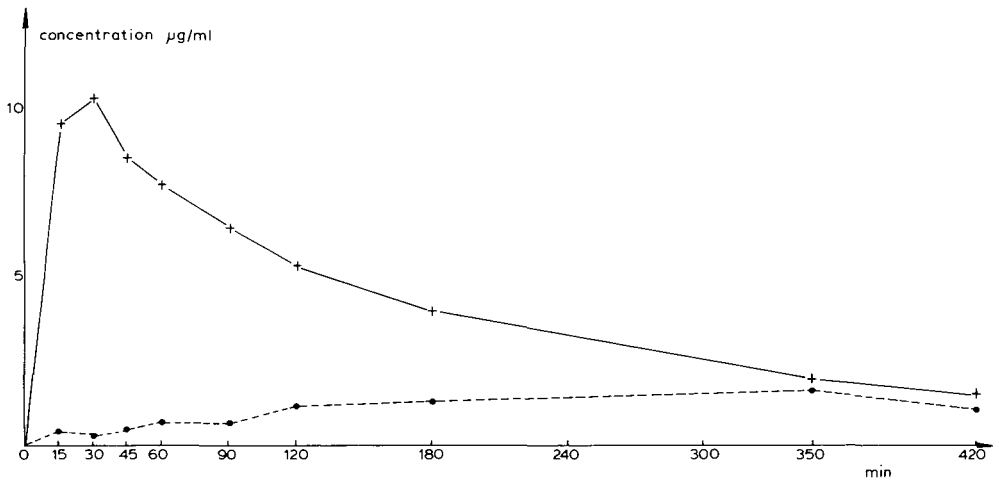


Fig. 5. Cinétique après prise orale unique de 5.2 mg/kg. ——, INH; — — —, Ac-INH.

TABLEAU IV

CONCENTRATION D'INH ET Ac-INH 3 h ET 6 h APRÈS PRISE ORALE DE 300 mg D'INH

		INH (µg/ml)	Ac-INH (µg/ml)
H.	3 h	2.25	3.44
	6 h	0.71	2.45
L.	3 h	3.59	2.77
	6 h	0.89	2.73
F.	3 h	4.88	1.38
	6 h	3.00	1.66
L.	3 h	6.20	3.05
	6 h	3.45	2.73

pourquoi, nous avons préféré une hydrolyse directe par l'acide chlorhydrique à chaud suivie d'un dosage différentiel de l'Ac-INH. Dans ces conditions, la transformation de l'Ac-INH en INH est complète, l'intégrité du pic est respectée, les blancs témoins sont plus faibles et surtout de valeur constante pour une même série de dosages (vérifiée sur 30 plasmas différents). La Fig. 5 illustre une étude de cinétique effectuée chez un malade après prise unique d'INH per os (5.2 mg/kg). Dans le Tableau IV sont regroupées les valeurs d'INH et d'Ac-INH obtenues 3 h et 6 h après prise orale de 300 mg d'INH chez des patients traités depuis un mois. Les autres antituberculeux employés en association (rifampicine, pyrazinamide, streptomycine, ethambutol) ne provoquent aucune interférence.

Par son extrême rapidité et sa simplicité de mise en oeuvre, cette technique peut avantageusement remplacer les méthodes spectrofluorimétriques qui, bien que précises, sont longues d'exécution. La grande sensibilité atteinte pour un temps de manipulation réduit et un volume de prélèvement très faible permet d'envisager son application à des études cinétiques après dose unique.

RÉSUMÉ

La technique chromatographie liquide à haute performance proposée pour le dosage de l'isoniazide et de l'acétylisoniazide ne nécessite qu'une prise d'essai réduite. La méthode est très rapide car ne comporte pas de phase d'extraction. L'isoniazide plasmatique est condensé avec l'aldehyde cinnamique après défécation trichloracétique. La technique a été corrélée de façon satisfaisante avec une méthode fluorimétrique. L'exactitude, la sensibilité et la précision sont excellentes. Les autres antituberculeux fréquemment utilisés en association n'interfèrent pas.

BIBLIOGRAPHIE

1 J.N. Vivien, R. Thibier et A. Lepeuple, Rev. Mal. Resp., 1 (1973) 753.
2 J. Grosset et G. Carnetti, Rev. Tuberc. Pneumol. (Paris), 26 (1962) 1077.
3 M. Le Lirzin, J.N. Vivien, A. Lepeuple et R. Thibier, Rev. Tuberc. Pneumol. (Paris), 26 (1962) 350.
4 J.R. Maher, J.M. Whitney, J.S. Chambers et J.D. Stotonis, Amer. Rev. Tuberc., 76 (1957) 852.
5 L. Eidus et E. Little, Amer. Rev. Resp. Dis., 85 (1962) 278.
6 L. Eidus et A. Harnanansingh, Clin. Chem., 17 (1971) 492.
7 E. Delacoux, M. Bresard, T. Evstigneff, D. Chansel et L. Noel, Thérapie, 34 (1979) 327.
8 E.M. Scott et R.C. Wright, J. Clin. Lab. Med., 70 (1967) 355.
9 R. Bourdon, J. Pollet et A.M. Nicaise, Ann. Biol. Clin., 34 (1976) 127.
10 B.H. Lauterburg, C.V. Smith et J.R. Mitchell, J. Chromatogr., 224 (1981) 431.
11 M. Galliot, R. Bourdon, G. Jotic., A. Funes et H. Sarmini, Ann. Biol. Clin., 40 (1982)
12 M. Guillaumont, M. Leclercq, Y. Frobert et B. Guise, J. Chromatogr., 232 (1982) 369.
13 M.A. Moulin, F. Albessard, J. Lacotte et R. Camsonne, J. Chromatogr., 226 (1981) 250.
14 D.W. Russel, Clin. Chim. Acta, 41 (1972) 163.
15 J.H. Peters et V.E. Hayes, Arch. Int. Pharmacodyn., 159 (1966) 328.
16 W. Wenner, J. Org. Chem., 18 (1953) 1333.
17 G.A. Ellard, P.T. Gammon et S.M. Willan, Biochem. J., 126 (1972) 449.

Journal of Chromatography, 307 (1984) 145—153
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2014

ACTIVATED α-ALKYL-α-ARYLACETIC ACID ENANTIOMERS FOR STEREOSELECTIVE THIN-LAYER CHROMATOGRAPHIC AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF CHIRAL AMINES*

HEIDRUN WEBER, HILDEGARD SPAHN and E. MUTSCHLER**

Department of Pharmacology, Faculty of Pharmacy and Biochemistry, University of Frankfurt/M., Frankfurt/M. (F.R.G.)

and

W. MÖHRKE

Röhm-Pharma, Weiterstadt (F.R.G.)

(First received September 5th, 1983; revised manuscript received November 15th, 1983)

SUMMARY

The separation of racemic benoxaprofen into the two benoxaprofen enantiomers by preparative high-performance liquid chromatography and the application of the activated enantiomers as derivatization reagents for the simultaneous stereoselective determination of chiral amines in biological material is described. Activated (+)- and (—)-benoxaprofen are both shown to be very sensitive and stable chiral fluorescence markers, applicable to thin-layer chromatography as well as to high-performance liquid chromatography.

INTRODUCTION

The chromatographic separation of optical isomers was already described by Kotake in 1951 [1] for the resolution of racemic amino acids using paper chromatography. In 1952 Dalgliesh postulated the so-called "three point attachment" necessary for the resolution of optical isomers [2]. Resolution of the enantiomers

*Part of the dissertation of H. Weber, in preparation.

**Address for correspondence and reprint requests: Prof. Dr. Dr. E. Mutschler, Pharmakologisches Institut für Naturwissenschaftler der Johann Wolfgang Goethe-Universität, Theodor-Stern-Kai 7, Gebäude 75A, D-6000 Frankfurt/M. 70, F.R.G.

0378-4347/84/$03.00

has been developed in two ways: (1) direct separation of enantiomers on an optically active stationary phase [3—5]; and (2) derivatization with a chiral reagent followed by chromatography of the diastereoisomers on a conventional stationary phase [6—8].

After the application of optically active drugs to humans or animals only small amounts of these substances can be found in biological material; sometimes the concentrations are in the picomole range or even lower. If the compounds do not have an intensive intrinsic fluorescence or strong chromophoric properties, it is necessary to have at one's disposal a chiral reagent that lowers the detection limit significantly. Beside adequate spectral properties, configurational purity and stability are demanded.

Up to now there has been no suitable fluorescence marker available for the determination of optically active amines in biological material that completely fulfils all these requirements. Chiral reagents used to form diastereoisomeric mixtures have some disadvantages: the tendency for racemization in a short period of time or insufficient fluorescent properties of the reagents themselves [9—12].

In the group of α-alkyl-α-arylacetic acids there are compounds that not only show a strong intrinsic fluorescence or ultraviolet (UV) absorbance or both, but also exist as racemates. For example, benoxaprofen [(*RS*)-2-(*p*-chlorophenyl)-α-methyl-5-benzoxazole acetic acid] turned out to be a fluorescence label well suited for the determination of amines and alcohols [13].

In this paper the separation of racemic benoxaprofen into the two benoxaprofen enantiomers and the application of the activated enantiomers as derivatization reagents for the simultaneous stereoselective determination of chiral amines in biological material will be described.

MATERIALS AND METHODS

Reagents and chemicals

Solvents (analytical grade), amphetamine, methamphetamine, thionyl chloride and thin-layer chromatographic (TLC) plates (silica gel) were obtained from E. Merck (Darmstadt, F.R.G.). Benoxaprofen was made available by Eli Lilly (Bad Homburg, F.R.G.), and tranylcypromine by Röhm Pharma (Weiterstadt, F.R.G.). α-Methylbenzylamine and α-methoxy-α-trifluoromethylphenylacetic acid were purchased from EGA (Steinheim, F.R.G.).

Apparatus

Melting points were obtained with a Büchi apparatus and are uncorrected. Infrared (IR) spectra were obtained in KBr disks with a Beckman Acculab 2 spectrophotometer.

Solutions were applied onto TLC plates using a Linomat III (Camag, Muttenz, Switzerland).

TLC plates were scanned with a chromatogram-spectrophotometer KM 3 (Carl Zeiss, Oberkochen, F.R.G.) and a recorder 56 (Perkin Elmer, Überlingen, F.R.G.).

A chromatograph LC 601 with a fluorescence detector 650-10 S (Perkin Elmer) and a preparative high-performance liquid chromatographic (HPLC) system 830 with a variable-wavelength UV spectrophotometer (DuPont,

Wilmington, DE, U.S.A.) were used for HPLC separations. Optical rotations were measured with a polarimeter Polartronic I (Schmidt + Haensch, Berlin). Gas chromatography (GC) was performed using a gas chromatograph F 22 (Perkin Elmer).

Separation of benoxaprofen enantiomers

Synthesis of benoxaprofen-α-methylbenzylamide. Benoxaprofen (600 mg, about 2 mmol) was dissolved in 50 ml of toluene. After slowly adding 5 ml of thionyl chloride (ca. a twenty-fold amount, freshly distilled over linseed oil) the solution was refluxed for 30 min and then evaporated to dryness. The crystalline residue was recrystallized from dichloromethane if necessary.

The benoxaprofen chloride was then dissolved in 50 ml of dichloromethane, and 10 ml of a solution of 1.5 ml of (*R*)-(+)-α-methylbenzylamine in 8.5 ml of dichloromethane were slowly added with stirring. After refluxing for 3 h the solution was washed first with 0.2 *M* hydrochloric acid, then with water, and dried over sodium sulphate. The solvent was evaporated and a white crystalline solid was recovered [13, 14]: m.p. 169°C (Lit. [14], 170°C), IR (cm^{-1}) 1640 (>C=O, amide). The solid gave one fluorescent spot (R_F 0.89) when examined by TLC in the solvent system chloroform—methanol—water—concentrated ammonia (70:30:5:1, v/v) [14], and two spots (R_F 0.28 and 0.16) in the solvent system toluene—dichloromethane—tetrahydrofuran (5:1:1, v/v), ammonia atmosphere.

Separation of the diastereoisomers using preparative HPLC. The diastereoisomeric mixture was separated into the two diastereoisomers: (*S*)-(+)-benoxaprofen-(*R*)-(+)-α-methylbenzylamide and (*R*)-(—)-benoxaprofen-(*R*)-(+)-α-methylbenzylamide. Chromatographic conditions were as follows: injection volume 2 ml (saturated solution of the diastereoisomeric mixture in mobile phase); guard column (DuPont) 50 mm × 4.6 mm I.D., packed with LiChrosorb Si 100 (30 μm); preparative HPLC column (DuPont) 250 mm × 21.2 mm I.D., stationary phase Zorbax-Sil, particle size 7 μm; mobile phase cyclohexane—dichloromethane—tetrahydrofuran (10:10:1, v/v); ambient temperature; flow-rate 15 ml/min (at a pressure of 5.0 MPa); detection by UV absorbance at 309 nm.

After collection of fractions of the column eluate, the solvent was evaporated to dryness. The residues were dried over phosphoric anhydride and recrystallized from acetone if necessary.

Hydrolysis of the amides. (*R*)-(—)-Benoxaprofen-(*R*)-(+)-α-methylbenzylamide (400 mg) was dissolved in 40 ml of toluene. After adding 40 ml of sulphuric acid (45%) the solution was refluxed for 1 h. The toluene phase, which contained only small amounts of benoxaprofen, was separated and evaporated to dryness. From the aqueous phase (*R*)-(—)-benoxaprofen was extracted at a pH value of 2—3 using dichloromethane. The dichloromethane phase was again evaporated to dryness. The residues from the dichloromethane and toluene phases were combined and dried over sodium sulphate. (*S*)-(+)-Benoxaprofen-(*R*)-(+)-α-methylbenzylamide was hydrolyzed in the same way. Benoxaprofen enantiomers can be recrystallized from methanol.

Activation of the benoxaprofen enantiomers to benoxaprofen chloride. The two enantiomers were activated to the respective acid chloride as described above.

Determination of the enantiomeric purity

In order to determine the enantiomeric purity of the benoxaprofen enantiomers, the enantiomeric purity of the substrates for derivatization (enantiomers of amphetamine, tranylcypromine, α-methylbenzylamine) must be known. The quantification of the enantiomeric purity of these amines was carried out by gas chromatography using activated α-methoxy-α-trifluoromethylphenylacetic acid, a reagent for the determination of the enantiomeric purity of alcohols and amines [15].

(+)-α-Methoxy-α-trifluoromethylphenylacetic acid (0.1 g) and 1 ml of distilled thionyl chloride were mixed and refluxed for 40 h. The excess thionyl choride was evaporated at 50°C in a stream of dry nitrogen and the residue dissolved in 10 ml of toluene. In each case 1 ml of this solution was added to about 10 mg of amine and the solution heated to 80°C for 1 h. The resulting amide was immediately assayed by gas chromatography.

Chromatographic conditions were as follows: injection volume 1 μl, split ratio 30:1; column OV-225, glass capillary, 25 m; carrier gas hydrogen; injection port temperature 230°C, oven 210°C, detector 250°C; flame-ionization detection. Under these conditions the enantiomeric purities were determined to be: 95.27% for (*S*)-(+)-amphetamine, 98.06% for (*S*)-(—)-tranylcypromine, 97.81% for (*R*)-(+)-tranylcypromine, 97.12% for (*S*)-(—)-α-methylbenzylamine, and 96.99% for (*R*)-(+)-α-methylbenzylamine.

After having obtained these results all the investigated amines were converted to the corresponding amide with (*S*)-(+)-benoxaprofen chloride or (*R*)-(−)-benoxaprofen chloride, in order to determine the enantiomeric purity of the benoxaprofen enantiomers themselves by HPLC (conditions described above).

General procedure for the determination of amines in biological material

Extraction and derivatization. One millilitre of plasma, 1 ml of 0.1 *M* sodium hydroxide solution and 3 ml of *n*-hexane were mixed in a centrifuge tube. After shaking (20 min) and centrifuging (10 min), 2 ml of the organic phase were transferred into another centrifuge tube and evaporated to dryness (using a vacuum centrifuge). Then 0.5 ml of reagent solution [1 mg of (*S*)-(+)-benoxaprofen chloride in 10 ml of dried dichloromethane] were added to the residue. The mixture was allowed to stand at room temperature for 30 min.

Chromatographic conditions. For TLC, the solutions were applied onto TLC plates (silica gel 60 without F_{254}) using a Linomat III. The volume applied was 10 μl, the band width 5 mm. Solvent systems were (I) toluene—dichloromethane—tetrahydrofuran (5:1:1, v/v), ammonia atmosphere, and (II) toluene—chloroform—tetrahydrofuran (5:4:1, v/v), ammonia atmosphere. Detection was by densitometric measurement of the fluorescence intensity using a chromatogram spectrophotometer KM 3; excitation wavelength was the 313-nm line of a mercury medium pressure lamp ST 41, slit 0.1 × 6 mm; emission, M 365 monochromatic filter; amplification, 1—10.

For HPLC, the injection volume was 10 μl. The analytical column (DuPont) was 250 mm × 4.6 mm I.D., with Zorbax-Sil (7 μm) as stationary phase. The mobile phase was cyclohexane—dichloromethane—tetrahydrofuran (5:1:1, v/v). Other conditions were: ambient temperature; flow-rate 1 ml/min (at a

pressure of 8.5 MPa); fluorimetric detection, excitation wavelength 312 nm, emission wavelength 365 nm.

RESULTS AND DISCUSSION

The results of all the investigations indicate that benoxaprofen enantiomers are well suited for the stereoselective determination of optically active compounds. The procedure for synthesizing the chiral reagent, which is described in this paper, can easily be carried out.

The liquid chromatographic separation of the synthesized diastereoisomeric mixture [(*RS*)-(±)-benoxaprofen-(*R*)-(+)-α-methylbenzylamide] using the preparative HPLC system described leads to diastereoisomers [(*S*)-(+)-benoxaprofen-(*R*)-(+)-α-methylbenzylamide and (*R*)-(−)-benoxaprofen-(*R*)-(+)-α-methylbenzylamide] of a high optical purity. Under the conditions described here retention times of 26 min for (*R*)-(−)-benoxaprofen-(*R*)-(+)-α-methylbenzylamide and 38 min for (*S*)-(+)-benoxaprofen-(*R*)-(+)-α-methylbenzylamide were observed. If 2 ml of a saturated solution of (*RS*)-(±)-benoxaprofen-(*R*)-(+)-α-methylbenzylamide in the mobile phase are injected, no tailing is observed and a baseline separation is always achieved. The difference in the chromatographic behaviour of the two diastereoisomers is more than sufficient for fractionated collection. The subsequent hydrolysis can be carried out in a short time without any detectable racemization.

The optical rotations ($[\alpha]_D^{20}$) of the enantiomers obtained [0.1% solution in chloroform—methanol (1:1, v/v)] were as follows: (*R*)-(−)-benoxaprofen −28.0°, (*S*)-(+)-benoxaprofen +27.5°, (*RS*)-(±)-benoxaprofen 0°.

Following GC and HPLC investigations the enantiomeric purity of the benoxaprofen antipodes was calculated to be 95.3% for (*S*)-(+)-benoxaprofen and 97.1% for (*R*)-(−)-benoxaprofen. When we tried using amino acid derivatives as optically active reagents, we could not obtain satisfactory results. In our experiments, the racemization rate of, for example, heptafluorobutyryl-prolyl chloride (the reagent was proposed as a sensitive chiral marker for GLC

TABLE I

CHROMATOGRAPHIC PROPERTIES (TLC AND HPLC) OF FOUR DIFFERENT AMINES AFTER DERIVATIZATION WITH (*S*)-(+)-BENOXAPROFEN CHLORIDE

	R_F (TLC)		HPLC retention time (min)
	Solvent system I	Solvent system II	
(*S*)-(+)-Amphetamine	0.14	0.53	9.5
(*R*)-(−)-Amphetamine	0.21	0.59	8.0
(*S*)-(+)-Methamphetamine	0.27	0.65	11.5
(*R*)-(−)-Methamphetamine	0.33	0.65	10.5
(*R*)-(+)-α-Methylbenzylamine	0.28	0.56	6.7
(*S*)-(−)-α-Methylbenzylamine	0.16	0.45	10.8
(*R*)-(+)-Tranylcypromine	0.16	0.39	10.7
(*S*)-(−)-Tranylcypromine	0.21	0.45	9.0

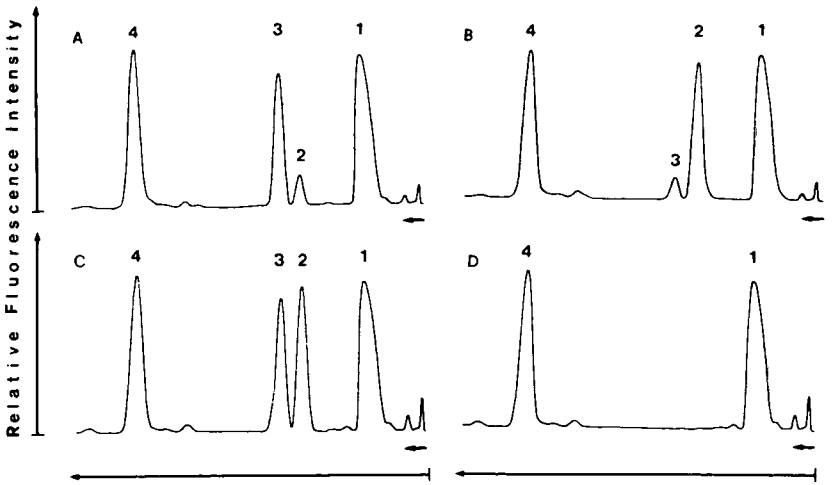


Fig. 1. Scans of thin-layer chromatograms (silica gel, solvent system I) of tranylcypromine (TCP) after extraction from plasma standards (5 μg/ml TCP) and derivatization with (*S*)-(+)-benoxaprofen chloride (1 = benoxaprofen, 2 = amide of (*R*)-(+)-TCP, 3 = amide of (*S*)-(—)-TCP, 4 = benoxaprofen chloride). (A) (*S*)-(—)-TCP; (B) (*R*)-(+)-TCP; (C) racemic TCP; (D) blank plasma.

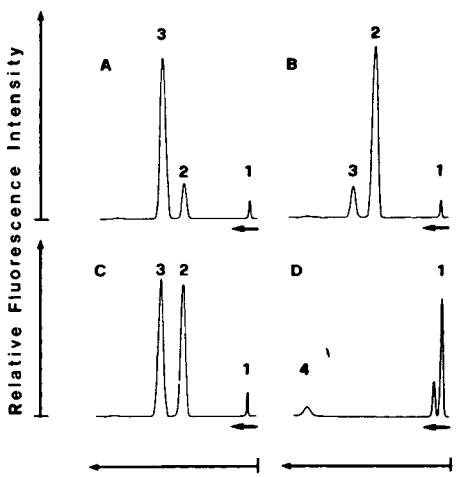


Fig. 2. TLC (silica gel, solvent system I) of α-methylbenzylamine (α-MBA) after extraction from plasma (5 μg/ml α-MBA) and derivatization with (*S*)-(+)-benoxaprofen chloride (1 = benoxaprofen, 2 = amide of (*S*)-(—)-α-MBA, 3 = amide of (*R*)-(+)-α-MBA, 4 = benoxaprofen chloride). (A) (*R*)-(+)-α-MBA; (B) (*S*)-(—)-α-MBA; (C) racemic α-MBA; (D) blank plasma.

[16]) was about 20%, although the experiments were carried out under mild conditions at very low temperatures (reaction temperature —70° C for 15 min).

The enantiomeric impurity of the benoxaprofen isomers themselves was found to be only 3% or 4%. If it is taken into account that the enantiomeric impurity of the (+)-α-methylbenzylamine that was used for the separation of the benoxaprofen isomers was about 3%, the racemization rate of (+)- or (—)-benoxaprofen during derivatization can be looked on as negligible.

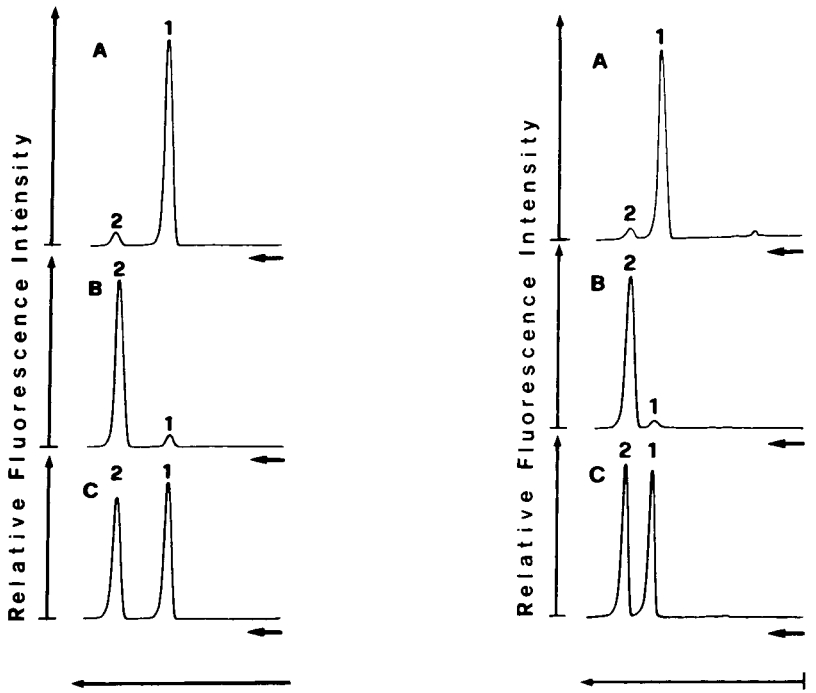


Fig. 3. Chromatograms of the HPLC determination (silica gel column; mobile phase cyclohexane—dichloromethane—tetrahydrofuran, 5:1:1, v/v) of α-methylbenzylamine (α-MBA) after extraction from plasma (5 μg/ml α-MBA) and derivatization with (*S*)-(+)-benoxaprofen chloride (1 = amide of (*R*)-(+)-α-MBA; 2 = amide of (*S*)-(—)-α-MBA). (A) (*R*)-(+)-α-MBA; (B) (*S*)-(—)-α-MBA; (C) racemic α-MBA.

Fig. 4. Chromatograms of the HPLC determination of tranylcypromine (TCP) after extraction from plasma (5 μg/ml TCP) and derivatization with (*S*)-(+)-benoxaprofen chloride (1 = amide of (*S*)-(—)-TCP; 2 = amide of (*R*)-(+)-TCP). (A) (*S*)-(—)-TCP; (B) (*R*)-(+)-TCP; (C) racemic TCP.

The chromatographic (TLC and HPLC) behaviour of several optically active amines after extraction from plasma and derivatization with (*S*)-(+)-benoxaprofen chloride is shown in Table I. Figs. 1—5 show chromatograms of different amines after extraction, derivatization and TLC or HPLC separation. It is evident that the resulting diastereoisomers differ in their R_F values, or retention times, or both. In Fig. 6 are shown calibration curves of (*R*)-(+)-tranylcypromine derivatized with (*S*)-(+)-benoxaprofen chloride and (*R*)-(+)-tranylcypromine derivatized with (*R*)-(—)-benoxaprofen chloride.

Apart from their chemical stability and intense fluorescence, the configurational stability of the (*R*)-(—)- and (*S*)-(+)-benoxaprofen chlorides is a marked advantage. (+)-Benoxaprofen and (—)-benoxaprofen can be stored at room temperature for at least twelve months without racemization.

In drug therapy numerous substances are administered as racemates. Often not only pharmacodynamics but also pharmacokinetics of the two optical isomers are different. Therefore it is essential to have analytical methods at one's disposal by means of which both enantiomers can be assayed separately in biological material. This can be accomplished in one analytical procedure

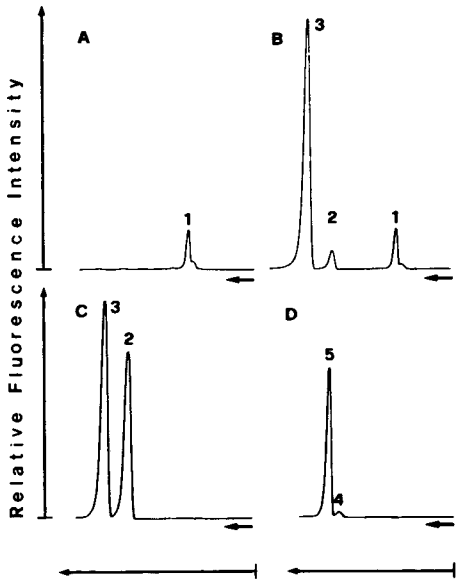


Fig. 5. HPLC determination of amphetamine and methamphetamine after extraction from plasma (5 μg/ml amine) and derivatization with (*S*)-(+)-benoxaprofen chloride (1 = benoxaprofen, 2 = amide of (*R*)-(—)-amphetamine, 3 = amide of (*S*)-(+)-amphetamine, 4 = amide of (*R*)-(—)-methamphetamine, 5 = amide of (*S*)-(+)-methamphetamine). (A) Blank plasma; (B) (*S*)-(+)-amphetamine; (C) racemic amphetamine; (D) (*S*)-(+)-methamphetamine.

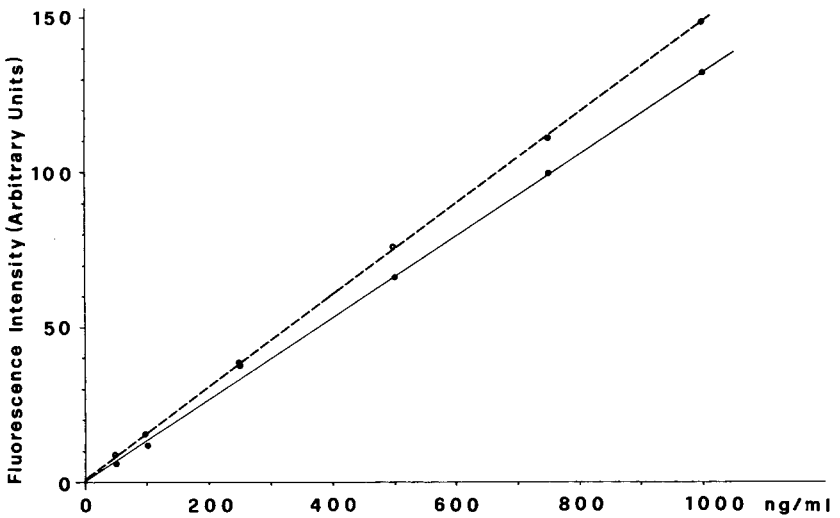


Fig. 6. Calibration curves of (*R*)-(+)-tranylcypromine extracted from different plasma standards and derivatized with (*S*)-(+)-benoxaprofen chloride (•——•) or (*R*)-(—)-benoxaprofen chloride (•- - -•) (TLC determination).

using our method. Because of insufficient or very complicated analytical methods the simultaneous determination of plasma levels and kinetic parameters of both enantiomers has up to now only been performed for a few optically active drugs. In particular, information is lacking on the extent to

which the optical isomers affect each other in their kinetics, and whether the transformation of one isomer into the other in the organism, which has been observed with some substances [17, 18], also occurs in other groups of drugs.

With activated benoxaprofen it is possible to derivatize not only substrates with amino groups but also those with hydroxyl groups [13]. Therefore this reagent can also be applied to the determination of optically active compounds with hydroxyl groups. Many racemic drugs contain either amino or hydroxyl groups or both. Thus, also β-adrenoceptor blocking agents (e.g. propranolol, metoprolol, atenolol) can be derivatized using activated benoxaprofen for their simultaneous stereoselective determination [19].

The described optically active fluorescent labels enable the quantitative determination of optically active amines and alcohols even at low concentrations [13] and in biological material. This is a marked advance in the development of efficient analytical assay methods for pharmacokinetic studies.

ACKNOWLEDGEMENTS

The work was supported by the Dr. Robert Pfleger-Stiftung (Bamberg, F.R.G.), the Deutsche Forschungsgemeinschaft, and Röhm Pharma (Weiterstadt, F.R.G.).

The authors wish to thank Eli Lilly (Bad Homburg, F.R.G.) for the supply of adequate amounts of benoxaprofen and A. and P. Weber for reading the manuscript.

REFERENCES

1 M. Kotake, J. Amer. Chem. Soc., 73 (1951) 3363—3366.
2 C.E. Dalgliesh, J. Chem. Soc., 137 (1952) 3940—3942.
3 W.H. Pirkle, D.W. House and J.M. Finn, J. Chromatogr., 192 (1980) 143—158.
4 G. Gübitz, W. Jellenz and W. Santi, J. Liquid Chromatogr., 4 (1981) 701—712.
5 R. Charles and E. Gil-Av, J. Chromatogr., 195 (1980) 317—327.
6 T. Tamegai, M. Ohmae, K. Kawabe and T. Tomoeda, J. Liquid Chromatogr., 2 (1979) 1229—1250.
7 R.W. Souter, Chromatographia, 9 (1976) 635—637.
8 G. Manius and R. Tscherne, J. Chromatogr. Sci., 17 (1979) 322—326.
9 J. Hermansson and C. von Bahr, J. Chromatogr., 221 (1980) 109—117.
10 B. Silber and S. Riegelman, J. Pharmacol. Exp. Ther., 215 (1980) 643—648.
11 J. Goto, N. Goto, A. Hikichi and T. Nambara, J. Liquid Chromatogr., 2 (1979) 1179—1190.
12 N. Nimura, H. Ogura and T. Kinoshita, J. Chromatogr., 202 (1980) 375—379.
13 H. Spahn, H. Weber, M. Möhrke and E. Mutschler, J. Chromatogr., submitted for publication.
14 R.J. Bopp, J.F. Nash, A.S. Ridolfo and E.K. Shepard, Drug Metab. Dispos., 7 (1979) 356—359.
15 J.A. Dale, D.A. Dull and H.S. Mosher, J. Org. Chem., 34 (1969) 2543—2549.
16 S. Wilk, S.E. Gitlow, M.J. Franklin and H.E. Carr, Clin. Chim. Acta, 10 (1964) 193—195.
17 S. Tamura, S. Kuzuna, K. Kawai and S. Koshimoto, J. Pharm. Pharmacol., 33 (1981) 701—706.
18 D.G. Kaiser, G.J. Vangiessen, R.J. Reischer and W.J. Wechter, J. Pharm. Sci., 65 (1976) 269—273.
19 G. Pflugmann, Thesis, Department of Pharmacology, University of Frankfurt/M., in preparation.

Journal of Chromatography, 307 (1984) 155—171
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2017

ISOMODAL COLUMN SWITCHING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC TECHNIQUE FOR THE ANALYSIS OF CIGLITAZONE AND ITS METABOLITES IN HUMAN SERUM

J.W. COX* and R.H. PULLEN

Drug Metabolism Research, Pharmaceutical Research and Development, The Upjohn Company, 301 Henrietta Street, Kalamazoo, MI 49001 (U.S.A.)

(First received September 6th, 1983; revised manuscript received November 11th, 1983)

SUMMARY

The application of isomodal column switching high-performance liquid chromatography as an alternative to gradient elution was investigated for the analysis of ciglitazone, a potential oral antidiabetic agent, and its monohydroxyl metabolites in human serum. A high-performance liquid chromatographic apparatus was designed to perform on-line fractionation of the serum extract into non-polar (drug) and polar (metabolite) fractions which were then automatically routed into individually optimized, isocratic, reversed-phase high-performance liquid chromatographic systems for simultaneous analysis. Sample fractionation was performed with a reversed-phase guard column, and solvent routing was accomplished with microprocessor-controlled switching valves. Serum was extracted for analysis by a one-step mode sequencing procedure using disposable bonded-phase columns, and quantitation was accomplished with spiked serum standards. Performance specifications of the method were defined for precision, accuracy, linearity, and sensitivity. The column switching method was found to be both expedient and reliable, and it may have general utility for the routine, quantitative analysis of drug/metabolite mixtures that cannot be assayed by simple isocratic elution methods.

INTRODUCTION

Ciglitazone is a potential oral antidiabetic agent intended for non-insulin-dependent diabetics [1, 2]. Both the drug and its known hydroxyl/oxo metabolites have shown pharmacological activity in insulin-resistant animal models, and it is therefore of interest to monitor serum levels of both ciglitazone and its metabolites in human clinical trials. As described below, resolution of the individual monohydroxyl metabolites by high-performance liquid chromatography (HPLC) was achievable only with a reversed-phase system, on which

0378-4347/84/$03.00

there was a large difference between the capacity factors of the parent drug and the metabolites.

This is an example of the general elution problem described by Snyder [3], i.e., the problem of analyzing a sample for multiple components that have widely spaced capacity factors. There are two general methods of dealing with this problem when analyzing multicomponent samples by HPLC. In the first, the sample or sample extract is divided into separate portions for individual isocratic analysis of the more poorly retained and strongly retained components. Late eluting peaks that otherwise might interfere with the poorly retained component assay are eliminated by either pre-analysis cleanup (e.g., by thin-layer chromatography) or column backflushing techniques [4, 5]. Because of the increased labor, sample, and equipment requirements imposed by making two sample injections, this approach is often unsuitable for routine, quantitative analysis.

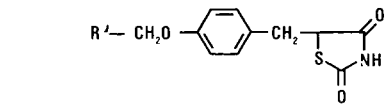
The second method of dealing with the general elution problem is to use techniques that require only a single sample injection. These include gradient elution procedures (programming the mobile-phase composition, flow-rate, or column temperature) [6], and column switching techniques [7—9]. The latter category includes isomodal sequencing methods, such as stationary-phase programming, that adjust component capacity factors by linking columns of the same type but with different surface area or loading [10] or length [11]. These methods are called isomodal because they employ a single chromatographic mode, or physical basis for separation. Heteromodal sequencing techniques, which greatly increase the peak capacity and selectivity of a system by linking different chromatographic modes [12, 13], have limited application to the general elution problem. The most cost-effective means of solving the general elution problem will depend on the application; for routine, quantitative assays, isomodal column switching methods were judged to be superior to other methods in terms of reliability, cost, and speed [6].

The general elution problem is frequently encountered in drug metabolism studies, where there can be large polarity differences between a drug and its metabolites. Nevertheless, the primary application of HPLC column switching in the pharmaceutical area has been intended to reduce analysis time when monitoring for the parent drug only and not to assist in multicomponent drug/metabolite assays [4, 9, 13—16]. The aim of the present study was to test the applicability of column switching techniques for the routine, quantitative analysis of ciglitazone and its monohydroxyl metabolites in human serum. The method employs a one-step serum extraction procedure and an automated, isomodal, column switching HPLC system designed for the simultaneous isocratic elution of the drug and its metabolites. The performance and dependability of the method are evaluated and its utility is demonstrated in a clinical trial.

EXPERIMENTAL

Materials

Ciglitazone {Fig. 1; 5-[4-(1-methylcyclohexylmethoxy)benzyl] thiazolidine-2,4-dione; U-63,287}, *cis*-4′-ol {5-[4-(*c*-4-hydroxy-1-methyl-*r*-1-cyclohexyl-



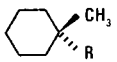
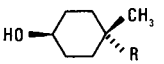
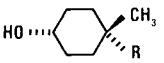
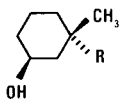
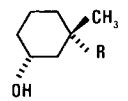
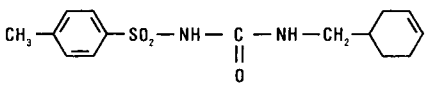
Name	R′
Ciglitazone	
trans-4′-ol	
cis-4′-ol	
trans-3′-ol	
cis-3′-ol	

Fig. 1. Structures of ciglitazone and its monohydroxyl metabolites.

Internal Standards

U-11,824



U-53,059

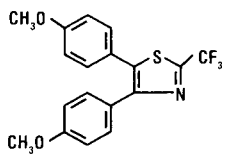


Fig. 2. Structures of the internal standards used for the metabolite assay (U-11,824) and the ciglitazone assay (U-53,059).

methoxy)benzyl] thiazolidine-2,4-dione}, *trans*-4′-ol {5-[4-(*t*-4-hydroxy-1-methyl-*r*-1-cyclohexylmethoxy)benzyl] thiazolidine-2,4-dione}, *cis*-3′-ol {5-[4-(*c*-3-hydroxy-1-methyl-*r*-1-cyclohexylmethoxy)benzyl] thiazolidine-2,4-dione}, and *trans*-3′-ol {5-[4-(*t*-3-hydroxy-1-methyl-*r*-1-cyclohexylmethoxy)benzyl]-thiazolidine-2,4-dione} were provided by Takeda Chemical Industries (Osaka, Japan). The internal standard for ciglitazone analysis, U-53,059 [Fig. 2; 4,5-bis(*p*-methoxylphenyl)-2-(trifluoromethyl)thiazole] and the internal standard for the metabolite analysis, U-11,824 [Fig. 2; 1-(3-cyclohexen-1-ylmethyl)-3-*p*-tolylsulfonylurea] were supplied by the Pharmaceutical Research and Development Labs. of Upjohn (Kalamazoo, MI, U.S.A.). Acetonitrile, hexane, methanol and water were UV or HPLC grade and were purchased from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Bond Elut™ C_{18} (100 mg/1.0 ml) and silica (500 mg/2.8 ml) extraction columns were purchased from Analytichem International (Harbor City, CA, U.S.A.).

Instrumentation

The HPLC apparatus consisted of two isocratic systems linked via a common guard column (Fig. 3). Injections were made automatically with an Upjohn μP autosampler (Upjohn; not commercially available). Pumps A and B (Beckman 112 solvent delivery modules, Beckman Instruments, Berkeley, CA, U.S.A.) were connected to Valco 10-port and 6-port air-actuated (4 bar)

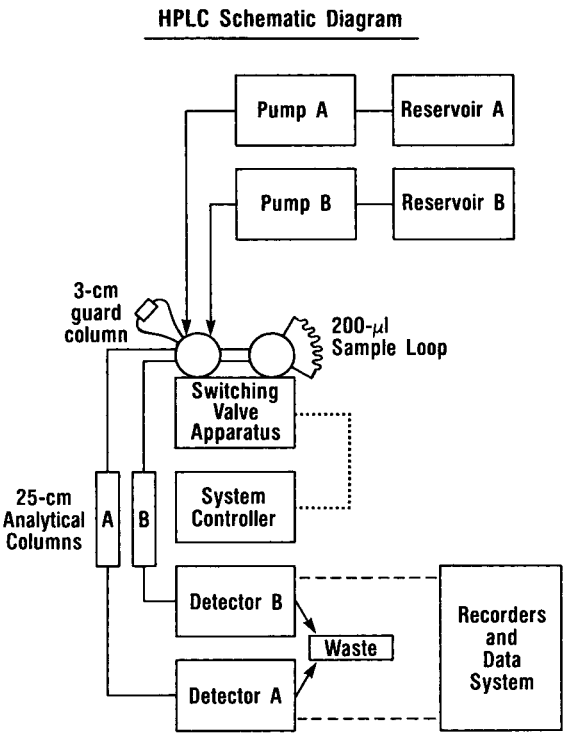
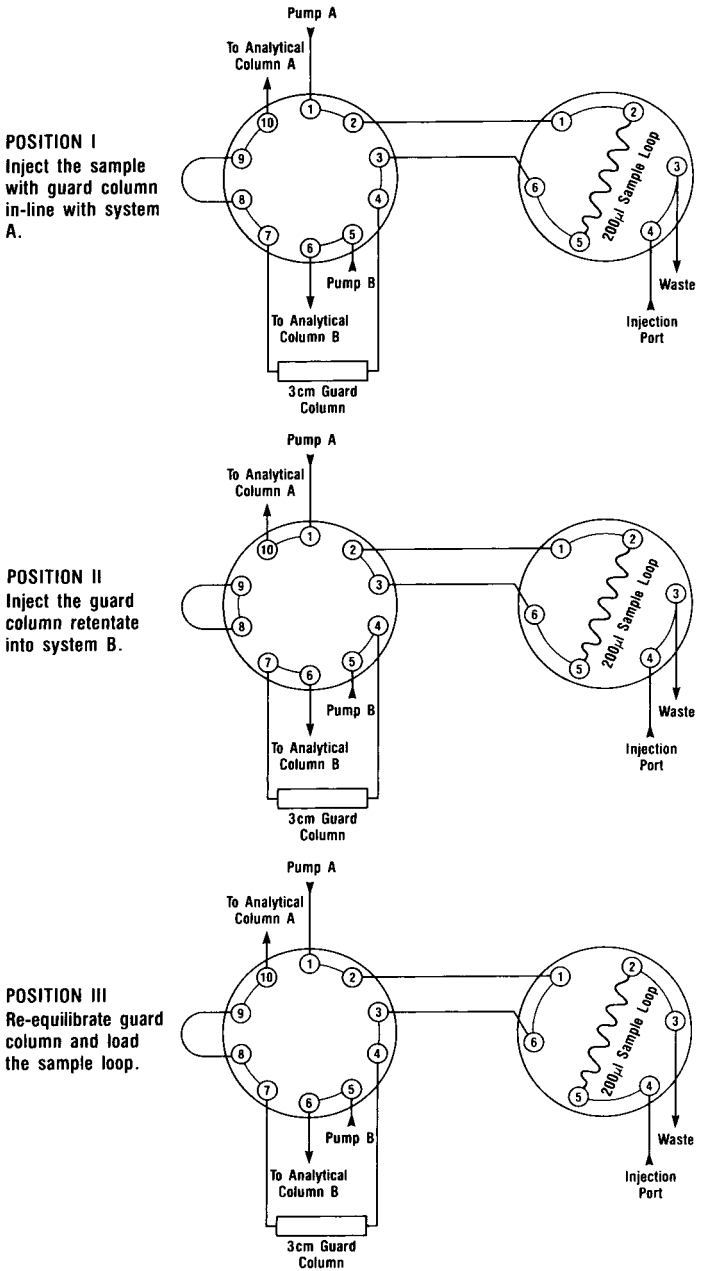


Fig. 3. Schematic representation of the HPLC apparatus.

switching valves (Valco Instruments, Houston, TX, U.S.A.). The position of these valves was controlled by a Beckman 421 system controller, which has built-in external flags for the control of external devices. The system controller was interfaced to the switching valves by a solenoid valve/solid state relay device assembled by the Research Technical Services Unit, Pharmaceutical Research and Development, Upjohn. Three switching valve positions were employed for this assay (Fig. 4): position III permitted loading of the sample loop and reconditioning of the guard column; position I injects the sample onto the guard column, with the guard column on-line in HPLC system A; and position II switches the guard column in-line with HPLC system B, and is used to inject guard column retentate from the initial injection into HPLC system B. The guard column was a Brownlee RP-18 Spheri-5, 3-cm cartridge (Brownlee Labs., Santa Clara, CA, U.S.A.). Analytical columns A and B were Supelcosil® LC-18, 5 μm, 250 × 4.6 mm I.D. (Supelco, Bellefonte, PA, U.S.A.). Detector A was an LDC UV Monitor III, Model 1203A, equipped with a Cd lamp (229 nm) (Laboratory Data Control, Riviera Beach, FL, U.S.A.). Detector B was a Beckman 165 variable-wavelength detector (229 nm).

Peak height and area measurements, response factor calculations, linear regression analysis, and unknown quantitation calculations for both detectors were performed using the Upjohn chromatography system on a Harris 500 computer.

Switching Valve Configurations



ANALYSIS PROGRAM
Position I: 0-5 min. (External Flag 1 at 0 min.)
Position II: 5-20 min. (External Flag 2 at 5 min.)
Position III: 20-25 min. (External Flags 3 and 4 at 20 min.)

Fig. 4. Schematic representation of the switching valve configurations used to complete one analysis cycle.

HPLC conditions

Mobile phase A (Fig. 3) was acetonitrile—7 m*M* phosphoric acid (aq.), pH 2.5 (4:6). Mobile phase B was acetonitrile—7 m*M* phosphoric acid (aq.), pH 2.5 (7:3). Phosphate buffer (pH 2.5) for the mobile phases was prepared by diluting 0.50 ml of 85% phosphoric acid to 1.0 l with water. The water (HPLC grade, Burdick and Jackson Labs.) used to prepare mobile phase A was extracted twice with a 0.1-volume of hexane in a separatory funnel in order to remove non-polar contaminants which otherwise accumulated on the guard column and interfered with the system B analysis. For the same reason, mobile phase A was not filtered through an ultrafiltration system prior to HPLC. Membrane filters from several suppliers were found to contaminate the mobile phase with non-polar components. Mobile phase B was filtered through 0.45-μm Nylon 66 membrane filters (Rainin Instrument, Woburn, MA, U.S.A.). Both mobile phases were degassed by helium purging. Mobile-phase flow-rates were 1.4 ml/min and 1.25 ml/min for mobile phases A and B, respectively, and both pumps operated continuously throughout the analysis cycle. The guard and analytical columns were at ambient temperature. The injection volume was 0.2 ml.

Serum extraction

Blood was collected from human volunteers by venipuncture and stored at 4°C for 3 h. Serum was collected by centrifugation in a refrigerated centrifuge for 15 min at 1300 *g*. Serum samples were transferred immediately to 15-ml screw-capped vials fitted with aluminium-lined caps and frozen at −20°C.

Bond Elut columns were prepared for serum extraction by the following procedure: C_{18} columns were washed with 2 ml of methanol followed by 2 ml of hexane-washed water (see HPLC conditions); silica gel columns were washed with 5 ml of hexane followed by 5 ml of methanol. The washed C_{18} columns were connected to the vacuum manifold (J.T. Baker, Phillipsburg, NJ, U.S.A.) and, without applying vacuum, 0.5 ml of 1.0 *M* phosphate buffer (pH 7.0) was added to each followed by 0.5 ml of the thawed serum specimen. The internal standard for the metabolite analysis was then added (20 μl of a 0.25-mg U-11,824 per ml solution in methanol) using a BDL positive displacement pipettor (Becton Dickinson Labware, Oxnard, CA, U.S.A.). After using the syringe action of the pipettor to mix the barrel contents, vacuum (660 mmHg internal manifold pressure) was applied and the eluent was discarded. The columns were washed with 0.5 ml water, twice (discarding the eluent), and aspirated for 1 min to remove excess water. The C_{18} columns were then removed from the vacuum manifold and replaced with silica gel columns, to the top of which were attached the loaded C_{18} columns using a special column-to-column adaptor (Analytichem International). The drug and metabolites were then eluted by vacuum aspirating two 0.5-ml washes of methanol through the connected columns. The methanol eluent was collected in 10 × 75 mm glass culture tubes and evaporated to dryness at 40°C under a stream of nitrogen. The resulting residue was reconstituted in 0.5 ml of 40% methanol by first adding 0.2 ml of a methanolic solution containing the internal standard for the ciglitazone analysis (2 μg of U-53,059 per ml in methanol) and vortexing for 45 sec, followed by adding 0.3 ml of water and vortexing for 15 sec. This reconstitution procedure

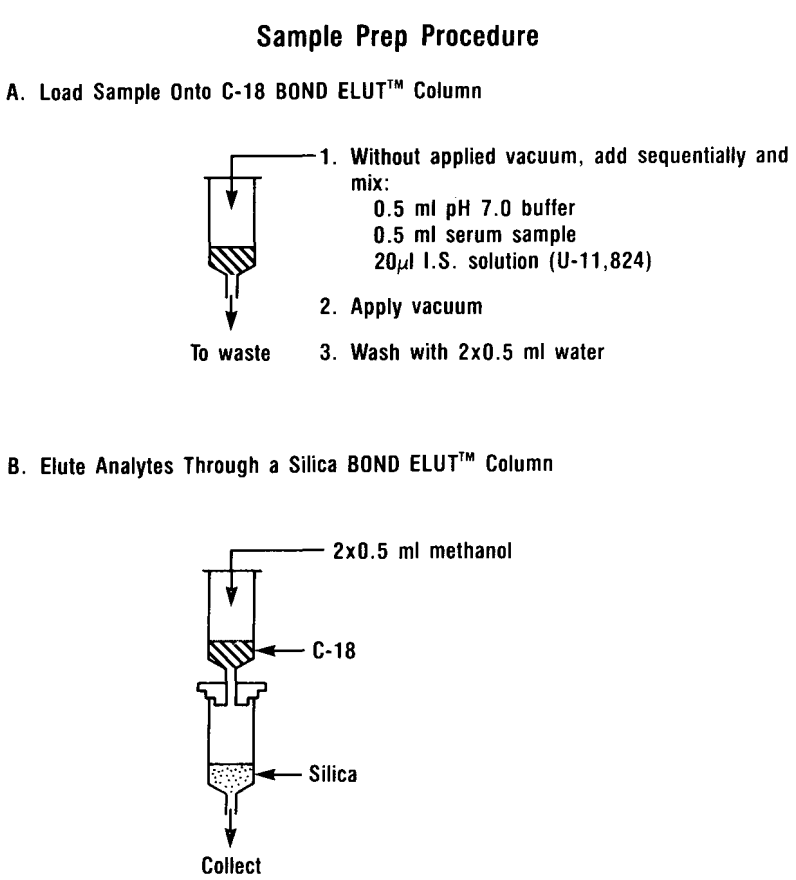


Fig. 5. Flow diagram of the serum extraction and cleanup procedure.

was necessary to ensure complete solubilization of the drug and metabolites. Using this extraction/cleanup procedure, summarized in Fig. 5, ten serum samples could be processed simultaneously in less than 1 h.

Standard preparation

Serum standards were prepared from pooled, blank serum specimens obtained from human volunteers. Accurately weighed amounts of ciglitazone and the four metabolite standards were dissolved in methanol to produce a stock concentration of 1 mg/ml for each metabolite and 0.5 mg/ml for ciglitazone. This solution was serially diluted with methanol to produce standard working solutions. Aliquots (10 ml) of fresh blank serum were then spiked with the standard solutions, keeping the methanol concentration of the resulting serum standards less than 2% (v/v). (This procedure produced more reliable results than were obtained by evaporating the standard solutions in a

vial and reconstituting with serum because of the limited solubility of ciglitazone in aqueous media.) After mixing, the serum standards were dispensed in 0.7-ml aliquots and frozen at −20° C until the time of analysis. Stability studies indicated no decline in potency after two weeks of storage.

Quantitation method

For quantitation purposes, the analysis for ciglitazone and its metabolites was treated as two independent assays, each having its own analytical system and internal standard. The internal standard (I.S.) for ciglitazone was U-53,059. The internal standard for the metabolites was U-11,824. Average response factors for each component were determined by analysis of serum standards that had been extracted and processed identically to unknown serum samples. The response factor for ciglitazone was calculated as the peak height ratio (ciglitazone/I.S.) divided by concentration, and the response factors for the metabolites were calculated as the peak area ratio (metabolite/I.S.) divided by concentration. The serum samples from each subject given the drug were analyzed as a set. Typically, each set consisted of fiteen samples and was analyzed with five serum standards having component concentrations dispersed evenly over the concentration range expected in the unknowns. The component concentrations in the unknowns were calculated by dividing the observed peak height or area ratio by the corresponding average response factor.

In vivo studies

Apparently healthy non-diabetic adult male volunteers were fasted 10 h prior to treatment and for 4 h following administration of four 250-mg compressed tablets of ciglitazone. Blood samples were collected at predetermined times ranging from 0 to 168 h post-dose. Serum was prepared as described above and stored frozen at −20° C.

RESULTS AND DISCUSSION

Chromatography

An isomodal column switching technique is described that permits the simultaneous quantitation of ciglitazone and its monohydroxyl metabolites by isocratic, reversed-phase HPLC (UV). The HPLC system consists of two isocratic units linked together via a common guard column (Fig. 3). The isocratic systems employ the same type of analytical column (C_{18}, 5 μm, porous particle), but use different strengths of mobile phase: system A is 40% acetonitrile; system B is 70% acetonitrile. The serum extract is injected with the guard column in-line with system A (Fig. 4). After the more polar components (metabolites and their internal standard, U-11,824) have cleared the guard column and been routed onto analytical column A, the guard column is switched in-line with system B for the analysis of the less polar components (ciglitazone and its internal standard, U-53,059). When the analysis on system A is complete, the guard column is switched back in-line with the weaker mobile phase to re-equilibrate for the next injection. The total run/recycle time for each injection is 25 min, and the column switching is done automatically with air-actuated valves and an event timer. The technique is called isomodal

because the entire analysis is conducted using one chromatographic mode (reversed-phase C_{18} columns with aqueous acetonitrile mobile phase).

The success of the system depends on the large difference in capacity factors between the parent drug and metabolites on the C_{18} guard column. Using mobile phase A, the metabolites and U-11,824 cleared the guard column in < 4 ml elution volume (determined by connecting the guard column directly to the detector). Under the same conditions, the elution volume of ciglitazone is 40 ml. Thus, at the 5-min run-time (7.0 ml elution volume) when the guard column is switched in-line with system B, the metabolites have completely eluted from the guard column while ciglitazone is fully retained. Since this separation is crucial for column routing, the ruggedness of the analytical system depends heavily on the ruggedness of the guard column. Using the Brownlee 3-cm Spheri-5 cartridge, it appears that a conservative estimate of column lifetime is 300 injection cycles. Guard column fatigue is detected as peak broadening or asymmetry rather than failure to retain ciglitazone. Typical component retention times and within-run relative standard deviations are given in Table I. Representative chromatograms of solution standards are shown in Fig. 6. Good system stability was indicated by retention time relative standard deviations for all components of $\leqslant 0.2\%$. The analytical columns exhibited approximately 12,000 theoretical plates for each component (N is uncorrected for peak skew). All components had peak asymmetry factors between 1.3 and 1.5 (ciglitazone = 1.4). Similar values were obtained when ciglitazone was analyzed on system B alone, indicating that the delayed elution with column switching did not adversely affect the chromatography. A relatively large injection volume (0.2 ml) was used because of autosampler requirements, but it was found that the 40% methanol sample solvent did not

TABLE I

ANALYTE RETENTION TIMES AND WITHIN-RUN RETENTION TIME PRECISION

Analyte name	Chromatographic system routing	Retention time* ± S.D. (min)	Relative standard deviation (%)
Trans-4′-ol	A	9.19 ± 0.02 (n = 20)	0.2
Cis-3′-ol	A	12.09 ± 0.02 (n = 20)	0.2
Trans-3′-ol	A	12.53 ± 0.01 (n = 9)	0.1
Cis-4′-ol	A	13.48 ± 0.02 (n = 20)	0.2
U-11,824 (I.S.)	A	17.98 ± 0.02 (n = 20)	0.1
Ciglitazone	B	17.07 (12.07)** ± 0.02 (n = 20)	0.2
U-53,059 (I.S.)	B	22.68 (17.68)** ± 0.03 (n = 15)	0.1

*Time reckoned from time 0-min injection.

**Parenthetical values are the retention times in system B reckoned from the time 5-min injection of the guard-column retentate.

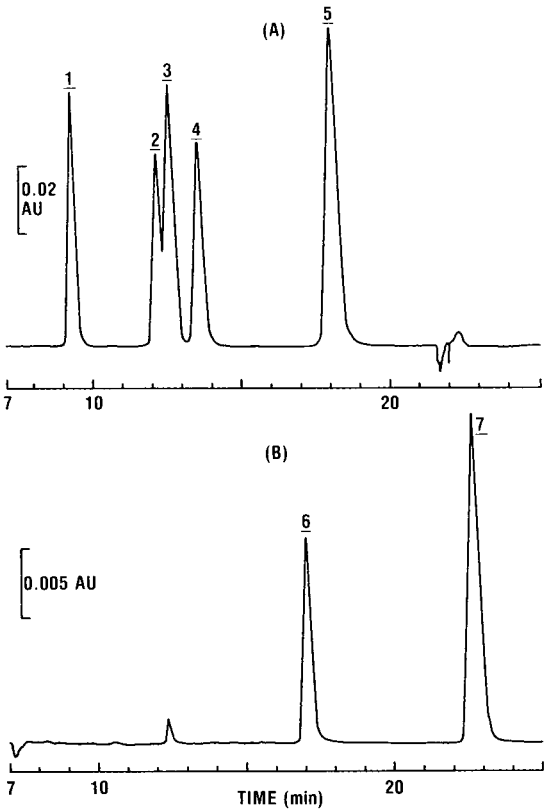


Fig. 6. Simultaneous chromatograms collected during the analysis of a solution standard containing approximately 5 μg/ml of each metabolite (A) and 0.5 μg/ml of ciglitazone (B). Peaks: (1) *trans*-4′-ol; (2) *cis*-3′-ol; (3) *trans*-3′-ol; (4) *cis*-4′-ol; (5) U11,824, I.S.; (6) ciglitazone; and (7) U-53,059, I.S. See text for chromatographic conditions.

contribute to band broadening when injected with mobile phase A (40% acetonitrile). All components were baseline resolved with the exception of *cis*-3′-ol and *trans*-3′-ol (resolution factor (R_s) = 0.9).

During the development of this method, other columns (silica, CN, TMS, C_8) were tested for their ability to compress the drug/metabolite separation into a single isocratic chromatogram. None was successful, nor did any of the columns resolve the *trans*-3′-ol and *cis*-3′-ol metabolites as well as the C_{18} column. Maximum resolution of these components was obtained with a C_{18} column and a mobile phase consisting of $\leqslant$ 40% acetonitrile at a flow-rate of $\leqslant$ 1.5 ml/min. (Acetonitrile was comparable to methanol for achieving this separation, and considerably better than tetrahydrofuran.) The pH of the mobile phase was adjusted to 2.5 to suppress ionization of the thiazolidinedione functional group (pK_a = 5.7).

Using a mobile-phase strength that gave acceptable capacity factors for the metabolites (k' = 3—6), the retention time of ciglitazone on the C_{18} column was greater than 2 h (k' = 40). This made it impractical to perform the analysis by isocratic elution. Torii et al. [17] overcame this problem by using thin-layer chromatography to separate the serum extract into parent drug and metabolite

fractions and then subsequently analyzing them with separate isocratic HPLC systems. Isomodal column switching techniques [6—10] offer a convenient alternative in this situation; as described here, the serum extract is fractionated into polar and non-polar components for subsequent isocratic HPLC analysis by an automated, on-line system. This approach combines the simplified sample workup used for gradient HPLC with the stability and sensitivity of isocratic HPLC, and is also relatively fast (step gradient run/recycle time of 45 min for this separation compared to the column switching run/recycle time of 25 min).

Serum extraction and percentage recovery

The serum extraction procedure was designed for rapid, one-step sample cleanup of both ciglitazone and its monohydroxyl metabolites. After buffering to pH 7.0, the serum was drawn through a C_{18} Bond Elut column (Fig. 5). The column was washed with water and then connected in series with a Bond Elut silica gel column, which was used to remove several polar components that interfered with metabolite quantitation. The analytes were eluted from both columns with two washes of methanol. The methanol was then evaporated and the extract was reconstituted for analysis.

Using this procedure, the recovery of the drug and metabolites from aqueous

TABLE II

PERCENTAGE RECOVERY OF CIGLITAZONE AND METABOLITES FROM HUMAN SERUM

Serum concentrations ranged from 0.1 to 20 μg/ml for the metabolites and 0.05 to 10 μg/ml for ciglitazone. Recovery was independent of concentration.

Analyte name	Parameter	Percent recovery			Pooled estimate of percent recovery
		Day 1	Day 2	Day 3	
Trans-4′-ol	$\bar{X}$	80.1	81.4	82.3	81.2
	S.D.	5.7	3.1	3.9	4.1
	R.S.D.	7.1	3.8	4.7	5.1
	n	8	8	8	24
Cis-3′-ol	$\bar{X}$	85.1	84.5	89.3	86.3
	S.D.	4.8	3.6	2.1	4.1
	R.S.D.	5.7	4.2	2.4	4.8
	n	8	8	8	24
Trans-3′-ol	$\bar{X}$	91.3	90.9	93.8	92.0
	S.D.	4.2	2.2	2.7	3.3
	R.S.D.	4.6	2.4	2.9	3.5
	n	8	8	8	24
Cis-4′-ol	$\bar{X}$	88.7	94.7	92.5	91.8
	S.D.	4.6	5.7	2.6	4.9
	R.S.D.	5.2	6.0	2.8	5.4
	n	7	7	7	21
Ciglitazone	$\bar{X}$	60.1	62.7	63.7	62.1
	S.D.	6.7	3.7	3.5	5.0
	R.S.D.	11.2	5.9	5.6	8.0
	n	8	8	8	24

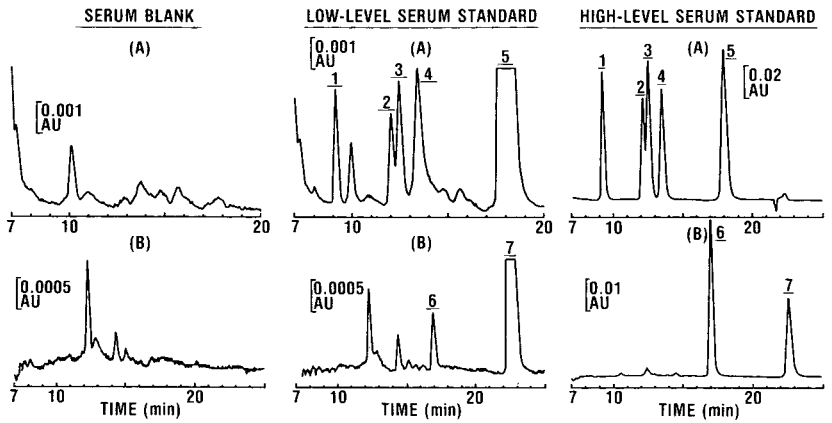


Fig. 7. Simultaneous chromatograms collected during the analysis of serum standards for metabolites (A) and ciglitazone (B). The low level standard was spiked with roughly 0.2 μg/ml of each metabolite and 0.1 μg/ml of ciglitazone; the high-level standard was spiked with 5 μg/ml of each metabolite and 2.5 μg/ml of ciglitazone. Peak identification as in Fig. 6.

solutions was quantitative. From serum, however, the absolute recovery was lower: 81—92% for the four metabolites and 62% for ciglitazone (Table II). The recovery of each component was determined at eight concentrations ranging from 0.1 to 20 μg/ml for the metabolites and 0.05 to 10 μg/ml for ciglitazone. Over this concentration range, the percent absolute recovery for each component was constant with a relative standard deviation of roughly 5% for the metabolites and 8% for ciglitazone (Table II, representative chromatograms are shown in Fig. 7). Additional methanol washes did not improve the ciglitazone recovery. Protein binding studies have shown the drug to be strongly bound (> 95% in plasma) [17] and this may interfere with the bonded-phase extraction process. Serum aging studies with serum standards indicated that there was no change in analyte absolute recovery from samples stored at −20° C for four weeks.

TABLE III

LINEARITY AND REPRODUCIBILITY OF SERUM STANDARD CALIBRATION CURVES

Serum standard concentrations ranged from 0.1 to 20 μg/ml for the metabolites and from 0.05 to 10 μg/ml for ciglitazone.

Parameter	*Trans*-4′-ol			*Cis*-3′-ol		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
Mean response factor* (X 100)	9.57	9.16	9.20	9.81	9.34	9.52
±S.D.	0.38	0.24	0.40	0.31	0.32	0.38
R.S.D.	3.9	2.7	4.3	3.2	3.5	4.0
Slope**	0.0967	0.0930	0.0929	0.0992	0.0946	0.0956
y-Intercept***	0.0023	−0.0027	0.0022	0.0001	0.0011	−0.0034
r	>0.999	>0.999	>0.999	>0.999	>0.999	>0.999

*Response factor = (peak area of analyte)/[(peak area of internal standard) X (concentration of analyte in μg/ml)].

**Obtained from best-fit linear regression analysis of a plot of peak height or area ratio (analyte/internal standard) versus the analyte concentration in μg/ml.

***The *y*-intercept was insignificant ($P > 0.05$) in all cases.

Internal standards

Since the analytical method employs two isocratic HPLC systems for the analysis of ciglitazone and its metabolites, two internal standards are required for quantitation. U-11,824 was found to be a suitable internal standard for the quantitation of the metabolites. It elutes in a region of the metabolite chromatogram free from interferences (see Fig. 7), it is extracted with similar efficiency from serum by a C_{18} extraction column (80% recovery), and factors that cause a change in the metabolite recovery also cause a similar change in U-11,824 recovery. It was therefore added to the serum prior to extraction.

Conversely, a suitable internal standard to control for the extraction variability of ciglitazone could not be located. Several sulfonylureas and related compounds were tested, but all had either unacceptably low extraction recovery (< 50%) or improper retention time. As a consequence, U-53,059 was selected as a chromatographic internal standard and was added during reconstitution of the extract for analysis.

Linearity

Data on the linearity and reproducibility of serum standard calibration curves on three separate days are given in Table III. The response factors for the metabolites were constant over the range 0.1—20 μg/ml, with relative standard deviations of roughly 4%. Plots of peak area ratio versus concentration for all of the metabolites had insignificant *y*-intercepts and correlation coefficients of ⩾ 0.999. The response factor for ciglitazone was also constant over the concentration range 0.05—10 μg/ml, with a relative standard deviation of roughly 8%. Plots of peak height ratio versus concentration had insignificant *y*-intercepts and correlation coefficients of ⩾ 0.994. The larger relative standard deviation and poorer linear correlation coefficient for ciglitazone compared to the metabolites are probably due to the poorer ciglitazone extraction efficiency and to the lack of an internal standard to account for ciglitazone extraction variability (U-53,059 is a chromatographic internal standard only). A minor increase in precision was obtained by using U-11,824 as the internal standard for both assays, but the improvement was not sufficient to justify the additional manual calculations required by limitations of the computer system.

Trans-3′-ol			*Cis*-4′-ol			Ciglitazone		
Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
11.8	11.2	11.4	10.6	10.4	10.2	76.4	80.6	81.3
0.4	0.4	0.4	0.5	0.5	0.3	8.6	4.8	4.5
3.4	3.3	3.4	4.4	5.2	2.7	11.2	5.9	5.6
0.115	0.110	0.112	0.106	0.103	0.102	0.855	0.796	0.793
0.005	0.006	0.004	0.004	0.007	0.000	−0.133	0.014	0.059
>0.999	>0.999	>0.999	0.999	>0.999	>0.999	0.994	0.998	0.998

Precision and accuracy

The intra-day and inter-day precision and accuracy of the assays were evaluated at three different concentration levels for each metabolite and ciglitazone by repeating triplicate assays of spiked serum samples on three days. The results of these experiments are presented in Table IV. The mean intra-day relative standard deviation for the assay over the concentration range 0.2—5 μg/ml for the metabolites and 0.1—2.4 μg/ml for ciglitazone appears to be roughly 6%. There appears to be no significant inter-day variation of assay results over this concentration range. There also appears to be no significant assay bias, since pooled estimates of assay error for both ciglitazone and the metabolites were (with one exception) less than ± 4%.

TABLE IV

ASSAY PRECISION AND ACCURACY

Serum samples were analyzed in triplicate on each of three successive days in order to determine both intra- and inter-day method variance. Analysis of variance procedures ($P > 0.05$) indicated that there was no significant interday difference in assay results for any of the analytes, and so the data for all three days ($n = 9$) were pooled.

Analyte name	Actual concentration of spiked serum of sample (μg/ml)	Mean assay result ($n = 9$) ± S.D. (μg/ml)	Relative standard deviation (%)	Percent error*
Trans-4'-ol	5.03	5.06 ± 0.29	5.7	0.6
	0.503	0.500 ± 0.020	4.0	—0.6
	0.202	0.195 ± 0.008	3.9	—3.5
Cis-3'-ol	4.55	4.68 ± 0.28	6.0	2.9
	0.455	0.466 ± 0.011	2.4	2.4
	0.182	0.181 ± 0.009	5.0	—0.5
Trans-3'-ol	6.21	6.33 ± 0.32	5.0	1.9
	0.621	0.645 ± 0.013	2.0	3.9
	0.249	0.257 ± 0.014	5.6	3.2
Cis-4'-ol	5.51	5.51 ± 0.27	5.0	0.0
	0.551	0.555 ± 0.019	3.4	0.7
	0.221	0.209 ± 0.012	5.7	—5.4
Ciglitazone	2.37	2.37 ± 0.09	3.7	0.0
	0.237	0.230 ± 0.015	6.7	—3.0
	0.095	0.094 ± 0.006	6.7	—1.4

*Percent error = [(observed — actual)/actual] × 100.

Sensitivity

The assay quantitation limit was defined as that concentration of analyte which produces a peak roughly ten times the size of serum blank peaks having retention times such that they may interfere with analyte measurement. The assay quantitation limit for the metabolites was roughly 0.1 μg/ml. The quantitation limit for ciglitazone was 0.05 μg/ml.

Application

The utility of the assay method was demonstrated by analyzing serum

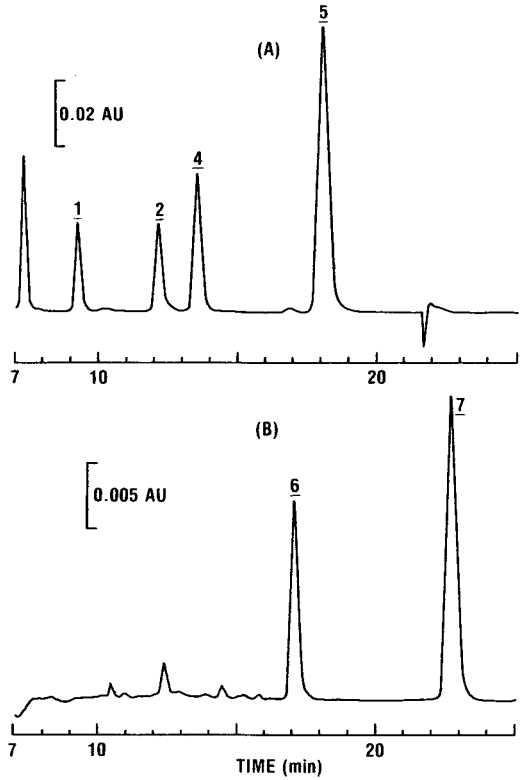


Fig. 8. Simultaneous chromatograms collected for the metabolites (A) and ciglitazone (B) during the analysis of a 3-h post-dose serum sample from a human subject given a single 1000-mg oral dose of ciglitazone. Peak identification as in Fig. 6.

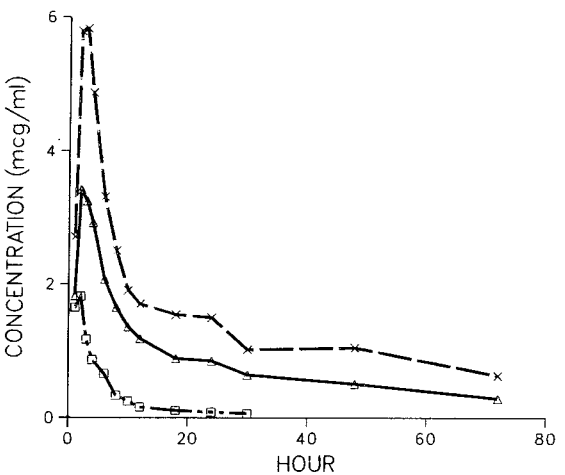


Fig. 9. Composite serum concentration—time profile from three human subjects given single 1000-mg oral doses of ciglitazone. (△), *cis*-3′-ol; (×), *cis*-4′-ol; and (□), ciglitazone.

samples from selected non-diabetic, human male volunteers participating in a ciglitazone phase I tolerance study. Based on coelution with authentic standards, measurable levels of ciglitazone, *cis*-4′-ol, *cis*-3′-ol, and *trans*-4′-ol were detected in serum following a 1000-mg oral dose (Fig. 8). The *trans*-3′-ol metabolite was not detected. (In order to form a distinct shoulder on the *cis*-3′-ol peak, the *trans*-3′-ol concentration must be > 20% of the concentration of the *cis*-3′-ol.) As shown in the composite serum concentration—time profile (Fig. 9), the assay method was sufficiently sensitive to assay the 78-h timepoint for the metabolites (2—3 times the $\beta t_{1/2}$) and the 30-h timepoint for ciglitazone (3 times the $\beta t_{1/2}$). Selected timepoints were assayed in duplicate and found to agree within the estimated method relative standard deviation.

CONCLUSION

The applicability of an isomodal, column switching HPLC technique was demonstrated for the routine, quantitative analysis of ciglitazone, a potential oral diabetic agent [1, 2], and its monohydroxyl metabolites in human serum. This analysis could not be conducted using a simple isocratic HPLC technique because of the polarity difference between ciglitazone and the metabolites. Resolution of the metabolite isomers (*cis*- and *trans*-isomers of 3′- and 4′-hydroxy metabolites) required a reversed-phase C_{18} HPLC system, on which the capacity factor ratio of the parent drug to metabolites was approximately ten to one. Rather than perform the analysis by gradient elution HPLC, an HPLC apparatus was designed to conduct on-line fractionation of the drug/metabolite serum extract into polar (metabolite) and non-polar (drug) fractions that were then automatically routed into individually optimized, isocratic, reversed-phase systems for analysis.

This column switching procedure performs essentially the same separation that could have been accomplished by a reversed-phase step gradient technique or by manually dividing the sample into equal portions for analysis on separate, reversed-phase, isocratic systems. The separation selectivity of all these methods is the same because they all rely on a single, identical, chromatographic mode. The advantage of the isomodal column switching approach over the manual two-injection procedure is that it conducts the same separation automatically and with a single sample injection. Compared to the gradient method, the column switching procedure is faster (25-min run/recycle time for column switching compared to 45-min run/recycle time for gradient), and it has the reliability and stability of isocratic elution methods.

For more general applications, this approach to multicomponent analysis could, in principle, be used with any chromatographic mode for which there is a large difference in capacity factors between the drug and metabolites. In this respect, isomodal column switching is equally versatile to gradient elution. A serious limitation of this column switching approach is that unlike gradient elution, which permits analysis for multiple analytes with uniformly distributed capacity factors over a broad range, the column switching method is restricted to the analysis of samples in which the analytes fall into one of two relatively narrow capacity factor ranges. For situations that meet this requirement, the isomodal column switching technique offers advantages over conventional

analysis procedures in terms of speed and reliability for routine, quantitative, drug/metabolite assays.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge Dave Gleason for construction of the solid-state relay device interfacing the system controller with the switching valves, and Linda Missias for assistance in the preparation of this manuscript.

REFERENCES

1 A.Y. Chang, B.M. Wyse, B.J. Gilchrist, T. Peterson and A.R. Diani, Diabetes, 32 (1983) 830.
2 A.Y. Chang, B.M. Wyse and B.J. Gilchrist, Diabetes, 32 (1983) 839.
3 L.R. Snyder, J. Chromatogr. Sci., 8 (1970) 692.
4 J.E. Paanaker, J.M.S.L. Thio and H.J.M. van Hal, Methodol. Surv. Biochem., 10 (1981) 214.
5 J.C. Suatoni, H.R. Garber and B.E. Davis, J. Chromatogr. Sci., 13 (1975) 367.
6 L.R. Snyder and J.J. Kirkland, Introduction to Modern Liquid Chromatography, Wiley, New York, 2nd ed., 1978, p. 662.
7 J.F.K. Huber, R. van der Linden, E. Ecker and M. Oreans, J. Chromatogr., 83 (1973) 267.
8 R.E. Majors, J. Chromatogr. Sci., 18 (1980) 571.
9 F. Erni, H.P. Keller, C. Morin and M. Schmitt, J. Chromatogr., 204 (1981) 65.
10 J.F.K. Huber and F. Eisenbeiss, J. Chromatogr., 149 (1978) 127.
11 R.J. Dolphin, L.P.J. Hoogeven, F.W. Wilmott and A.D. Mills, J. Chromatogr., 122 (1976) 259.
12 D.H. Freeman, Anal. Chem., 53 (1981) 2.
13 J.A. Apffel, J.V. Alfredson and R.E. Majors, J. Chromatogr., 206 (1981) 43.
14 D.L. Conley and E.J. Benjamine, J. Chromatogr., 257 (1983) 337.
15 J.C. Gfeller and M. Stockmeyer, J. Chromatogr., 198 (1980) 162.
16 C.M. Riley, L.A. Sternson, A.J. Repta and R.W. Siegler, J. Chromatogr., 229 (1982) 373.
17 H. Torii, K. Yoshida, T. Tsukamoto and S. Tanayama, Xenobiotica, in press.

Journal of Chromatography, 307 (1984) 172—179
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2015

Note

Capillary gas chromatographic investigation of plasma lipid alcoholysis during alcohol extraction

MENDEL TUCHMAN* and WILLIAM KRIVIT

Department of Pediatrics, University of Minnesota, Box 391, Mayo Memorial Building, 516 Delaware Street S.E., Minneapolis, MN 55455 (U.S.A.)

(First received August 11th, 1983; revised manuscript received November 15th, 1983)

Alcoholysis of lipids has been known since 1846 when Rochleder [1] obtained fatty acid methyl esters using castor oil, methanol and dry hydrochloric acid. Other methods for alcoholysis have been described using hydrochloric acid methanol [2], hydrochloric acid and sulfuric acid [3], boron trifluoride methanol [4], potassium carbonate [5], sodium methoxide [6] and potassium hydroxide ethanol [7].

Alcohols are commonly used as solvents for extraction of plasma and tissue lipids. Chloroform—methanol extraction is most commonly used and is known as the method of Folch et al. [8]. Several authors have reported the formation of fatty acid alcohol esters as an artifact when using alcohols for extraction of lipids [9—14]. Using capillary gas chromatography (GC) we investigated the formation of fatty acid alcohol esters when treating plasma with alcohol. The high efficiency of the capillary columns enabled us to separate the various derivatives of fatty acids.

EXPERIMENTAL

Materials

Lauric acid (dodecanoic), myristic acid (tetradecanoic), palmitic acid (hexadecanoic) and stearic acid (octadecanoic) were purchased from Poly Science (Niles, IL, U.S.A.). Pentadecanoic acid, linoleic acid (9,12-octadecadienoic), oleic acid (*cis*-9-octadecanoic) and arachidonic acid (6,10,14,18-eicosatetraenoic) were obtained from Sigma (St. Louis, MO, U.S.A.). Chloroform, ethanol, ethyl acetate, methanol and propanol were obtained from Spectrum Chemical Manufacturing (Redondo Beach, CA, U.S.A.). N,O-Bis(trimethylsilyl)-trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) were from

0378-4347/84/$03.00

Pierce (Rockford, IL, U.S.A.). L-α-Lecithin (bovine liver) and L-α-dipentadecanoyl lecithin were purchased from Avanti Polar Lipids (Birmingham, AL, U.S.A.). Tripentadecanoin and cholesteryl pentadecanoate were from Nu-Chek-Prep (Elysian, MN, U.S.A.).

Methods

Standard solutions of free fatty acids were prepared by dissolving 20 mg of free acid in 20 ml of ethyl acetate. Standard solutions of triglycerides, phospholipids, and cholesterol esters were prepared by dissolving 10 mg of the lipid in 1 ml of chloroform. All standard solutions were stored below 0°C.

Different amounts (10, 25 and 50 μg) of lauric (C_{12}), myristic (C_{14}), palmitic (C_{16}), linoleic ($C_{18:2}$), oleic ($C_{18:1}$), stearic (C_{18}), and arachidonic ($C_{20:4}$) acids were derivatized with 25 μl of BSTFA—TMCS (10:1). A 2-μl aliquot of the resulting solution was injected into the capillary column of the gas chromatograph and peaks were obtained for each acid as trimethylsilyl (TMS) derivatives.

We used a Model 5880A gas chromatograph (Hewlett-Packard, Palo Alto, CA, U.S.A.) equipped with two split-mode capillary injection ports and two flame ionization detectors. The chromatograph was controlled by a Level 4 Hewlett-Packard computer terminal. We used a 25 m × 0.31 mm I.D. cross-linked fused-silica capillary column, with a 0.52-μm thick film, and a stationary phase of 5% methyl silicone (Ultra-1). The flow-rate (linear velocity) of the carrier gas helium was 25 cm/sec. The injection split ratio was 1:100, and the injection port and detector temperatures were 250°C and 300°C, respectively.

The initial programmed oven temperature was 150°C, increasing by 5°C/min to a final temperature of 275°C, after which the oven temperature was increased to 325°C and held for 15 min before cooling to 150°C.

Retention times and peak areas were determined by the computer. Methylene units were calculated with a program stored on tape.

Heparinized blood was obtained by venipuncture from laboratory personnel. The plasma was immediately separated and if not analyzed, stored at −70°C. A 1-ml sample of plasma was slowly mixed with 6 volumes of alcohol (methanol, ethanol, or propanol). After centrifugation, the alcoholic supernatant was dried under a stream of nitrogen at 50°C. To the dry residue, 1 ml of deionized water was added and the fatty acids were extracted with 3 ml of ethyl acetate. The ethyl acetate layer was separated by centrifugation and dried under nitrogen. The dry residue was derivatized with 25 μl of BSTFA—TMCS (10:1) at 80°C for 15 min. A 2-μl aliquot of the resulting solution was injected into the capillary column of the gas chromatograph.

Ethanol precipitation was used for the following investigations:

In order to check whether esterification of free fatty acids occurs, different amounts (10, 25 and 50 μg) of long-chain fatty acids were added to plasma which was treated as described above. The peak areas of the fatty acids TMS and ethyl ester derivatives were compared to those of the plasma with no fatty acids added to assess whether any of the added fatty acids was converted to ethyl ester. Recovery percentages of the free fatty acids added to plasma were calculated.

In order to find out which lipid class is affected by transesterification, we added to three 1-ml aliquots of the same plasma, 1 mg of tripentadecanoin,

1 mg of L-α-dipentadecanoyl lecithin and 1 mg of cholesteryl pentadecanoate. The samples were then treated as described above and we looked for TMS and ethyl ester derivatives peak areas of pentadecanoic acid on the chromatograms to assess the degree of alcoholysis (pentadecanoic acid, being an odd number carbon fatty acid, was not detected in normal plasma with our method).

The effect of room temperature on plasma lipid alcoholysis was determined by extracting two 1-ml aliquots of the same plasma. One sample was dried at room temperature while the other was dried at 50°C (drying time 2 h). We compared the peak areas of the TMS and ethyl ester derivatives of the fatty acids in both samples.

We prepared three 0.5-ml aqueous solutions to each of which we added 1 mg of L-α-lecithin (bovine liver); to two of these we added 2.5 mg of sodium carbonate and adjusted the pH of one to neutrality with 1 *M* hydrochloric acid solution. These samples were treated as plasma and were analyzed by capillary GC.

Finally, we analyzed two 1-ml aliquots of the same plasma. To one of the samples we added 0.05 mequiv. of hydrochloric acid to eliminate sodium bicarbonate; then we added to both samples 1 mg of L-α-dipentadecanoyl lecithin and treated them as described before. All identifications of the fatty acid derivatives were confirmed by mass spectrometry. All samples were tested in duplicate.

RESULTS

Fig. 1 shows the chromatogram obtained from free fatty acids standards as their TMS derivatives on the Ultra-1 column. Fig. 2 shows three chromatograms of plasma to which L-α-dipentadecanoyl lecithin was added and which was treated with methanol (a), ethanol (b), or propanol (c). Methyl, ethyl, and propyl esters were obtained for palmitic, linoleic, oleic, stearic acids (derived from plasma lipids) and for pentadecanoic acid (derived from L-α-dipentadecanoyl lecithin added). TMS derivatives of lauric, myristic, palmitic, linoleic, oleic, stearic, arachidonic and pentadecanoic acids were also obtained. No ethyl esters were obtained from free fatty acids added to plasma. These acids were recovered exclusively as TMS derivatives and the recovery percentage was close to 100%.

It appears that most of the fatty acid TMS and alcohol ester derivatives were obtained from phospholipids, as added L-α-dipentadecanoyl lecithin resulted in much larger peaks of pentadecanoic TMS and pentadecanoic ethyl ester than added tripentadecanoin, the ratio being 30:1. We obtained no pentadecanoic acid peaks from cholesteryl pentadecanoate.

Table I compares the peak areas obtained for the fatty acid derivatives when the extract was dried at room temperature and at 50°C. Avoiding the warming of the extract did not result in less alcoholysis.

In aqueous solutions, alcoholysis occurred only when we added sodium carbonate to the solution (pH 11). When we adjusted the pH to 7 with hydrochloric acid the reaction did not occur.

Adding 0.05 mequiv. of hydrochloric acid to plasma before alcohol extraction (pH 5.5) resulted in no ethyl esters formation and no hydrolysis of phos-

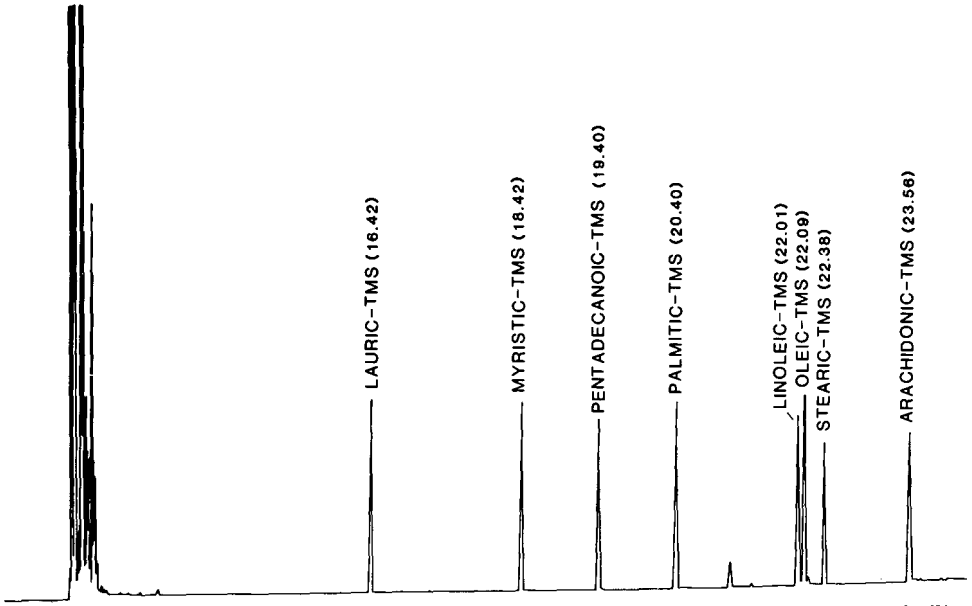


Fig. 1. Long-chain fatty acids as their TMS derivatives separated on a 5% methyl silicone (Ultra-1) capillary column temperature program 150°C to 275° C at 5°C/min. The number in brackets indicates the methylene units of the acid derivative.

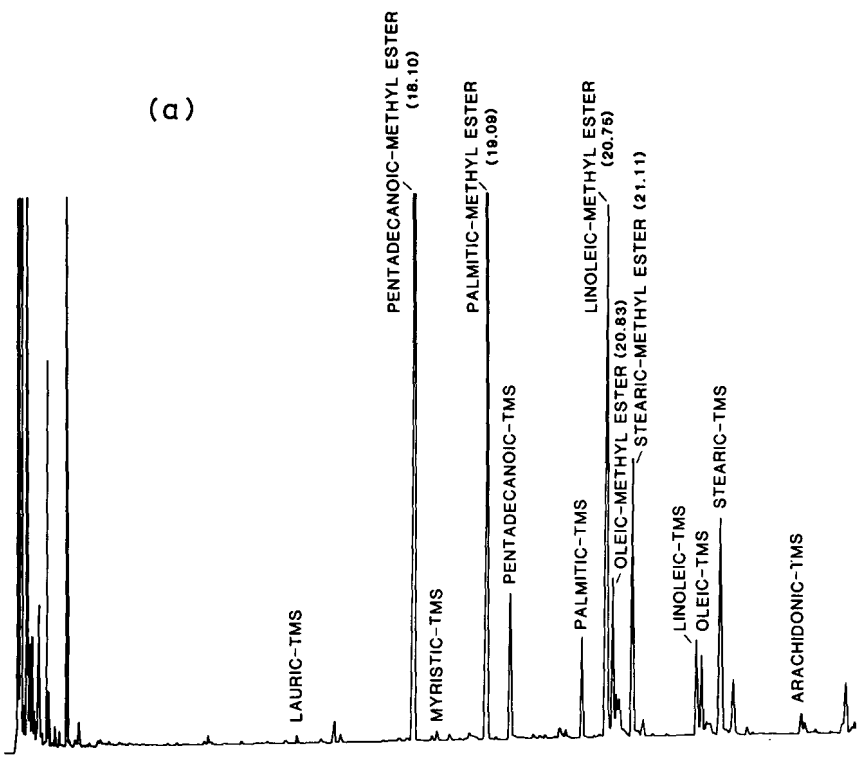


Fig. 2.

(Continued on p. 176)

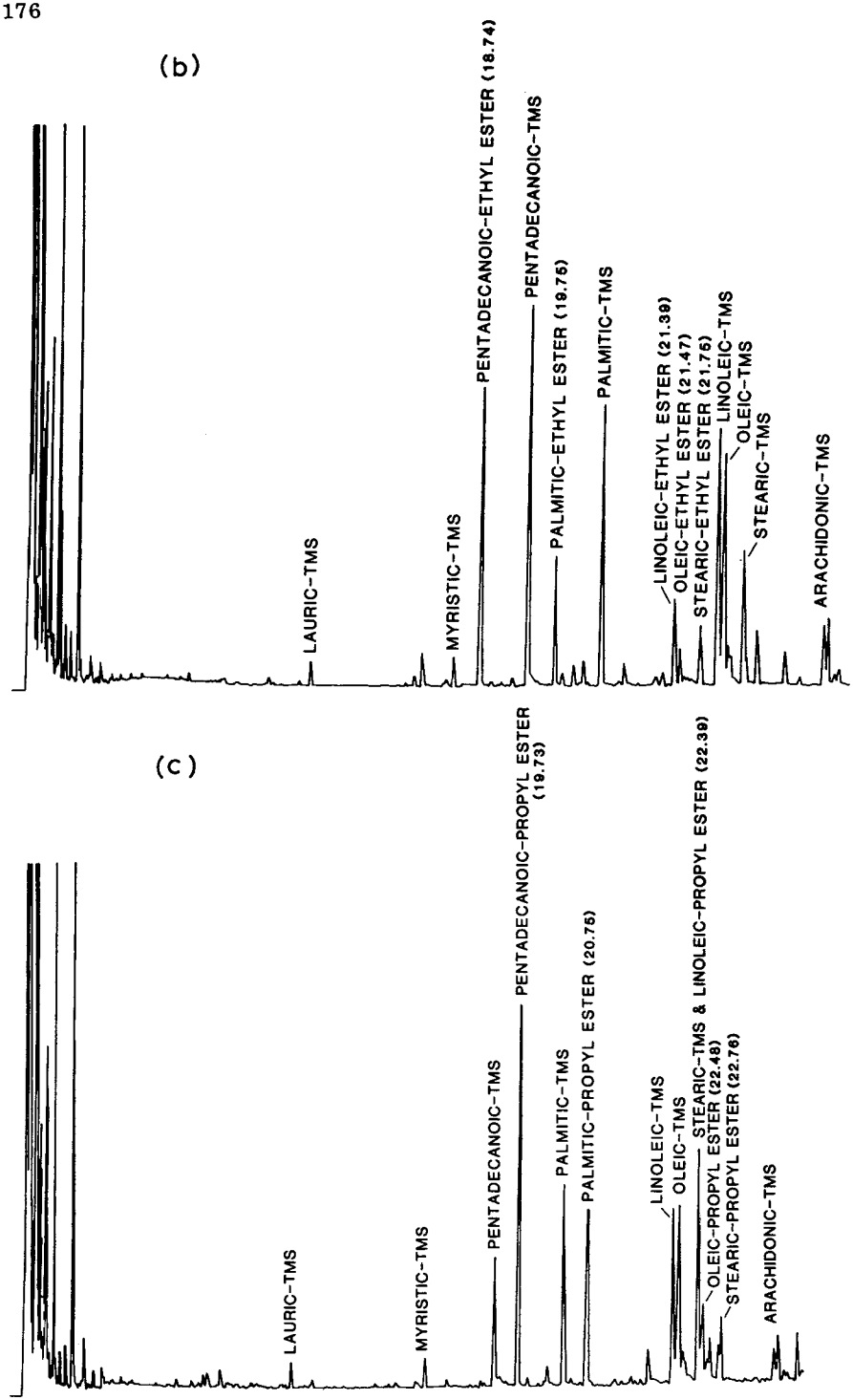


Fig. 2. Chromatograms obtained from plasma with L-α-dipentadecanoyl lecithin added, using methanol (a), ethanol (b), and propanol (c). The chromatograms were obtained on an Ultra-1 capillary column. The number in brackets indicates the methylene units of the acid derivative.

TABLE I

FATTY ACID ESTERS OBTAINED FROM THE SAME PLASMA EXTRACTED AT DIFFERENT TEMPERATURES

Fatty acid derivative	Peak areas	
	Room temperature	50°C
Palmitic ethyl ester	36.30	25.29
Palmitic TMS	185.32	170.29
Linoleic ethyl ester	29.01	25.12
Linoleic TMS	236.37	236.27
Oleic ethyl ester	8.30	5.54
Oleic TMS	237.68	218.48
Stearic ethyl ester	19.44	11.97
Stearic TMS	72.33	71.91

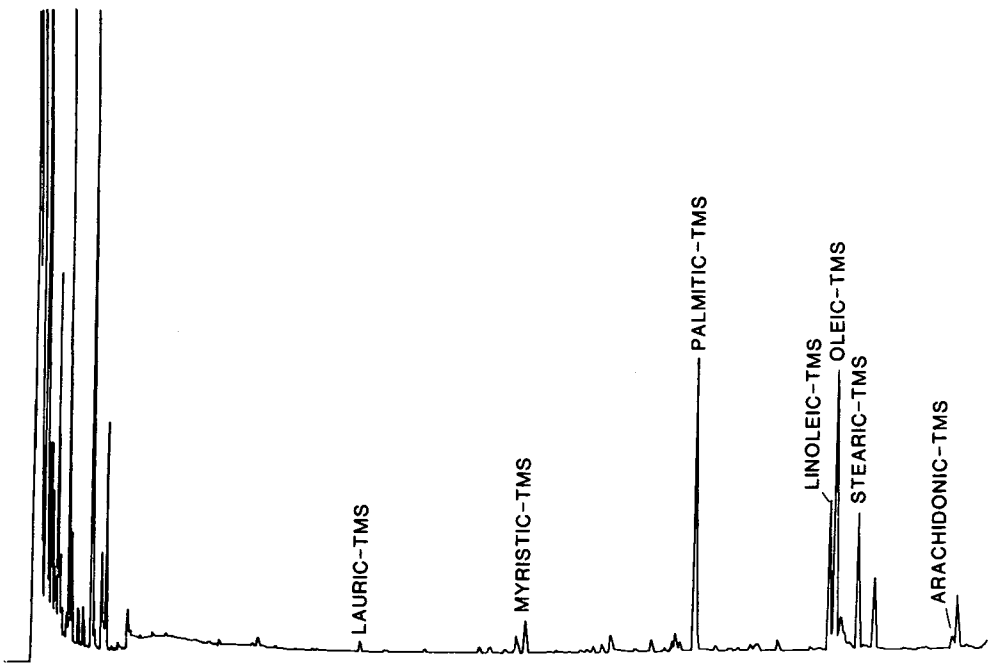


Fig. 3. A chromatogram obtained from plasma with L-α-dipentadecanoyl lecithin added, eliminating the plasma sodium bicarbonate prior to extraction.

pholipids and free fatty acid release as indicated by the lack of pentadecanoic acid peaks.

In this experiment we obtained peaks of free fatty acids as their TMS derivatives that were probably the original plasma free fatty acids. Fig. 3 displays the chromatogram obtained.

DISCUSSION

Several authors have noticed that during extraction of plasma or tissue lipids with alcohols, alcoholysis of these fats occurs. Newsome and Rattay [15] sug-

gested that free fatty acids may be esterified in the presence of alcohol by an enzymatic process. We did not obtain alcohol esters from free fatty acids added to plasma, thus precluding alcohol esterification of free fatty acids as a major source of this artifact.

Our experiments show that hydrolysis and transesterification of fatty acids from phospholipids, and to a much lesser extent, from triglycerides, are the explanation of this observation. No hydrolysis or transesterification from cholesterol esters occurred. These observations are contradictory to those of Lough et al. [10] who obtained most of the alcohol esters from triglycerides.

Analysis of our samples involved drying at 50°C, but when the extraction was performed at room temperature, there was no decrease in the extent of hydrolysis or transesterification of plasma fats.

Lough et al. [10] and Fukuda et al. [11] obtained transesterification of triglycerides by adding sodium carbonate. They postulated that heating the plasma or lymph lipid extracts produces sodium carbonate from sodium bicarbonate the former catalyzing the reaction.

We obtained significant hydrolysis and transesterification of phospholipids in plasma or in aqueous solutions with sodium carbonate added; this effect disappeared when hydrochloric acid was added to plasma or when the solution pH was neutralized with hydrochloric acid probably due to conversion of sodium carbonate to carbon dioxide. Gordon et al. [13], reported alcoholysis when storing lipids in acid alcohol solutions which precludes sodium carbonate as a catalyzing factor in that case.

It is possible that other yet unknown factors present in plasma or tissues are involved in alcoholysis of lipids. The fact that no alcoholysis of lipids was obtained after eliminating sodium bicarbonate with hydrochloric acid favors the findings of Lough et al. [10] and Fukuda et al. [11]. The present study indicates that it may be possible to determine plasma free fatty acids from alcoholic extracts using a direct method, providing the alcoholysis of plasma lipids can be avoided.

CONCLUSIONS

Artifact formation of free fatty acids and fatty acid alcohol esters occurs when plasma is treated with alcohols. This alcoholysis occurs mainly from phospholipids, and to a much lesser degree, from triglycerides. This can be avoided by pre-eliminating plasma sodium bicarbonate using hydrochloric acid.

ACKNOWLEDGEMENTS

Special thanks to Dr. Charles Sweeley, from the Department of Biochemistry, Michigan State University, East Lansing, Michigan, for his help and guidance. This work was supported by the University of Minnesota Graduate School Grants-in-Aid, Kroc Radio and Television Stations in Rochester, MN, U.S.A., and Order of the Eastern Star.

REFERENCES

1 F. Rochleder, Ann. Chem. Pharm., 59 (1846) 260.
2 A.Z. Rollet, Physiol. Chem., 61 (1909) 210.
3 G.Y. Shinowara and J.B. Brown, Oil Soap, 15 (1938) 151.
4 H. Bowlus and J.A. Nieuwland, Amer. Chem. Soc., 53 (1935) 3835.
5 A. Zorawski, Tluszcze Jadalne, 17 (1973) 182.
6 S.F. Herb, P. Magidman, F.E. Luddy and R.W. Riemenschneider, J. Amer. Oil. Chem. Soc., 39 (1962) 142.
7 H.P. Burchfield and E.E. Storrs, Biochemical Applications of Gas Chromatography, Academic Press, New York, 1962, p. 528.
8 J. Folch, M. Lees and G.H. Sloan Stanley, J. Biol. Chem., 226 (1957) 497.
9 E. Fischer, Ber. Deut. Chem. Ges., 53 (1920) 1634.
10 A.K. Lough, L. Felinski and G.A. Garton, J. Lipid Res., 3 (1962) 478.
11 J.I. Fukuda, E. Mizukami and K. Imaichi, J. Biochem., 61 (1967) 657.
12 A.K. Lough and G.A. Garton, Lipids, 3 (1967) 321.
13 S.G. Gordon, F. Philippon, K.S. Borgen and F. Kern, Biochim. Biophys. Acta, 218 (1970) 366.
14 J. Arnaud and J. Boyer, Biochim. Biophys. Acta, 270 (1972) 189.
15 W.H. Newsome and J.B.M. Rattay, Canad. J. Biochem., 43 (1965) 1223.

Journal of Chromatography, 307 (1984) 180—184
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2020

Note

Analysis of carbohydrates in lens, erythrocytes, and plasma by high-performance liquid chromatography of nitrobenzoate derivatives

MICHAEL PETCHEY and M. JAMES C. CRABBE*

University of Oxford, Nuffield Laboratory of Ophthalmology, Walton Street, Oxford OX2 6AW (U.K.)

(First received September 27th, 1983; revised manuscript received November 25th, 1983)

Many methods for the qualitative and quantitative analysis of carbohydrates using high-performance liquid chromatography (HPLC) have been developed (see, e.g., ref. 1). Where sensitivity of detection is of prime importance, ultraviolet-absorbing derivatives [2] have been used with success.

Preparation of biological samples for analysis of free carbohydrates, by removal of protein, has been performed using various methods (Somogyi [3]; trichloroacetic acid; perchloric acid; ethanol; acetonitrile) which we have found to interfere with the reaction forming the UV-absorbing *p*-nitrobenzoate esters.

The work presented here gives a suitable method for sample preparation, and subsequent quantitative analysis of sugars and sugar alcohols in human and rat lens, erythrocytes, and plasma.

EXPERIMENTAL

Chromatographic system

A DuPont Series 850 liquid chromatograph was used (DuPont, U.K.; Stevenage, U.K.) equipped with an 850 gradient pump (used isocratically), a column compartment (kept at 35°C) with Rheodyne syringe loading injector, and an 852 variable-wavelength UV spectrophotometer (set at 260 nm).

The column was stainless steel (25 cm × 4.6 mm I.D.) packed with Zorbax SIL, particle size 6 μm (DuPont).

Chemicals

Pyridine (BDH, analytical grade) was refluxed for 3 h with sodium hydroxide pellets (BDH, analytical grade), distilled, and stored over sodium

0378-4347/84/$03.00

hydroxide pellets. *p*-Nitrobenzoyl chloride (Aldrich) was recrystallised from light petroleum (BDH, analytical grade, boiling range 60—80°C). Carbohydrate standards were obtained from Sigma. Solvents for the mobile phase were all HPLC grade (Fisons). All other chemicals were analytical grade.

Sample preparation and chromatography

Cataractous human lenses (obtained from Oxford Eye Hospital) were homogenised in 4 ml of sodium fluoride (2 mg/ml). Homogenate (1 ml) was filtered using the Centrifree system (Amicon, Stonehouse, U.K.), in an angle-head rotor at 2400 *g* for 30 min. Aliquots (200 μl of filtrate were pipetted into separate tubes, 10 μl of internal standard added (perseitol, i.e., α-mannoheptitol) (1 mg/ml), mixed, and lyophilised. Rat lenses were homogenised in 1 ml of sodium fluoride (2 mg/ml) and filtered; plasma samples (1 ml) were filtered undiluted; 600 μl of frozen and thawed erythrocytes were diluted with 400 μl of water to aid haemolysis, and filtered.

Standards were prepared by lyophilising 200 μl of aqueous solutions with internal standard.

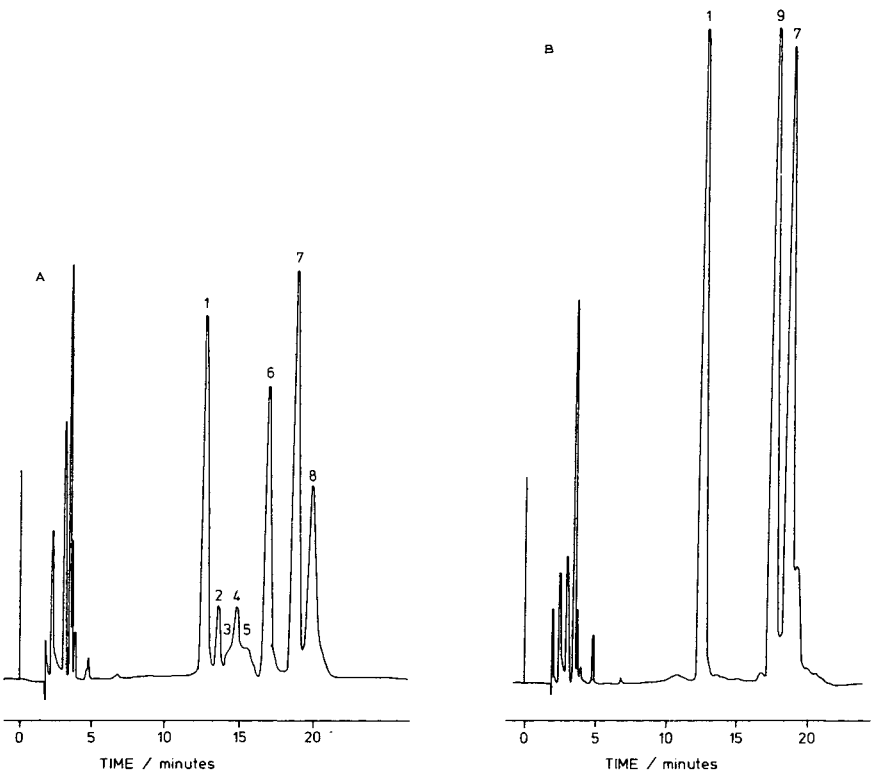
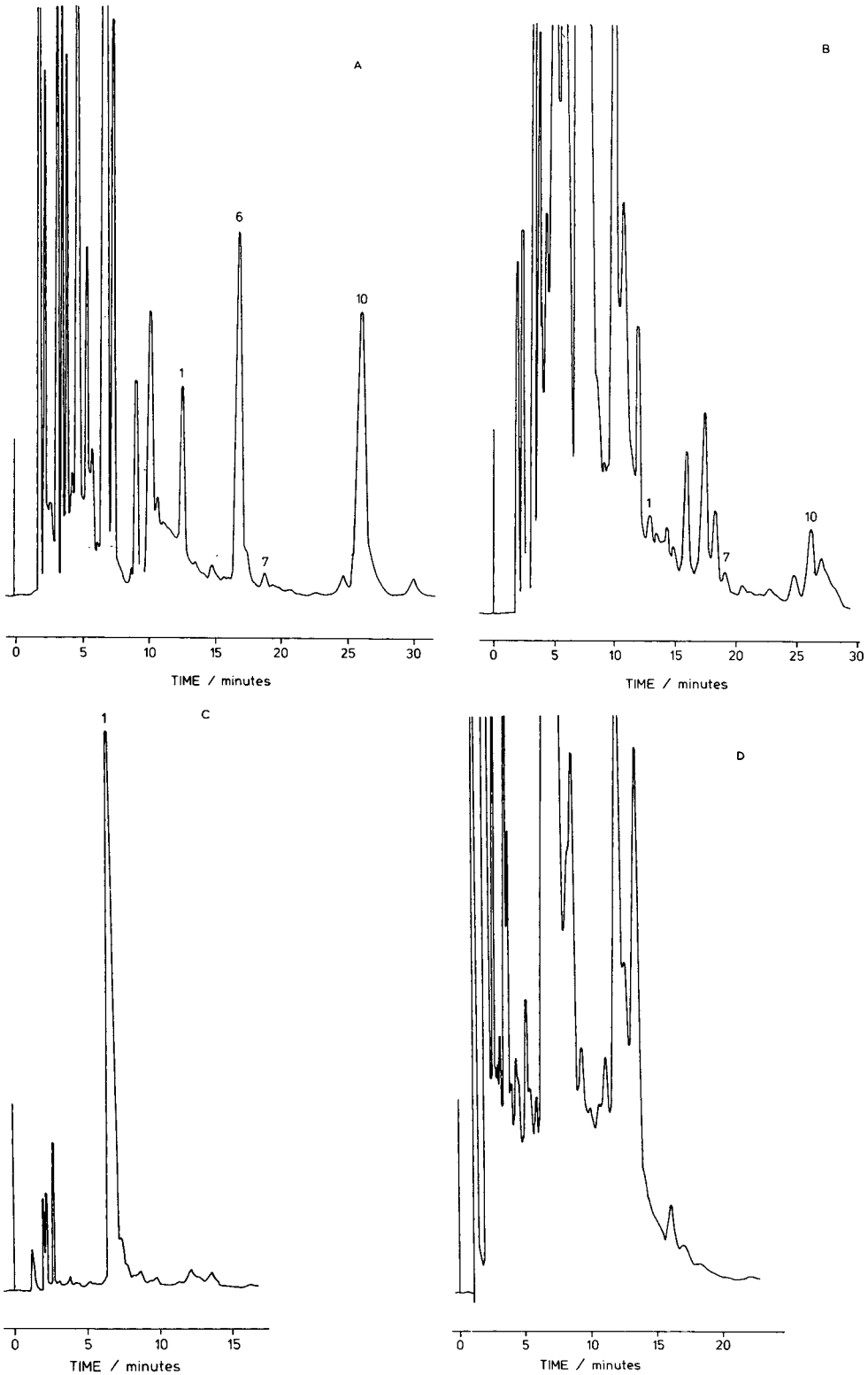


Fig. 1. HPLC of sugar standards (4-nitrobenzoate derivatives). (A) Peaks: 1, D-glucose (α- and β-); 2—5 and 8, isomers of D-fructose; 6, myo-inositol; 7, D-sorbitol; (B) separation of 9, mannitol, from 7, sorbitol. Mannitol is also resolved from inositol by this solvent mixture (see text).


A
6
10
1
7
0
5
10
15
20
25
30
TIME / minutes
B
1
10
7
0
5
10
15
20
25
30
TIME / minutes
C
1
0
5
10
15
TIME / minutes
D
0
5
10
15
20
TIME / minutes

The freeze-dried residues were taken and 200 μl of *p*-nitrobenzoyl chloride in pyridine (100 mg/ml) added, and reacted in the stoppered tubes at 60°C for 1 h. One drop of water was added to stop the reaction, and the products dissolved in 2 ml of chloroform. This was washed twice with 2 ml of sodium bicarbonate (5%) by vortex mixing for 1 min, and centrifuging for 30 sec to separate the layers prior to aspirating the aqueous layer. A further two washes, with 3 ml of 1 *M* hydrochloric acid, were performed [2].

A 50-μl aliquot of the chloroform layer was injected into the chromatograph, and eluted with hexane—chloroform—acetonitrile (10:3:1.9) with 0.1% water added at a flow-rate of 1.5 ml/min.

Recovery experiments were carried out by spiking lens homogenate with various amounts of glucose and sorbitol, and comparing to spiked sodium fluoride standards taken through the complete procedure.

RESULTS AND DISCUSSION

Sample preparation

The ultrafiltration method used has been found to provide a lens extract which does not interfere with the derivatising reaction by producing spurious peaks (Somogyi extract [3]), hydrolysis of reagent by excess acid (trichloroacetic acid, perchloric acid), or partial derivatisation by leaving significant quantities of protein in solution (perchloric acid, ethanol, acetonitrile). The method has also been found to be suitable for use with plasma and haemolysed erythrocytes.

A similar ultrafiltration method has been used to prepare hexoses and hexosamines from bovine lens [4].

Chromatography

Carbohydrate standards could be resolved as single peaks or anomers, dependent on the solvent used. Glucose eluted as a single peak which was more easily quantified, but fructose was separated into its anomers (Fig. 1A). Of the sugar alcohols, mannitol was completely resolved from sorbitol (Fig. 1B).

Calibration and precision

Use of perseitol as an internal standard (not found in lens) gave linear calibration graphs (r = 0.99) of peak height ratios with glucose and sorbitol over a range of initial concentrations from 3 to 60 μg/ml, the amounts detected being 15 to 300 ng, and the quantitative detection limit being in the region of 1—2 ng of these carbohydrates at 0.005 a.u.f.s.

The recovery of glucose and sorbitol from lens homogenate was found to be 105.7% (± 0.9) and 104.8% (± 5.4), respectively. No significant deviation in recovery was noted for plasma and erythrocyte samples.

Fig. 2. HPLC of biological tissue extracts. (A) Human cataractous lens homogenate; (B) human erythrocytes; (C) human plasma, showing major glucose peak (15 cm × 4.6 mm I.D. column, 2.56 a.u.f.s.); (D) human plasma, showing minor components, (0.08 a.u.f.s.). Peaks: 1, glucose; 6, inositol; 7, sorbitol; 10, perseitol.

* J. Chromatogr. Biomed. Appl. 311 (2) 1984 : 468

Carbohydrate extracts

Chromatograms of typical elution profiles are shown (Fig. 2). The levels of carbohydrates in human cataractous lenses (n=8) were found to be (mg per g lens wet weight ± S.D.): glucose, 1.01 ± 0.77; sorbitol, 0.35 ± 0.21; inositol, 11.74 ± 8.16. These values are comparable to those found by previous workers using paper chromatography [5].

This technique provides a useful method for the analysis of small quantities of carbohydrates in biological tissues using HPLC, and negates any artefacts produced by chemical methods of deproteinising. The method is currently being used for analysing the effects of aldose reductase inhibitors on carbohydrate levels in tissues prone to diabetic sequelae.

ACKNOWLEDGEMENTS

We thank the Medical Research Council, the National Institutes of Health, U.S.A., Fight-For-Sight Inc., New York, U.S.A., and Pfizer Research, U.K. for financial assistance, and Mr. A.J. Bron for the provision of laboratory facilities.

REFERENCES

1 L.A.Th. Verhaar and B.F.M. Kuster, J. Chromatogr., 220 (1981) 313.
2 F. Nachtmann and K.W. Budna, J. Chromatogr., 136 (1977) 279.
3 M. Somogyi, J. Biol. Chem., 160 (1945) 69.
4 A.V. Johnson, W.A. Szarek and D.J. Walton, Exp. Eye Res., 35 (1982) 391.
5 A. Pirie and R. van Heyningen, Exp. Eye Res., 3 (1964) 124.

Journal of Chromatography, 307 (1984) 185—189
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1997

Note

A rapid, sensitive method for detecting different arachidonic acid metabolites by thin-layer chromatography: the use of autoradiography

ASKO RIUTTA, ERKKI SEPPÄLÄ and HEIKKI VAPAATALO*

Department of Biomedical Sciences, University of Tampere, Box 607, SF-33101 Tampere 10 (Finland)

(Received September 26th, 1983)

Thin-layer chromatography (TLC) is widely used for the separation and identification of a number of arachidonic acid metabolites. There are descriptions of many TLC methods by which a variety of prostanoids can be separated [1—8]. Various spray reagents have been used for the detection of prostaglandins (PGs) following TLC separation. However, they require micro- or nanogram quantities of the compound to be detected [1, 4, 9—12].

Instead of spray reagents, direct scanning and autoradiography of labelled prostaglandins on TLC plates have been used, but these techniques have low sensitivity [13] or are time-consuming [14]. Thus large quantities of the isotope are necessary, especially with ^{3}H-labelled prostaglandins. For the detection of different arachidonic acid metabolites on TLC we have developed a rapid, sensitive autoradiographic method, in which 24 h is enough for visualization of a spot with an activity of 2400 dpm.

MATERIALS AND METHODS

LKC_{18} preadsorbent reversed-phase TLC plates were purchased from Whatman (Maidstone, U.K.), and were used without activation or a washing procedure. [^{3}H]PGE_2 (specific activity 160 Ci/mmol), 6-keto-[^{3}H]$PGF_{1\alpha}$ (specific activity 150 Ci/mmol) and [^{3}H]$PGF_{2\alpha}$ (specific activity 16.2 Ci/mmol) were from The Radiochemical Centre (Amersham, U.K.), and [^{3}H]-TXB_2 (thromboxane B_2, specific acitivity 100 Ci/mmol) was from New England Nuclear (Boston, MA, U.S.A.). Diethyl ether was from E. Merck (Darmstadt, F.R.G.), acetonitrile and chloroform were from Rathburn Chemicals (Walkerburn, U.K.). 2,5-Diphenyloxazole (PPO) was from Packard, (Downers

0378-4347/84/$03.00

Grove, IL, U.S.A.), and the film Kodak X-OMAT AR from Eastman-Kodak (Rochester, NY, U.S.A.).

The four arachidonic acid metabolites (activity of 8000 dpm) and their mixture were applied in 30 μl of chloroform onto the preadsorbent area with a micro-syringe using a brush-like downward stroke starting 3 mm below the reversed-phase/preadsorbent interface. The samples were dried under nitrogen and the plate was put in the rectangular chamber. The mobile phase was water—acetonitrile (70:30, v/v), and its pH was adjusted to 3.5 with 17 m*M* orthophosphoric acid phosphate buffer. The solvent system was 0.5 *M* in respect to sodium chloride. The amount of the mobile phase was chosen so that the solvent only wet the lowest 3 mm of the plate. For good saturation,

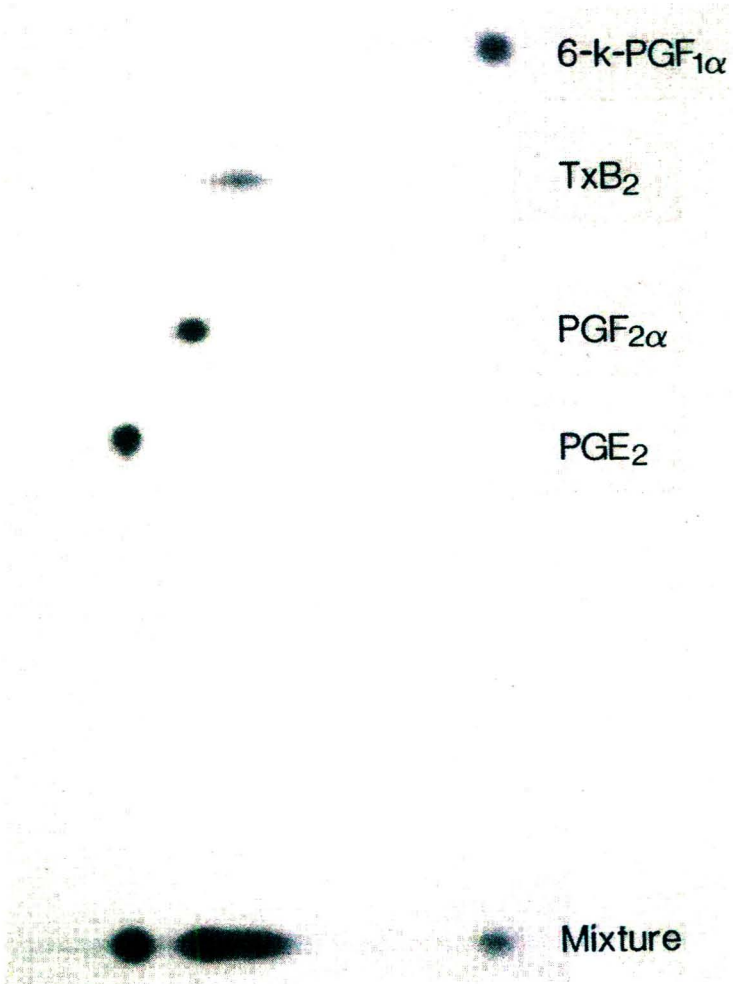


Fig. 1. The autoradiogram of 6-keto-[^{3}H]PGF$_{1\alpha}$, [^{3}H]PGF$_{2\alpha}$, [^{3}H]TXB$_2$ and [^{3}H]PGE$_2$. The radioactivity of each compound by application was 8000 dpm. The film was exposed for 24 h.

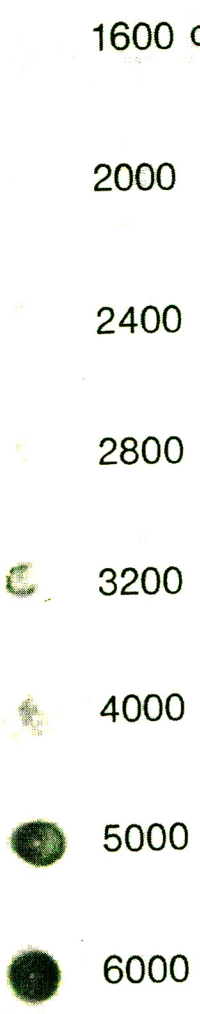


Fig. 2. The detection of spots with different activities of [^{3}H]PGE_2 in autoradiogram. The spot diameters were 10 mm. The figures represent the radioactivities applied. The film was exposed for 24 h.

TABLE I

R_F VALUES OF FOUR ARACHIDONIC ACID METABOLITES

Compound	R_F
PGE_2	0.13
$PGF_{2\alpha}$	0.19
TXB_2	0.21
6-keto-$PGF_{1\alpha}$	0.39

the walls of the chamber were lined with Whatman 3 MM filter paper. The mobile phase was used only once, and it was equilibrated 2 h before use. When the solvent front had migrated 15 cm from the application line, the plate was removed from the chamber and dried under a stream of air. Thereafter the autoradiography was performed. We also applied [^{3}H]PGE_2 samples of various activities directly onto the plate as spots, after which the autoradiography was performed. This was to test the real sensitivity of the method, independent of the different TLC techniques.

A solution of the scintillator (7% PPO in chloroform) was sprayed over the dry chromatogram (35 μl/cm^2), and the solvent was evaporated. All further operations were carried out in a dark room under proper lighting conditions. The dry chromatogram was placed in contact with Kodak X-OMAT AR film and kept between two glass plates. This package was wrapped in aluminium foil and stored at −70°C for 24 h. The film was developed in a Kodak RP X-OMAT M8 processor.

RESULTS AND DISCUSSION

A scintillator was added on the chromatogram to convert the very low energy of ^{3}H β-particles to light. The scintillation autoradiography together with the exposure of the film at a low temperature makes this method 100 times more efficient than the conventional one [15]. Fig. 1 shows the autoradiogram of four arachidonic acid metabolites. The mobilities of the compounds expressed as their R_F values are shown in Table I. The detection of spots with different activities of [^{3}H]PGE_2 are presented in Fig. 2. For clear visualization radioactivity of only 2400 dpm is needed when the scintillator has been dissolved in chloroform and sprayed onto the plate.

Tritium scintillator autoradiography has been used before [16—18], but we have applied it for the detection of different arachidonic acid metabolites. Since it is important to get a uniform distribution of the scintillator over the

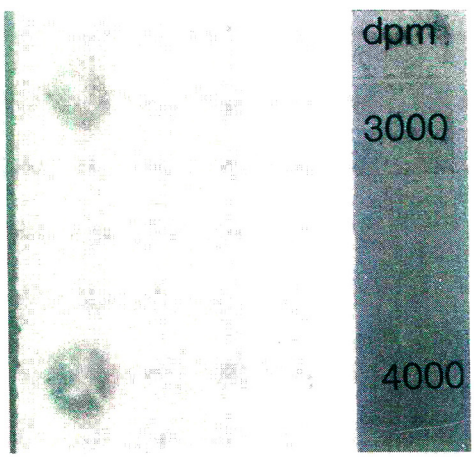


Fig. 3. Distribution of PPO on TLC plate when chloroform (on the left) or diethyl ether (on the right) was used. The figures represent the radioactivities applied.

desired chromatographic area [15, 19], a 7% solution of PPO in diethyl ether was used, and it was poured onto the chromatogram [8]. We have noted that dissolving the scintillator in chloroform and spraying the solution onto the chromatogram give a more uniform distribution of PPO and keep it on the desired area on the TLC plate (Fig. 3).

Thin-layer chromatography has been used for the identification of arachidonic acid metabolites, and nowadays, to an increasing extent, for their purification. The detection systems for prostaglandins on TLC have been so far insensitive and/or time-consuming. Here we present a rapid and sensitive alternative for analytical detection of the prostanoids.

ACKNOWLEDGEMENT

This study was supported by a grant from the Academy of Finland.

REFERENCES

1 K. Green and B. Samuelsson, J. Lipid Res., 5 (1964) 117.
2 M. Hamberg and B. Samuelsson, J. Biol. Chem., 241 (1966) 257.
3 P.W. Ramwell and E.G. Daniels, in G.W. Marinetti (Editor), Lipid Chromatographic Analysis, Vol. 2, Marcel Dekker, New York, 1969, p. 313.
4 N.H. Andersen, J. Lipid Res., 10 (1969) 316.
5 P.C. Isakson, A. Raz, S.E. Denny, E. Pure and P. Needleman, Proc. Nat. Acad. Sci. U.S., 74 (1977) 105.
6 C. Pace-Asciak and L.S. Wolfe, Biochemistry, 10 (1971) 3657.
7 G.C. Millar, Prostaglandins, 7 (1974) 207.
8 S.K. Goswami and J.E. Kinsella, J. Chromatogr., 209 (1981) 334.
9 P.W. Ramwell, J.E. Shaw, G.B. Clarke, M.F. Grostic, D.G. Kaiser and J.E. Pike, in R.T. Holman (Editor), Progress in the Chemistry of Fats and Other Lipids, Vol. 9, Pergamon Press, London, 1968, p. 233.
10 H.C. Kiefer, C.R. Johnson and K.L. Arora, Anal. Biochem., 68 (1975) 336.
11 S.K. Goswami and J.E. Kinsella, Lipids, 16 (1981) 759.
12 F.B. Ubatuba, J. Chromatogr., 161 (1978) 165.
13 J.C. Touchstone and M.F. Dobbins, Practice of Thin Layer Chromatography, Wiley, New York, 1978, p. 293.
14 E. Granström, Methods Enzymol., 86 (1982) 493.
15 A.W. Rogers, Practical Autoradiography, Review 20, Radiochemical Centre, Amersham, 1979, p. 35.
16 A.T. Wilson, Nature, 182 (1958) 524.
17 A.T. Wilson and D.J. Spedding, J. Chromatogr., 18 (1965) 76.
18 A.T. Wilson, Biochim. Biophys. Acta, 40 (1960) 522.
19 K. Randerath, Anal. Biochem., 34 (1970) 188.

Journal of Chromatography, 307 (1984) 190—193
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2005

Note

Assay of the antiarrhythmic compound stirocainide in plasma by fused-silica gas—liquid chromatography and nitrogen-selective detection

JÜRGEN BACKHAUS*, ELISABETH DINGLER and ROLAND WEYHENMEYER*

Abteilung Humanpharmakologie der Thiemann Arzneimittel GmbH, Im Wirrigen 25, D-4355 Waltrop (F.R.G.)

(First received July 11th, 1983; revised manuscript received December 15th, 1983)

Stirocainide, 2-benzal-1-(2-diisopropylaminoethoxyimino)cycloheptane hydrogen fumarate (Fig. 1), is an antiarrhythmic compound currently under clinical development. Its electrophysiological characteristics as a class I agent resemble partly lidocaine and partly quinidine [1—3]. Its chemical structure is unique within this class of antiarrhythmics. Preliminary pharmacokinetic studies in humans showed that concentrations down to several ng/ml must be assayed precisely in order to establish its pharmacokinetic profile.

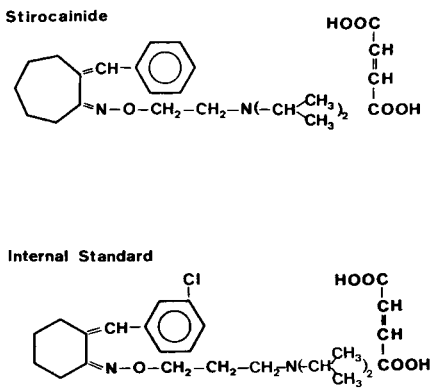


Fig. 1. Structures of stirocainide and of the internal standard.

*Present address: Abteilung B-DFS, Boehringer Mannheim GmbH, Sandhofer Strasse 116, D-6800 Mannheim 31, F.R.G.

0378-4347/84/$03.00

EXPERIMENTAL

Chemicals

Stirocainide and the internal standard 2-(*m*-chlorobenzal)-1-(3-diisopropylaminopropoxyimino)cyclohexane hydrogen fumarate (Fig. 1) were synthesized by Egyt Pharmaceutical Works (Budapest, Hungary). Methanol and *n*-hexane, both LiChrosolv, were from Merck (Darmstadt, F.R.G.). $LiAlH_4$ analysed was from Aldrich (Beerse, Belgium). Hexane was washed with sulphuric acid and water and distilled over $LiAlH_4$. Glassware was rinsed with methanol before use. Water was distilled twice in a quartz apparatus.

Equipment

The gas chromatograph F 22 from Perkin-Elmer (Überlingen, F.R.G.) with a nitrogen-selective flame-ionization detector was equipped with a capillary injection system from Gerstel Labormechanik (Mülheim, F.R.G.) in the splitless mode. We used a DB-5 fused-silica column from J&W Scientific (Rancho Cordova, U.S.A.), 60 m × 0.32 mm I.D., and film thickness 0.25 μm. The carrier and make-up gas was helium with flow-rates of 2.3 and 38 ml/min, respectively. The temperatures were oven 230°C, injector 270°C, and detector 300°C. The flow-rates of the fuel gases were 120 ml/min for synthetic air and 1.5 ml/min for hydrogen.

Peak areas were integrated by the data system 3352 B from Hewlett-Packard (Palo Alto, U.S.A.).

Assay

Internal standard, 10—100 ng in 25 μl of methanol, was added to 1 ml of plasma and extracted twice with 3 ml of hexane (10 min, 100 rpm, lab shaker by Braun, Melsungen, F.R.G.). After centrifugation, the organic phase was evaporated at 30°C under nitrogen in conical tubes. The residue was redissolved with 200 μl of methanol and transferred to 0.3-ml conical vials (Wheaton Scientific, Millville, U.S.A.). After evaporation, the residue was redissolved in 20 μl of methanol and 2 μl were injected into the gas chromatograph. Quantification was based on the peak area ratios of analyte to internal standard.

Since stirocainide and the internal standard are light-sensitive, especially in organic solvents, care was taken to protect the samples, extracts, and standard solutions.

RESULTS

In Fig. 2A the chromatogram of a blank extract is shown and in Fig. 2B the chromatogram of a clinical plasma sample containing 8.0 ng/ml stirocainide and 100 ng/ml internal standard. A peak from human plasma close to stirocainide may be used as a crude quality check for the performance of the system. If it cannot be separated anymore, the glass insert of the injector and/or the column have to be cleaned (the column may be rinsed with a few millilitres of hexane and the first centimeters of it are discarded). Usually about 50 plasma samples may be analysed before cleaning becomes necessary.

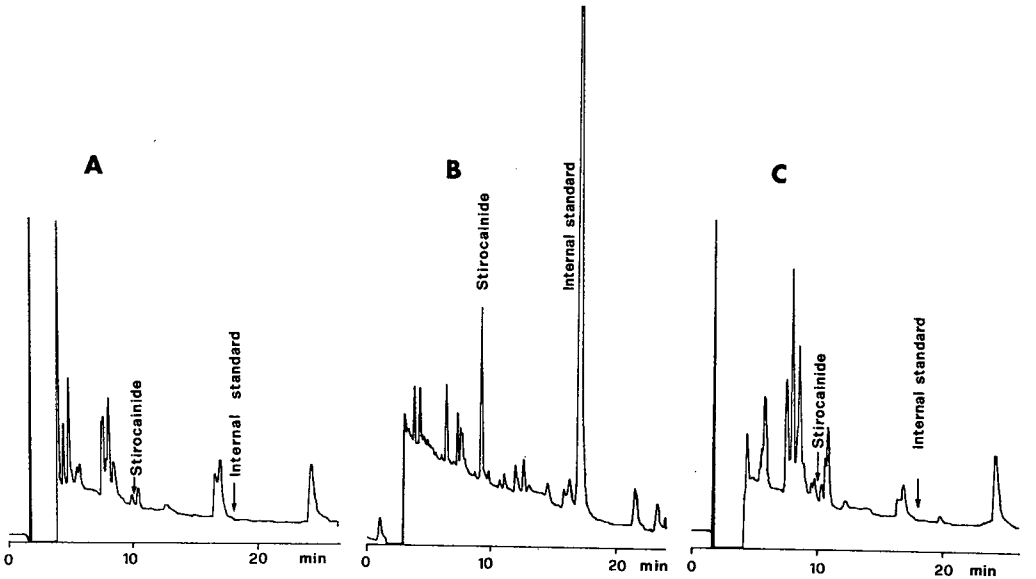


Fig. 2. Gas chromatograms of extracts of human plasma: (A) drug-free plasma; (B) clinical sample containing 8.0 ng/ml stirocainide and 100 ng/ml internal standard; (C) drug-free plasma stored for 5 min in a plastic syringe.

Because of this separation problem, high-quality fused-silica columns have to be used. If the column temperature is increased, stirocainide is no longer separated from the unknown plasma component. Since this component is missing in extracts from rabbit or rat plasma, the assay reported here is at least as sensitive in these species as it is in man. Dog plasma is comparable to man's. Plasticizers represents another problem. In Fig. 2C, the chromatogram of an extract is shown where blank plasma was left in a plastic syringe for 5 min. These syringes and catheters may be used only if the contact time with the sample is kept below 5 min. Otherwise, the amount of plasticizer increases to such an extent that separation of trace amounts of stirocainide will be impossible.

Five calibration curves were analysed on five different days, each in duplicate, in the range 5—80 ng/ml. Linear-regression analysis resulted in the following equation: $Y = 0.514\ (\pm\ 0.437) + 0.948\ (\pm\ 0.057)X$. The correlation coefficient was 0.9986 ± 0.0016.

The coefficients of variation between days calculated from these data are shown in Table I. Also shown are coefficients of variation for analyses on the same day. From these results we conclude that our detection limit is about 2 ng/ml. The extraction yield was $98.4 \pm 9.2\%$ ($n = 4$) for stirocainide (2—25 ng/ml) and $83.8 \pm 9.8\%$) ($n = 4$) for the internal standard (100 ng/ml). Stability of stirocainide was tested by adding 20 μg of stirocainide to 100 ml of heparinized blood at 37°C. Aliquots of 5 ml each were centrifuged immediately and stored at −20°C for up to one month. Approximately 35 ml were left at 37°C for up to 1 h before being centrifuged. Samples analysed immediately had a concentration of 320 ng/ml. All other samples were in the range

TABLE I

REPRODUCIBILITY AND LINEARITY OF THE ASSAY OF STIROCAINIDE

Human plasma was spiked with stirocainide in the concentrations indicated and with 100 ng of internal standard per ml of plasma.

Concentration (ng/ml)		C.V. (%)
Added	Found	
Day-to-day variation*		
5	5.05	9.8
10	9.88	2.6
20	19.04	3.3
40	38.67	5.5
80	76.36	5.8
Within-day variation**		
2	2.66	11.8
5	4.78	5.9
10	12.17	9.9
25	28.01	2.2

*Five calibration curves were run in duplicate on five different days. Each coefficient of variation is based on five duplicate measurements.
**Within-day variation of ten spiked plasma samples.

293—344 ng/ml, except blood left for 1 h before being centrifuged. In this sample, haemolysis occurred and a concentration of 261 ng/ml only was found. In another test for stability human plasma was spiked with 20 ng/ml stirocainide and stored at −20° C. Up to now six samples have been analysed during a period of three months. A mean of 19.5 ng/ml was found (C.V. = 4.7%) with no tendency for declining concentrations. All samples were within the 95% confidence limit of the regression line $Y = 19.2 + 0.01X$. These results show that stirocainide is stable in whole blood at 37° C for approximately 30 min and that it is stable in plasma at −20° C for at least three months.

Currently we are using the assay to study the pharmacokinetics of stirocainide in steady state. Initial results from human volunteers who received 60 mg b.i.d. for 14 days show trough levels of approximately 10 ng/ml and maximum levels of about 50 ng/ml.

REFERENCES

1 D. Mayer, H.J. Reiermann, W. Rück, H. Stock and L. Szekeres, Z. Kardiol., 69 (1980) 696.
2 H.J. Rüdiger, H. Homburger and H. Antoni, Naunyn-Schmiedeberg's Arch. Pharmacol., 317 (1981) 238—244.
3 J. Thale, H. Gülker, B. Brisse, H. Heuer, G. Hübner and D. Rümke, Herz/Kreisl., 14 (1982) 7—14.

Journal of Chromatography, 307 (1984) 194—199
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2013

Note

Haloperidol determination in serum and cerebrospinal fluid using gas—liquid chromatography with nitrogen—phosphorus detection: application to pharmacokinetic studies

DARRELL R. ABERNETHY, DAVID J. GREENBLATT*, HERMANN R. OCHS, CHRISTOPHER R. WILLIS, DEL D. MILLER and RICHARD I. SHADER

**Division of Clinical Pharmacology, Departments of Psychiatry and Medicine, Tufts University School of Medicine and New England Medical Center Hospital, 171 Harrison Avenue, Boston, MA 02111 (U.S.A.); the Medizinische Universitätsklinik, Bonn (F.R.G.); and Case Western Reserve School of Medicine and University Hospitals of Cleveland, OH 44106 (U.S.A.)*

(First received September 7th, 1983; revised manuscript received November 15th, 1983)

Haloperidol, a widely used major tranquilizer of the butyrophenone class [1] has been measured by gas—liquid chromatography (GLC) using electron-capture detection [2], GLC using mass spectroscopy [3] and nitrogen—phosphorus detection [4—6], liquid chromatography with electrochemical detection [7] and by radioreceptor assay [8, 9]. With the exception of radio-receptor assays, these techniques are specific but are limited by the number of analyses that can be performed in a working day. Furthermore, many of the methods lack adequate sensitivity to detect very low haloperidol serum or plasma levels. This is of importance since steady-state concentrations are often in the 1—10 ng/ml range, and studies of pharmacokinetics and bioequivalence may require sensitivity of less than 1.0 ng/ml [10, 11]. Here we describe a method that is selective, separating haloperidol from metabolites and plasma contaminants, and sensitive to 0.5 ng/ml using GLC with nitrogen—phosphorus detection. When coupled with an automated injection system, up to 60 samples per 24 h may be analyzed after a straightforward plasma extraction which requires only 3—4 h technical time.

EXPERIMENTAL

Apparatus and chromatographic conditions

The analytic instrument is a Hewlett-Packard Model 5840A gas

0378-4347/84/$03.00

chromatograph equipped with a nitrogen—phosphorus detector and electronic integrator, and fitted with an optional logic board (Hewlett-Packard No. 07670-60840) to permit injection of small volume samples with alternate wash vials. The column is coiled glass, 1.22 m × 2 mm I.D., packed with 3% SP-2250 on 80—100 mesh Chromosorb WHP (Packing 1-1767, Supelco, Bellefonte, PA, U.S.A.). The carrier gas is ultra high purity helium (Matheson Gas Products, Gloucester, MA, U.S.A.) at a flow-rate of 30 ml/min. The detector purge is ultra high purity hydrogen (Matheson) at a flow-rate of 3 ml/min mixed with dry air (Matheson) at a flow-rate of 50 ml/min. Operating temperatures are: injection port, 310°C, column, 270°C, detector, 275°C. Before being connected to the detector, a new column is conditioned at 280°C for 48 h with carrier flow-rate of 30 ml/min.

At the beginning of each work day, the column is primed with 2 μg of phospholipid (asolectin) in benzene.

Reagents

The following reagents are used: certified 99% pure *n*-hexane (Mallinckrodt, St. Louis, MO, U.S.A.), analytical reagent-grade toluene (Fisher Scientific, Fairlawn, NJ, U.S.A.), certified isoamyl alcohol (Fisher), HPLC grade methanol (Fisher), analytical reagent grade concentrated hydrochloric acid (Mallinckrodt), and analytical reagent-grade sodium carbonate and sodium bicarbonate (Mallinckrodt). Isoamyl alcohol is glass-distilled prior to use. Other organic solvents are used without further distillation. All aqueous solvents (0.25 *M* sodium hydroxide, 0.1 *M* hydrochloric acid, 1 *M* carbonate—bicarbonate buffer at pH 11.5) are washed five times with hexane—isoamyl alcohol (98:2) prior to use.

Reference standards

Pure standards of haloperidol and the internal standard McN-JR-1854 (chlorohaloperidol, Fig. 1) were kindly provided by McNeil Labs. (Ft. Washington, PA, U.S.A.). Standards of each are prepared by dissolving 100 mg in 100 ml methanol. Sequential dilutions to 1 μg/ml are made. The solutions are stored in the dark in glass-stoppered bottles at 4°C and are stable for at least twelve months.

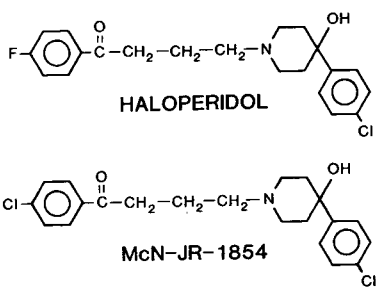


Fig. 1. Structural formulae of haloperidol and the internal standard, McN-JR-1854.

Preparation of samples

McN-JR-1854 (chlorohaloperidol) is used as the internal standard for all

analyses. A 100-μl volume of stock solution (1 μg/ml), containing 100 ng of McN-JR-1854, is added to a series of 15-ml round-bottom glass culture tubes, with PTFE-lined screw-top caps. A 0.25—2.0 ml sample of unknown serum or plasma is added to each tube. Calibration standards for haloperidol are prepared by adding 1, 2.5, 5, 10, 25, 50, and 100 ng of drug to consecutive tubes. Drug-free control serum or plasma is added to each of the calibration tubes. One blank sample, taken from the experimental animal prior to drug administration, is analyzed with calibration standards and each set of unknown samples.

Extraction procedure

To each tube 1 ml of 0.25 *M* sodium hydroxide solution is added, followed by 5 ml hexane—isoamyl alcohol (98:2). The tubes are agitated gently in the upright position on a vortex mixer for 15 min, then centrifuged at room temperature for 5 min at 400 *g*. The organic layer is transferred to another 15-ml glass culture tube which contains 1.2 ml of 0.1 *M* hydrochloric acid. This mixture is agitated gently in the upright position on a vortex mixer for 10 min, and again centrifuged at room temperature for 5 min at 400 *g*. The upper organic layer is discarded. The aqueous layer is transferred by a 23-cm pipet to a conical 13-ml screw-top centrifuge tube. Great care is taken to transfer only the aqueous layer uncontaminated with organic residue. To this 0.5 ml of 1 *M* carbonate—bicarbonate buffer (pH 11.5) is added. The final organic extraction is done by adding 80 μl toluene—isoamyl alcohol (85:15) to the conical centrifuge tube. This mixture is agitated gently in the upright position on a vortex mixer for 15 min. The samples are again centrifuged at room temperature for 5 min at 400 *g*. After freezing in a dry ice—acetone bath to break emulsions formed, with a 23-cm disposable pipet passed through the organic layer, the entire aqueous layer is removed leaving the small volume (80 μl) of organic phase containing haloperidol and internal standard. This is transferred to a 0.3-ml Wheaton automatic sampling micro vial (Wheaton Scientific, Millville, NJ, U.S.A.). A 6-μl aliquot is injected into the gas chromatograph using the automatic injection sampling system.

Animal study

An adult mongrel dog (17 kg) was anesthetized with intravenous pentobarbital (30 mg/kg), intubated, and ventilated with a Harvard respirator to maintain arterial oxygen tension within normal limits [12, 13]. Body temperature was maintained with a heating pad, and fluid losses were approximately replaced with intravenous 0.9% sodium chloride solution. A 7.5-cm spinal needle (19 gauge) was inserted into the cisternum magnum to allow repeated sampling of cerebrospinal fluid (CSF). A single 5-mg intravenous bolus of haloperidol (McNeil) was administered through a glass syringe. Multiple venous blood (4—5 ml) and CSF (1—2 ml) samples were drawn into additive-free tubes over the following 8 h. Concentrations of haloperidol in all samples were determined by the method described above. CSF samples were extracted and analyzed identically to serum.

Serum haloperidol concentrations were analyzed by iterative weighted nonlinear least-squares regression analysis [14]. The following serum pharmaco-

kinetic variables were determined: distribution half-life, elimination half-life, central compartment volume, total volume of distribution, and total clearance. CSF disappearance half-life was determined by least-squares regression analysis.

RESULTS

Evaluation of the method

Under the described conditions, retention times for haloperidol and McN-JR-1854 were approximately 3.5 and 7.0 min, respectively (Fig. 2). The relation between haloperidol concentrations and the peak height ratio (versus internal standard) is linear at least to 100 ng/ml. Analysis of ten standard curves over a 4-month period indicates that the correlation coefficient is always 0.99 or greater. Day-to-day coefficient of variation in the slopes of the calibration curves was 7.8%.

The sensitivity limit of the method is 0.5 ng/ml for a 2-ml sample. Within-day coefficients of variation for identical samples were: at 1 ng/ml, 9.6%; 2.5 ng/ml, 13.6%; 5 ng/ml, 7.6%; 10 ng/ml, 6.7%; and 25 ng/ml, 3.6%. Residue

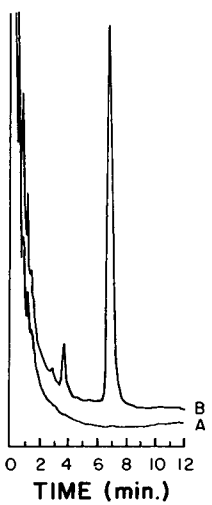


Fig. 2. Gas—liquid chromatograms of (A) extract of 1 ml serum obtained from dog prior to receiving haloperidol and (B) calibration standard spiked with 5 ng haloperidol (3.5 min) and 100 ng internal standard, MCN-JR-1854 (7.0 min).

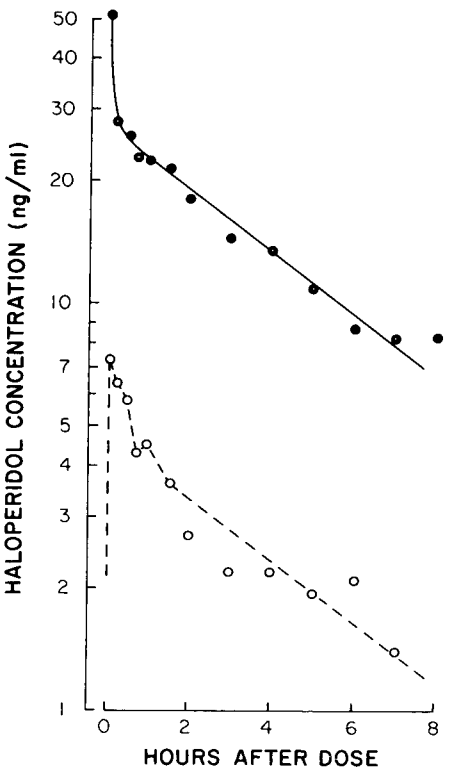


Fig. 3. Serum (•—•) and CSF (○— — —○) haloperidol concentrations and pharmacokinetic functions following intravenous haloperidol (5 mg) administration to a mongrel dog. See Table I for derived kinetic variables.

analysis indicated the extraction efficiency of haloperidol is greater than 95% at 10 ng/ml plasma concentrations.

The retention time of haloperidol was considerably longer than that of a number of other commonly used psychotropic drugs including benzodiazepines (diazepam, desmethyldiazepam, desalkylflurazepam, oxazepam, lorazepam) and antidepressants (imipramine and metabolites, desipramine and metabolites). Trazodone, on the other hand, had considerably longer retention than haloperidol. Therefore these compounds did not interfere with the assay.

Pharmacokinetic study

Fig. 3 shows serum and CSF haloperidol concentrations and pharmacokinetic functions for the experimental animal. Derived pharmacokinetic parameters are listed in Table I. Haloperidol was extensively distributed, with a total volume of distribution of more than 10 l/kg. Haloperidol rapidly entered CSF, and was eliminated in parallel from both serum and CSF after attainment of distribution equilibrium. The mean CSF to total serum haloperidol concentration ratio averaged only 0.17, probably due to serum protein binding of haloperidol.

TABLE I

DERIVED HALOPERIDOL SERUM AND CSF PHARMACOKINETIC PARAMETERS AFTER A 5-mg INTRAVENOUS DOSE ADMINISTERED TO A 17-kg MONGREL DOG

Parameter	Plasma	CSF
Distribution half-life (min)	3.0	—
Elimination half-life (h)	3.9	3.9
Central compartment volume (l/kg)	2.8	—
Total volume of distribution (l/kg)	10.6	—
Total metabolic clearance (ml/min/kg)	31.6	—
Mean CSF/total plasma concentration ratio*	0.17	

*Mean of values 1—7 h after the dose.

DISCUSSION

This report describes a reliable, selective method for the quantitation of haloperidol in serum or plasma and in CSF using GLC with nitrogen—phosphorus detection. Sensitivity is adequate to carry out single-dose pharmacokinetic studies and evaluate entry of haloperidol into CSF. A basic extraction from serum or plasma, acidic back-extraction, subsequent adjustment of the aqueous phase to a basic pH, and final organic extraction into a small volume for direct injection into the gas—liquid chromatograph is the method employed. This method produces blank serum and CSF samples that are consistently free of contaminants in the areas corresponding to the retention times for haloperidol and the internal standard. Dihydrohaloperidol, a metabolite of haloperidol [7], also is resolved from both haloperidol and the internal standard under these conditons. However we did not attempt to quantitate dihydrohaloperidol since a pure reference standard was not available.

The value of this method includes the reasonable time required for sample preparation, as well as its sensitivity which is adequate for single-dose serum and CSF pharmacokinetic studies. The method is potentially applicable to monitoring of steady-state haloperidol concentrations during clinical use of haloperidol, and we have analyzed a large number of clinical samples using this procedure.

ACKNOWLEDGEMENTS

This work was supported in part by Grants AM-32050 and MH-34223 from the United States Public Health Service and by Grant Oc 10-6-/3 from Deutsche Forschungsgemeinschaft.

REFERENCES

1 A. DiMascio and R.I. Shader (Editors), Butyrophenones in Psychiatry, Raven Press, New York, 1972.
2 A. Forsman, E. Martensson, G. Nyberg and R. Ohman, Naun. Schmied. Arch. Pharmacol., 286 (1974) 113—124.
3 M.A. Moulin, R. Camsonne, J.P. Davy, E. Poilpre, P. Morel, D. Debruyne, M.C. Bigot, M. Dedieu and M. Hardy, J. Chromatogr., 178 (1979) 324—329.
4 H. Dekirmenjian, J.I. Javaid, B. Duslak and J.M. Davis, J. Chromatogr., 160 (1978) 291—296.
5 M. Franklin, Clin. Chem., 136 (1980) 1367—1368.
6 G. Bianchetti and P.L. Morselli, J. Chromatogr., 153 (1978) 203—209.
7 E.R. Korpi, B.H. Phelps, H. Granger, W.H. Chang, M Linnoila, J.L. Meek and R.J. Wyatt, Clin. Chem., 29 (1983) 624—628.
8 B.M. Cohen, M. Herschel, E. Miller, H. Mayberg and R.J. Baldessarini, Neuropharmacology, 19 (1980) 663—668.
9 R.E. Poland and R.T. Rubin, Life. Sci., 29 (1981) 1837—1845.
10 M.A. Moulin, J.P. Dary, D. Debruyne, J.C. Andersson, M.C. Bigot, R. Camsonne and E. Poilpre, Psychopharmacology, 76 (1982) 346—350.
11 F.O. Holley, J.R. Magliozzi, D.R. Stanski, L. Lombrozo and L.E. Hollister, Clin. Pharmacol. Ther., 33 (1983) 477—484.
12 H.R. Ochs, D.J. Greenblatt, B.L. Lloyd, E. Woo, M. Sonntag and T.W. Smith, Amer. Heart J., 100 (1980) 341—346.
13 D.J. Greenblatt, H.R. Ochs and B.L. Lloyd. Psychopharmacology, 70 (1980) 89—93.
14 D.R. Abernethy and D.J. Greenblatt, Clin. Pharmacol. Ther., 29 (1981) 757—761.

Journal of Chromatography, 307 (1984) 200—205
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2016

Note

Simultaneous determination of lidocaine and its deethylated metabolites using gas—liquid chromatography with nitrogen—phosphorus detection

CHRISTOPHER R. WILLIS, DAVID J. GREENBLATT*, DAVID M. BENJAMIN and DARRELL R. ABERNETHY

**Division of Clinical Pharmacology, Tufts-New England Medical Center, Boston, MA 02111 (U.S.A.); The Medical Department, Astra Pharmaceutical Products, Worcester, MA 01606 (U.S.A.); and the Section on Hypertension-Clinical Pharmacology, Baylor College of Medicine, Houston, TX 77030 (U.S.A.)*

(First received October 5th, 1983; revised manuscript received November 15th, 1983)

Lidocaine is extensively used in the treatment of cardiac arrhythmias and as a local and regional anesthetic agent [1]. Biotransformation of lidocaine in vivo leads to two major metabolic products, monoethylglycinexylidide (MEGX) and glycinexylidide (GX), both of which have pharmacologic acitivity [2, 3].

Gas chromatography (GC) [4—11], GC—mass spectroscopy [3, 11] and liquid chromatography [12—15] have been used to quantitate lidocaine in plasma, and some of these methods simultaneously measure one or both metabolites. The present report describes an improved GC methodology using nitrogen—phosphorus detection (NPD) which can simultaneously quantitate lidocaine, MEGX, and GX in plasma without derivitazion or sample clean-up. An automated injection system allows analysis of up to 100 samples per 24 h.

EXPERIMENTAL

Apparatus and chromatographic conditions

The analytic instrument was a Hewlett-Packard Model 5840A gas chromatograph equipped with a nitrogen—phosphorus detector, electronic data processor integrator and automatic sampler (Model 7672A). The column was coiled glass, 3.05 m × 2 mm I.D., packed with 3% SP-2250 on 80—100 mesh Supelcoport (Packing 1-1767, Supelco, Bellefonte, PA, U.S.A.). The carrier gas was ultra high purity helium (Matheson Gas Products, Gloucester, MA, U.S.A.) at a flow-rate of 30 ml/min. The detector purge was ultra high purity hydrogen

0378-4347/84/$03.00

(Matheson) at a flow-rate of 3 ml/min mixed with dry air (Matheson) at a flow-rate of 50 ml/min. Operating temperatures were: injection port, 310°C; column, 200°C; detector, 275°C. Before being connected to the detector, a new column was conditioned at 220°C for 48 h with a carrier flow-rate of 30 ml/min. At the beginning of each work day the column was primed with 2—4 μg of purified soy phosphatides (asolectin) in benzene [16].

Reagents

The following reagents were used: analytical reagent-grade ethyl acetate; analytical reagent-grade toluene; certified isoamyl alcohol; HPLC-grade methanol, all obtained from Fisher Scientific (Fair Lawn, NJ, U.S.A.); analytical-reagent grade sodium hydroxide (Mallinckrodt, St. Louis, MO, U.S.A.); and Baker-Analyzed acetone (J.T. Baker, Phillipsburg, NJ, U.S.A.).

Reference standards

Pure standards of lidocaine, MEGX and GX, as well as the internal standard ethylmethylglycinexylidide (EMGX), were kindly provided by Astra Pharmaceutical Products (Worcester, MA, U.S.A.). Standards of each were prepared by dissolving 100 mg of free base in 100 ml methanol. Sequential dilutions were made to 10 μg/ml. Solutions were stored in the dark in glass-stoppered bottles at 4°C and were stable for at least two months.

Preparation of samples

Extraction tubes were 15-ml round-bottomed glass culture tubes with PTFE-lined screw-top caps. Tubes were rinsed with acetone and air-dried prior to use. To each tube, 0.5 μg (50 μl of the 10 μg/ml solution) of EMGX was added as the internal standard. Calibration standards for lidocaine, MEGX and GX were prepared by adding varying amounts (0.1 to 3 μg) of all three compounds to consecutive tubes. The tubes were placed in a vacuum oven and the solvent evaporated to dryness at 40—45°C under reduced pressure. Drug-free control plasma (1 ml) was added to each calibration tube and 1 ml of unknown plasma added to all other tubes containing only internal standard.

Extraction procedure

To each tube 200 μl of 5 *M* sodium hydroxide and 2 ml of ethyl acetate were added. The tubes were capped and agitated gently in the upright position on a vortex mixer for 30 sec. The samples were centrifuged at room temperature for 5 min at 400 *g*. The organic layer was transferred to standard 2-ml Wheaton automatic sampling vials (Wheaton Scientific, Millville, NJ, U.S.A.), which were then placed in a vacuum oven and evaporated to dryness at room temperature. The residue was reconstituted with 250 μl of a toluene—isoamyl alcohol mixture (85:15), capped with aluminum foil and gently vortexed. The automatic sampler was programmed to inject 2 μl of each sample.

Pharmacokinetic studies

A healthy male volunteer participated after giving written informed consent. A 300-mg oral dose of lidocaine hydrochloride, as 15 ml of a 2 g per 100 ml solution (Xylocaine Viscous, Astra) was administered every 3 h for eight

consecutive doses. Venous blood samples were drawn into heparinized Venoject tubes prior to each dose, and at multiple points after the first and last doses. Plasma samples were separated and frozen until the time of assay as described above.

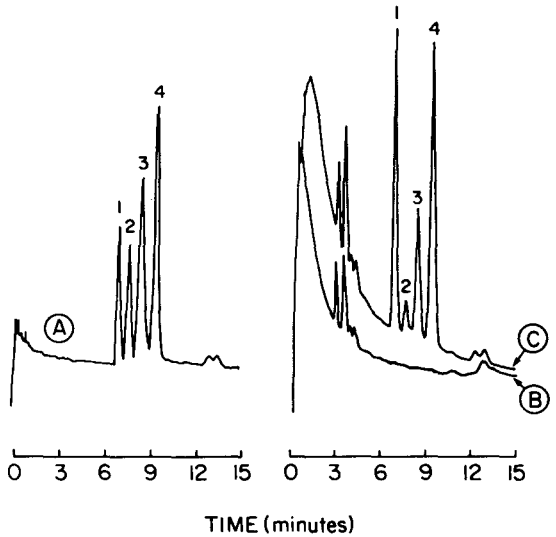


Fig. 1. (A) Chromatogram of an extract of a calibration standard containing 0.5 μg/ml of EMGX (the internal standard) and 1.0 μg/ml of GX, MEGX, and lidocaine; (B) chromatogram of a drug-free control plasma extract; (C) chromatogram of a sample from a subject after oral administration of lidocaine. Peaks and concentrations determined were: 4 = lidocaine, 0.56 μg/ml; 3 = MEGX, 0.32 μg/ml; 2 = GX, 0.09 μg/ml; 1 = EMGX, internal standard.

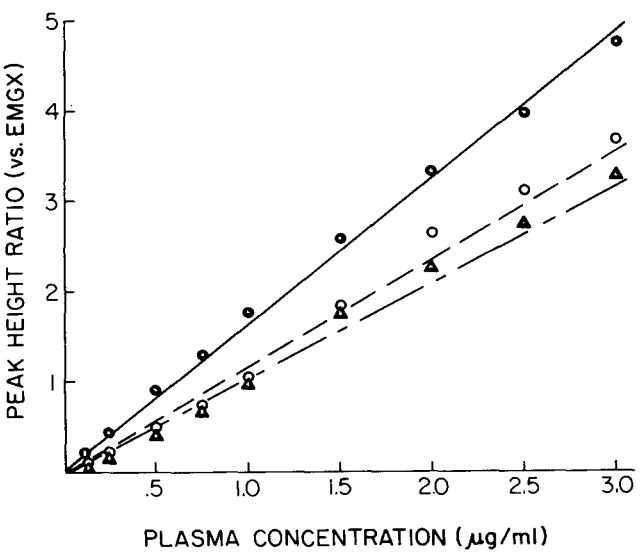


Fig. 2. Calibration curve showing relation of plasma concentration of lidocaine and its two metabolites versus the peak height ratio of drug to internal standard. (●——●), Lidocaine; (○ - - ○), MEGX; (▲— — ▲), GX.

RESULTS

Evaluation of the method

Under the described chromatographic conditions, lidocaine, its two metabolites, and the internal standard gave well resolved chromatographic peaks (Fig. 1). Drug-free blank plasma samples were free of contaminating peaks (Fig. 1). Plasma concentrations of lidocaine, MEGX, and GX were linearly related to the peak height ratio of each compound versus the internal standard (Fig. 2). The sensitivity limits are approximately 0.05 μg of each compound per ml of plasma. Table I shows replicability of identical samples at various concentrations. Residue analysis indicated greater than 90% recovery of all three compounds.

After extraction and reconstitution, lidocaine and GX are stable at room temperature for at least 48 h. However, degradation of MEGX is noted after 24 h. Therefore samples should be chromatographed within 24 h of extraction.

TABLE I

REPLICABILITY OF IDENTICAL SAMPLES AT VARIOUS CONCENTRATIONS

At each concentration $n = 4–6$.

Plasma concentration (μg/ml)	Coefficient of variation* (%)		
	Lidocaine	MEGX	GX
0.1	7.1	16.5	13.6
0.25	2.6	5.9	8.3
0.5	4.8	8.0	3.9
0.75	2.9	4.0	7.4
1.0	2.8	6.7	11.3
1.5	3.9	7.4	3.6
2.0	1.0	3.8	3.8
2.5	4.0	7.8	3.0
3.0	1.0	5.2	4.3

*Standard deviation divided by mean, in percent.

Pharmacokinetic results

Fig. 3 shows plasma concentrations of lidocaine, MEGX, and GX in the volunteer subject. Concentrations of lidocaine and MEGX reached similar levels during multiple-dose therapy. After termination of treatment, washout of MEGX was slower than that of lidocaine. GX concentrations were considerably lower than those of other two compounds.

DISCUSSION

The present report describes a rapid, sensitive, automated method for simultaneous quantitation of lidocaine and its two metabolites in plasma. The straightforward extraction procedure allows one person to prepare a large number of samples in a standard working day. With the automated sampler, 100 or more samples can be chromatographed in a 24-h period with no

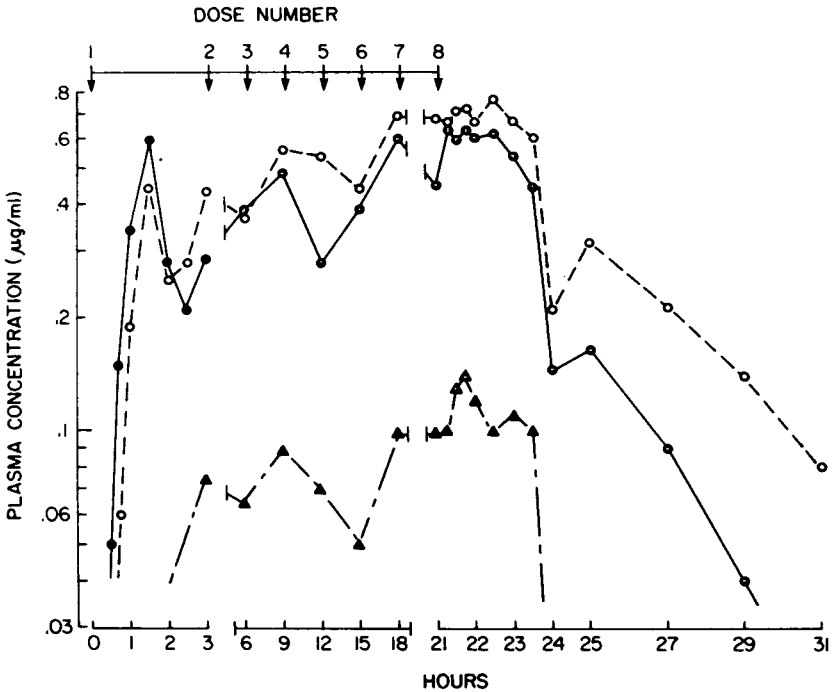


Fig. 3. Plasma concentrations of lidocaine and its two metabolites in the volunteer subject. (•——•), Lidocaine; (○ - - ○), MEGX; (▲— — ▲), GX.

technical personnel in attendance. The method is sensitive enough for therapeutic monitoring and for most pharmacokinetic studies. The two metabolites of lidocaine, MEGX and GX, both have pharmacologic activity [3, 4]. MEGX, in particular, has activity similar to that of the parent compound, and may contribute to antiarrhythmic activity and/or toxicity. Thus the capacity for quantitation of lidocaine metabolites as well as the parent compound is of considerable importance.

A parallel clinical pharmacokinetic study in eighteen volunteers confirmed previous reports that concentrations of lidocaine in the systemic circulation after oral administration are relatively low [17—19]. This is probably explained by extensive first-pass hepatic extraction. During repeated oral dosage with lidocaine, levels of the principal metabolite, MEGX, were similar to those of the parent compound. Levels of GX were considerably lower than those of either lidocaine or MEGX, confirming that GX is a quantitatively less important metabolic product [15].

ACKNOWLEDGEMENTS

We are grateful for the collaboration of Medical and Technical Research Associates, Boston and Needham, MA, U.S.A. This work was supported in part by Grant MH-34223 and AM-MH-32050 from the United States Public Health Service.

REFERENCES

1 K.A. Collinsworth, S.M. Kalman and D.C. Harrison, Circulation, 50 (1974) 1217—1230.
2 R.G. Burney, C.A. DiFazio, M.J. Peach, K.A. Petrie and M.J. Silvester, Amer. Heart J., 88 (1974) 765—769.
3 J.M. Strong, D.E. Mayfield, A.J. Atkinson, B.C. Burns, F. Raymon and L.T. Webster, Clin. Pharmacol. Ther., 17 (1975) 184—194.
4 K.K. Adjepon-Yamoah and L.F. Prescott, J. Pharm. Pharmacol., 26 (1974) 889—893.
5 R.L. Nation, E.J. Triggs and M. Selig, J. Chromatogr., 116 (1976) 188—193.
6 D.R. Abernethy, D.J. Greenblatt and H.R. Ochs, J. Chromatogr., 232 (1982) 180—185.
7 B. Levine and R. Blanke, J. Anal. Toxicol., 7 (1983) 123—124.
8 B.E. Pape, R. Whiting, K. Parker and R. Mitra, Clin. Chem., 24 (1978) 2020—2022.
9 H. Breuer, J. Chromatogr., 231 (1982) 65—72.
10 E. Zylber-Katz, L. Granit and M. Levy, Clin. Chem., 24 (1978) 1573—1575.
11 C.E. Hignite, C. Tschanz, J. Steiner, D.H. Huffman and D.L. Azarnoff, J. Chromatogr., 161 (1978) 243—249.
12 J.L. Wisnicki, W.P. Tong, D.B. Ludlum, Clin. Chim. Acta, 93 (1979) 279—282.
13 R.L. Nation, G.W. Peng and W.L. Chiou, J. Chromatogr., 162 (1979) 466—473.
14 J. Hill, A. Roussin, J. Lelorier and G. Caille, J. Pharm. Sci., 69 (1980) 1341—1343.
15 D.E. Drayer, B. Lorenzo, S. Werns and M.M. Reidenberg, Clin. Pharmacol. Ther., 34 (1983) 14—22.
16 W.E. Leitch, L.P. Stuart and E. Forchielli, Anal. Biochem., 56 (1973) 580—583.
17 R.N. Boyes, D.B. Scott, P.J. Jebson, M.J. Godman and D.G. Julian, Clin. Pharmacol. Ther., 12 (1971) 105—116.
18 M.C.O. Fehmers and A.J. Dunning, Amer. J. Cardiol., 29 (1972) 514—519.
19 E. Perucca and A. Richens, Brit. J. Clin. Pharmacol., 8 (1979) 21—31.

Journal of Chromatography, 307 (1984) 206—209
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2018

Note

Silica capillary gas chromatographic determination of ibuprofen in serum

LIISA HEIKKINEN

Department of Pharmaceutical Chemistry, School of Pharmacy, University of Helsinki, SF-00170 Helsinki 17 (Finland)

(First received October 3rd, 1983; revised manuscript received November 25th, 1983)

Ibuprofen (Fig. 1, I), 2-(4-isobutylphenyl)propionic acid, is a drug possessing analgesic, anti-inflammatory and antipyretic effects. Several analytical methods, including gas chromatography [1—4], have been presented for the determination of ibuprofen in serum. The method described in this paper is based on the determination of ibuprofen as the free acid on a fused-silica capillary column. Derivatization is not required. Ibufenac (Fig. 1, II) is used as the internal standard.

This procedure has been devised for rapid estimation of the drug at therapeutic levels and in cases of possible overdosage.

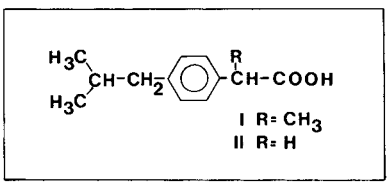


Fig. 1. Chemical structure of ibuprofen (I) and of the internal standard (II).

EXPERIMENTAL

Materials and reagents

Ibuprofen was kindly supplied by Medipolar (Oulu, Finland) and ibufenac by the Boots Company (U.K.). The identity and purity of the substances were verified by different chromatographic (thin-layer, gas) and spectrometric (ultraviolet, infrared) methods. The control serum, M + D Moni-trol IE, was obtained from Dade-Fennica Oy (Helsinki, Finland). The following reagents and stock

0378-4347/84/$03.00

solutions were used: 1 *M* hydrochloric acid (Orion, Finland), as the extraction solvent, petroleum ether (b.p. 40—60°C) (May & Baker, U.K.), and the stock solutions ibuprofen 100 μg/ml and the internal standard, ibufenac, 100 μg/ml in petroleum ether.

Gas chromatography

Gas chromatographic analyses were performed with a Fractovap 4200 gas chromatograph (Carlo Erba, Italy) equipped with a flame-ionization detector and connected to a Hewlett-Packard 3380A peak integrator. The fused-silica capillary column was OV-351 (25 m × 0.32 mm I.D.) with a film thickness of 0.20 μm (Orion Analytica, Finland). The operating temperatures were column 220°C, injection port and detector 250°C. Helium was employed as the carrier gas (156.9 kPa) and the splitting ratio was 1:20. Hydrogen (39.2 kPa) and compressed air (78.5 kPa) flow-rates were adjusted to give maximum response. Under these conditions the retention times of ibuprofen and ibufenac were 4.53 and 4.84 min, respectively. The calibration curves were calculated using a Hewlett-Packard 85 computer.

Sample preparation

First 0.30 ml (30 μg) of the internal standard solution was pipetted into each of series of tubes and evaporated to dryness with a gentle stream of nitrogen. A 1.0-ml sample of serum was added to each tube and mixed well. Samples were acidified with 0.25 ml of 1 *M* hydrochloric acid and extracted with 6.0 ml of petroleum ether by vortexing for 30 sec. The solutions were centrifuged for 5 min at 2600 *g*, after which 4.0 ml of the petroleum ether layer were transferred to a fresh tube and evaporated to dryness with a gentle stream of nitrogen. The residue was dissolved in 100 μl of petroleum ether and 0.5—1.0 μl of this solution was injected into the gas chromatograph.

A calibration curve was constructed at ibuprofen concentrations of 5.0—50.0 μg/ml control serum and the peak area ratios of I to internal standard were plotted against the amount of ibuprofen added.

RESULTS AND DISCUSSION

Gas chromatographic determination of acidic drugs in serum involves two major problems. Because of the strong polarity of these compounds they usually have to be derivatized before gas chromatographic analysis [1, 4]. Furthermore, it is often impossible to extract them from serum with sufficient recovery without extracting interfering compounds. The polar OV-351 fused-silica capillary column gives good separation of ibuprofen and ibufenac under the conditions used and permits the determination of ibuprofen as the free acid. The OV-351 column is specially designed for the determination of free fatty acids and phenols. Fig. 2 shows gas chromatograms of the serum extracts. Clearly the extraneous peaks do not interfere with the peaks of I and II.

The precision of the method is demonstrated in Table I. Results are based on at least five determinations of each ibuprofen concentration, ranging from 5.0 to 50.0 μg/ml, which were treated as described in the experimental part. The calibration curves are linear over the range 5.0—50.0 μg/ml and can be expressed

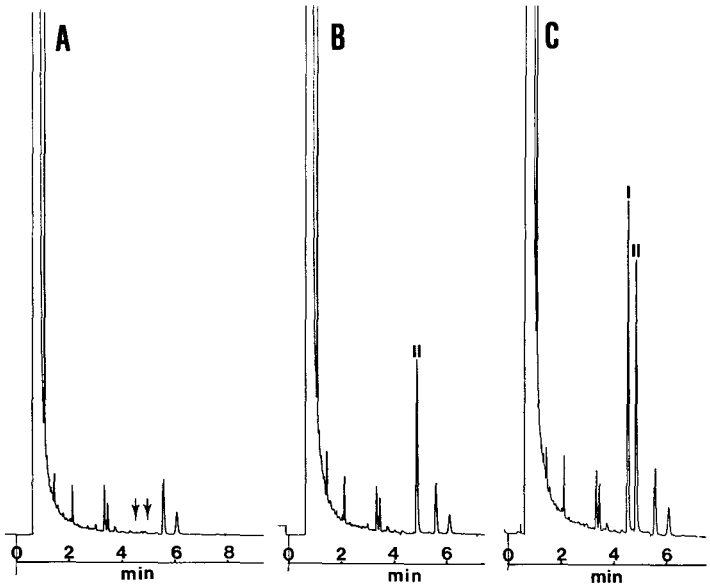


Fig. 2. Chromatograms of serum extracts. (A) Serum blank; (B) serum blank containing internal standard (II); (C) serum containing 25 μg/ml ibuprofen and 30 μg/ml ibufenac. Peaks: I = ibuprofen; II = ibufenac (internal standard).

TABLE I

PRECISION IN MEASUREMENT OF IBUPROFEN (I) ADDED TO SERUM

$Y = 0.037X + 0.019$, $r = 0.999$.

Amount of I added (μg/ml)	n	Mean peak area ratio	S.D.	R.S.D. ± %
5.0	6	0.189	0.004	2.12
10.0	5	0.416	0.008	1.92
20.0	6	0.781	0.013	1.66
30.0	6	1.063	0.016	1.50
40.0	5	1.506	0.032	2.12
50.0	6	1.880	0.025	1.33

TABLE II

RECOVERY OF IBUPROFEN (I) AND IBUFENAC (II) FROM SERUM

Recovery of I and II from serum was studied by adding 25 μg of I or II to 1.0 ml of blank serum, by extracting and adding the internal standard at the end. The reference samples were prepared by first extracting blank serum and then by adding 25 μg of I or II and the internal standard at the end. The peak area ratios of the reference samples were designated as the 100% value.

Amount added (μg/ml)	Recovery*		R.S.D. ± %
	μg	%	
I: 25.0	16.9	67.6	4.2
II: 25.0	13.5	53.9	3.1

*Each result is the mean of six determinations.

by the equations $Y = 0.036X + 0.008$, $r = 0.999$ for pure substance, and $Y = 0.037X + 0.019$, $r = 0.999$ for the spiked serum specimens.

The accuracy of the method was studied by analysing six samples, each containing an equal amount of ibuprofen (25 μg/ml) under the experimental conditions described above. The accuracy of the method was 98.1% for the spiked serum specimens, with a relative standard deviation of 1.1%.

The results of the recovery studies are presented in Table II. The recovery of ibuprofen is only about 70%, but the method gives very good precision (1.33—2.12%) and accuracy (98.1%). The extraction solvent, isopropanol—dichloromethane (1:20, v/v), gives slightly better recovery, but it extracts interfering compounds from serum. Petroleum ether is better for routine analysis because the layer of petroleum ether is the upper phase, which makes the analysis quicker to perform in practice. Speed and simplicity of the sample preparation methods are great benefits in routine clinical chemistry work.

Use of the fused-silica capillary column makes it possible to determine ibuprofen as the free acid without derivatization. The total procedure is simple and specific and allows rapid estimation of ibuprofen at therapeutic and overdose levels.

REFERENCES

1 K.K. Midha, J.K. Cooper, J.W. Hubbard and I.J. McGilveray, Can. J. Pharm. Sci., 12 (1977) 29.
2 L.P. Hackett and L.J. Dusci, Clin. Chim. Acta, 87 (1978) 301.
3 D.J. Hoffman, J. Pharm. Sci., 66 (1977) 749.
4 L. Heikkinen, Acta Pharm. Fenn., 92 (1983) 275.

Journal of Chromatography, 307 (1984) 210—215
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2019

Note

Gas chromatographic determination of gemfibrozil and its metabolites in plasma and urine

EDWARD J. RANDINITIS*, A.W. KINKEL, C. NELSON and T.D. PARKER, III

Pharmacokinetics/Drug Metabolism Department, Warner-Lambert/Parke-Davis Pharmaceutical Research, Ann Arbor, MI 48105 (U.S.A.)

(First received September 22nd, 1983; revised manuscript received November 25th, 1983)

Gemfibrozil, (Lopid®, Parke-Davis), 5-(2,5-dimethylphenoxy)-2,2-dimethylpentanoic acid, is a recently marketed lipid regulating agent [1—4]. Its metabolism has been studied using radiolabelled material as described by Okerholm et al. [5]. A gas chromatographic (GC) procedure has been described and a metabolism scheme has been proposed [6]. The major metabolite has been shown to be metabolite III, the benzoic acid metabolite. The bioavailability of the capsule has been reported by Smith [7] but no method of assay was described in this report. Prior to its marketing several pharmacokinetic and biopharmaceutic studies were conducted to determine the optimum dose and dosage form. In order to carry out such studies a GC method [8] was developed to monitor plasma concentrations of gemfibrozil and urinary excretion of unchanged gemfibrozil as well as its major metabolite, and their glucuronides. The previously reported method used a N,O-bis(trimethylsilyl)acetamide (BSA) derivative which was unstable in our hands.

This report describes the GC method for the determination of gemfibrozil and its major metabolite, 3-[(4-carboxy-4-methylpentyl)oxy]-4-methylbenzoic acid from plasma, as well as gemfibrozil, and their glucuronides from plasma and urine. The method involves in situ derivatization by methylation with trimethylanilinium hydroxide in methanol (TMAH) prior to its GC analysis. The method has been employed in several pharmacokinetic studies and some typical results will be reported to demonstrate the applicability of the method.

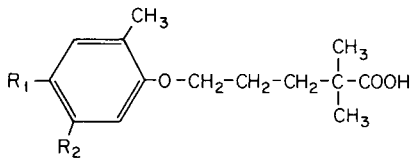
EXPERIMENTAL

Reagents

Acetic acid, hydrochloric acid, and sodium hydroxide were analytical rea-

0378-4347/84/$03.00

gent grade. Diethyl ether and chloroform were absolute reagent ACS grade. Chloroform was freshly redistilled in glass prior to its use. Glucuronidase-sulfatase (Glusulase, Endo Labs., Garden City, NY, U.S.A.) and TMAH (0.2 *M* trimethylanilinium hydroxide in methanol, Supelco, Bellefonte, PA, U.S.A.) were also used. Gemfibrozil (I), metabolite III (II), and internal standard (III) were synthesized in the Warner-Lambert/Parke-Davis Pharmaceutical Research Labs. (Ann Arbor, MI, U.S.A.). The structures of gemfibrozil, metabolite III, and internal standard are presented in Fig. 1.



	R_1	R_2
GEMFIBROZIL	$-H$	$-CH_3$
METABOLITE III	$-H$	$-COOH$
INTERNAL STANDARD	$-CH_3$	$-CH_3$

Fig. 1. Structures of gemfibrozil, metabolite III, and internal standard.

Stock solutions of gemfibrozil and internal standard, 2,2-dimethyl-5-(2,4,6-trimethylphenoxy)pentanoic acid (1 mg/ml and 100 μg/ml, respectively) and metabolite III (1 mg/ml) were prepared in 0.1 *M* sodium hydroxide. Plasma standard curves were prepared by adding 0.025, 0.05, 0.1, 0.2, and 0.3 ml of the 100 μg/ml stock solution of gemfibrozil to 1 ml of blank human plasma. Urine standard curves were prepared by adding 0.025, 0.05, 0.1, 0.2, and 0.3 ml of the 1 mg/ml stock solutions of gemfibrozil and metabolite III to 1 ml of blank human urine.

Extraction

For the assay of plasma samples or standards for free gemfibrozil, 0.2 ml of internal standard (at 100 μg/ml in 0.1 *M* sodium hydroxide), 1 ml of water, 1 ml of 1 *M* hydrochloric acid, and 10 ml of chloroform are added to 1 ml of plasma. The mixture is shaken on a reciprocating shaker for 15 min, centrifuged, and the aqueous phase discarded. The organic layer is transferred to a clean tube and evaporated to dryness in a 60°C water bath with the aid of a current of air. The residue is dissolved in 75 μl of TMAH and 100 μl of diethyl ether, and 1—2 μl are injected onto the GC column.

The urine samples are analyzed for total (free plus conjugated) gemfibrozil and metabolite III by hydrolyzing the glucuronides present in 1 ml of urine with 25 μl of glucuronidase-sulfatase adding 1 ml of water, 0.15 ml of internal standard (at 1 mg/ml in 0.1 *M* sodium hydroxide), and 1 ml of 2 *M* acetate buffer at pH 5.2. After overnight hydrolysis at 37°C, 2 ml of 1 *M* hydrochloric acid, and 10 ml of chloroform are added and the mixture is shaken for 15 min on a reciprocating shaker. After centrifugation the aqueous layer is discarded and 5 ml of 0.1 *M* sodium hydroxide are added to the organic phase. This mixture is shaken for 15 min, centrifuged, and the aqueous phase discarded. The

organic phase is removed to a clean tube and evaporated to dryness using a water bath at 60°C and a current of air. The residue is dissolved in 100 μl of TMAH and 200 μl of diethyl ether, and 1—2 μl are injected onto the gas chromatograph.

Apparatus

A Varian Model 2100 gas chromatograph equipped with a flame ionization detector was used for the assays. For the plasma samples, a 1.8 m × 2 mm I.D. glass column packed with 3% OV-22 on 80—100 mesh support was used (Applied Science, Deerfield, IL, U.S.A.). The column temperature was 180°C and the injector and detector temperatures were 230°C and 250°C, respectively. For the urine samples, a 1.8 m × 2 mm I.D. glass column packed with 10% Poly I-110 on 80—100 mesh GCQ (Supelco), maintained at 240°C was used. The injector and detector temperatures were 280°C. Nitrogen was used as a carrier gas at a flow-rate of 50 ml/min. A Shimadzu C-RIA recording integrator was used to quantitate the results using peak heights and peak height ratios.

RESULTS AND DISCUSSION

Typical chromatograms for plasma extracts are shown in Fig. 2. The retention times for the gemfibrozil and internal standard are 3.8 and 5.0 min, respectively. A peak at a retention time of about 3.1 min occurred in all of the plasma samples including the blank. This peak was well resolved from the gemfibrozil peak and did not interfere using the OV-22 column. This column was not utilized for the determination of gemfibrozil and metabolite III in urine because of the long retention time of metabolite III.

Typical chromatograms for the urine extracts are shown in Fig. 3. The retention times for the gemfibrozil, internal standard, and metabolite III are 2.8,

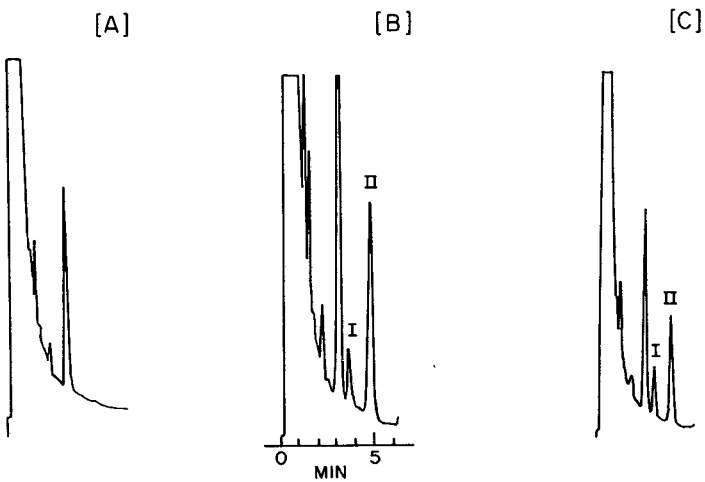


Fig. 2. Typical chromatograms of plasma extracts. A = Blank plasma; B = spiked plasma, 5 μg/ml; C = plasma sample 3 h after a 300-mg oral dose of gemfibrozil. Peaks: I = gemfibrozil; and II = internal standard.

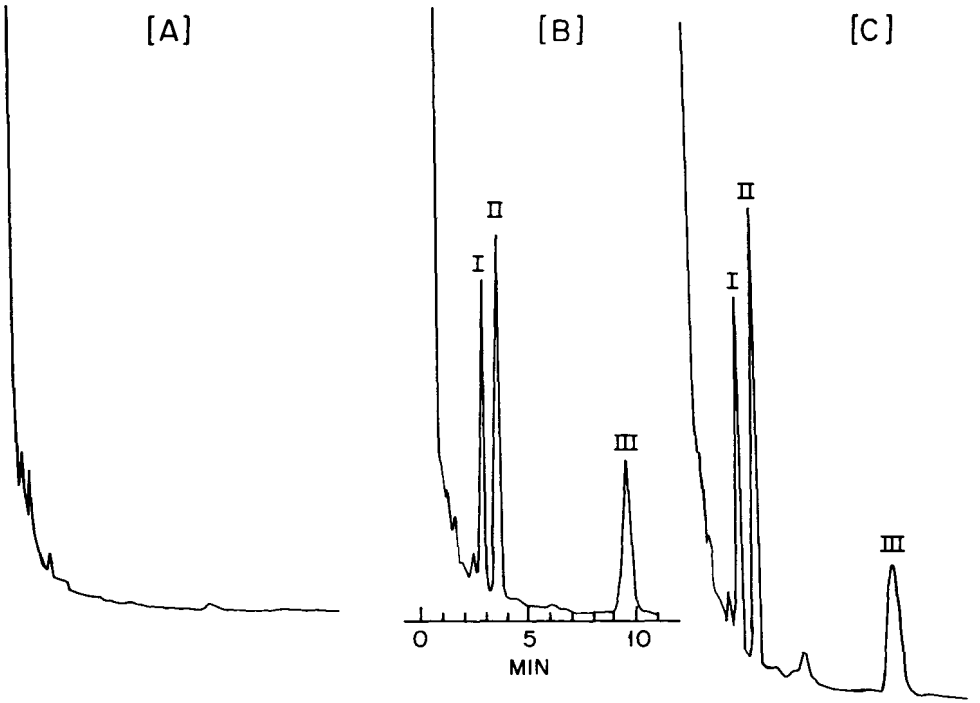


Fig. 3. Typical chromatograms of urine extracts. A = Blank urine; B = spiked urine, 100 μg/ml each of gemfibrozil and metabolite III; C = 12—24 h urine sample after a 300-mg dose of gemfibrozil. Peaks: I = gemfibrozil; II = internal standard; and III = metabolite III.

3.5, and 9.5 min, respectively. Urine extracts did not contain any interfering peaks, and the retention time for the metabolite was reasonable, therefore the urine was assayed for both gemfibrozil and metabolite III on the same injection. This column was not used for the plasma samples due to an interfering peak at the retention time of gemfibrozil.

Extraction recovery as measured against a non-extracted standard solution of gemfibrozil was about 85% and consistent over the range of concentrations occurring in plasma and urine. The minimum detectable concentration of gemfibrozil was 0.5 μg/ml from plasma and 5 μg/ml from urine as measured by three times the standard deviation of the lowest concentration used in the precision studies. Similarily the minimum detectable concentration of the metabolite was determined to be 7 μg/ml from urine.

Calibration curves were linear ($r > 0.99$) for gemfibrozil in plasma in the range 2.5—30 μg/ml and for gemfibrozil and metabolite III in urine in the range 25—300 μg/ml. The results of the precision studies are listed in Table I. Precision was assessed by the determination of percent R.S.D. on a within-day basis as well as an overall basis. The relative standard derivations were less than 10% in both measures of precision and the method was considered valid.

The method was used in several studies including the assessment of bioavailability of a 300-mg capsule formulation. Complete details of the bioavailability study will be reported elsewhere. The mean plasma concentration—

TABLE I

PRECISION STUDIES

Added	Mean backcalculated (percent R.S.D.)*							
	Day No. 1	(n=3)	Day No. 2	(n=3)	Day No. 3	(n=3)	Overall	(n=9)
Plasma concentrations (μg/ml) gemfibrozil								
0	0		0		0		0	
2.5	2.7	(9.2)	2.8	(4.1)	2.9	(7.3)	2.8	(6.6)
5	5.0	(2.0)	5.2	(1.1)	5.2	(1.1)	5.1	(2.4)
10	10.2	(3.1)	10.1	(1.7)	10.0	(1.7)	10.1	(2.2)
20	19.8	(2.6)	19.8	(1.3)	19.8	(1.3)	19.8	(1.6)
30	29.8	(1.9)	29.6	(1.1)	29.6	(0.3)	29.7	(1.1)
Urine concentrations (μg/ml) gemfibrozil								
0	0		0		0		0	
25	21.0	(10.0)	19.5	(5.5)	21.5	(8.5)	20.7	(8.4)
50	45.4	(9.4)	46.4	(6.7)	39.6	(2.5)	43.8	(9.6)
100	97.5	(2.4)	99.3	(4.6)	92.6	(4.0)	96.5	(4.5)
200	200.0	(3.3)	204.3	(1.4)	204.8	(2.3)	203.8	(5.1)
300	311.2	(1.6)	301.3	(3.7)	316.4	(1.1)	309.6	(3.0)
Urine concentrations (μg/ml) metabolite III								
0	0		0		0		0	
25	24.3	(2.2)	20.3	(9.7)	22.1	(7.6)	22.3	(9.8)
50	54.7	(7.5)	50.9	(4.6)	45.6	(1.8)	50.4	(9.2)
100	112.3	(9.6)	99.7	(7.4)	98.7	(6.4)	103.6	(9.5)
200	198.0	(6.3)	219.7	(3.0)	196.5	(8.9)	204.8	(7.8)
300	279.7	(1.7)	294.9	(1.5)	310.3	(6.8)	294.9	(5.9)

*Percent R.S.D. was assessed on both a within-day and overall basis.

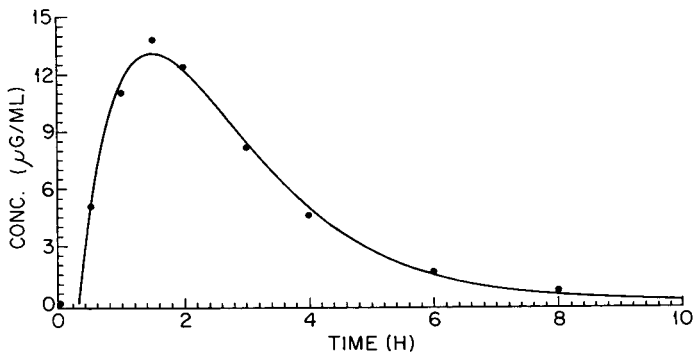


Fig. 4. Plasma concentration—time profile for gemfibrozil following a 300-mg oral capsule. The points are mean of twelve subjects. The line in the computer best-fit to a mono-exponential elimination process following first-order absorption.

time profile of a 300-mg capsule in twelve subjects is given in Fig. 4. The plasma concentrations follow mono-exponential elimination kinetics with an elimination half-life of about 1.4 h. The mean maximum plasma concentration was 16.1 μg/ml (range 10—27 μg/ml). The 48-h urinary excretion of total (free plus conjugated) gemfibrozil and metabolite III was about 27.4% (range 14—45%) and 33.2% (range 19—57%) of the dose, respectively. The relative bioavailability of the capsule as compared to the oral solution, as measured by both area

under the plasma concentration—time curve and urinary excretion data was about 0.97.

CONCLUSION

A simple rapid GC method for the analysis of gemfibrozil in plasma and total (free plus conjugated) gemfibrozil and metabolite III in urine has been developed and validated. The method is reproducible (percent R.S.D. $< 10\%$) with a minimum detectable concentration of gemfibrozil in plasma of 0.5 μg/ml and of gemfibrozil and metabolite III in urine of 5 μg/ml and 7 μg/ml, respectively. The method has been employed in several pharmacokinetic and bioavailability studies of single and multiple doses of gemfibrozil in man and an example of such a study is presented to demonstrate the applicability of the method.

REFERENCES

1 A.K. Jain, J.R. Ryan, W.S.S. Lacorte and F.G. McMahan, Clin. Pharmacol. Ther., 29 (1981) 254.
2 Anonymous, Med. World News, 23 (1982) 40.
3 Anonymous, J. Amer. Med. Ass., 247 (1982) 1540.
4 H.D. Peabooy, Jr., Cardiovasc. Rev. Rep., 3 (1982) 1194.
5 R.A. Okerholm, F.J. Keeley, F.E. Peterson and A.J. Glazko, Fed. Proc., Fed. Amer. Soc. Exp. Biol., 35 (1976) Abstract 675.
6 R.A. Okerholm, F.J. Keeley, F.E. Peterson and A.J. Glazko, Proc. R. Soc. Med., 69 Suppl 2 (1976) 11.
7 T.C. Smith, Proc. R. Soc. Med., 69 Suppl 2 (1976) 24.
8 E.J. Randinitis, A.W. Kinkel, C. Nelson and T.D. Parker, III, Abstracts of Papers, 33rd National Meeting Academy of Pharmaceutical Sciences, San Diego, CA, November 15, 1982, Vol. 12, No. 2, 1982, p. 159.

Journal of Chromatography, 307 (1984) 216—219
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2006

Note

Analysis of riboxamide in urine by high-performance liquid chromatography

N.M. MELTZER and L.A. STERNSON*

Department of Pharmaceutical Chemistry, The University of Kansas, Lawrence, KS 66045 (U.S.A.)

(First received July 27th, 1983; revised manuscript received November 15th, 1983)

We have recently described an analytical method for the determination of the experimental antineoplastic agent riboxamide (TCAR, see Fig. 1) in plasma [1]. The procedure, based on high-performance liquid chromatography (HPLC), utilizes a column-switching configuration consisting of two different solvent generated anion-exchange columns which exhibit different retention characteristics toward the analyte. Initial pharmacokinetic evaluation of the drug has revealed it to be primarily excreted in urine [2]. Unfortunately, the procedure reported through our earlier communication [1] is not directly applicable to urine analysis of riboxamide. In this note, a modification of that method is described which permits routine urinary monitoring of the drug in clinical settings.

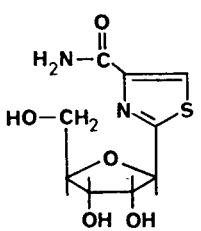


Fig. 1. Chemical structure of riboxamide (TCAR).

MATERIALS AND METHODS

Riboxamide (TCAR) was obtained from the National Cancer Institute. μBondapak C_{18} columns (10-μm particles, 300 × 4.6 mm) were purchased from Waters Assoc. (Milford, MA, U.S.A.) and ODS Hypersil bulk packing (5-μm particles) obtained from HETP (Macclesfield, U.K.) was slurry packed [3]

0378/4347/84/$03.00

into 150 × 4.6 mm I.D. columns. The chromatographic apparatus was configured as described by Riley et al. [1] except that a μBondapak C_{18} column was used as column 1 and an ODS Hypersil column was used as column 2. Urine samples were filtered through 3-μm Millipore filters and the filtrate applied directly to the chromatographic system. Column 1 was eluted with 0.1 *M* phosphate buffer (pH 2.1) and column 2 was eluted with 0.1 *M* phosphate buffer (pH 7.0). Following elution of TCAR from column 1, the eluent strength (for column 1) was increased to methanol—phosphate buffer (pH 2.1) (20:80) and maintained there for 10 min to purge the column of strongly retained urinary contaminants. The column was then allowed to re-equilibrate for 10 min with the purely aqueous mobile phase prior to the application of the next sample. Eluent was monitored spectrophotometrically at 254 nm.

RESULTS AND DISCUSSION

Urine samples were prepared for HPLC analysis by simply subjecting them to a single filtration of the biological fluid through a 3-μm Millipore filter and direct injection of the filtrate onto the HPLC system. This procedure was also applicable to plasma and represents a significant simplification of the clean-up step reported previously [1] for plasma analysis of TCAR. This modification saves ca. 20 min per sample in total analysis time. The recovery of TCAR from urine was 98 ± 0.6% and 97 ± 0.8% when spiked at the 10 and 1 μg/ml levels, respectively. This indicates that TCAR is not retained to a significant extent by the filter apparatus. Similar recoveries were noted with plasma samples.

Whereas plasma analysis involved separation on a dynamic anion-exchange system [1], an ion-exchange system was not required to chromatographically resolve TCAR from urinary components. Resolution was achieved using a column-switching configuration involving two columns packed with different reversed-phase (C_{18}) materials which in turn provided different selectivities toward the analyte. This selectivity difference was amplified by eluting the components with buffers differing only in pH. Optimal resolution was achieved using the basic dual-column hardware employed earlier [1] but achieving initial separation on a μBondapak C_{18} column eluted with 0.1 *M* phosphate buffer (pH 2.1) followed by transfer of the eluent slice containing TCAR ($k' = 4.11$) onto an ODS Hypersil column and elution of the analyte with 0.1 *M* phosphate buffer (pH 7.0). The overall retention time for TCAR in this system was 21.5 min (Fig. 2).

The peak height of TCAR was linearly related to the amount of solute injected, q, over the range 1—200 μg/ml according to the equation $P = 1.01q - 0.54$ ($r = 0.999$). Peak heights (P in mm) were corrected for changes in detector attenuation, using 0.02 a.u.f.s. as the reference. The response factor was independent of detector attenuation (0.005 to 0.160) at constant injection volumes [although a slight decrease in response factor was observed with large ($\geqslant$ 200 μl) injection volumes].

The day-to-day reproducibility (expressed as coefficient of variation, $n \geqslant 5$) of the response factor was 7%. To maximize precision, the response factor was recalculated after analysis of every fourth urine sample using an external standard. Within a single day, the coefficient of variation of the peak heights of

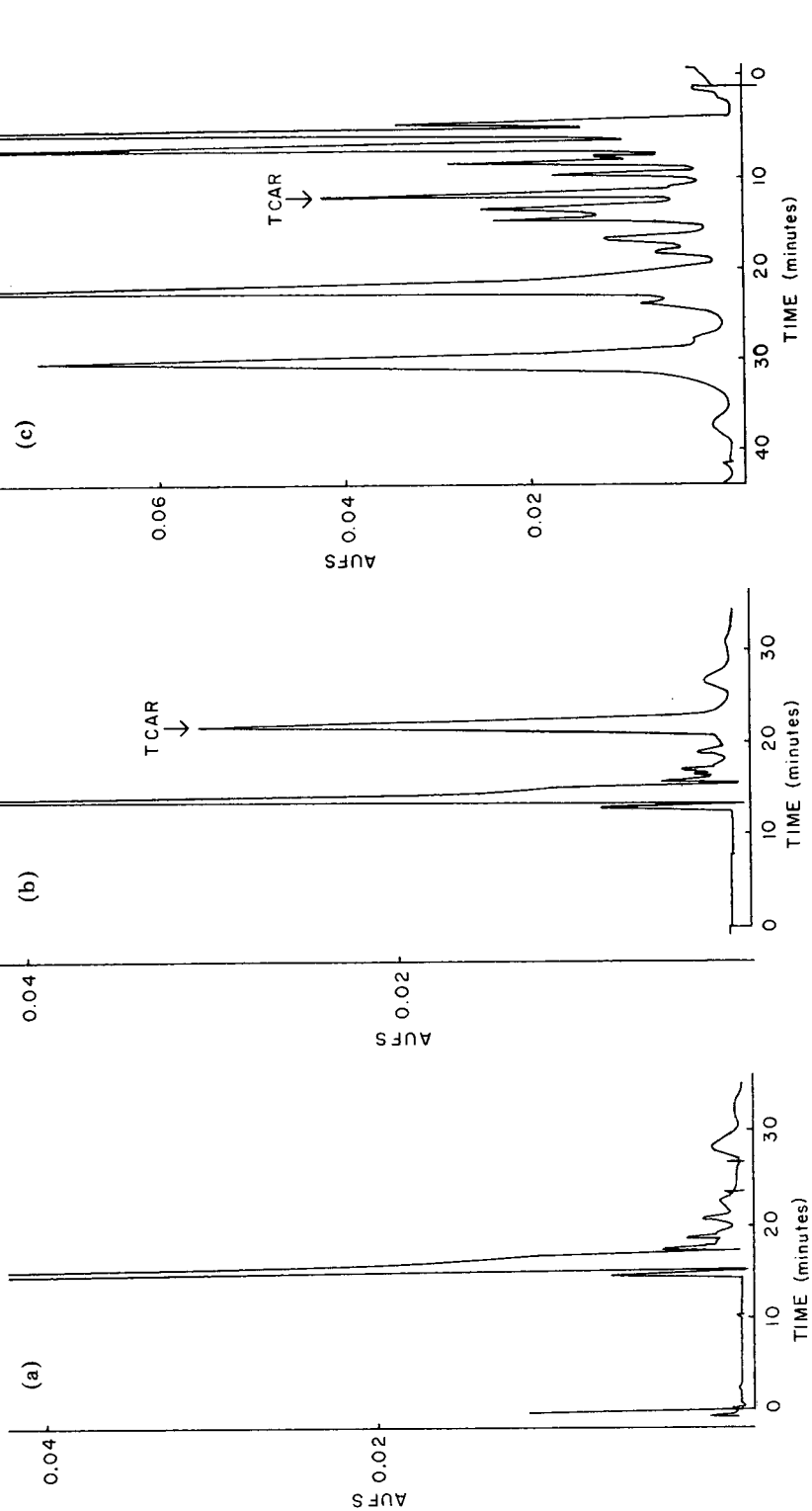


Fig. 2. Chromatograms obtained during analysis of patient urine for TCAR. (a) Urine blank; and (b) urine sample containing TCAR (500 ng/ml) carried through the column-switching procedure described in this report; (c) TCAR in urine obtained on a single μBondapak C_{18} column eluted with 0.1 *M* phosphate buffer (pH 2.1).

the external standards was $\leqslant$ 3% (n = 8) for 20-μl injections. The limit of detection for TCAR in urine was 200 ng/ml which gave a signal-to-noise ratio of 3:1 with an injection volume of 20 μl.

APPLICATION

The urinary excretion of TCAR was monitored in a patient receiving a daily intravenous dose of 550 mg/m^2 for five consecutive days. Urine collections (24 h) were analyzed for drug (Table I). Over a 24-h period, approximately 30% of the administered dose is excreted in the urine as unchanged drug, which can be expressed as an average excretion rate of 13.65 ± 2.17 μg/ml/h. Thus, a facile, rapid assay for riboxamide in urine is presented and, through a modification of the initial clean-up step, the plasma assay [1] is greatly simplified.

TABLE I

DAILY URINARY EXCRETION OF RIBOXAMIDE IN A MALE PATIENT RECEIVING DRUG ON FIVE CONSECUTIVE DAYS

TCAR administered as a single daily intravenous dose of 550 mg/m^2 on days 1—5.

Day	Total urinary excretion* (μg TCAR per ml)	Average hourly excretion rate (μg/ml h)
1	286.21	11.92
2	285.86	13.14
3	379.31	14.54
4	393.10	17.75
5	272.41	11.35
6	332.76	14.47
7	310.34	12.41

*Values represent the average of three determinations of each 24-h pooled urine specimen.

ACKNOWLEDGEMENT

The work was supported in part by NIH training grant CA-09242 from the National Cancer Institute.

REFERENCES

1 C.M. Riley, L.A. Sternson and A.J. Repta, J. Chromatogr., 276 (1983) 93.
2 L.A. Sternson, D.D. von Hoff and J. Kuhn, Invest. New Drugs, in press.
3 P.A. Bristow, P.N. Brittain, C.M. Riley and B.F. Williamson, J. Chromatogr., 131 (1977) 57.

Journal of Chromatography, 307 (1984) 220—223
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2011

Note

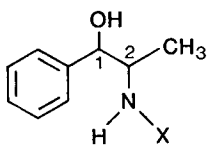
Resolution of the enantiomers of ephedrine, norephedrine and pseudoephedrine by high-performance liquid chromatography

JOSEPH GAL

Departments of Medicine and Pharmacology, Division of Clinical Pharmacology, Box C237, University of Colorado Health Sciences Center, 4200 East Ninth Avenue, Denver, CO 80262 (U.S.A.)

(First received August 17th, 1983; revised manuscript received November 15th, 1983)

Ephedrine [1-phenyl-2-(methylamino)-1-propanol, EPH, Fig. 1] and the related compounds pseudoephedrine (PSE, Fig. 1) and norephedrine (NOR, Fig. 1) are adrenergic agents widely available in asthma, ophthalmic, cold, and allergy products. The hydroxypropylamino structural element of EPH, PSE and NOR contains two asymmetric centers, and each of these compounds exists as a pair of enantiomers (Fig. 1). Reasons for the interest in the stereochemistry of EPH and related compounds are several, including differences between the enantiomers in their pharmacological actions [1, 2], and reports of stereoselective metabolism of these compounds [3]. Studies on the chromatographic



X	Compound	Absolute Configuration
CH_3	ephedrine	1R,2S-(-)
CH_3	pseudoephedrine	1R,2R-(-)
H	norephedrine	1R,2S-(-)

Fig. 1. The chemical structures and absolute configuration of the compounds studied. The numbering of C_1 and C_2 of the propyl group is also shown.

0378-4347/84/$03.00

resolution of the enantiomers of EPH, PSE and NOR have been few, and, with one exception, limited to gas—liquid chromatography (GLC). Beckett and Testa [4, 5] studied the resolution of the enantiomers of EPH, PSE and NOR as their N-[N'-(trifluoroacetyl)-L-prolyl] derivatives. Poor resolution of the enantiomers of EPH and PSE was attained [4, 5], and another disadvantage of the technique is that the chiral reagent used undergoes partial racemization during its synthesis and/or storage [6, 7]. Frank et al. [8] investigated the resolution of the enantiomers of EPH as their N,O-bis(pentafluoropropionyl) derivatives by GLC on glass capillary columns coated with a chiral stationary phase. Baseline resolution of the EPH enantiomers was not achieved [8]. König and Benecke [9] synthesized new chiral GLC stationary phases and used them in glass capillary columns to separate the enantiomers of a variety of compounds including NOR but not including EPH or PSE.

Wainer et al. [10] recently described the use of a chiral high-performance liquid chromatography (HPLC) column for the resolution of EPH. The procedure involves a 2-h reaction of EPH with 2-naphthaldehyde followed by recrystallization of the oxazolidines produced. The diastereomeric oxazolidines are incompletely resolved by the chromatography system used [10]. PSE and NOR were not studied [10].

In this communication, a new, simple and rapid procedure for the resolution of the enantiomers of EPH, PSE and NOR is described. The method is based on derivatization with the chiral reagent 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) and separation of the resulting diastereomeric thioureas by reversed-phase HPLC.

EXPERIMENTAL

Chemicals and reagents

(±)-Norephedrine hydrochloride was obtained from Eastman Organic Chemicals (Rochester, NY, U.S.A.); (±)-ephedrine hydrochloride, (−)-ephedrine, (−)-pseudoephedrine, and (+)-pseudoephedrine were purchased from Sigma (St. Louis, MO, U.S.A.); (+)-norephedrine was from Aldrich (Milwaukee, WI, U.S.A.); triethylamine and ammonium phosphate (monobasic) were obtained from J.T. Baker (Phillipsburg, NJ, U.S.A.); GITC was purchased from Polysciences (Warrington, PA, U.S.A.), acetonitrile (distilled-in-glass grade) from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.).

Chromatography

A Waters Assoc. (Milford, MA, U.S.A.) HPLC system consisting of a Model M-6000 solvent delivery system, a Model U6K injector, and a Model 440 absorbance detector was used. Separations were carried out on a Beckmann Instruments (Berkeley, CA, U.S.A.) 150 mm × 4.6 mm HPLC column packed with Ultrasphere ODS of 5-μm particle size. The mobile phase was prepared by mixing 400 ml of acetonitrile with 600 ml of water containing 1.4 g of monobasic ammonium phosphate. The two components were vacuum-filtered before mixing. The mobile phase was delivered at 1.0 ml/min, and the column effluent was monitored at 254 nm. Chromatograms were recorded using a Perkin-Elmer (Norwalk, CT, U.S.A.) Model 56 recorder.

Preparation of derivatives

A 1-mg sample of the compound to be derivatized, in the free-base form or as the hydrochloride, was treated with 200 μl of acetonitrile in a test tube. If the compound was in the hydrochloride form, 20 μl of triethylamine was added. A 50-μl aliquot of acetonitrile containing 3 mg of GITC was then added, the mixture was briefly swirl-mixed, and allowed to stand at room temperature for 10 min. Acetonitrile, 500 μl, was added, and 3—5-μl aliquots were injected into the HPLC system.

RESULTS

Fig. 2 shows the resolution of EPH, PSE and NOR. The following values were obtained for the separation factor α [11] and resolution factor R [12]: EPH: $\alpha = 1.19$, $R = 2.73$; PSE: $\alpha = 1.09$, $R = 1.32$; NOR; $\alpha = 1.12$, $R = 1.71$. The diastereomer derived from GITC and the levorotatory enantiomer of EPH, PSE, or NOR elutes before the derivative formed from the corresponding (+)-enantiomer (Fig. 2). The retention times are given in Fig. 2.

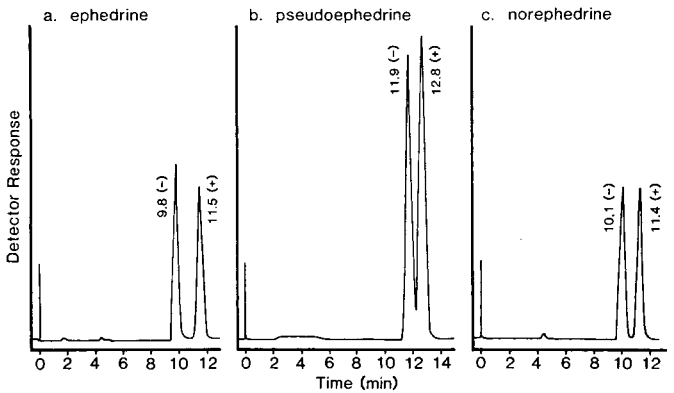


Fig. 2. Resolution of a, (±)-EPH; b, (±)-PSE; and c, (±)-NOR. The retention times in min and the enantiomeric identity of each peak are given.

DISCUSSION

GITC has been shown recently to be a useful chiral reagent for the HPLC resolution of a variety of amines and amino acids [13—15]. Using this reagent, baseline resolution of the enantiomers of EPH and NOR was achieved (Fig. 2). The extent of separation of the enantiomers of PSE was smaller (Fig. 2). If the resolution factor $R = 1.0$, the resolution of two equal-area peaks is approximately 98% complete [12]. The value of $R = 1.32$ achieved in the resolution of (±)-PSE, therefore, represents a near-complete separation of the peaks. Fig. 3 shows the structure of the derivative of NOR formed with GITC.

The GITC derivative of the levorotatory enantiomer of each of the three compounds studied eluted before that of its dextrorotatory antipode. Since all three levorotatory compounds possess the R-configuration at C_1 (Fig. 1), it appears that, at least in this series, the order of elution is governed by the configuration around the hydroxyl-bearing C_1 rather than by that around the amino-bearing C_2.

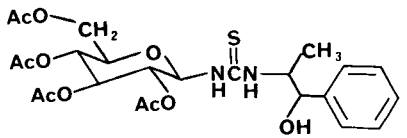


Fig. 3. The chemical structure of the derivative of NOR formed with GITC.

The procedure described has significant advantages: the derivatization method is extremely simple; the chiral reagent is commercially available and is chemically and stereochemically stable; the resolution of EPH, PSE and NOR achieved with GITC is considerably better than that described in previously published GLC [4, 5, 8, 9] or HPLC [10] methods. Thiourea derivatives of GITC have high UV absorption [13, 14], and therefore the present method may be adaptable to the determination of the enantiomers in biological fluids. Such an extension of the procedure would be a useful alternative to the stereospecific radioimmunoassays for (+)-PSE [16] and for the enantiomers of EPH [17].

In conclusion, GITC is a useful reagent for the resolution of the enantiomers of EPH, PSE and NOR by reversed-phase HPLC.

REFERENCES

1 P.S. Portoghese, Ann. Rev. Pharmacol., 10 (1970) 51.
2 E.E. Smissman, in C.O. Wilson, O.G. Gisvold and R.F. Doerge (Editors), Textbook of Organic Medicinal and Pharmaceutical Chemistry, Lippincott, Philadelphia, PA, 7th ed., 1977, p. 441.
3 P. Jenner and B. Testa, Drug Metab. Rev., 2 (1973) 117.
4 A.H. Beckett and B. Testa, J. Chromatogr., 69 (1972) 285.
5 A.H. Beckett and B. Testa, J. Pharm. Pharmacol., 25 (1973) 382.
6 D.E. Nichols, C.F. Barfknecht, D.B. Rusterholz, R. Benington and R.D. Morin, J. Med. Chem., 16 (1973) 480.
7 J. Gal, J. Pharm. Sci., 66 (1977) 169.
8 H. Frank, G.J. Nicholson and E. Bayer, J. Chromatogr., 146 (1978) 197.
9 W.A. König and I. Benecke, J. Chromatogr., 209 (1981) 91.
10 I.W. Wainer, T.D. Doyle, Z. Hamidzadeh and M. Aldridge, J. Chromatogr., 261 (1983) 123.
11 L.R. Snyder and J.J. Kirkland, Introduction to Modern Liquid Chromatography, Wiley, New York, 2nd ed., 1979, p. 840.
12 H. McNair and E.J. Bonelli, Basic Gas Chromatography, Varian Aerograph, Walnut Creek, CA, 1969, p. 33.
13 T. Kinoshita, Y. Kasahara and N. Nimura, J. Chromatogr., 210 (1981) 77.
14 N. Nimura, Y. Kasahara and T. Kinoshita, J. Chromatogr., 213 (1981) 327.
15 A.J. Sedman and J. Gal, J. Chromatogr., 278 (1983) 199.
16 J.W.A. Findlay, J.T. Warren, J.A. Hill and R.M. Welch, J. Pharm. Sci., 70 (1981) 624.
17 K.K. Midha, J.W. Hubbard, J.K. Cooper and C. Mackonka, J. Pharm. Sci., 72 (1983) 736.

Journal of Chromatography, 307 (1984) 224—229
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1999

Note

Liquid chromatographic determination of amikacin in serum with spectrophotometric detection

POKAR M. KABRA*, PRADIP K. BHATNAGER* and MARLA A. NELSON

Department of Laboratory Medicine, School of Medicine, University of California, San Francisco, San Francisco, CA 94143 (U.S.A.)

(First received March 30th, 1983; revised manuscript received November 15th, 1983)

Amikacin, an aminoglycoside antibiotic, is widely used against aerobic gram-negative bacillary infections, and, like other aminoglycoside antibiotics, has the potential for nephrotoxicity and ototoxicity if not carefully monitored [1, 2]. To minimize these toxicities and to assure therapeutic serum concentrations, frequent and careful monitoring of serum amikacin levels is essential.

Methods for measuring serum amikacin include microbiological [3], radioenzymatic [4], and immunoassays [5]. The microbiological assays are inexpensive and simple, but may suffer from several deficiencies such as slow turnaround time, limited specificity because of interferences by other antimicrobial agents, and variable precision. The enzymatic and immunoassays can be more specific and accurate, but they also depend on the purity of the enzyme and the specificity of the antibodies. Interferences and cross-reactions have been reported for both techniques [6, 7].

Various liquid chromatographic (LC) procedures have been reported for the measurement of amikacin [8—10], most require either pre-column or post-column derivatization for fluorescence detection [8, 10]. These methods may not be suitable for routine clinical laboratory application because of the complicated procedures, the need for specialized equipment, and the length of the procedure. Liquid chromatographs equipped with a variable-wavelength spectrometric detector are routinely used in many clinical laboratories for therapeutic drug monitoring, and have advantages over methods involving the more expensive and complicated pre- and post-column fluorescence equipment.

We describe a method that is well suited for the routine assay of amikacin

*Present address: Beckman, Bio-Product Division, 1050 Page Mill Road, Palo Alto, CA 94304, U.S.A.

0378-4347/84/$03.00

with commonly available LC equipment. The method is simple, precise, sensitive, selective and results correlate well with existing radioimmunoassay methods.

MATERIALS AND METHOD

Instrumentation

The chromatographic system consisted of a Series 2 or Series 3 liquid chromatograph, a Model LC-100 column oven, a Model LC-75 variable-wavelength detector, a Sigma 10 data system (all from Perkin-Elmer, Norwalk, CT, U.S.A.), and a reversed-phase 25 cm × 4.6 mm column packed with 5-μm reversed-phase octyl packing material (Ultrasphere Octyl, Altex Scientific, Berkeley, CA, U.S.A.) mounted in the oven. The sample was injected into a Model 7105 valve (Rheodyne, Cotati, CA, U.S.A.). The mobile phase consisted of acetonitrile—phosphate buffer (52:48). The flow-rate was maintained at 2.0 ml/min at 50° C, and the column effluent was monitored at 340 nm. Polypropylene tubes, 1.5-ml capacity, and an eppendorf Model 5412 centrifuge were from Brinkmann Instruments (Westbury, NY, U.S.A.). Vac Elut^{TM} vacuum chamber and Bond-Elut^{TM} C_{18} extraction columns, were purchased from Analytichem International (Harbor City, CA, U.S.A.).

Reagents

All chemicals used were of reagent grade. Acetonitrile and methanol, all distilled-in-glass, were obtained from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Mobile phase was a solution of 520 ml of acetonitrile in 480 ml of 20 mmol/l phosphate buffer, pH 3.0. Phosphate buffer, 20 mmol/l, pH 3.0, was prepared by dissolving 2.68 g of potassium dihydrogen phosphate in 1 l of distilled water. The solution was titrated to pH 3.0 with phosphoric acid. Tris buffer, 2 mol/l, pH 10.3, was prepared by dissolving 24.2 g of Trizma base (Sigma, St. Louis, MO, U.S.A) in 100 ml of distilled water. This solution is stable for at least a year at 4°C. 2,4,6-Trinitrobenzene-1-sulfonic acid, 250 g/l, was prepared by dissolving 2.5 g of trinitrobenzene sulfonic acid (TNBS, Sigma) in 10 ml of acetonitrile. This solution is stable for one month at 4°C. Wash buffer, 10 mmol/l potassium hydrogen phosphate, was prepared by dissolving 1.82 g of potassium hydrogen phosphate in 1 l of distilled water. The pH was adjusted to 8.6 with phosphoric acid. This solution is stable for at least six months at ambient temperature.

Drug standards. Amikacin and kanamycin sulfate were obtained from Bristol Labs. (Syracuse, NY, U.S.A.). The amikacin stock standard, 25 mg in 100 ml of water, is stable at 4°C for at least six months. The stock internal standard, 25 mg of kanamycin in 100 ml of acetonitrile, is stable at 4°C for at least six months. The working internal standard, prepared by diluting the stock internal standard ten-fold with acetonitrile, is stable for one month at 4°C. The working amikacin serum standards (5, 10, and 25 mg/l) were prepared by diluting 200, 400, and 1000 μl of stock standard with 9.8, 9.6 and 9.0 ml of drug-free serum. The serum standards are stable for at least one month at 4°C.

Procedure

Pipette 50 μl of serum standards, controls, or unknown into 1.5-ml polypropylene tubes. Add 25 μl of 2 mol/l Tris buffer, and 100 μl of working internal standard (kanamycin, 16.0 mg/l) to each tube. Vortex-mix and centrifuge all tubes for 1 min in an eppendorf centrifuge at 15,000 *g*. Decant the supernate into a second set of appropriately labeled polypropylene tubes, then add 30 μl of TNBS solution. Cap, vortex-mix, and place the tubes in a 70°C heating block for 30 min. For each sample, place a Bond-Elut C_{18} extraction column on the top of the Vac-Elut chamber, and connect the vacuum to the chamber. Pass two column volumes of methanol and two column volumes of water through each column. Disconnect the vacuum, and fill each column with 700 μl of working wash solution, followed by approximately 250 μl of derivatized sample. Reconnect the vacuum to the chamber, and pass three column volumes of working wash solution through each column. Disconnect the vacuum, and place a rack of labeled 10 × 75 mm glass tubes in the Vac-Elut chamber, corresponding to each column and connect the vacuum. Pipette 300 μl of acetonitrile onto each column and connect the vacuum. After collecting the eluate in the tubes, remove the rack from the vacuum chamber, shake the tubes to mix the eluate, and inject 50 μl of each eluate onto the liquid chromatograph.

RESULTS AND DISCUSSION

Optimum conditions for derivatization

The optimum conditions for derivatization were derived by varying reagent concentration, reaction temperature, reaction time, pH, and the composition of buffers. A large excess of derivatizing agent, TNBS (18,000—20,000 molar ratio) was necessary to yield a single amikacin derivative quantitatively in less than 30 min. TNBS reacts with primary amino groups of amino acid and peptide in the aqueous solution at pH 8 and at room temperature without any undesirable side-reactions. The resulting trinitrophenyl derivative has a high molar absorptivity at 340 nm [11]. The TNBS solution was dissolved in acetonitrile to expedite the derivatization, deproteinize the serum, and solubilize the nonpolar amikacin derivative. At temperatures lower than 70°C and reaction time shorter than 30 min, multiple derivatives of amikacin formed, due to incomplete reaction. At 70°C, the derivatization was complete in less than 30 min. The pH of the reaction mixture was critical for complete derivatization. Below pH 9, derivatization was incomplete and slow because of the basic nature of amikacin molecule. The optimum pH for this reaction is between 9.5 and 10.0.

Several different buffers were tried for the derivatization reaction. Carbonate, phosphate, and borate buffers at pH 9.5—10 were unsuitable because of their low buffering capacity in this pH range, insolubility in organic solvents, or complexation of amikacin hydroxyl groups with borate buffer. The high molarity of this Tris buffer (2 mol/l) was necessary to maintain the reaction pH above 9.5 in the presence of a large excess of strong acids (TNBS, and picric acid formed during the reaction) used.

Extraction of amikacin derivative

When 50 μl of the crude derivatized sample was injected onto a reversed-

phase octyl column, a large void volume peak was observed, due to the presence of a large excess of polar constituents (TNBS and picric acid) in the crude mixture. The amikacin sample was eluted from the reversed-phase octyl column with acetonitrile—phosphate buffer (52:48) at a flow-rate of 2.0 ml/min at 50°C, and the total analysis time was about 15 min. The solvent front was significantly reduced by extracting the amikacin and kanamycin, internal standard, derivatives from the crude mixture using a Bond-Elut C_{18} reversed-phase column. The solid-phase extraction procedure simplified the process and eliminated the large solvent front. A batch of ten samples could be extracted in less than 5 min. This extraction procedure increased the useful life of the analytical column.

Chromatography

A number of chromatographic parameters were investigated to optimize the separation in the shortest time. The composition of the mobile phase, the pH of the mobile phase, column temperature, and detection wavelength were varied to achieve optimum chromatographic conditions.

Mobile-phase variation included various ratios of acetonitrile—tetrahydrofuran—phosphate buffer, namely 45:5:50, 48:4:48, 50:4:46, and acetonitrile—phosphate buffer (52:48). The greatest resolution and sharpest peaks between amikacin and kanamycin were obtained with a mobile phase of acetonitrile—phosphate buffer (52:48). To reduce tailing, the pH of the phosphate buffer was adjusted to 3.0. At a higher pH substantial peak tailing was observed. We elected to use above ambient temperature (50°C) to avoid variations in the retention time and to reduce the solvent viscosity for optimum column efficiency.

Detection wavelength. The λ_{max} value of the trinitrophenyl derivatives of amikacin and kanamycin is 340 nm, a wavelength at which interference from exogenous and endogenous serum constituents was also minimized.

Sensitivity. The limit of detection of the assay was <0.5 mg/l ,when 50 μl of serum was used.

Analytical variables

Precision. Repeated analysis of serum specimens containing amikacin at two different concentrations gave the results shown in Table I. The within-day coefficients of variation (C.V.) ranged from 3.5 to 6.9%; the day-to-day C.V. ranged from 2.8 to 3.1%.

TABLE I

PRECISION OF ASSAYS FOR AMIKACIN IN SERUM

	Range ± S.D. (mg/l)	C.V. (%)
Within-day (n = 10)	9.0 ± 0.542	6.0
	24.0 ± 0.846	3.5
Day-to-day (n = 15)	9.49 ± 0.298	3.1
	23.63 ± 0.668	2.8

Background. Several drug-free sera and plasma samples were processed as described above to ascertain the level of background peak interference at the elution times corresponding to the amikacin and kanamycin. The background calculated from these samples was < 0.1 mg/l, and did not interfere with the analysis.

Analytical recovery and linearity. Amikacin was added to drug-free serum in amounts equivalent to 2.5—50.0 mg/l. A constant amount of internal standard (kanamycin) was added to each sample and processed as described above. Concentration and peak height ratios were linearly related over this range. Analytical recoveries for amikacin from low therapeutic to toxic concentrations ranged from 92.8 to 98.4% (Table II).

TABLE II

ANALYTICAL RECOVERY OF AMIKACIN FROM SERUM (n = 5)

Added (mg/l)	Recovered (mg/l)	Recovery (%)
2.5	2.5	98.4
5.0	4.7	94.0
10.0	9.6	96.1
25.0	23.6	94.4
50.0	46.4	92.8

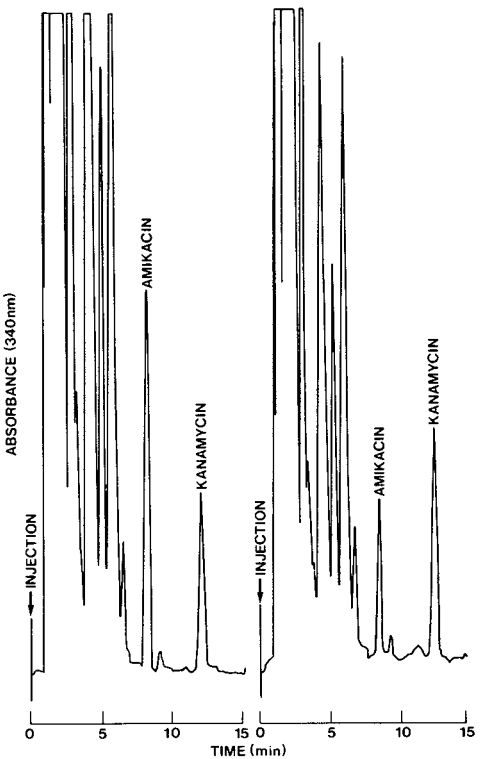


Fig. 1. Chromatograms of patient serum containing left: 26.2 μg/ml of amikacin; and right: 8.6 μg/ml of amikacin.

Interference. Potential interference with the analysis of amikacin was evaluated by chromatographing pure drug solutions and/or serum standards individually, noting retention time for each. Drugs tested but not detected under these conditions were: acetaminophen, acetazolamide, amobarbital, ampicillin, amitriptyline, caffeine, cafamandole, cefoxime, cefoxitin, cephalothin, clindamycin, chloramphenicol, chlordiazepoxide, diazepam, erythromycin, ethosuximide, nitrofurantoin, penicillin G, pentobarbital, phenobarbital, phenytoin, primidone, procainamide, N-acetylprocainamide, quinidine, salicylate, secobarbital, tetracycline, theophylline, and vancomycin. Other aminoglycoside antibiotics (gentamicin and tobramycin) did not interfere with the analysis.

Comparison with radioimmunoassay

To assess the accuracy of the method, we compared our results for 25 sera from patients receiving amikacin with results obtained with established commercially available radioimmunoassay (Amikacin ^{125}I kit, American Diagnostic, Newport Beach, CA, U.S.A.). The regression data comparing our LC method with radioimmunoassay method were: $n = 25$, $r = 0.999$, y-intercept = 0.398 mg/l, and slope = 1.047. The correlation study indicates that the LC method is accurate and selective for the determination of amikacin in serum. Sample chromatograms are illustrated in Fig. 1.

REFERENCES

1 S.K. Maitra, T.T. Yoshikawa, L.B. Guze and M.C. Schotz, Clin. Chem., 25 (1979) 1361.
2 J.P. Anhalt, in P.M. Kabra and L.J. Marton (Editors), Liquid Chromatography in Clinical Analysis, Humana Press, Clifton, NJ, 1981, p. 163.
3 G.P. Bodey, R.F. Valdivieso and V. Rodrighez, Antimicrob. Ag. Chemother., 5 (1974) 508.
4 W.W. Kirby, J.T. Clarke, R.D. Libke and C.R. Regamey, J. Infect. Dis., 134 (suppl) (1976) S312.
5 P. Stevens, L.S. Young and W.L. Hewitt, Antimicrob. Ag. Chemother., 7 (1975) 374.
6 P. Stevens, L.S. Young and W.L. Hewitt, J. Lab. Clin. Med., 86 (1975) 349.
7 B.H. Minshew, R.K. Holmes and C.R. Baxter, Antimicrob. Ag. Chemother., 7 (1975) 107.
8 J.P. Anhalt and S.D. Brown, Clin. Chem., 24 (1978) 1940.
9 S.E. Back, I. Nilssen-Ehle and P. Nilssen-Ehle, Clin. Chem., 25 (1979) 1222.
10 S K. Maitra, T.T. Yoshikawa, C.M. Steyn, L.B. Guze and M.C. Schotz, Antimicrob. Ag. Chemother., 14 (1978) 880.
11 K. Satake, T. Okuyama, M. Ohashi and T. Shinoda, J. Biochem., 47 (1960) 654.

Journal of Chromatography, 307 (1984) 230—234
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2021

Note

Determination of prenalterol in plasma and urine by liquid chromatography with electrochemical detection

PER-OLOF LAGERSTRÖM* and PATRICIA CARLEBOM

Department of Analytical Chemistry, AB Hässle, S-431 83 Mölndal (Sweden)

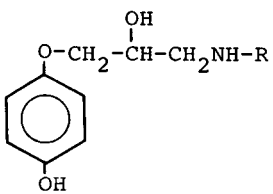
and

ANTHONY F. CLARKE and DAVID B. JACK

Department of Therapeutics and Clinical Pharmacology, Medical School, Birmingham (U.K.)

(First received September 9th, 1983; revised manuscript received December 1st, 1983)

Prenalterol is a selective beta-1-adrenoceptor agonist in man [1] and animals [2]. Prenalterol is structurally related to beta-adrenoceptor blocking agents (Fig. 1) and has been assayed in biological fluids after perfluoroacylation by gas chromatography with electron-capture [3] or mass spectrometric [4] detection. The latter technique allows determination of plasma concentrations down to less than 5 nmol/l. Recently, a liquid chromatographic method



	R
Prenalterol	$-CH(CH_3)_2$
H 133/12 (internal standard)	$-C(CH_3)_3$

Fig. 1. Chemical structures of prenalterol and internal standard (H 133/12).

0378-4347/84/$03.00

offering equivalent sensitivity with fluorometric detection has been suggested by Oddie et al. [5].

In the present paper, liquid chromatography was combined with electrochemical detection for the determination of prenalterol in plasma samples in concentrations as low as 2 nmol/l.

EXPERIMENTAL

Reagents

Prenalterol and the internal standard, H 133/12, were used as hydrochlorides and obtained from the Department of Organic Chemistry, AB Hässle (Mölndal, Sweden). Stock solutions (100 μmol/l) were prepared in 0.01 *M* hydrochloric acid and could be stored at 4°C for more than a month. More dilute working standards, 2 μmol/l for prenalterol and 4 μmol/l for H 133/12, were prepared weekly from the stock solutions. HPLC grade methanol was used in the mobile phase (E. Merck, Darmstadt, F.R.G., or Fisons, Loughborough, U.K.). Diethyl ether (May and Baker, p.a. quality) was distilled before use. Hexane was HPLC grade from Rathburn Chemicals (Walkerburn, U.K.). All other reagents were prepared from analytical grade chemicals.

All glassware was washed in an automatic dishwasher with detergent at pH 12, rinsed with phosphoric acid solution (pH 2) and repeatedly with distilled water and finally dried at 70°C before use.

Chromatographic system

The liquid chromatograph consisted of an LDC Model 71147 pump with an extra pulse-dampener or a Waters M6000 pump, a Rheodyne injection valve with a 150-μl sample loop, a stainless-steel column (150 × 4.5 mm) and a BAS LC-4 electrochemical detector (Bioanalytical Systems, Indiana, U.S.A.) with a glassy carbon working electrode, operated at +0.70 V. The separation column was packed with LiChrosorb RP-8, 5-μm average particle diameter (Merck). The mobile phase was a citrate–acetate buffer (pH 3.5) containing 15% methanol and 10^{-2} mol/l propylamine. The composition of the buffer was sodium acetate 106 mmol/l, sodium hydroxide 59 mmol/l and citric acid 200 mmol/l. The flow-rate was 1 ml/min, giving a retention time of about 6 min for prenalterol and 9 min for the internal standard H 133/12.

Analytical procedure

The frozen plasma sample was allowed to thaw at room temperature and was mixed and centrifuged. A 1.00-ml volume of the sample or reference sample (100 nmol/l) was transferred to a 15-ml centrifuge tube, 0.4 g of sodium chloride and 100 μl of carbonate buffer were added (final pH 9.5–10.0) and prenalterol was extracted with 6.00 ml of diethyl ether by shaking for 10 min.

After centrifugation for 5 min, 5.00 ml of the organic phase were transferred to a 5-ml tapered centrifuge tube and prenalterol was back-extracted with shaking for 10 min into 250 μl of orthophosphoric acid 0.10 mol/l. After centrifugation the ether phase was sucked off and the aqueous phase was shaken twice with 1.5 ml of hexane for 2 min for removal of dissolved diethyl ether. After centrifugation 100 μl of the aqueous phase were injected via the injection valve onto the chromatographic column.

Calibration and accuracy

Calibration was effected by adding 50 μl of working standard (2 μmol/l) of prenalterol to 1 ml of blank plasma and taking these standards (n = 4) through the analytical procedure. The peak heights for prenalterol in the reference samples in the chromatograms were measured and used to calculate the drug concentratation in the authentic samples. The recovery was determined by comparison with a directly injected reference solution of prenalterol. When internal standard (H 133/12) was used to compensate for minor variations in detector response and for volume changes, 50 μl of the working standard (4 μmol/l) were added to the plasma sample. The ratios between the peak heights of prenalterol and the internal standard in the chromatograms from the reference samples were then used to evaluate the content of prenalterol in the authentic samples.

Assay of prenalterol in urine

In urine unchanged prenalterol was determined, using a similar method to that in plasma; 0.5 ml of sample was taken and 0.5 ml of sodium carbonate 0.5 mol/l was used to buffer the sample to pH 9.5. A lower potential (+0.55 V) was used to decrease the influence from interfering endogenous compounds.

RESULTS AND DISCUSSION

Extraction

The extraction of prenalterol from aqueous solution was thoroughly investigated in a previous paper [4]. Due to the zwitterionic character of the compound, maximum extraction is achieved at pH 9.5. Different organic solvents were tested and the extraction recovery was found to be improved significantly by addition of sodium chloride to the aqueous phase. The purification of the final phosphoric acid extract by mixing with hexane was found to be necessary since diethyl ether dissolved in the aqueous phase interfered severely in the chromatographic procedure.

Liquid chromatography

Acetate–citrate buffer was preferred to phosphate buffer in the mobile phase, since about 50% higher detector response was obtained. Propylamine was included as modifier in the mobile phase to improve the chromatographic behaviour of prenalterol. A chromatogram from a blank plasma sample is shown in Fig. 2 and from the same sample after addition of prenalterol (Fig. 3A), at a concentration in the proximity of the sensitivity of the method. A chromatogram from an authentic plasma sample is illustrated in Fig. 3B.

Recovery and precision

The recovery of prenalterol from spiked plasma samples was 100% at the 4 μmol/l level and 97% at the 15 nmol/l level, the same figure as that calculated from distribution constants. The repeatability (S.D., %) on analysing replicate samples (n = 8) was 2.4 and 1.6, respectively, at these two concentration levels. The minimum determinable concentration (S.D. $\leqslant$ 10%) was 2 nmol/l.

In urine the recovery was 100% and 103%, respectively, with relative

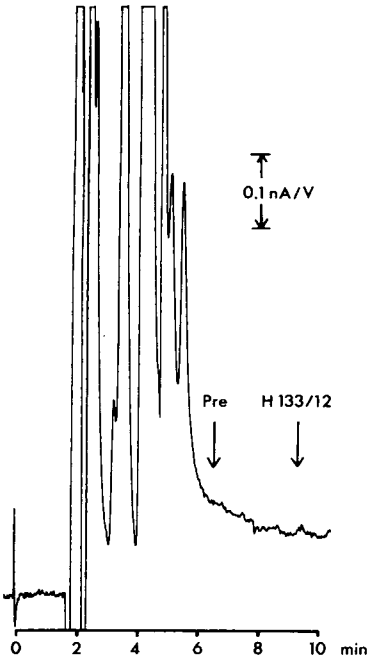


Fig. 2. Chromatogram from blank plasma sample. Stationary phase: LiChrosorb RP-8, 5 μm. Mobile phase: acetate—citrate buffer, pH 3.5, containing 10^{-2} M propylamine and 10% methanol. Potential: +0.70 V.

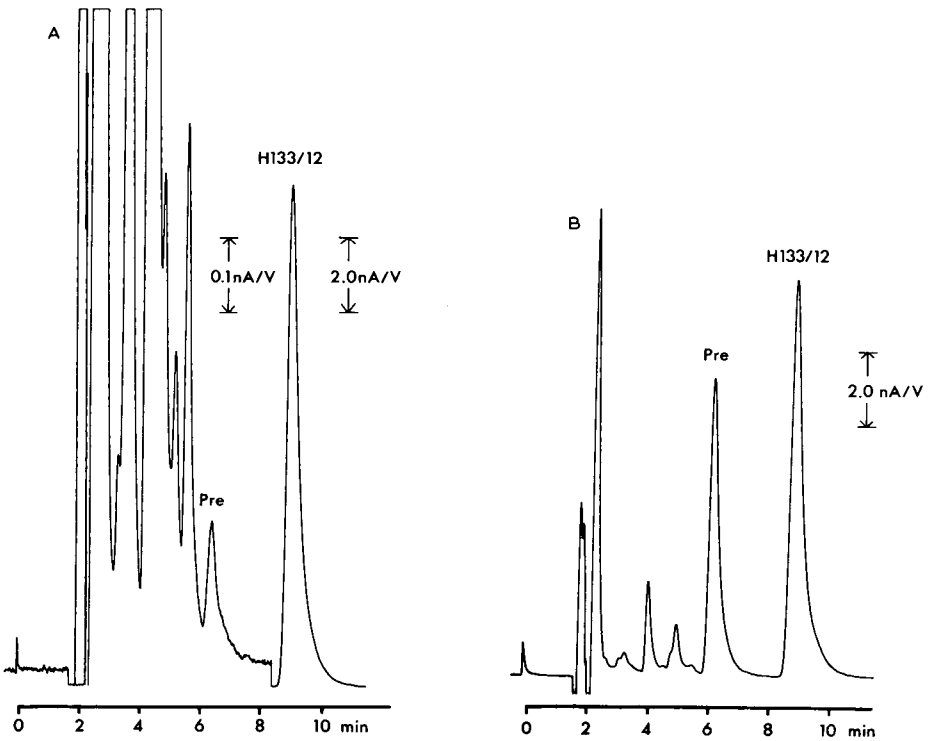


Fig. 3. Chromatogram from plasma. Chromatographic conditions the same as in Fig. 2. (A) Spiked plasma sample containing 2 nmol/l prenalterol. (B) Authentic plasma sample containing 80 nmol/l prenalterol.

standard deviations of 2.5% at concentration levels of 0.2 and 2 μmol/l of urine. The minimum determinable concentration (S.D. ⩽ 10%) was 20 nmol/l.

Stability

The chromatographic system and the electrochemical detector showed good long-term stability. Intermittently the upper part (1—2 mm) of the column packing was replaced with new material. No interference from plasma constituents or drugs expected to be co-administered was observed. This analytical method was evaluated against a gas chromatographic—mass spectrometric method as reported previously [4] and showed a good correlation. The stability of prenalterol in plasma samples on storage at −18°C was also examined [4] and no changes were found over a period of six months.

REFERENCES

1 O. Rönn, E. Fellenius, C. Graffner, G. Johnsson, P. Lundborg and L. Svensson, Eur. J. Clin. Pharmacol., 17 (1980) 81.
2 E. Carlsson, C.-G Dahlöf and A. Hedberg, Naunyn-Schmiedeberg's Arch. Pharmacol., 300 (1977) 101.
3 P.H. Degen and M. Ervik, J. Chromatogr., 222 (1981) 437.
4 M. Ervik, K. Kylberg-Hanssen and P.-O. Lagerström, J. Chromatogr., 229 (1982) 87.
5 C.J. Oddie, G.P. Jackman and A. Bobik, J. Chromatogr., 231 (1982) 473.

Journal of Chromatography, 307 (1984) 235—239
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2012

Note

High-performance liquid chromatographic determination of gossypol in plasma

NISON SATTAYASAI*

Department of Chemistry, Faculty of Science, Khon Kaen University, Khon Kaen (Thailand)

JINTANA SATTAYASAI

Department of Pharmacology, Faculty of Medicine, Khon Kaen University, Khon Kaen (Thailand)

and

VIWAT HAHNVAJANAWONG

Department of Chemistry, Faculty of Science, Khon Kaen University, Khon Kaen (Thailand)

(First received August 22nd, 1983; revised manuscript received November 15th, 1983)

Gossypol, a yellowish phenolic compound occurring in the pigment glands of the seeds of the plants in genus *Gossypium* [1] has been advocated recently by Chinese scientists as a new antifertility agent for males [2, 3]. Oral administration of gossypol—acetic acid to male rats at a dose level of 15—40 mg/kg/day for 2—4 weeks induced infertility. Because of its instability, the existence of gossypol in plasma should be determined. Tang et al. [4] detected plasma gossypol using a radioactive labelling method. However, this method can not really show the existence of gossypol in plasma. This report describes a high-performance liquid chromatographic (HPLC) method that was developed for the quantitative and qualitative analysis of plasma gossypol. The method can show the occurrence of gossypol in plasma after oral administration.

EXPERIMENTAL

Materials

Gossypol (1,1′,6,6′,7,7′-hexahydroxy-5,5′-diisopropyl-3,3′-dimethyl-2,2′-

0378-4347/84/$03.00

binaphthalene-8,8′-dicarboxaldehyde) was purchased as gossypol–acetic acid from Sigma (St. Louis, MO, U.S.A.) and standard solutions were freshly made in methanol (HPLC grade) before use. HPLC solvents were obtained from J.T. Baker (Phillipsburg, NJ, U.S.A.). Glacial acetic acid (RPE) was purchased from Carlo Erba (Milan, Italy). Ethylenediaminetetraacetic acid (EDTA) disodium salt (AR) and benzene (AR) were products of Koch-Light Labs. (Colnbrook, U.K.) and Mallinckrodt (Paris, KY, U.S.A.), respectively.

Gossypol extraction from plasma

Rats were fed with a single dose of gossypol–acetic acid at a dose level of 10, 30 and 100 mg per kg body weight. Blood samples were collected from tails at 0, 1.5, 2, 3, 5, 6, 8 and 9 h after the oral administration of gossypol. Heparin was used as anticoagulant. For extraction of gossypol, 0.15 ml of plasma separated from the blood was added with 0.15 ml of absolute ethanol to precipitate proteins and then followed with 1 ml of saturated solution of EDTA disodium salt. Gossypol was extracted from the water–ethanol phase by adding 1 ml of benzene. After vortex-mixing for 4 min the sample was centrifuged for 10 min at 800 *g* at 10°C. The benzene layer was transferred to another tube. The water–ethanol phase was extracted again by the same method. The combined benzene phases were evaporated to dryness in a stream of nitrogen. The residue was redissolved in 100 μl of absolute methanol (HPLC grade). A 5–15-μl volume of the extract was injected into the column. The same procedure was also applied to smaller plasma samples (down to 100 μl).

High-performance liquid chromatography

A Water Assoc. liquid chromatograph consisting of M 6000A solvent delivery system, M 440 absorbance (UV) detector, a U6K universal injection system and a reversed-phase μBondapak C_{18} column (30 cm × 3.9 mm I.D., particle size 10 μm) were used. Solvents were filtered through Millipore membrane filters, type FH, pore size 0.5 μm, and degassed in an ultrasonic bath prior to use. Standard and extracted gossypol were injected in 5–15 μl of methanol and eluted from the column isocratically at room temperature. The mobile phase consisted of methanol–water–glacial acetic acid (77:20:3). The flow-rate was 2 ml/min. Gossypol was detected and quantitated by monitoring the ultraviolet (UV) absorbance of the column eluates at 254 nm. The peaks of substances were drawn by an Omni-Scribe recorder (Houston Instruments).

RESULTS AND DISCUSSION

Chromatogram

One of the prime objectives of this study was to develop a simple analytical HPLC method that could be used for qualitative and quantitative analysis of gossypol in plasma after oral administration. Since gossypol has many ionizable phenolic groups, the mobile phase has to be acidified to obtain a good performance peak. Abou-Donia et al. [5] added phosphoric acid to the mobile system to decrease ionization of gossypol. But the gossypol used in the experiment was in the form of gossypol–acetic acid (a loosely bound complex of one molecule of gossypol and one molecule of acetic acid [1]), therefore

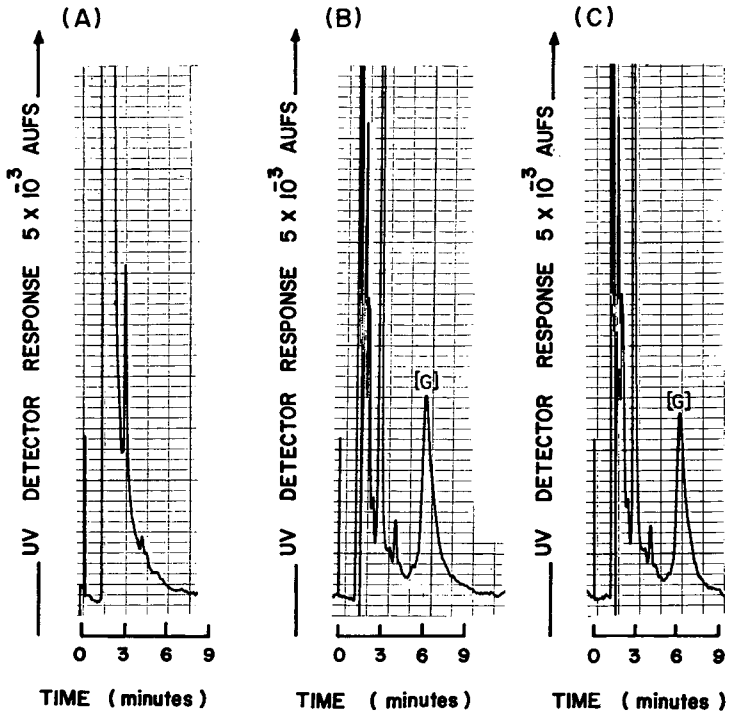


Fig. 1. Chromatograms of HPLC analysis of plasma extracts on a μBondapak C_{18} column of (A) blank plasma extract, (B) plasma extract spiked with 20 ng of gossypol—acetic acid [G], and (C) a 1-h plasma sample obtained from a rat taking a single dose of 10 mg/kg gossypol—acetic acid (corresponding to a plasma level of about 2.44 μg/ml).

acetic acid should be more suitable than phosphoric acid. Fig. 1A and B show typical chromatograms of extracts from plasma samples without and with gossypol—acetic acid as internal standard. The chromatogram of the extract from the plasma of the rat at the first hour after oral administration (force-feed) of 10 mg gossypol—acetic acid per kg body weight is shown in Fig. 1C. Standard gossypol and gossypol extracted from rat plasma have the same retention time. It is 6 min and varied by at most 2% from day to day. The relationship between the amount of gossypol injected and the peak areas was linear and detection by UV at 254 nm was very sensitive.

Percentage recovery and precision

Gossypol can form complexes with many kinds of metal ions [1]. This is why gossypol can not be easily extracted from plasma. However, some chelating agents such as EDTA can break the complexes into free gossypol that can be easily extracted. Benzene was used for the extraction because it can dissolve gossypol rapidly. The intra-assay linearity and precision of the method was evaluated over a concentration range of 1.5—9 μg gossypol—acetic acid per ml of plasma (225—1350 ng gossypol—acetic acid per 0.15 ml) are shown in Table I. Triplicate samples at each concentration of the compound were added to 0.15 ml of plasma and taken through the analytical procedure. The data

TABLE I

LINEARITY AND INTRA-ASSAY PRECISION OF THE HPLC ASSAY FOR GOSSYPOL–ACETIC ACID IN PLASMA

Correlation coefficient (r) = 0.999.

Concentration range	N	Conc. added (μg/ml)	Mean conc. found ± S.D. (μg/ml)	Coefficient of variation (%)	Mean recovery (%)
1.5—9 μg/ml	3	1.50	1.31 ± 0.10	7.63	87.50
	3	3.00	2.48 ± 0.15	6.05	82.72
	3	4.50	3.61 ± 0.18	4.99	80.15
	3	6.00	5.10 ± 0.17	3.33	84.95
	3	9.00	7.35 ± 0.45	6.12	81.62
Mean				5.62	83.39 ± 4.90

TABLE II

LINEARITY AND INTER-ASSAY PRECISION OF THE HPLC ASSAY FOR GOSSYPOL–ACETIC ACID IN PLASMA

Correlation coefficient (r) = 0.999. Each determination was combined from four different dates of assay.

Concentration range	N	Conc. added (μg/ml)	Mean conc. found ± S.D. (μg/ml)	Coefficient of variation (%)	Mean recovery (%)
1.5—16.5 μg/ml	4	1.50	1.33 ± 0.09	6.77	88.50
	5	3.00	2.52 ± 0.12	4.76	84.12
	5	4.50	3.72 ± 0.20	5.38	82.61
	4	6.00	5.04 ± 0.18	3.57	84.00
	5	9.00	7.31 ± 0.44	6.02	81.17
	3	16.5	14.28 ± 0.41	2.87	85.71
Mean				4.90	84.20 ± 4.58

indicated the high degree of linearity of the method with a correlation coefficient (r) of 0.999. The method showed an average coefficient of variation of 5.62%. The recovery of gossypol from plasma was 83.39 ± 4.90%.

Table II shows the inter-assay linearity and precision for the method with a correlation coefficient (r) of 0.999 and average coefficient of variation of 4.90%. The overall recovery was 84.20 ± 4.58%.

Another chromatographic system was also tested in order to ensure that the peaks in the system described above are of gossypol itself and not its degradation products. The mobile phase was changed to a more polar system by increasing the percentage of water. With this system, the retention times of standard gossypol and the extracted gossypol were extended but it is still the same time. However, the peak of gossypol in this system was broader than in the first one.

Gossypol in plasma

Concentrations of gossypol in plasma at different times that were

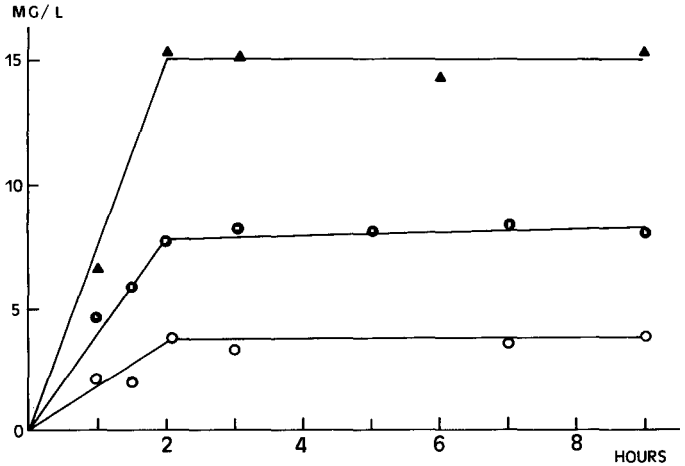


Fig. 2. Gossypol—acetic acid concentrations in plasma from three rats after single oral administration of gossypol—acetic acid: (○), 10 mg/kg body weight; (●), 30 mg/kg body weight; (▲), 100 mg/kg body weight.

quantitated by peak height are presented in Fig. 2. The maximum levels of plasma gossypol appear between the second and the third hour after oral administrations and the levels are maintained for many hours. The maintenance of the level may result from complex formation between gossypol and some metal ions in plasma [1].

ACKNOWLEDGEMENTS

The authors wish to thank Miss Angkana Pootako for typing this manuscript and Mr. Rangsan Niamsanit for assistance.

REFERENCES

1 R. Adams, T.A. Geissman and J.D. Edwards, Chem. Rev., 60 (1960) 555.
2 Anonymous, Chin. Med. J., 4 (1978) 417.
3 R.-X. Dai, S.-N. Pang, X.-K. Lin, U.-B. Ke, Z.-L. Lui and R.-H. Gong, Acta Biol. Exp. Sinica, 11 (1978) 1 (in Chinese with English Summary).
4 X.-C. Tang, M.-K. Zhu and Q.-X. Shi, Yao Hsueh Hsueh Pao, 15 (1980) 212.
5 S.A. Abou-Donia, J.M. Lasker and M.B. Abou-Donia, J. Chromatogr., 206 (1981) 606.

PUBLICATION SCHEDULE FOR 1984

Journal of Chromatography (incorporating *Chromatographic Reviews*) and *Journal of Chromatography, Biomedical Applications*

MONTH	D 1983	J	F	M	A	M	J	
Journal of Chromatography	282	283 284/1	284/2 285/1	285/2 285/3 286 287/1	287/2 288/1 288/2 289	290 291 292/1	292/2 293	The publication schedule for further issues will be published later
Chromatographic Reviews		300/1						
Bibliography Section		304/1	304/2			304/3		
Biomedical Applications		305/1	305/2	306	307/1	307/2	308	

INFORMATION FOR AUTHORS

(Detailed *Instructions to Authors* were published in Vol. 264, No. 3, pp. 491–494. A free reprint can be obtained by application to the publisher.)

Types of Contributions. The following types of papers are published in the *Journal of Chromatography* and the section on *Biomedical Applications:* Regular research papers (Full-length papers), Short communications and Notes. Short communications are preliminary announcements of important new developments and will, whenever possible, be published with maximum speed. Notes are usually descriptions of short investigations and reflect the same quality of research as Full-length papers, but should preferably not exceed four printed pages. For review articles, see page 2 of cover under Submission of Papers.

Submission. Every paper must be accompanied by a letter from the senior author, stating that he is submitting the paper for publication in the *Journal of Chromatography.* Please do not send a letter signed by the director of the institute or the professor unless he is one of the authors.

Manuscripts. Manuscripts should be typed in double spacing on consecutively numbered pages of uniform size. The manuscript should be preceded by a sheet of manuscript paper carrying the title of the paper and the name and full postal address of the person to whom the proofs are to be sent. Authors of papers in French or German are requested to supply an English translation of the title of the paper. As a rule, papers should be divided into sections, headed by a caption (*e.g.,* Summary, Introduction, Experimental, Results, Discussion, etc.). All illustrations, photographs, tables, etc., should be on separate sheets.

Introduction. Every paper must have a concise introduction mentioning what has been done before on the topic described, and stating clearly what is new in the paper now submitted.

Summary. Full-length papers and Review articles should have a summary of 50–100 words which clearly and briefly indicates what is new, different and significant. In the case of French or German articles an additional summary in English, headed by an English translation of the title, should also be provided. (Short communications and Notes are published without a summary.)

Illustrations. The figures should be submitted in a form suitable for reproduction, drawn in Indian ink on drawing or tracing paper. Each illustration should have a legend, all the *legends* being typed (with double spacing) together on a *separate sheet.* If structures are given in the text, the original drawings should be supplied. Coloured illustrations are reproduced at the author's expense, the cost being determined by the number of pages and by the number of colours needed. The written permission of the author and publisher must be obtained for the use of any figure already published. Its source must be indicated in the legend.

References. References should be numbered in the order in which they are cited in the text, and listed in numerical sequence on a separate sheet at the end of the article. Please check a recent issue for the layout of the reference list. Abbreviations for the titles of journals should follow the system used by *Chemical Abstracts.* Articles not yet published should be given as "in press", "submitted for publication", "in preparation" or "personal communication".

Proofs. One set of proofs will be sent to the author to be carefully checked for printer's errors. Corrections must be restricted to instances in which the proof is at variance with the manuscript. "Extra corrections" will be inserted at the author's expense.

Reprints. Fifty reprints of Full-length papers, Short communications and Notes will be supplied free of charge. Additional reprints can be ordered by the authors. An order form containing price quotations will be sent to the authors together with the proofs of their article.

Advertisements. Advertisement rates are available from the publisher on request. The Editors of the journal accept no responsibility for the contents of the advertisements.