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CHROMATOGRAPHY '77

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High Performance Thin-Layer Chromatography

edited by **A. ZLATKIS,** University of Houston, Houston, Texas, and **R.E. KAISER,** Institute of Chromatography, Bad Dürkheim

JOURNAL OF CHROMATOGRAPHY LIBRARY, Vol. 9

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CONTENTS: Simplified theory of TLC. The separation number in linear and circular TLC. Advantages, limits and disadvantages of the ring development technique. The U-chamber. Dosage techniques in HPTLC. HPTLC: development, data and results. Consideration on the reproducibility of TLC. Potential and experience in quantitative HPTLC. Application of a new high performance layer in quantitative TLC. Appendix. Subject Index.

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Biomedical Applications

CHROMBIO. 042

QUANTITATIVE ANALYSIS OF TRIFLUOROACETIC ACID IN BODY FLUIDS OF PATIENTS TREATED WITH HALOTHANE

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(Received October 15th, 1976)

SUMMARY

A simple procedure for the quantitative analysis of trifluoroacetic acid (TFA) in urine and serum from patients narcotized with halothane is described. This involves addition of sodium hydroxide to the body fluid, evaporation of the aqueous phase and esterification of TFA in concentrated sulphuric acid with 2,2,2-trichloroethanol. The gaseous phases above the reaction mixture were then analyzed by gas chromatography with a nickel-63 electron-capture detector. The detection limit was 1 μg of TFA per millilitre of body fluid (200 μg of body fluid are analysed) and the relative standard deviation was ±6%. Patients treated with ethrane, another commercial anaesthetic, did not produce any detectable TFA.

INTRODUCTION

The narcotic halothane (2-bromo-2-chloro-1,1,1-trifluoroethane) is partly metabolized in man to trifluoroacetic acid (TFA) [1, 2]. As TFA could be responsible for the observed toxicity of halothane [3], it is desirable to develop methods for the quantitative analysis of this highly water-soluble metabolite.

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Earlier attempts to detect TFA in urine involved formation of the methyl ester, extraction with diethyl ether and final gas chromatographic (GC) [4] or infrared [5] analysis or paper chromatography and colorimetric analysis of fluorine in the combusted sheets [6]. We report here a simple and more sensitive procedure in which the 2,2,2-trichloroethyl ester of TFA is quantified by head-space analysis and by gas chromatography with electron-capture detection. No extraction, separation, sample transfer or other isolation procedures are needed owing to the specifity of detection.

EXPERIMENTAL

Materials

Urine and serum were collected from 35 individuals at various time intervals after anaesthesis with halothane. Details of the medical treatments and results will be reported elsewhere [7]. Halothane (Hoechst, Frankfurt/M, G.F.R.), ethrane (Abbot, S. Pasadena, Calif., U.S.A.) and TFA (Merck, Darmstadt, G.F.R.) were used without further purification. 2,2,2-Trichloroethanol (zur Synthese, Merck) and heptafluorobutyric acid (HFB) (Pierce, Rockford, Ill., U.S.A.) were distilled before use. Trifluoroacetyl-2,2,2-trichloroethyl ester was prepared by adding TFA anhydride to 2,2,2-trichloroethanol. The product was distilled three times.

Gas chromatography

A Perkin-Elmer F 20 FE gas chromatograph equipped with a nickel-63 electron-capture detector (ECD) (10 mCi) was used throughout. The injection port temperature was 150°, the column temperature 80° and the detector temperature 260°. The carrier gas nitrogen flow-rate was 20 ml/min, the purge gas flow-rate 40 ml/min, the pulse interval 10 μsec, the attenuation × 8 and the range 25. The column employed in all studies was a 2 m × 2 mm I.D. glass column packed with 4% OV-17 on Chromosorb W HP, 100—120 mesh (Perkin-Elmer, Norwalk, Conn., U.S.A.).

Gas chromatography—mass spectrometry (GC—MS)

The GC separation conditions were identical with those described above, but helium was used as the carrier gas. The glass column was coupled by a 1/4-in. Swagelok union drilled to 1/4 in. via a membrane separator to an AEI MS 30 mass spectrometer [8]. This was coupled with a DS-50 computer system (AEI) to obtain mass spectra continuously at intervals of 7 sec. The separator was kept at 230° and the temperature of the source was about 240°. The accelerating voltage was 4 kV and the electron energy 24 eV.

Derivatization of urine and serum samples

Samples of 200 μl of serum or urine were mixed with 100 μl of HFB standard (100 mg in 100 ml of water) and 200 μl of 0.1 *N* sodium hydroxide solution in 30-ml Minnert vials (Precision Sampling Corp., Baton Rouge, La., U.S.A.). The samples were frozen with liquid nitrogen and lyophilized in a desiccator (sodium hydroxide pellets) for ca. 4 h at room temperature. Then 200 μl of trichloroethanol were added and the inside of the upper part of the

vessel was wetted with six drops of concentrated sulphuric acid and quickly closed before the acid reached the bottom. The reaction mixture was equilibrated at 50° for 15 min and 200 μl of the vapour phase were injected into the port of the gas chromatograph.

Calibration

Various amounts of TFA and HFB standard were added to urine and serum blanks to yield TFA solutions with 13 concentrations in the range from 1 μg/ml to 2 mg/ml. These samples were esterified with trichloroethanol in a procedure identical with that described above. The relative standard deviations of the peak heights in various samples of identical TFA concentrations were 6%. Using the calibration graph, TFA concentrations in urine and serum samples from narcotized patients were determined graphically from the ratio of the heights of TFA and HFB peaks. Owing to the non-linear detector response, a linear relationship between peak height and sample size was not obtained.

A graph of recovery for the esterification was also determined by comparison of urines with known TFA concentrations with samples of pure TFA 2,2,2-trichloroethyl ester in diethyl ether solution. It was found that the yields were highly dependent on concentration (Table I). Measurements should be taken in the linear range from 0 to 100 μg/ml. In the higher concentration range of trifluoroacetic acid (starting at ca. 150 μg/ml), appreciable amounts (up to 10%) of trifluoroacetic anhydride were formed during the derivatization procedure. All effects that had an influence on the yield of the trichloroethyl ester and therefore also an quantification of trifluoroacetic acid were eliminated by the calibration method described above.

RESULTS AND DISCUSSION

We chose the ECD for quantitative analysis of TFA in the GC peaks because of its high sensitivity and selectivity for the detection of halogenated carbon compounds. However, the electron affinity of the trifluoromethyl group was not sufficient for the determination of TFA in serum and urine without elaborate work-up procedures. Therefore, we prepared esters of TFA with the following

TABLE I

RECOVERY OF THE 2,2,2-TRICHLOROETHYL ESTER AT VARIOUS CONCENTRATIONS OF TRIFLUOROACETIC ACID IN THE SAMPLES

TFA in urine (μg/ml)	Yield (%)	TFA in urine (μg/ml)	Yield (%)
5	12	145	30
40	12	160	38
70	12	175	45
110	18	200	49
		225	57

halogenated alcohols: 2,2,2-trichloroethanol, 1,1,1,3,3,3-hexafluoroisopropanol, 1,1,2,2-tetrahydroperfluorohexanol and 1,1-dihydroperfluoroheptanol. Esterification with 2,2,2-trichloroethanol yielded the best results and we used that derivatization method throughout the study. 2,2,2-Trichloroethanol has also been used by other workers for the determination of aromatic and aliphatic acids other than TFA [9,10].

With the detector used, 1 μg/ml of TFA in urine could still be detected in a sample volume of 200 μl; larger samples yielded higher sensitivity. The only limiting factors were the large detector noise level produced in the detector, its non-linear response and the presence of negative peaks. Preliminary measurement with a later gas chromatograph (Perkin-Elmer F22) equipped with a frequency-modulated ECD yielded higher sensitivity and better reproducibility. For our purposes, however, the accuracy obtained was sufficient to allow the use of the less sensitive detector which we had available at that time. Measurements with the GC—MS—computer system led to approximately the same results.

The conditions used in the esterification procedure proved to be critical. After salt formation with TFA and evaporation of water, the esterification with 2,2,2-trichloroethanol takes place in concentrated sulphuric acid. This acid should come into contact with the TFA—trichloroethanol mixture only after the reaction vessel has been closed. The reaction mixture has to be equilibrated at 50° for at least 15 min; longer reaction times (up to a few hours) did not change the results, but lower temperatures led to a decrease in reproducibility. We found it unnecessary to separate the residual trichloroethanol

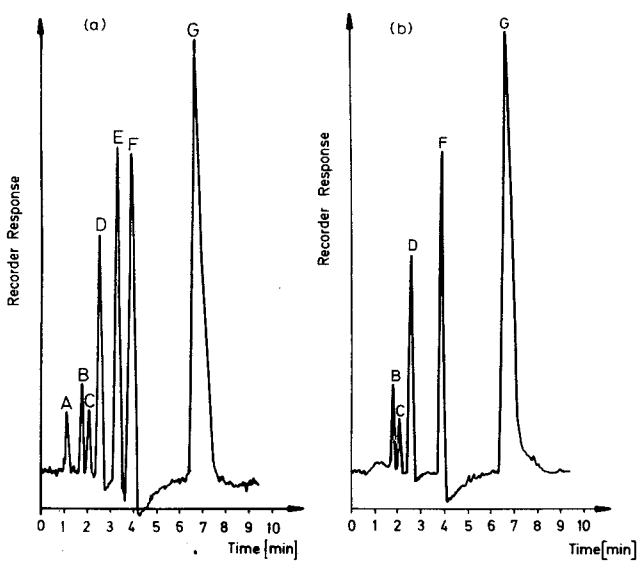

Fig. 1. (a) Gas chromatogram of 200 μl of the head-space of 200 μl of lyophilized urine from a halothane-treated patient, reacted with 200 μl of 2,2,2-trichloroethanol and approximately 70 μl of concentrated sulphuric acid. Peaks: A = TFA anhydride; B = $CHCl_3$; C = unknown; D = CCl_3CHO; E = 2,2,2-trichloroethyl trifluoroacetate; F = 2,2,2-trichloroethyl heptafluorobutyrate (internal standard); G = 2,2,2-trichloroethanol. (b) Blank; identical experiment as in (a), but with urine from an untreated patient.

and other compounds from the other constituents. We rather used the total mixture and found the following components (in order of their elution times): trifluoroacetic anhydride, chloroform, chloral (or its hydrate), a product from 2,2,2-trichloroethanol that could not be identified, 2,2,2-trichloroethyl trifluoroacetate, 2,2,2-trichloroethyl heptafluorobutyrate and 2,2,2-trichloroethanol (Fig. 1).

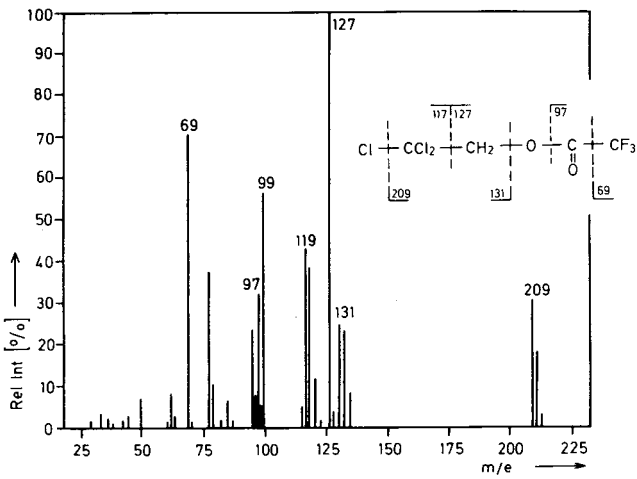

Fig. 2. Mass spectrum of 2,2,2-trichloroethyl trifluoroacetate.

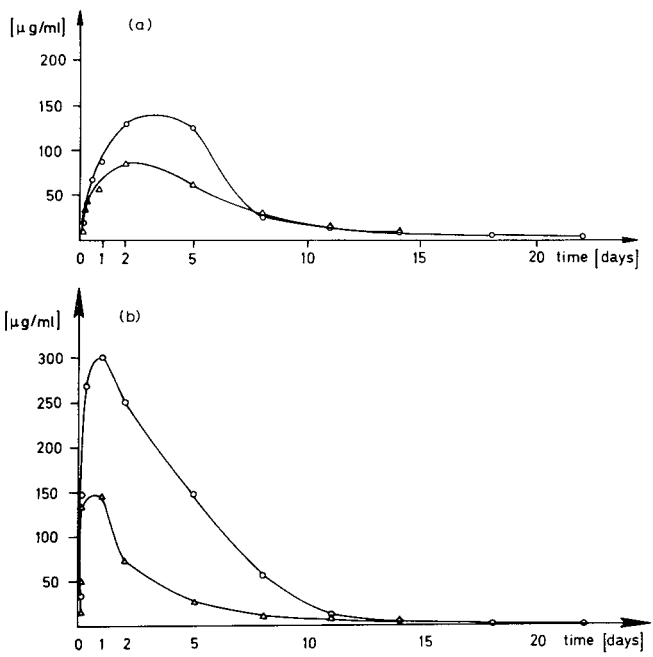

Fig. 3. Time-dependent increase of concentrations of trifluoroacetic acid in the urine (○) and serum (△) of halothane-treated patients. The curves represent approximately the upper (b) and lower (a) limits of TFA in the body fluids of 16 patients.

The mass spectrum of the 2,2,2-trichloroethyl ester of TFA is shown in Fig. 2. The spectrum does not contain a molar peak (M^+; *m/e* 244), but a peak at *m/e* 209 that corresponds to a loss of one chlorine atom. More important peaks are observed for the CF_3 group (*m/e* 69) and for fragments that are formed by loss of CCl_3 (*m/e* 127 base peak) and CCl_3—CH_2O (*m/e* 97).
The *m/e* 99 peak ($C_2H_2F_3O$) corresponds to a loss of CCl_3 and of C=O by skeletal rearrangement [4]. This fragmentation pattern is in accordance with those of other highly halogenated carbon compounds [11]. All but one GC peak have been identified by mass spectrometry and by comparison with pure compounds, and originate from solvolytic and oxidation reactions of TFA and trichloroethanol in warm sulphuric acid. Quantitative analysis of the TFA trichloroethylester was not hampered by any of the other components. The total mixture can be analyzed and pure GC fractions resulted for the TFA and HFB derivatives owing to the specificity of the ECD. An even more specific method was MS single-ion recording at *m/e* 69, which detected only TFA and HFB derivatives.

Typical experimental results are displayed in Fig. 3, and agree with earlier paper chromatographic results [6]. Patients were narcotized with halothane and both urine and serum showed a considerable rise in TFA concentration for a period of about 8 h. After 2 days a maximum of ca. 130—300 μg/ml of TFA in urine had been reached. In the body fluids of patients treated with ethrane under otherwise identical conditions, no TFA or other acids [12] were found, down to the detection limit of about 1 μg/ml.

ACKNOWLEDGEMENTS

The technical assistance of Ms. J. Fuhrhop and Mr. J. Rudolph is gratefully acknowledged.

REFERENCES

1 A. Stier, Biochem. Pharmacol., 13 (1964) 1544.;
2 D.A. Blake, J.Q. Barry and H.F. Cascorby, Anesthesiology, 36 (1972) 152.
3 A. Stier, H.W. Kunz, A.K. Walli and H. Schimassek, Biochem. Pharmacol., 21 (1972) 2181.,
4 D. Karashima, S. Takahashi, A. Shigematsu, T. Furukawa, T. Kuhara and I. Matsumoto, Biomed. Mass. Spectrom., 3 (1976) 41.
5 K. Gossler, K.H. Schaller and H.G. Essing, Z. Anal. Chem., 279 (1976) 112.
6 K. Rehder, I. Forbes, H. Alter, O. Hessler and A. Stier, Anesthesiology, 28 (1967) 711.
7 A. Doenicke, B. Grote, A. Bauer, L. Witte, H. Nau and J.-H. Fuhrhop, in preparartion.
8 H. Nau, J. Chromatogr., 121 (1976) 376.
9 R.V. Smith and S.L. Tsai, J. Chromatogr., 61 (1971) 29.
10 C.C. Alley, J.B. Brooks and G. Choudhary, Anal. Chem., 48 (1976) 387.
11 H. Budzikiewicz, C. Djerassi and D.H. Williams, Interpretation of Mass Spectra, Holden Day, San Francisco, Calif., 1969, pp. 125 *ff.*
12 G. Loew, H. Motulsky, J. Trudell, E. Cohen and L. Hjelmeland, Molec. Pharmacol., 10 (1974) 406.

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CHROMBIO. 045

RAPID MICRODETERMINATION OF FATTY ACIDS IN BIOLOGICAL MATERIALS BY GAS—LIQUID CHROMATOGRAPHY*

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(Received November 3rd, 1976)

SUMMARY

Total and free fatty acids in general ranging from lauric to nervonic acid were separated and quantitated based on an internal standard method as methyl esters by "on column" methylation with trimethyl-(α,α,α-trifluoro-*m*-tolyl) ammonium hydroxide (TMTFTH) in a gas chromatographic system. This study represents an application of a method published by MacGee and Allen and a change to an internal standard technique. For the determination of the total fatty acids the samples were saponified with KOH—CH_3OH, acidified with H_3PO_4, and then the fatty acids were extracted into hexane. An aliquot of the hexane extract was then extracted with TMTFTH and chromatographed. For determination of free fatty acids the sample was acidified with H_3PO_4, immediately extracted with hexane and processed as described earlier. The relative standard deviation of 1.4 to 4.2% illustrates the precision of the method and the recovery of the fatty acids ranged from 88.5 to 100.5%. This method was applied to the determination of fecal fatty acids in conjunction with an interdepartmental study on "High protein diet in colon cancer" at the University of Missouri. In addition, the applicability of the analytical procedure (with small modifications) was shown for a wide variety of biological materials (serum, milk, skin tissue, fungal spores, food homogenates, beef tissues, and tumor cell cultures). The analyses were performed on different gas chromatographs by different analysts.

INTRODUCTION

A wide variety of analytical techniques have been developed and used for the separation and quantitation of fatty acids in biological materials [1, 2]. These techniques have usually produced reliable data. In conjunction with a nutritional study at the University of Missouri, concerned with the effect of high protein (beef) diet on anaerobic and aerobic flora and chemical components of human feces, a large number of fecal specimens and food homogenates were

*Contribution from the Missouri Agricultural Experiment Station. Journal Series No. 7686. Approved by the Director.

analyzed for total and free fatty acids. A summary of the results is published by Hentges et al. [3] and this study is described in further detail by Flynn et al. [4].

A method that allowed for the rapid reliable determination of fatty acids from a large number of samples by "on column" methylation in a gas chromatographic system was published by MacGee and Allen [5]. After saponification of the sample with KOH—CH_3OH, acidification with H_3PO_4, and extraction of saponifiable fatty acids into hexane, the acids were extracted from hexane with a small amount of trimethyl-(α,α,α-trifluoro-*m*-tolyl)ammonium hydroxide (TMTFTH) as their quaternary ammonium salts. The salt solutions were injected together with a mixture of methyl propionate—methanol into a gas chromatograph at an injection port temperature of ca. 260°. This procedure was adapted for the analysis of fatty acids in fecal specimens, food homogenates, and modified to an internal standard method (heptadecanoic acid was used as internal standard). In addition, a variety of other biological materials were analyzed including serum (human, dog, and rat), tumor cell culture media, milk (shrew and cow), rat skin, and fungal spores.

EXPERIMENTAL

Apparatus

During the course of fatty acid analyses the following gas chromatographs, equipped with hydrogen flame detectors, were used: Barber-Colman 5000 (Barber-Colman, Rockford, Ill., U.S.A.); Tracor 222 (Tracor, Austin, Texas, U.S.A.), and Packard 7300 (Packard Instrument, Downers Grove, Ill., U.S.A.). The electrometers of these instruments were interfaced with a Hewlett-Packard 3352B laboratory data system (Hewlett-Packard, Avondale, Pa., U.S.A.) for identification and quantitation of the fatty acids.

The samples were analyzed on a column packed with 15% HI-EFF-1BP on 100—120 mesh Chromosorb W (AW), glass, 2 m × 2 mm I.D., or 2.5 m × 2 mm I.D. The injection port temperature was maintained, in general, at 260°, the detector temperature at 245°. The column oven temperature was usually kept at 155°, and varied slightly (140—155°) depending on the instrument used and the type of analysis performed. In general, a linear carrier gas flow (Nitrogen) of 20 cm/sec was used. To determine the average linear carrier gas flow, methane was injected into the column. Methane gas traveled practically with the same speed as the carrier nitrogen gas. The velocity was calculated by dividing the length of the column (cm) by the time (sec) measured with a stop watch from injection to detection.

The samples were stored in a Revco Ultra-low-temperature freezer at -70° (Revco Freezer from Scientific Products, St. Louis, Mo., U.S.A.).

Reagents

All fatty acids (of highest purity available), the pre-tested phase HI-EFF-1BP (diethylene glycol succinate) and Chromosorb W AW were obtained from

Applied Science Labs. (State College, Pa., U.S.A.). Potassium hydroxide (ACS grade), methanol (ACS grade), and silver oxide (purified) were purchased from Fisher Scientific (St. Louis, Mo., U.S.A.). Iodomethane (99% purity) and 3-aminobenzotrifluoride, 99+% (α,α,α-trifluoro-*m*-toluidine) were purchased from Aldrich (Milwaukee, Wis., U.S.A.). Methyl propionate was obtained from Eastman-Kodak (Rochester, N.Y., U.S.A.). Phosphoric acid (85%, analytical reagent) was obtained from Mallinckrodt (St. Louis, Mo., U.S.A.). Hexane (high purity) was purchased from Phillips Petroleum (Borger, Texas, U.S.A.). TMTFTH was prepared as described by MacGee and Allen [5].

Procedure

Analysis of saponifiable fecal fatty acids. All specimens were stored at - 70°. For the analysis, samples were thawed, homogenized, ca. 100 mg weighed, the exact weight recorded, and then placed in a 12-ml centrifuge tube with a PTFE-lined cap. The original sample was immediately refrozen at - 70°. The internal standard (I.S.), heptadecanoic acid (50 μg) in hexane, was then added and the hexane completely evaporated using a stream of pure nitrogen gas at room temperature. To the sample were added 2 ml of KOH—CH_3OH solution (15 g KOH in 100 ml CH_3OH). After purging with nitrogen gas, the centrifuge tube was capped tightly, sonicated briefly and saponified in a dry-bath at 65° for 1 h. During the saponification the contents of the tubes were mixed briefly on a Vortex-Genie every 10 min.

After 1 h the centrifuge tubes were cooled to room temperature and 3 ml of 1 *M* H_3PO_4 were added. The fatty acids were then extracted three times with 5-ml portions of hexane. For each extraction the centrifuge tube was shaken vigorously for 1 min by hand and centrifuged for 10 min. The upper hexane layer was drawn off with a Pasteur pipet and the extracts were pooled in a ca. 20-ml culture tube, and mixed well. A 5.0-ml aliquot was transferred into a 5-ml glass-stoppered centrifuge tube (Pyrex No. 8061), and 10 μl of TMTFTH (0.5 *M*) were added. The centrifuge tube was then shaken vigorously for 1 min and centrifuged at approximately 600 *g* for 1 min. For chromatography 1 μl of the TMTFTH extract was taken from the bottom of the centrifuge tube with a 10-μl syringe. The 1-μl TMTFTH extract was sandwiched between a methyl propionate—methanol mixture (1:2) and injected into a gas chromatograph at an inlet temperature of 260°.

Analysis of free fecal fatty acids. The samples were prepared as described for the determination of total or saponifiable acids with the exception that the KOH—CH_3OH saponification step was omitted, then the sample was acidified with 3 ml of 1 *M* H_3PO_4. The centrifuge tube was closed tightly and carefully mixed on a Vortex-Genie, and sonicated briefly. Distilled water (2 ml) was added to the sample, mixed again, and then extracted with hexane and processed as described previously.

Total fatty acids in food homogenate. The 100-mg samples of food homogenates were analyzed for saponifiable fatty acids in the same manner as described for saponifiable fecal fatty acids.

Saponifiable fatty acids in spores of fungus Helminthosporium. A mixture of 100 mg of spores and 100 μg heptadecanoic acid (I.S.) was saponified with 2

ml KOH—CH_3OH at 75° for 2 h and then treated as described for saponifiable fatty acids.

Total fatty acids in shrew milk. Shrew milk (100 mg) was processed as described for saponifiable fatty acids.

Total fatty acids in rat serum and rat skin. To 100 μl rat serum or 100 mg rat skin and 50 μg heptadecanoic acid (I.S.) 0.5 ml KOH—CH_3OH (15 g KOH in 100 ml CH_3OH) were added. The centrifuge tube was purged with pure nitrogen gas, capped tightly, and saponified at 65° for 30 min. After cooling, 0.7 ml of 1 *M* H_3PO_4 were added, and the assay was carried out as described for saponifiable fecal fatty acids.

Total fatty acids in cell culture medium. A 2.00-ml sample containing 50 μg heptadecanoic acid (I.S.) was lyophilized to dryness, then 1 ml of KOH—CH_3OH (ca. 2 *N*) was added. The 12-ml centrifuge tube was purged with pure nitrogen gas, capped tightly, and heated at 65° in a dry-bath for 30 min. The samples were then treated as described for saponifiable fecal fatty acids.

RESULTS AND DISCUSSION

Calculations

The quantitation of the fatty acids (FA) was based on an internal standard method using heptadecanoic acid as internal standard.

The amount of each fatty acid in a sample was calculated as follows:

$$\mu g \text{ FA per sample} = \frac{\text{Area}_{FA} \times \mu g_{IS}}{\text{Area}_{IS} \times \text{RWR}_{FA/IS}} \quad \text{(samples)}$$

where:

$$\text{RWR}_{FA/IS} = \frac{\text{Area}_{FA} \times \mu g_{IS}}{\mu g_{FA} \times \text{Area}_{IS}} \quad \text{(standard)}$$

The relative weight response (RWR) values of each fatty acid from C12:0 to C20:4 were determined by repeated analyses of calibration standards of the combined fatty acids.

A computer interfaced with the GC instrumentation determined the peak area of the identified fatty acids and was programmed to calculate the amount of each fatty acid per sample.

Precision and recovery

The reliability of the method was continuously monitored during the course of analysis of the different biological materials. In Table I the relative standard deviations (R.S.D.) are listed from independent analyses of five types of biological samples: food homogenates, fecal specimens, shrew milk, rat serum, and tumor cell media.

The last column in Table I shows the average of five R.S.D. values. These

TABLE I

PRECISION OF FATTY ACID ANALYSIS IN DIFFERENT BIOLOGICALS

In most samples C20:1, C20:2, C20:3, and C20:4 appeared in trace to undetectable amounts. NA = Not analyzed, ND = not detected, TR = trace amount.

Fatty acid	R.S.D. (%)					
	Food homogenate	Fecal specimen	Shrew milk	Rat serum	Cell culture	Mean*
C12:0	2.9	6.4	0.7	NA	NA	3.3
C14:0	2.0	4.1	1.6	NA	NA	2.6
C16:0	3.2	2.2	1.1	1.5	1.1	1.8
C16:1	8.3	TR	1.3	5.2	1.8	4.2
C18:0	7.2	2.4	1.6	2.3	0.3	2.7
C18:1	6.0	1.2	1.4	1.0	2.2	2.4
C18:2	0.5	5.4	1.4	0.5	2.8	2.1
C18:3	2.8	ND	0.0	ND	ND	1.4
C20:1	ND	ND	TR	ND	ND	—
C20:2	ND	ND	0.0	ND	ND	—
C20:3	ND	ND	NA	ND	TR	—
C20:4	ND	ND	2.8	0.9	7.0	3.5

*Represents the average of relative standard deviations of five different biological samples. The R.S.D. for each individual biological is calculated from at least three independent analyses. The different biologicals were analyzed on different gas chromatographs with different columns (same type of packing) on different days by different analysts.

R.S.D. values ranged from 1.4 to 4.2%. The values of the precision study were obtained from at least three independent analyses of each different biological sample performed on different gas chromatographs with different types of columns (same type of packing) by different analysts on different days.

The percent recovery for each fatty acid added to five different biologicals is listed as the average in Table II which ranged from 88.5 to 100.5%. Columns three and four in this table show the standard deviation of the averaged recovery values and R.S.D. ranging from 3.3 to 7.5%.

Chromatography and analytical results

A good separation of all fatty acids of interest was achieved on a pre-tested HI-EFF-1BP (diethylene glycol succinate) liquid phase as illustrated in Figs. 1—4. The applicability of the analytical procedure was shown for a wide variety of biological materials with different matrices in Table III.

Fig. 2 shows a representative chromatogram of a standard mixture with fatty acids ranging from capric to nervonic. Shrew milk was analyzed over a wide range from capric to nervonic acids. The fatty acids of longer chain-length than arachidonic appeared usually in trace to undetectable amounts and are not listed in Tables I, II and III.

Generally, the samples were chromatographed isothermally at 140 or 155°. To determine the fatty acids in shrew milk ranging from capric (C10:0) to nervonic acids (C24:1), chromatography was started at 65°. The temperature was then raised by 7°/min to 175° to obtain well shaped peaks especially for

TABLE II

RECOVERY OF FATTY ACIDS ADDED TO DIFFERENT BIOLOGICALS

Fatty acid	Mean recovery (%)*	Standard deviation	R.S.D. (%)
C12:0	88.5	2.9	3.3
C14:0	89.8	6.3	7.0
C16:0	95.0	5.2	5.4
C16:1	95.0	5.5	5.8
C18:0	97.3	6.2	6.3
C18:1	95.6	5.1	5.4
C18:2	97.7	6.9	7.1
C18:3	100.5	6.4	6.4
C20:1	98.8	6.0	6.1
C20:2	99.3	7.4	7.5
C20:3	96.9	5.8	6.0
C20:4	97.4	3.2	3.3
AV.	96.0	5.6	5.8

*Represents at least two independent runs for each of five different biological samples performed on different gas chromatographs with different columns (same type of packing), on different days by different analysts.

the long chain acids in a reasonable time. To avoid excessive column bleeding and shifting of the baseline the column temperature was not increased higher than 175°.

A good example for the efficiency of the method by MacGee and Allen [5] is shown in Fig. 1 depicting the fatty acid composition of a fecal specimen. Practically no interference from background was observed. The extraction of the fatty acids from the acidic methanol—water phase into the hexane phase and the back-extraction of the acids from the supernatant hexane into the aqueous system containing Oakes and Willis base TMTFTH [6] functions as a very efficient cleanup step. A further advantage inherent in this method is the concentrating effect [5] by extracting the fatty acids out of 5 ml hexane into 10 μl TMTFTH. This is illustrated in Fig. 4, where low levels of fatty acids were determined in cell culture media. To ensure complete saponification of fungal spores the samples were heated at 75° for 2 h. The completeness of saponification of the pellet was determined by resaponifying and analyzing. No fatty acids were found. For the determination of the fatty acids in rat serum, rat skin tissue, and all culture media samples were saponified for 30 minutes which was sufficient.

Fig. 3 shows the chromatogram of the fatty acid pattern in rat serum. Only small samples were necessary for the determination. The samples were obtained from Dr. Boyd L. O'Dell who is conducting nutritional fatty acid deficiency studies; note in Fig. 3, the low level of 8,11,14-eicosatrienoic acid (C20:3ω6) and the high level of abnormal 5,8,11-eicosatrienoic acid (C20:3ω9) which appears when essential fatty acid deficiency in certain mammals is induced (for the explanation of the abbreviated fatty acid formulae see legend to Fig. 3 and Press et al. [7]. Since 5,8,11-eicosatrienoic acid was not available as a reference compound we had to deduce the presence of this abnormal acid

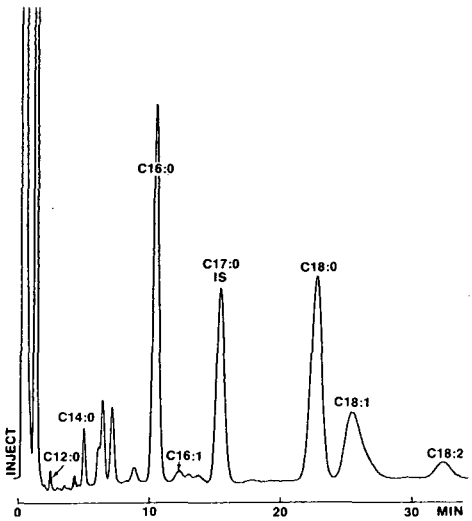

Fig. 1. Gas—liquid chromatographic analysis of saponifiable fecal (human) fatty acids as methyl esters. Sample, 100 mg (amount of sample injected into instrument, ca. 3 mg). Column: 15% HI-EFF-1BP on 100—120 mesh Chromosorb W AW, 2.5 m × 2 mm I.D., glass; volume injected, 1 μl of TMTFTH extract sandwiched between a mixture of methyl propionate—methanol (1:2); column temperature, 140° (isothermal); detector temperature, 210°; and injection temperature, 240°. Gas chromatograph: Barber-Colman 5000.

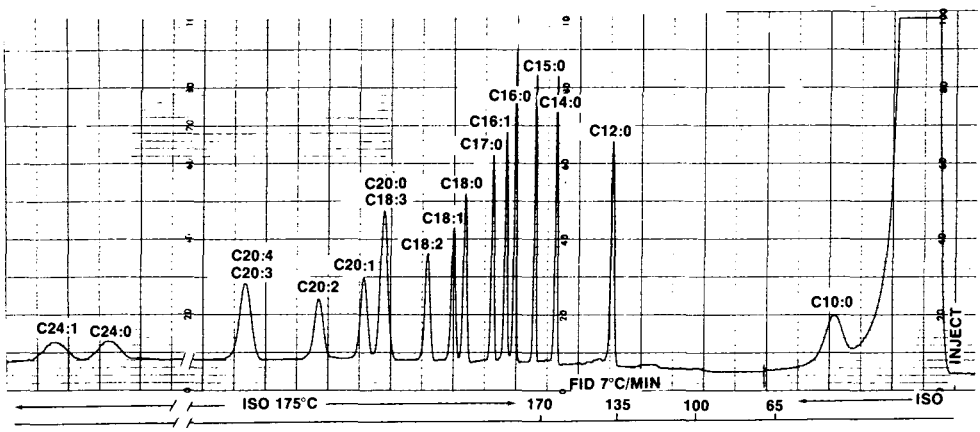

Fig. 2. Gas—liquid chromatography of fatty acids as methyl esters in a standard mixture, ranging from capric to nervonic acid. Volume injected, 1 μl of TMTFTH extract, 200 ng of each fatty acid. Column and sandwiching as in Fig. 1. Initial column temperature 65°, isothermal for 28 min, programmed 7°/min, final temperature 175°. Injector temperature 250°, detector temperature 210°. Instrument Tracor 222.

TABLE III

SAPONIFIABLE FATTY ACID COMPOSITION IN BIOLOGICAL SAMPLES

ND = Not detected, NA = not analyzed.

Fatty acid	Food homogenates, dry wt. (mg/g)	Fecal specimen, dry wt. (mg/g)	Spores *Helminthosporium*, dry wt. (mg/g)	Shrew milk, wet wt. (mg/g)	Rat serum (mg/100 ml)	Rat skin, wet wt. (mg/100 g)	Tumor cell culture (μg/ml)
Lauric C12:0	1.08	0.29	ND	8.31	NA	NA	NA
Myristic C14:0	4.25	1.15	0.06	10.11	NA	NA	NA
Palmitic C16:0	20.66	11.79	6.11	13.47	46.6	248.9	31.3
Palmitoleic C16:1	1.35	0.34	0.26	1.29	18.8	91.7	6.9
Stearic C18:0	12.77	11.92	1.20	2.00	23.1	91.1	22.9
Oleic C18:1	82.99	12.39	6.31	8.47	86.8	459.3	40.3
Linoleic C18:2	10.32	1.21	12.74	8.90	8.2	486.5	55.8
Linolenic C18:3	1.36	0.17	1.22	0.73	ND	8.3	7.5
11-Eicosenoic C20:1	ND	ND	ND	0.31	ND	9.0	0.7
11,14-Eicosadienoic C20:2	ND	ND	ND	0.38	ND	17.4	ND
8,11,14-Eicosatrienoic C20:3	ND	ND	ND	ND	38.0*	5.4	3.1
Arachidonic C20:4	ND	ND	ND	0.32	11.5	81.7	7.8

*5,8,11-eicosatrienoic acid.

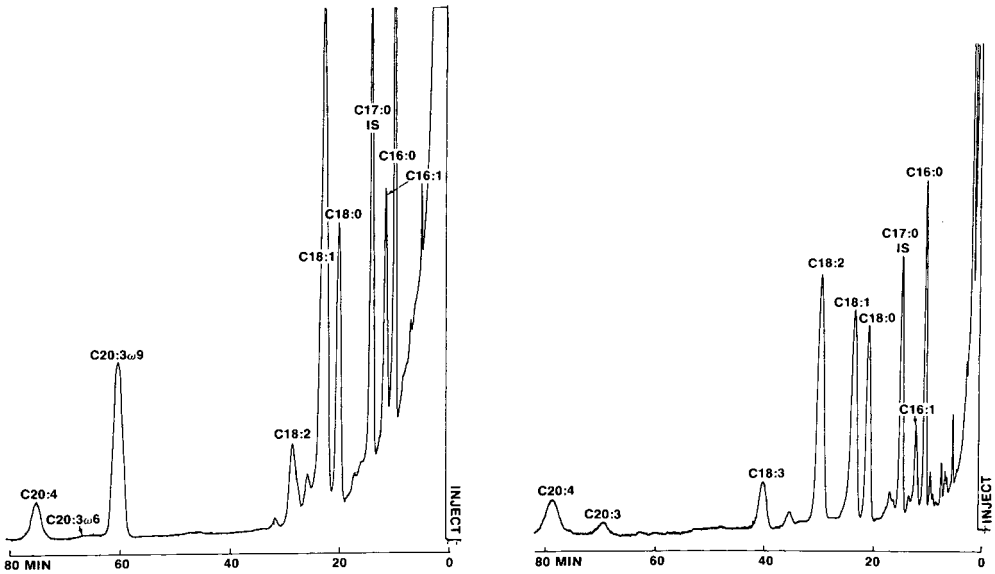

Fig. 3. Gas—liquid chromatography of saponifiable fatty acids as methyl esters in serum of rats with essential fatty acid deficiency manifested by the presence of abnormal 5,8,11-eicosatrienoic acid (C20:3ω9), the practical disappearance of 8,11,14-eicosatrienoic acid (C20:3ω6) and a rather low level of arachidonic acid (C20:4). Sample: 100 μl, sample size injected ca. 3 μl. Chromatographic conditions: volume injected, 1 μl TMTFTH extract; the 2 m × 2 mm I.D. glass column was packed with 15% HI-EFF-1BP on Chromosorb W AW (100—120 mesh); column temperature 155° (isothermal), detector temperature 240° and inlet temperature 260°. Gas chromatograph: Packard 7300. The abbreviated formula (C20:3ω9) indicates the number of carbon atoms and the numbers of double bonds. The position of the double bond nearest to the methyl terminus, counting CH_3 as 1, is indicated by the symbol ω.

Fig. 4. Gas—liquid chromatographic analysis of saponifiable fatty acids as methyl esters in a cell culture medium of prostate (human) cancer. Sample: 2.00 ml, sample size injected ca. 50 μl. Lyophilized to dryness; volume injected, 1 μl TMTFTH extract. Gas chromatograph: Packard 7300. See legend to Fig. 3 for further details.

Based on the elution pattern of a chromatogram published by Press et al. [7]. The 5,8,11-eicosatrienoic acid was quantitated on the assumption that its RWR factor would be similar to that of the isomer 8,11,14-eicosatrienoic acid. Based on our experience, this should be a reasonable approach.

Occasionally "memory" peaks were observed when samples of relative high fatty acid concentration were analyzed prior to those of lower concentration. Repeated injections of a mixture of TMTFTH and methyl propionate—methanol eliminated the "ghosting" effect.

When the hexane solution of the internal standard has been added to the sample it is very important to remove all hexane by use of a gentle stream of pure nitrogen gas to avoid incomplete saponification under the given experimental conditions.

CONCLUSION

The described internal standard method proved to be rapid and reliable for the determination of fatty acids at low levels in a variety of biologicals. Sample preparation and analysis requires approximately 1½ h. The TMTFTH extraction of the acids from the organic phase functions as a cleanup and concentration step. Good precision and accuracy of the method was demonstrated by use of different instrumentation and different analysts.

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REFERENCES

1 G.V. Marinetti (Editor), Lipid Chromatographic Analysis, Vol. 3, Marcel Dekker, New York, 1976.
2 A. Kuksis, J. Chromatogr., 143 (1977) 3.
3 D.J. Hentges, M.A. Flynn, G.C. Burton, J.M. Franz, C.W. Gehrke, K.O. Gerhardt, B.R. Maier, R.K. Tsutakawa and R.L. Wixom, Proc. 3rd Int. Symp. on Detection and Prevention of Cancer, New York, Marcel Dekker, New York, in press.
4 M.A. Flynn, B.R. Maier, C.W. Gehrke, R.K. Tsutakawa and D.J. Hentges, J. Amer. Diet. Ass., in press.
5 J. MacGee and K.G. Allen, J. Chromatogr., 100 (1974) 35.
6 T.R. Oakes and C.E. Willis, Amer. Assoc. Clin. Chem. 24th National Meeting, August 20—25, 1972, Abstr. 023, p. 25.
7 M. Press, H. Kikuchi, T. Shimoyama and G.R. Thompson, Brit. Med. J., 2 (1974) 247.

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Biomedical Applications

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POLYAMINES—AN IMPROVED AUTOMATED ION-EXCHANGE METHOD*

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SUMMARY

An accurate, precise, and improved automated cation-exchange chromatographic method with ninhydrin detection for the analysis of di- and polyamines (putrescine, cadaverine, spermidine, and spermine) has been developed. We have shown that different types of biological fluids such as urine, blood plasma, blood sera, tissue extracts, and cancer cell culture media can be analyzed under identical chromatographic conditions. The simplicity and precision of the method was achieved by eliminating the sample pre-separation and using an internal standard technique. Thus, not only has the sample preparation been simplified, but the accuracy and precision and sensitivity of the method have been greatly improved. Twenty-four unattended analyses were performed each day. With minor modifications of the instrument a two-fold analytical output can be achieved with analysis time cut to 30 min. The ruggedness and applicability of the method has been demonstrated in our laboratory during the past six months. More than two thousand urine and hundreds of other physiological samples have been analyzed by this method with a relative standard deviation from 3.3 to 7.8%, and recoveries of 94 to 97%.

This automated ion-exchange chromatographic method for the polyamines will be useful to researchers in biological markers programs for monitoring the course of cancer and effectiveness of chemotherapy.

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INTRODUCTION

The role of di- and polyamines (putrescine, cadaverine, spermine, and spermidine) as biological markers, whether for diagnosis of neoplasia [1—3] or as a means of monitoring efficacy of cancer chemotherapy [4], continues to attract the attention of cancer investigators. Their research has generated an ever increasing number and type of biological samples which have necessitated the continual development of more sensitive and expeditious polyamine analytical methods.

Most research emphasis has been placed upon the urinary excretion level of polyamines. Investigative work has now expanded to the point where the analysis of additional biological samples — blood serum and plasma, tissue extracts, and the media of cultured neoplastic cells are being requested by various researchers.

Numerous techniques have been developed for the determination of di- and polyamines; among them high-voltage paper electrophoresis, radioimmunoassay, enzymatic assay, and thin-layer chromatography. In most cases, however, not all of the polyamines are measured by each procedure, nor is adequate sensitivity and specificity obtained [5]. Precise and accurate determinations have not been adequately demonstrated with any of the methodologies.

Gas—liquid chromatography (GLC) [6] has proven sensitivity and resolution in polyamine analyses. The limitations of this method are encountered in the extensive sample cleanup procedures which make routine application of GLC impractical without the development of some automation.

In terms of accuracy, precision, simplicity, and rapidity of analyses, cation-exchange chromatography (CIE) utilizing an automated amino acid analyzer with ninhydrin detection apparently lends itself most readily to the analyses of a large number of samples. In 1971, Bremer et al. [7] first used such an instrument in the determination of polyamines. This method was significantly improved in sensitivity, separation, and speed by Marton et al. [8], Gehrke et al. [9], and Veening et al. [10].

The sample run time, for all three of these investigators, still remained at 90 to 120 min. Marton's method encountered interference problems and baseline shifts at high sensitivities, while Gehrke's procedure lacked the necessary sensitivity to analyze low levels of polyamines. Elution inconsistencies and relatively high minimum detection quantity for spermine were reported by Veening, nor had the method been substantiated by extensive application.

To answer the demand for the analysis of an ever increasing number and kind of biological samples for polyamines, this research has been directed toward the development of an improved method. Versatility and simplicity of analysis along with the required precision and accuracy were the goals of the investigation.

EXPERIMENTAL

Apparatus

A Beckman Model 121M amino acid analyzer (Beckman, Palo Alto, Calif., U.S.A.) provided with a 4.0 × 0.28 cm column packed with Beckman AA-20

resin, was used for the analyses. Column temperature was maintained at 65° throughout the analysis. The detector was set at a wavelength of 570 nm with a 1.2 cm flow cell with an output of 0.1 O.D./100 mv.

The data handling system was a Hewlett-Packard 3352B laboratory data system consisting of: a Hewlett-Packard 2100 computer with 16 K of memory; 18652A analog-to-digital converters (A/D); ASR33 teletype; and a 2748B high-speed photo reader (Hewlett-Packard, Maryland Heights, Mo., U.S.A.).

The urine sample aliquots and the deproteinized cell culture media were placed in 16 × 75 mm PTFE-lined screw cap Pyrex culture tubes obtained from Corning Glass Works (Corning, N.Y., U.S.A.). The blood plasma and sera aliquots were put into Corex (Corning) 30-ml round-bottom centrifuge tubes. Samples and buffered hydrolysates were shaken on a Vortex Genie mixer purchased from Scientific Products (Evanston, Ill., U.S.A.). Centrifugation was done in an automatic Servall superspeed centrifuge (Ivan Servall, Norwalk, Conn., U.S.A.) or in an Eppendorf Model 3200/30 microcentrifuge (Brinkman, Westbury, N.Y., U.S.A.).

Hydrolysis of samples was done in either a constant temperature oven from Aloe Scientific (Division of Brunswick, St. Louis, Mo., U.S.A.) or in a custom built proportional temperature controlled heating block (Science Instrument Shop, University of Missouri, Columbia, Mo., U.S.A.). An aluminum block (37 long × 15 wide × 8 high cm) with 44 holes (1.8 cm diameter × 3.5 cm deep) was heated by four CE 200-X 400 W heaters from Watlow (St. Louis, Mo., U.S.A.). The temperature was maintained at 145° ± 1° by a RFL temperature controller from RFL Industries (Boonton, N.J., U.S.A.).

Samples and buffered hydrolysates were dried either on a Temp-Blok module heater from Lab-Line Instruments (Melrose Park, Ill., U.S.A.) or on a Vaspilator rotary evaporator purchased from Rinco (Greenville, Ill., U.S.A.). Water was distilled from a Corning Model AG-11 all glass still equipped with a 45 l automatic collection system (9-035-65).

Sterilization of the water used in buffer preparation was accomplished by passage through a twin-90 (0.22 μm) filter from Millipore (Bedford, Mass., U.S.A.). The buffers were filtered through a Millipore GSWP04700 (0.22 μm) filter supported by a Millipore disc filter holder (XX1004730). The buffered hydrolysates were filtered through a DAWP01300 filter (0.65 μm). The deproteinized cell culture media, blood plasma, and blood sera were filtered through Millipore GSWP01300 filters (0.22 μm).

Reagents

Putrescine, cadaverine, spermidine, and spermine were obtained as hydrochloride salts from Calbiochem (Los Angeles, Calif., U.S.A.). 3,3′-Diaminodipropylamine was purchased from Aldrich (Milwaukee, Wis., U.S.A.). Ninhydrin and hydrindantin were obtained from EM Labs (Elmsford, N.Y., U.S.A.). Lithium hydroxide · H_2O, glacial acetic acid, dimethylsulfoxide, and sodium chloride, all reagent grade, were purchased from J.T. Baker (Phillipsburg, N.J., U.S.A.). Reagent-grade sodium citrate · H_2O, disodium ethylenediamine tetraacetate, and 5-sulfosalicylic acid were obtained from Fisher Scientific (St. Louis, Mo., U.S.A.).

Buffers. Two liters each of the buffers were prepared using deionized,

TABLE I

COMPOSITION OF EACH BUFFER

Reagent	Buffer per l					
	Sample	A	B	C	D	NaOH
pH, ±0.03	5.90	8.20	7.00	6.00	6.00	—
Na^+ Concentration (*N*)	0.20	0.20	1.60	2.50	3.50	0.2
Sodium citrate · $2H_2O$ (g) (0.2 *N*)	19.6	19.6	19.6	19.6	19.6	0
Sodium chloride (g)	0	0	81.83	134.4	192.9	0
Phenol (ml)	1.0	1.0	1.0	0	0	0
Na_2EDTA (g)	20.0	0	0	0	0	10.0

doubly distilled water that had been sterilized by passage through a Millipore twin-90 filter (0.22 μm). Glassware and buffer containers were treated with Chlorox (Chlorox, Oakland, Calif., U.S.A.), washed with 1 *N* HCl, and finally rinsed with the sterilized deionized doubly distilled water, prior to use.

All of the buffer solutions were boiled for 30 min, then filtered through a 0.22-μm filter before final pH and volume adjustments were made. Adjustments of pH were made with 6 *N* HCl. See Table I.

Ninhydrin. The ninhydrin solution was prepared according to the method of Moore [11]. Lithium acetate buffer (4 *M*) was first made by adding 336 g of LiOH · H_2O to 800 ml of doubly distilled water in a beaker. The solution was stirred until half of the LiOH · H_2O was dissolved, then 568 ml of glacial acetic acid were added. The lithium acetate solution was cooled, transferred to and brought almost to volume in a 2-l volumetric flask. A 1.0-ml aliquot of the buffer was withdrawn, diluted with 3.0 ml of doubly distilled water and its pH determined. If the pH was not 5.20 ± 0.05, it was adjusted with 2 g LiOH · H_2O or 2 ml acetic acid per 2.0 liters for each 0.01 pH unit. The lithium acetate buffer was made to volume, filtered through a 0.22-μm filter and 225 ml of the buffer were combined with 675 ml of dimethylsulfoxide (DMSO). Nitrogen containing <10 ppm of oxygen was then bubbled through the solution for at least 15 min.

Ninhydrin (18.0 g) was added to the above DMSO—lithium acetate solution while stirring. The bubbling of nitrogen was continued until all of the ninhydrin was in solution. Just prior to use the ninhydrin was reduced by the addition of 0.56 g of hydrindantin while stirring and bubbling the nitrogen through the solution for ca. 3 h.

Instrument and chromatography conditions

Beckman AA-20 resin with a bed size of 40.0 × 2.8 mm was used to separate the di- and polyamines. Flow-rates of the buffers and the ninhydrin were 8.8 and 4.4 ml/h, respectively. A column temperature of 65° and a reaction bath temperature of 100° ± 1° was maintained. The polyamines reacted with the ninhydrin for 2.8 min and were then detected at 570 nm in a 12.0 mm flow cell. Sensitivity was set at 0.1 a.u.f.s. A 50-μl volume of the sample filtrate or supernatant was injected on the column for analysis.

TABLE II

PREPARATION OF SINGLE STOCK SOLUTIONS

Polyamine	MW	g/100 ml	μmoles/ml
Pu·2HCl	161.1	1.611	100.0
Cd·2HCl	175.1	1.751	100.0
I.S.*	131.2	1.312	100.0
Sd·3HCl	254.6	1.273	50.0
Sp·4HCl	348.2	1.161	33.3

*3,3'diaminodipropylamine free base.

Calibration standard solutions

The hydrochloride salts of the polyamines used for the preparation of the standard solutions were stored under refrigeration at 4° and dried for one day in a desiccator with reduced pressure of less than 70 μ at room temperature prior to weighing.

Single compound stock solutions were prepared to yield concentrations of 100 μmoles/ml each of putrescine (Pu), cadaverine (Cd), internal standard (I.S.) (3,3'-diaminodipropylamine), 50 μmoles/ml of spermidine (Sd), and 33 μmoles/ml spermine (Sp). Exactly 10.0 ml of each of the single compound stock solutions were made to a volume of 2000 ml with 0.1 *N* HCl. Exactly 5.0 ml of this solution was brought to a volume of 100 ml with 0.05 *N* HCl. Final polyamine concentrations as the free bases are given in Table II. Working standard solutions were prepared of 25.0 nmoles/ml for Pu, Cd, and I.S.; 12.5 nmoles/ml for Sd; and 8.35 nmoles/ml for Sp.

It was observed that the stock solutions were not stable when stored for a few days if pH >7. Storage at 4° and at pH 2 was required. At these conditions the polyamine solutions were stable for three months.

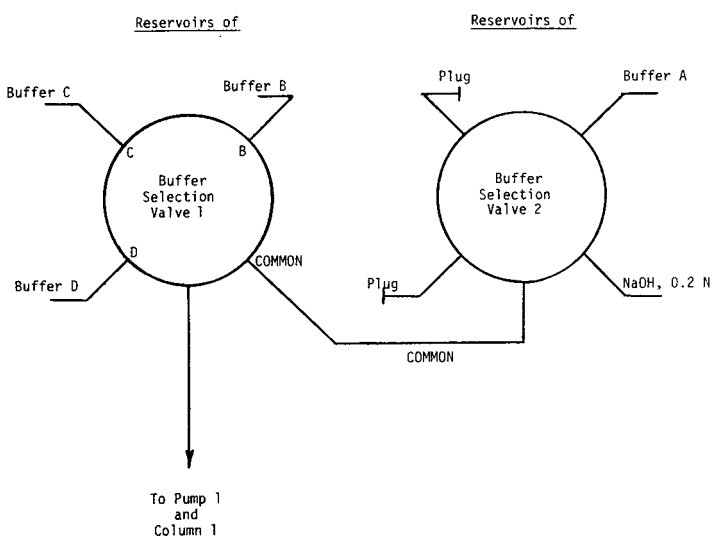

Fig. 1. CIE buffer selection valve sequence. For buffer composition see Table I.

CIE analysis sequence

Table III describes the sequence of events for the automated CIE analysis and Fig. 1 shows the modifications of the buffer selection valves employed in the instrumentation for polyamine analysis.

Procedure

Urine. Homogeneous 2.00-ml aliquots of 24-h urine collections were placed in 16 × 75 mm Pyrex screw-cap tubes and evaporated to dryness under purified nitrogen gas at 65 to 70° in a heating block. Exactly 2.00 ml of 6 *N* HCl containing 50 nmoles of 3,3'-diaminodipropylamine (I.S.) were added to each dried sample. The tubes were tightly capped with PTFE-lined screw caps, and then placed in a 110° oven for 16 h. After hydrolysis the HCl was evaporated from the samples to dryness at 70° under a gentle stream of nitrogen gas followed by the addition of 1.00 ml of the sample buffer. The buffered hydrolysates were thoroughly mixed and then placed under refrigeration at 4° for a minimum of 4 h.

TABLE III

CIE INSTRUMENTAL ANALYSIS SEQUENCE

Step 1 for starting instrument only. Steps 2—12 are the actual analytical time. Steps 13 and 14 are for instrument shut down only.

Step	Step time (min)	Event
1	15.0	Buffer pump No. 1 on. Ninhydrin pump on. Buffer selection valve 1 to buffer D position. Ninhydrin diverter valve to reaction coil position. Column No. 1 effluent to reaction coil.
2	0.1	Stop program recycling. Sample transfer pump on. Buffer selection valve 2 to NaOH position. Buffer selection valve 1 to common.
3	4.9	This completes sample transfer to the injection loop.
4	3.0	Buffer selection valve 2 to buffer A position to equilibrate column.
5	7.0	Ninhydrin diverter valve to drain position. Buffer pump No. 2 on.
6	0.1	Start sample transfer to column. Start computer. Start data reduction system.
7	0.9	Complete transfer of sample from injection loop to column. Column No. 1 effluent to drain. Column No. 2 effluent to reaction coil.
8	13.0	Buffer selection valve 1 to buffer B position.
9	2.0	Column No. 1 effluent to reaction coil. Buffer pump No. 2 off.
10	18.0	Ninhydrin diverter valve to reaction coil position. Buffer selection valve 1 to buffer C position.
11	10.9	Buffer selection valve 1 to buffer B position.
12	0.1	Start program recylcing.
13	8.0	Buffer selection valve 1 to common. Buffer selection valve 2 to buffer A position.
14	15.0	Ninhydrin pump off. Ninhydrin diverter valve to drain. Program shut down.

Total Run time: 60.0

The samples were filtered through a Millipore 0.45—0.65-μm filter or centrifuged on the Eppendorf Microcentrifuge for 5 min at 12,000 *g*, and then 50 μl of the filtrate or supernatant were placed on the CIE column for analysis.

Cell culture media. Homogeneous 5.00-ml aliquots of cell culture media were placed in 30 ml centrifuge tubes to which were added 1.00 ml of 20% (w/v) sulfosalicylic acid. The tubes were then thoroughly shaken, heated at 70° for 30 min, and than centrifuged at 12,100 *g* for 10 min at 4°. The deproteinized supernatants were transferred to 16 × 75 mm Pyrex screw-cap tubes and then evaporated to dryness in a 70° heating block under a nitrogen gas sweep. Exactly 2.00 ml of 6 *N* HCl containing 5.00 nmoles of I.S. were added to the dried samples. They were then tightly capped with PTFE-lined screw caps and hydrolyzed at 120° for 40 h. The samples were periodically shaken during hydrolysis.

After hydrolysis, the tubes were placed under refrigeration for 30 min. While maintaining a temperature of 0° with an ice bath, the hydrolysates were filtered through glass wool plugs into 16 × 75 mm screw-cap Pyrex tubes. The hydrolysis tubes and the glass-wool plugs were both washed with ca. 1 ml of ice cold 6 *N* HCl, and the washes were combined with the filtrates and then taken to dryness at 70° under a purified nitrogen gas flow. The dried samples were redissolved with sonication in 1.00 ml of doubly distilled water, prior to CIE analysis, then 50 μl placed on the analyzer.

Blood plasma and serum. Same sample preparation method as for cell culture media except 16 h of hydrolysis at 110° was used.

Pork tissue extracts. Ground tissue portions (100 g) were homogenized in a blender with 150 ml of 0.6 *N* $HClO_4$ and then centrifuged at 2000 *g* for 20 min. The tissue residues were homogenized once again with an additional 150 ml of 0.6 *N* $HClO_4$, centrifuged, and the two supernatants were combined and refrigerated at 4°. Filtration through glass-wool to remove suspended fat followed, and the pH of the filtrates was then adjusted to 6 with 6 *N* KOH. To facilitate the precipitation of the perchlorate salts, the sample solutions were cooled (4°) and the salts removed with filtration through Whatman No. 5 filter paper. The filtrates were then frozen, lyophilized, and redissolved in 50 ml of doubly distilled water. The salts were once again removed by filtration and the filtrates made to volumes of 100 ml with doubly distilled water (extract A).

Free polyamines. Determinations of the free polyamines in the tissue extracts were made by taking exactly 1.00 ml aliquot of extract A and adding an internal standard solution of exactly 1.00 ml of 0.1 *N* HCl containing 50.0 nmoles of 3,3′-diaminodipropylamine. Then 50 μl were placed on the CIE column for analysis.

Total polyamines. The total polyamine contents of the extracts were determined by placing exactly 1.00 ml aliquots of extract A in 16 × 75 mm Pyrex screw-cap tubes and then evaporating to dryness at 70° under a nitrogen gas sweep in a heating block. Exactly 1.00 ml of 6 *N* HCl containing 50.0 nmoles of internal standard was then added to the dried samples. After being tightly capped with PTFE-lined screw caps, the tubes were hydrolyzed at 110° for 16 h and then dried at 70° in a heating block under a nitrogen gas flow. The dried hydrolysates were redissolved in 2.00 ml of doubly distilled water for CIE analysis, and 50 μl were placed on the CIE column for analysis.

Precision, recovery and accuracy

Initially the precision of the CIE chromatography and the instrumentation was established by the multi-analysis of a working standard solution. The precision of the method was then demonstrated by the repeated analysis of two normal pooled urine collections. Additionally, during the routine application of the method in analyses of urine from cancer patients, 10% of the samples were chosen at random for duplicate independent analyses.

Recovery experiments were routinely made by determining the initial level of polyamines in a random 10% of the samples and then spiking these samples with known amounts of all the polyamines and re-analysis by CIE.

Drying time and temperature

The effects of time and temperature of drying on the polyamines in hydrolysates were investigated. Twelve aliquots of a urine hydrolysate were prepared and divided into four sets of three each. The first set was taken just to dryness at 70°, the second set was taken to dryness at 70° and then allowed to remain in the heating block for an additional hour. The third set was taken just to dryness at 100° and then held in the heating block for an additional hour. At all times during the drying all of the hydrolysate aliquots were kept under a gentle stream of nitrogen. The dried hydrolysates were then taken through the remainder of the sample preparation procedure and analyzed.

Hydrolysis

Ten replicate aliquots of a pooled normal urine collection were drawn and divided into two equal groups. One group was hydrolyzed at 110° for 16 h while the five other aliquots were hydrolyzed at 150° for 4 h. Sample preparation for both groups was then completed followed by CIE analysis of the polyamines.

In addition, a group of twelve 24-h urine collections from cancer patients were also used to evaluate the modified hydrolysis. Duplicate aliquots were drawn from each of the samples and one taken through the routine hydrolysis method (110° for 16 h) while the other was hydrolyzed at 150° for 4 h. Determination of the polyamines followed completion of the sample preparation.

RESULTS AND DISCUSSION

Fig. 2 shows the separation, resolution and order of elution of the various di- and polyamines at the nmole level. Marton et al. [12], in their analyses of cerebrospinal fluids, reported the virtual co-elution of histamine with cadaverine between putrescine and spermidine. In our method, histamine elutes well ahead of not only cadaverine, but putrescine as well, leaving cadaverine clearly resolved.

The sensitivity of analysis with the Beckman 121M instrumentation was demonstrated. Quantification at the ngram level for the calibration mixture of di- and polyamines was done at a recorder setting of 0.01 a.u.f.s. with negligible noise interference. Linearity of the ninhydrin color response was observed from 17 pmoles to 25 nmoles placed on the column.

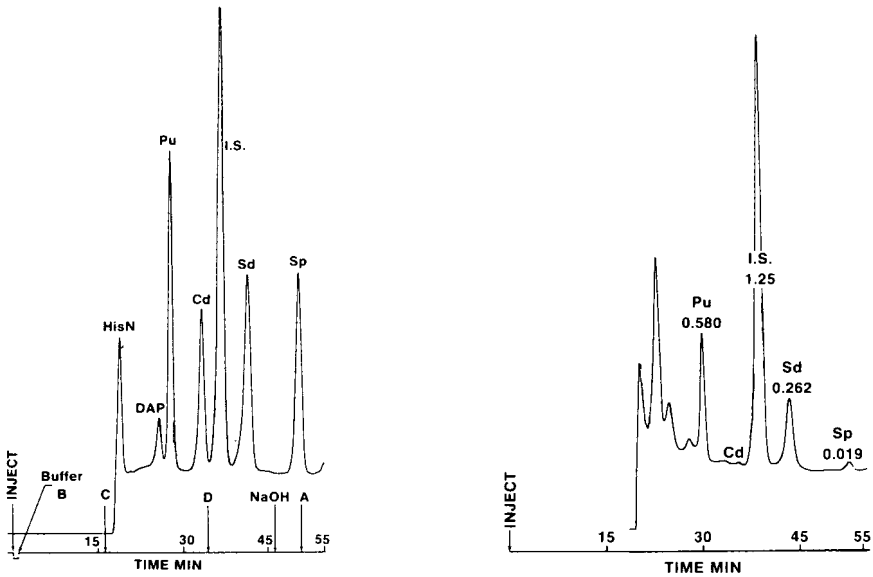

Fig. 2. CIE chromatogram of a calibration mixture of di- and polyamines. Conditions: injection, 50 μl; detection, 570 nm, 0.10 a.u.f.s.; column, 40 × 2.8 mm, packed with Beckman AA-20 resin, temperature, 65°; flow-rate, 8.8 ml/h; reaction, 10-in. coil, 2.8 min, 100°. Sample units: Pu, 1.25; Cd, 1.25; I.S., 1.25; Sd, 0.625; Sp, 0.418 nmoles.

Fig. 3. CIE chromatogram of polyamines in urine of breast cancer patient. Sample, 0.10 ml; further conditions as in Fig. 2. The sample units at the peaks are given in nmoles.

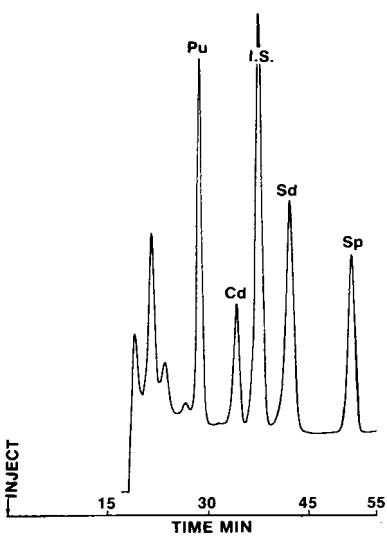

Fig. 4. CIE chromatogram of breast cancer patient urine spiked with polyamines. Sample 0.10 ml; further conditions as in Fig. 2.

COMPUTER REPORT FOR POLYAMINE ANALYSIS IN URINE

REPORT NO. 286 — 121M PA's ANAL.
CHAN# 6 METHOD: PA'S — SAMPLE: URINE
ISTD = 25.000,R NM/ML

RT	AREA	NM/ML	NAME
26.53	16508	3.142	DAP
27.89	161815	22.664	PU
32.62	15908	3.084	CD
36.24	426304		&IS
40.06	107073	6.289	SD
49.47	21850	1.098	SP

TOTAL AREA = 749, 458

COMPUTER PROGRAM FOR POLYAMINE ANALYSIS

```
1. CHAN, PROC, RPRT, RDVC
   6, ISTD, ME, T1
2. SAMP, UNTS, TITLE
   URINE, NM/ML, 121M PA'S ANAL.
3. #PKS, RTM, PRG
   25, 54.00, YES,
4. MIN AR, MV/M, DLY, DVT, DIL-FTR%
   2000, .020, 22.0, 0.00, 100.00
5. REF-RTW, %RTW, ID-LVL, RF-UNK
   .50, 1, 2000, 0.000
6. # KWN PKS
   7
```

#	TIME	AMT		FACTOR		NAME
1	26.54	1.0000E	1 =	3.2457E	0	DAP
2	27.90	2.5000E	1 =	2.3883E	0	PU
3	32.61	2.5000E	1 =	3.3055E	0	CD
4	36.25	2.5000E	1 =	1.0000E	0	&IS
5	40.01	1.2500E	1 =	1.0015E	0	SD
6	49.42	8.3500E	0 =	8.5654E-	0	SP
7	60.00	1.0000E	0 =	0.0000E	0	#DUMMY

```
7. # EVENTS
   5
```

	TIME,	EVENT	
1	24.00	B	Reset baseline at end of peak
2	26.20	B	
3	27.90	B	
4	44.00	I	Reset baseline
5	49.40	B	

Fig. 5. Computer report and program for polyamine analysis in urine. The parameters of the computer method for polyamine analysis are divided into seven categories.

(1) Calculation procedure: CHAN: channel assigned to this method (No. 6); PROC: calculation procedures (internal standard method); RPRT: report types (Medium type); RDVC: report device (Teleprinter No. 1).

(2) Sample identification: SAMP: sample description (urine); UNTS: units of concentration to be reported (nmoles/ml); TITLE: title of the method (Polyamine analysis Beckman 121M).

(3) Chromatography conditions: NO. OF PKS: the maximum number of peaks presented in the analysis (25 peaks); RTM: computer run time (54.0 min); PRG: chromatography program mode (stepwise program).

(4) Integration parameters: MIN AR: minimum area of a peak (2000 $\mu V \cdot sec$); MV/M: slope threshold (0.02 mV/min); DLY: delay of integration (22.0 min); DVT: dead volume holding time (0.00 min); DIL-FTR%: %dilution (factor in 100%).

(5) Identification procedures: REF-RTW: reference peak (I.S. peak) retention time window (0.50 min), %RTW: retention time window for known peaks (1.0%); ID-LVL: identification-level of the known peaks (2000 $\mu V \cdot sec$); RF-UNK: response factor for unknown peaks (0.00).

(6) Information for the identification and calculation of known peaks: TIME: absolute retention time of known peaks (min); AMT: concentration of known peaks (nmoles/ml); FACTOR: response factor of known peaks as 1/RWR,

$$\frac{1}{RWR} = \frac{\text{(Area of I.S.)}}{\text{(Area of compound)}} \times \frac{\text{Conc. of Compound}}{\text{Conc. of I.S.}}$$

NAME: name of known compounds.

(7) The integrator time events: B: force baseline next valley; I: force baseline immediately.

Fig. 3 is a chromatogram of a typical urine sample; in this case that of a diagnosed breast cancer patient. It is noted that potentially interfering diaminopropane has been adequately separated from putrescine to allow accurate analysis. Fig. 4 shows the same urine sample spiked with di- and polyamines prior to hydrolysis. A representative computer report and method for a urine sample analysis is presented in Figure 5. The computer method parameters are also defined and presented.

The CIE chromatograms of polyamines in blood plasma and sera are presented in Figures 6 and 7, respectively, and show quantitation at much lower levels of the di- and polyamines. 1,6-Diaminohexane commonly encountered in plasma and sera must be separated from spermine to allow for an accurate analysis. A chromatogram of spiked blood serum is presented in Fig. 8.

The in vitro culturing of bladder cancer cells in Minimum Essential Medium (MEM) yielded the chromatogram shown in Fig. 9. The supernatant of the cultured tumor cells was analyzed to determine the excreted polyamines.

The versatility of the method is once again demonstrated in the analyses of meat tissue extracts, typical chromatograms of which are presented in Figures 10 and 11. Both free and total di- and polyamine determinations were made on extracts of pork tissue. Relatively high levels of spermine are seen in the unhydrolyzed extract. The conjugated components of spermidine and spermine add significantly to their total in the hydrolyzed extract.

Excellent precision and accuracy for the chromatography and instrumentation were established by the ten replicate analyses of the working standard solution. The statistical parameters of the analyses are presented in Table IV.

The precision of the method was determined in two aspects. The sample matrix independent precision is presented in Table V in which five independent

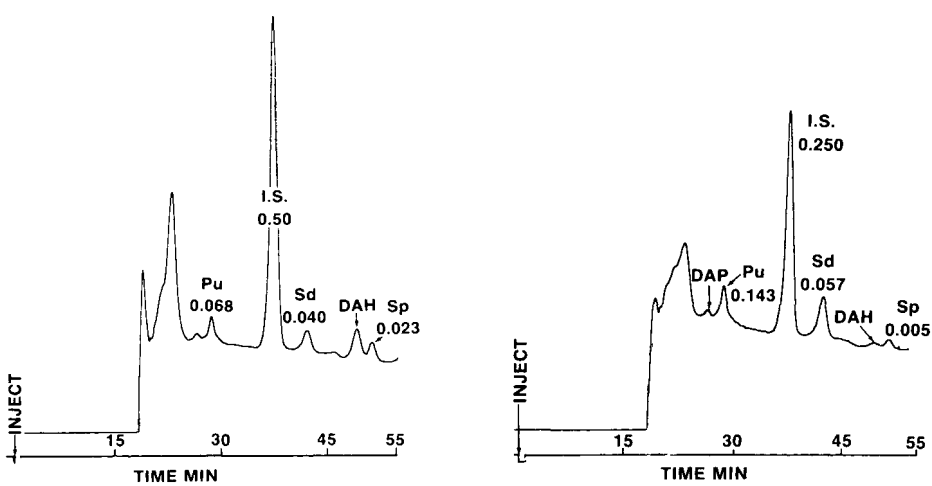

Fig. 6. CIE chromatogram of polyamines in pooled human plasma. Sample, 0.25 ml; further conditions as in Fig. 2. The sample units at the peaks are given in nmoles.

Fig. 7. CIE chromatogram of polyamines in pooled human serum. Sample, 0.25 ml; further conditions as in Fig. 2. The sample units at the peaks are given in nmoles.

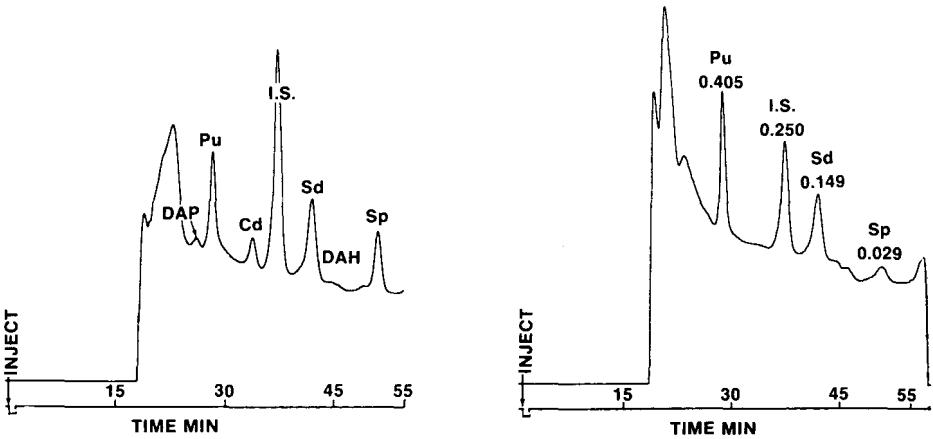

Fig. 8. CIE chromatogram of pooled human serum spiked with polyamines. Sample, 0.25 ml; further conditions as in Fig. 2.

Fig. 9. CIE chromatogram of polyamines in control media cultured with bladder cancer cells. Sample, 0.25 ml of MEM media; further conditions as in Fig. 2. The sample units at the peaks are given in nmoles.

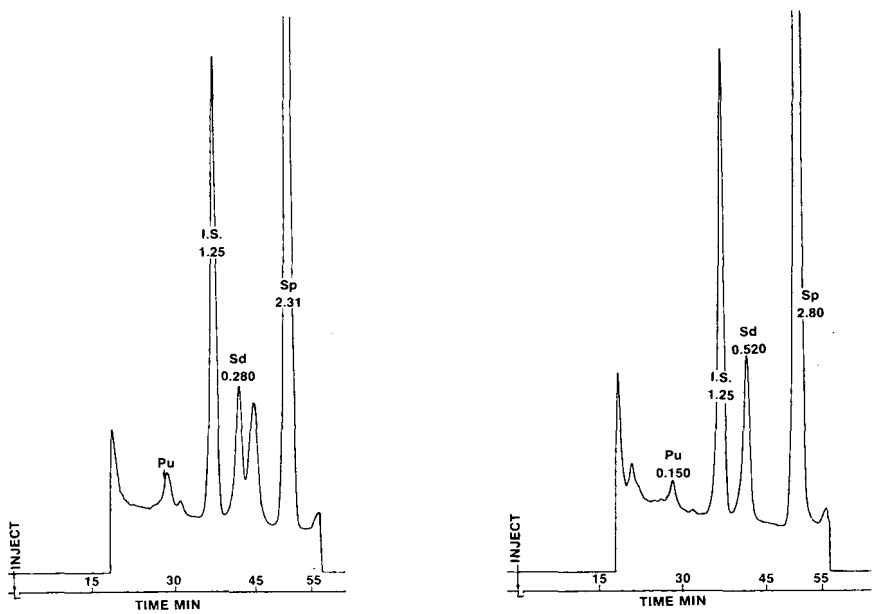

Fig. 10. CIE chromatogram of free polyamines in meat tissue extract. Sample, 25 mg; further conditions as in Fig. 2. The sample units at the peaks are given in nmoles.

Fig. 11. CIE chromatogram of polyamines in hydrolyzed meat tissue extract. Sample, 25.0 mg; further conditions as in Fig. 2. The sample units at the peaks are given in nmoles.

TABLE IV

PRECISION OF RETENTION TIME AND RESPONSE (n = 10)

	Retention time (min)				Response (nmoles/ml)			
	Pu	Cd	Sd	Sp	Pu	Cd	Sd	Sp
$\bar{x}$	27.90	32.61	40.01	49.43	24.93	25.12	12.53	8.45
S.D.	0.008	0.010	0.004	0.010	0.144	0.244	0.035	0.070
R.S.D. (%)	0.03	0.03	0.02	0.02	0.58	0.90	0.28	0.80

TABLE V

PRECISION STUDY ON THE SAME AND DIFFERENT SAMPLES FOR POLYAMINES IN URINE

$\sigma = \sqrt{[\Sigma(x_1 - x_2)^2]/2P}$; R.S.D. (%) = $\sigma/\bar{x} \times 100$, P = number of pairs, $\bar{x}$ = mean for a population.

Sample 1 (n = 5)	nmoles/ml			
	Pu	Cd	Sd	Sp
$\bar{x}$	10.40	2.36	4.99	0.522
σ	0.092	0.057	0.068	0.010
R.S.D. (%)	0.88	2.4	1.4	1.9
Sample 2 (n= 5)				
$\bar{x}$	35.48	30.10	16.93	10.60
σ	0.430	0.202	0.169	0.114
R.S.D. (%)	1.2	0.67	1.0	1.1
Different samples (n = 21)				
$\bar{x}$	15.5	3.44	7.35	0.994
σ	0.806	0.113	0.407	0.078
R.S.D. (%)	5.2	3.3	5.5	7.8

TABLE VI

RECOVERY OF POLYAMINES FROM CANCER PATIENT URINE

Sample (n = 17)	Recovery (%)			
	Pu	Cd	Sd	Sp
$\bar{x}$	93.8	96.8	96.0	97.2
σ	6.46	4.29	5.11	5.55
R.S.D. (%)	6.89	4.43	5.32	5.71

analyses were made on the same sample on different days, and that for the sample matrix dependent precision for analyses on different samples in Table V. As we expected the sample matrix affected the precision of the method. The relative standard deviations for the sample matrix independent analyses ranged from 0.67 to 2.4%; whereas, the relative standard deviations for the sample matrix dependent analyses were from 3.3 to 7.8%.

Consistent recoveries of 94 to 97% were achieved in day to day routine operation (Table VI) with a relative standard deviation of 4 to 7%.

During the analyses of over 1000 urine samples less than 1% of the samples have shown interfering unknown peaks requiring manual correction. In these instances chemotherapeutic drugs or their metabolites are possibly the cause. A systematic study of the various cancer chemotherapeutic drugs and their effects on polyamine analysis might well be of value in identification of the interferences occasionally encountered in polyamine determinations.

Quantitation of putrescine and spermine in the 16 h, 110° cell culture media hydrolysate was difficult due to the elution of interfering peaks at nearly the same retention time. The interferences were successfully removed on hydrolysis for 40 h at 120 ± 1°. The stability of the polyamines under these rigorous conditions was verified by the recovery (89—107%) of all polyamines added to urine and processed as for samples.

Only a limited number of blood plasma and sera samples have been analyzed,

TABLE VII

THE EFFECT OF TIME AND TEMPERATURE ON DRYING URINE HYDROLYSATES

Three aliquots of a pooled urine control were evaporated at each of the conditions given.

Polyamine	nmoles/ml			
	Drying at 70° and 100°			
	70 *	70 + 1 h **	100° *	100° + 1 h **
Pu				
$\bar{x}$	10.50	10.80	10.73	11.14
σ	0.06	0.05	0.08	0.02
R.S.D. (%)	0.5	0.5	0.8	0.1
Cd				
$\bar{x}$	2.54	2.54	2.56	2.67
σ	0.11	0.06	0.06	0.13
R.S.D. (%)	4.2	2.6	2.3	4.9
Sd				
$\bar{x}$	5.15	4.98	5.12	5.10
σ	0.06	0.06	0.04	0.06
R.S.D. (%)	1.2	1.2	0.8	1.2
Sp				
$\bar{x}$	0.51	0.51	0.52	0.47
σ	0.04	0.04	0.03	0.04
R.S.D. (%)	8.8	8.8	5.8	8.8

*The samples were taken just to dryness at 70° and 100°.

**The samples were held at 70° and 100° for 1 h after dryness.

however, sensitivity and recoveries (96—105%) associated with the serum polyamine analyses appear superior to those obtained with other methodologies. Previous investigations [13, 14] employed a tedious *n*-butanol extraction of the serum hydrolysates with 50% recoveries reported. By elimination of the alcohol extraction step the method was greatly simplified and made more rapid, accurate, and reliable as well.

An extremely high spermine to putrescine ratio was observed in all the meat tissue extracts. This is a reversal of the spermine to putrescine ratio consistently found in urine. This observation may indicate different metabolic pathways of these two molecules.

The ruggedness of the analytical part of the method was demonstrated by drying of the hydrolysates at different time and temperatures (Table VII). No noticeable difference in the polyamine results was observed on drying at 70° or 100° and holding at either temperature for 1 h.

Roach and Gehrke [15] reported that the maximum yield for all of the protein amino acids was obtained on hydrolysis at 145 ± 2° for the minimal time of 4 h. An attempt was made to apply this rapid method to the hydrolysis of conjugated polyamines in urine [16].

Somewhat higher results were obtained on hydrolysis at 150°—4 h as compared to the commonly used 110°—16 h (Table VIII). A systematic hydrolysis study is underway to determine the significance of hydrolysis time and temperature.

A program is underway on the use of a fluorescent detection system with the collaboration of Beckman Instrument Company to determine sensitivity and reliability by measurement at low levels.

Comments on the method

All glassware and containers used in the preparation of buffers and their storage must be maintained scrupulously clean and sterilized to avoid mold contamination.

To retard mold formation and maintain stability of standards and buffers, they should always be kept under refrigeration at 4°.

An alternative procedure for the preparation of the sodium citrate buffer solution is to prepare a 2.00 *N* stock solution which is then boiled for ca. 30 min for sterilization. This will prolong the mold-free lifetime of the buffers. Ten fold dilutions can then be made to prepare the other working buffer solutions.

Adjustments of the pH of the buffers should be made to within 0.03. This is especially important for buffer B which affects the separation of diaminopropane and putrescine.

The complete dissolution of the hydrindantin generally requires ca. 3 h of constant stirring under nitrogen at room temperature. Complete solubility can be verified by looking at the solution with a strong light during mixing.

Working standard solutions of the di- and polyamines should be freshly prepared every two weeks.

To assure accuracy and precision of this method, thoroughly mixed samples for homogeneity, accurate aliquots, and exact amounts of internal standard are critical.

TABLE VIII

THE EFFECT OF HYDROLYSIS TIME AND TEMPERATURE ON THE ANALYSIS OF POLYAMINES IN URINE

Urine	Concentration (nmoles/ml), hydrolysis temperature and time							
	Pu		Cd		Sd		Sp	
	110°, 16 h	150°, 4 h	110°, 16 h	150°, 4 h	110°, 16 h	150°, 4 h	110°, 16 h	150°, 4 h
Normal, 1								
$\bar{x}$ (n = 4)	10.90	11.78	2.36	2.45	4.93	5.22	0.471	0.513
σ	0.13	0.20	0.06	0.15	0.11	0.06	0.02	0.04
R.S.D. (%)	1.2	1.7	2.6	6.2	2.2	1.1	4.1	7.1

If only limited volumes of cell culture media, blood plasma, or sera are available 2.00 ml can be analyzed, if the sample is reconstituted in 0.40 ml of buffer prior to analysis.

The 100 g of pork tissue cited in the method were necessary to obtain a representative sample. Routine polyamine analyses were made on 1.0 g of tissue.

To ensure accurate and precise analyses from day to day, a pooled, normal control sample with pre-determined polyamine levels was prepared and analyzed with each group of samples (ca. 50).

Internal standard solutions of 3,3′-diamino-dipropylamine containing 5.00 and 50.0 nmoles/ml were prepared on dilution of the stock solution. This solution is very stable under acidic conditions.

Time and temperature conditions for the drying of hydrolysates were found not to be critical.

Each day check all instrument controls for proper settings of flow-rate, temperature of reaction coil and chromatographic column, and column pressure.

In our laboratory the ion-exchange column has been used for more than one thousand analyses without increased pressure or deterioration.

CONCLUSIONS

An improved chromatographic method for the automated CIE analysis of the di- and polyamines, putrescine, cadaverine, spermidine, and spermine has been developed utilizing the highly sensitive and versatile Beckman Model 121M amino acid analyzer and ninhydrin detection. The unique chromatographic conditions and sample preparation enable the analysis of polyamines in a wide array of biological samples in less than 1 h per analysis. The precision, accuracy, and routine applicability of the method has been demonstrated. We believe this method provides the precise, accurate, and simple means of determining polyamines in biological fluids in support of research for bio-markers in cancer.

ACKNOWLEDGEMENT

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REFERENCES

1 U. Bachrach, Function of Naturally Occurring Polyamines, Academic Press, New York, 1967.
2 S.S. Cohen, Introduction to the Polyamines, Prentice-Hall, Englewood Cliffs, N.J., 1971.
3 D.H. Russell (Editor), Polyamines in Normal and Neoplastic Growth, National Cancer Institute Symposium Volume, Raven Press, New York, 1973.
4 D.H. Russell, G.M. Durie and S.E. Salmon, Lancet, ii (1975) 797.
5 J. Savory and J.R. Shippe, Ann. Clin. Lab. Sci., 5 (1975) 110.
6 C.W. Gehrke, K.C. Kuo, R.W. Zumwalt and T.P. Waalkes, in D.H. Russell (Editor), Polyamines in Normal and Neoplastic Growth, Raven Press, New York, 1973, p. 343.
7 H.J. Bremer, E.J. Kohne and W. Endres, Clin. Chim. Acta., 32 (1971) 407.

8 L.J. Marton, O. Heby and C.B. Wilson, FEBS Lett., 46 (1974) 305.
9 C.W. Gehrke, K.C. Kuo and R.W. Zumwalt, J. Chromatogr., 89 (1974) 231.
10 H. Veening, W.W. Pitt, Jr. and G. Jones, Jr., J. Chromatogr., 90 (1974) 129.
11 S.J. Moore, J. Biol. Chem., 243 (1968) 62.
12 L.J. Marton, Cancer Res., 36 (1976) 973.
13 L.J. Marton, D.H. Russell and C.C. Levy, Clin. Chem., 19 (1973) 923.
14 K. Nishioka and M.M. Romsdahl, Clin. Chim. Acta., 57 (1974) 155.
15 D. Roach and C.W. Gehrke, J. Chromatogr., 52 (1970) 393.
16 C.W. Gehrke, K.C. Kuo and R.W. Zumwalt, Unpublished results.

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DETERMINATION OF CHLORDIAZEPOXIDE, DIAZEPAM, AND THEIR MAJOR METABOLITES IN BLOOD OR PLASMA BY SPECTROPHOTODENSITOMETRY*

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SUMMARY

An analytical procedure was developed for the determination of chlordiazepoxide, diazepam and their major metabolites in blood or plasma. Demoxepam, a metabolite of chlordiazepoxide, is determined by spectrofluorometry after selective extraction. The remaining compounds are determined by spectrophotodensitometry after thin-layer chromatographic separation.

The sensitivity limit of the spectrofluorometric determination of demoxepam is 0.1 to 0.2 μg while that of the spectrophotodensitometric determination of chlordiazepoxide, diazepam and their N-desmethyl metabolites is 0.05 to 0.2 μg. The sensitivity and specificity of the assay renders it suitable for monitoring plasma levels of chlordiazepoxide and its major metabolites following single or chronic oral administration of chlordiazepoxide hydrochloride. The sensitivity limit for diazepam and nordiazepam, its major metabolite, renders the assay useful only for the determination of plasma concentrations resulting from high dosage of diazepam. The assay was used to determine chlordiazepoxide and its metabolites following oral administration of Librium. The data showed a significant correlation to those obtained on the same specimens by differential pulse polarography and by radioimmunoassay.

INTRODUCTION

Chlordiazepoxide hydrochloride, 7-chloro-2-methylamino-5-phenyl- 3H-1, 4-benzodiazepine-4-oxide [I] hydrochloride, is the active ingredient in Librium which is marketed as an antianxiety agent. Metabolic studies in man [1—5] have shown that the compound is biotransformed to form three major metabolites which are present in the blood or plasma; N-desmethylchlordiazepoxide [II], demoxepam [III], and nordiazepam [V] (Fig. 1).

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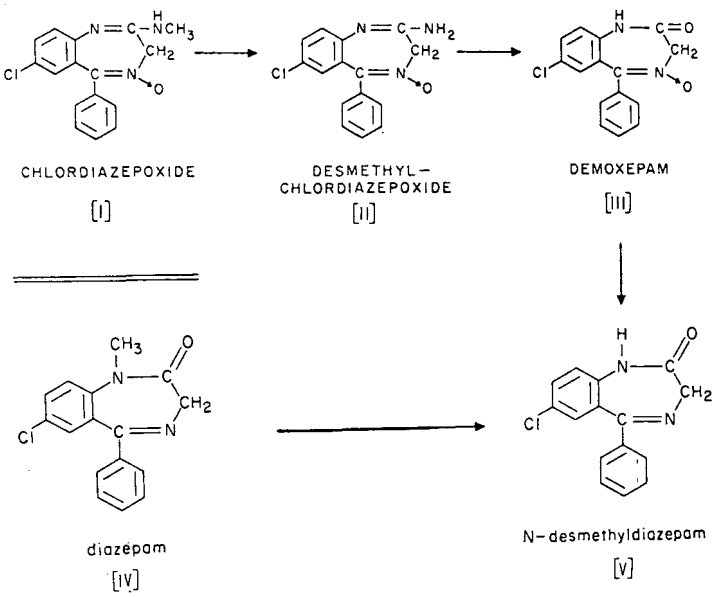

Fig. 1. Chemical structures of compounds I—V.

Spectrofluorometric methods employing selective extraction procedures followed by mild acid hydrolysis and photochemical rearrangement to yield fluorescent derivatives have been used for the specific determination of therapeutic levels of chlordiazepoxide, N-desmethylchlordiazepoxide and demoxepam [1,3]. An electron-capture gas—liquid chromatographic (GLC—ECD) method was reported [6] for the specific determination of intact chlordiazepoxide; however, it does not measure the major metabolites. A differential pulse polarographic (DPP) method was also reported [7] which is capable of measuring therapeutic levels of chlordiazepoxide and its metabolites. It is specific by virtue of thin-layer chromatographic (TLC) separation. Each compound is eluted separately and quantitated by the reduction of the azomethine [$>C_5N_4$-] peak. Recently a radioimmunoassay (RIA) was developed for the specific determination of chlordiazepoxide per se. This assay has been applied in bioavailability studies and to monitor patients undergoing chronic therapy [8]; however, it does not measure any metabolites.

Diazepam, 7-chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4 benzodiazepin-2-one [IV], is the active ingredient in Valium, which is marketed as a psychotropic agent and muscle relaxant. The compound undergoes biotransformation in man by N-demethylation to nordiazepam, the major blood metabolite [9] (Fig. 1).

GLC—ECD [10,11] is the method of choice for measuring blood concentrations of diazepam and nordiazepam following either single or chronic oral dosing, while DPP [12,13] and RIA [14] have proved useful in toxicological analysis.

Densitometric techniques for measuring compounds in situ after TLC [15, 16] either by intrinsic UV absorption [17] or by fluorescence emission [18, 19] have been shown to be rapid and sensitive methods of analysis. An analytical method was developed for chlordiazepoxide, diazepam and their major

metabolites in plasma utilizing reflectance spectrophotodensitometry. The assay involves selective extraction of the compounds into diethyl ether from plasma buffered to pH 9.0 with 1 *M* phosphate buffer. Demoxepam is determined by selective extraction into 0.1 *N* NaOH and photolytic reaction to a quinazoline derivative which is determined by spectrofluorometry. The other benzodiazepines are separated by TLC and then analyzed in situ by spectrophotodensitometry at 260 nm. The assay was used to determine chlordiazepoxide and its metabolites following oral administration of Librium. The TLC assay data showed a significant correlation to those obtained by DPP [7] and to results for chlordiazepoxide obtained by RIA [8]. The assay was also used to determine diazepam and nordiazepam following suspected ingestion of overdoses of Valium. The results were compared to those obtained by DPP and/or GLC—ECD.

EXPERIMENTAL

The reagents used in this assay are the same as those described for DPP analysis [7].

Standard solutions

Weigh out 25.0 mg each of chlordiazepoxide [7-chloro-2-methylamino-5-phenyl-3H-1,4-benzodiazepine-4-oxide, $C_{16}H_{14}N_3OCl$ (mol.wt. = 299.71; m.p. = 236—236.5°)]; N-desmethylchlordiazepoxide [2-amino-7-chloro-5-phenyl-3H-1,4-benzodiazepine-4-oxide, $C_{15}H_{12}N_3OCl$ (mol.wt. = 285.73, m.p. = 255—256°)]; demoxepam [7-chloro-1,3-dihydro-2H-1,4-benzodiazepin-2-one-4-oxide, $C_{15}H_{11}N_2O_2Cl$ (mol.wt. = 286.72, m.p. = 235—236°)]; diazepam [7-chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one; $C_{16}H_{13}ON_2Cl$ (mol. wt. = 284.74; m.p. = 131—135°)]; and nordiazepam [7-chloro-1,3-dihydro-5-phenyl-2H-1,4-benzodiazepin-2-one; $C_{15}H_{11}ON_2Cl$ (mol. wt. = 270.72; m.p. = 216—217°)] into separate 25-ml volumetric flasks and dissolve in 25 ml of methanol to give stock solutions containing 1 mg/ml. Prepare a working solution containing all five compounds by transferring 1 ml of each stock solution into a 100-ml volumetric flask and diluting to volume with acetone to give a working solution containing 10.0 μg of each compound per ml. A fresh working solution should be prepared every two weeks.

TLC separation is performed on E. Merck silica gel F_{254} G-60 TLC plates (manufactured by E. Merck, Darmstadt, G.F.R., and marketed by Brinkmann, Westbury, N.Y., U.S.A.).

Instrumentation

Fluorescence measurements were performed on a Farrand Mark I Spectrofluorometer (Farrand, Valhalla, N.Y, U.S.A.). A TLC spectrophotodensitometer (Model 137190 visual—UV chromatogram analyzer (Farrand) equipped with a 150W d.c. xenon arc energy source was used for in situ spectrophotometric analysis in the 45° reflectance mode [20].

used for in situ spectrophotometric analysis in the 45° reflectance mode [20].

Light source for conversion of demoxepam to fluorescent product. The light source used is a Pyro-Lux R-57 lamp (Luxor, New York, N.Y., U.S.A.), with the samples placed 12 in. from the lamp inside an aluminum foil lined reflector box. UV protective glasses should be worn when working with this lamp.

Instrumental parameters for the chromatogram analyser

Absorption (Vis—UV) Mode. The excitation monochromator was set at 260 nm when assaying for chlordiazepoxide and its metabolites and at 250 nm when assaying for diazepam and its metabolite. The energy is incident upon the plate through the beam condenser lens (200—375 nm) in the exciter leg containing a 0.625-in. reducer (spacer) and no optical filters. The analyzer leg was used with the 200—300 nm lens, in conjunction with a 0.125-in. reducer without an optical filter. A 10-nm entrance slit was used with an RCA 1P-28. photomultiplier. The single beam mode of scanning was used. The chromatoplate was scanned spatially at a rate of 4 in./min in the single-beam recording mode, with the recorder chart-speed also set at 4 in./min.

Calibration. Aliquots of the working solution were applied to each TLC plate to provide known concentrations of each benzodiazepine to calibrate the response of the instrument according to the expected range of the unknowns and to provide a standard for each plate to compensate for inter-plate variability.

Assay in plasma for chlordiazepoxide, diazepam and their metabolites

Procedure. Prior to each analysis pre-develop the necessary number of chromatoplates for 15 to 20 cm ascending in a vapor-saturated chamber using chloroform as the developing solvent to clean the surface of hydrocarbon ("octoils") impurities.

Into a 50-ml glass-stoppered centrifuge tube, add 1 (or 2) ml of blood or plasma sample, 5 ml of 1 *M* potassium phosphate buffer (pH 9.0), and 7 ml of fresh diethyl ether. Along with the samples, process three separate specimens of control blood or plasma containing 0.5, 1.0, and 2.0 μg each of chlordiazepoxide, N-desmethylchlordiazepoxide, demoxepam, diazepam, and nordiazepam as internal standards, prepared by adding 0.05, 0.1, and 0.2 ml of the working standards into 50-ml centrifuge tubes. The solutions are then evaporated to dryness in a 65° water-bath under a stream of nitrogen, and 1 ml of control blood or plasma specimen is added to the residues.

Stopper the tube with a PTFE stopper, shake on a reciprocating shaker (Eberbach, Ann Arbor, Mich., U.S.A.) for 10 min at a moderate speed, and then centrifuge for 5 min at 2300 rpm (1300 *g*) at 0—5° in a refrigerated centrifuge (Model PR-J with a No. 253 rotor; Damon/IEC Division, Needham, Mass., U.S.A.). Carefully remove the diethyl ether layer using a 10 ml serological pipet and transfer into a 50-ml glass-stoppered centrifuge tube. Re-extract the sample with a second 7-ml aliquot of diethyl ether as described above. Combine the second extract with the residue of the first extract in the 50-ml tube.

Assay for demoxepam. Add 4.0 ml of 0.1 *N* NaOH into the 50-ml tube containing the combined ether extracts and extract demoxepam into the alkaline phase. Centrifuge the samples and transfer the ether supernatant into a fresh 15-ml centrifuge tube and evaporate to dryness in a 40° water-bath (Evapomix; Buchler, Fort Lee, N.J., U.S.A.). This residue is used for the analysis of chlordiazepoxide, N-desmethylchlordiazepoxide, diazepam and nordiazepam. While this step is being completed, the NaOH fraction containing demoxepam is processed as follows. The 0.1 *N* NaOH phase is washed once with 10 ml of

ether, centrifuged, the ether removed by aspiration, and the tubes warmed in a 50° bath to expel any residual ether. The tubes are stoppered and exposed to UV light from a Pyro-Lux R-57 lamp contained in an aluminum foil lined reflector box placed in a single row approximately 12 in. from the light source for 20—30 min. Read the fluorescence in a 1-cm path quartz cell in a spectrofluorometer (Farrand Mark I or equivalent) at 450 nm, exciting at 380 nm and determine the demoxepam concentration as described previously [1,3].

Assay for chlordiazepoxide, diazepam and their respective N-desmethyl metabolites. Dissolve the residue of the combined extract containing chlordiazepoxide, diazepam and their respective N-desmethyl metabolites in 100 μl of diethyl ether and transfer quantitatively on to a pre-developed 20 × 20 cm Merck silica gel F_{254} G-60 plate, applying the samples not less than 2.5 cm apart (7 samples per 20-cm plate). Rinse the tube with two successive 50-μl aliquots of ether and transfer on to the chromatoplate. Apply mixtures (0.50 μg and 2.0 μg) of authentic standards of the benzodiazepines to each chromatoplate as reference standards for calibrating the instrument and as markers for locating the benzodiazepines in the biological extracts. Develop the plate twice in a vapor-saturated chamber using chloroform until the solvent front has ascended 15 cm. Air-dry the plate after each development. Then develop the plate using chloroform—acetone (1 : 1) until the solvent front has ascended 15 cm. Air-dry the plate and examine under short-wave UV, and identify the areas on the silica gel corresponding to chlordiazepoxide ($R_F \approx 0.3$), N-desmethylchlordiazepoxide ($R_F \approx 0.15$), diazepam ($R_F \approx 0.6$), and nordiazepam ($R_F \approx 0.5$) by comparison to the R_F of the authentic standards run alongside the sample extracts (Fig. 2).

If the determination of diazepam and nordiazepam is of primary importance then use benzene—*n*-propanol—conc. ammonium hydroxide (80 : 20 : 1) as the developing solvent to achieve optimal resolution of diazepam ($R_F \approx 0.58$) and nordiazepam ($R_F \approx 0.47$) from extracted lipids. Under these conditions the resolution of chlordiazepoxide ($R_F \approx 0.38$), N-desmethylchlordiazepoxide ($R_F \approx 0.24$) and demoxepam ($R_F \approx 0.28$) is unsatisfactory for densitometric analysis.

Spectrodensitometry. Set the absorption range to give 90% full-scale negative deflection on the recorder pen for the 2-μg standard of chlordiazepoxide, with the baseline set at 90—95% full scale on the recorder. Scan the chromatoplate in the absorption mode using the parameters described and record the UV absorption of each compound as symmetrical peaks. The peak area [peak height (centimeters) × width (centimeters) at half-height], which is determined using either the slope baseline technique or electronic digital integration, is proportional to concentration of the benzodiazepine. In order to obtain greater sensitivity, set the absorption range to give 90% full-scale negative deflection for the 0.5 μg standard. Re-scan the chromatoplate as before.

Construct a calibration curve of peak area versus concentration of each of the respective added authentic standards of chlordiazepoxide, diazepam and their respective N-desmethyl metabolites. Determine the concentration of these compounds in the unknowns by interpolation. Determine the overall recovery of the added authentic standards of these compounds by comparison of the absorption (as peak area) of the respective benzodiazepines recovered from

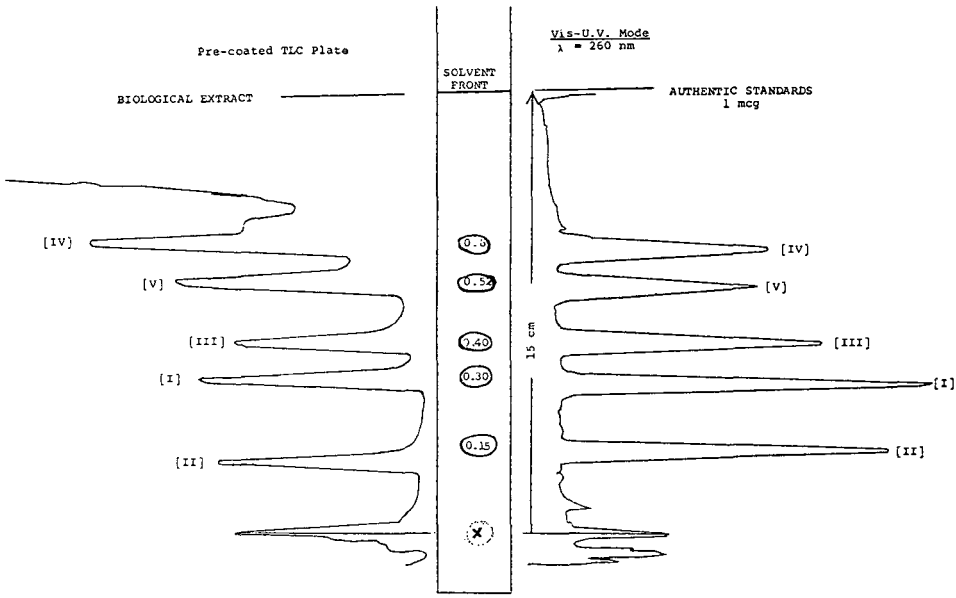

Fig. 2. TLC separation and spectrophotodensitometric analysis of compounds I—V. The chromatoplate was developed twice in chloroform to move endogenous lipids up to the solvent front, followed by a final development in chloroform—acetone (1 : 1) to move the compounds.

plasma against that of the respective authentic standards scanned on the chromatoplate. Percent recovery should be determined routinely as a check on the reproducibility of the assay. After spectrodensitometry, the sample areas may be scraped, eluted and analyzed by other techniques such as either spectrofluorometry [3] or DPP [7].

RESULTS AND DISCUSSION

Analytical parameters

The Farrand spectrophotodensitometer used can be operated only in the reflectance mode [20] and not in the transmission mode, which is utilized in the Schoeffel and Zeiss spectrophotodensitometers. The most significant advantages of spectrophotodensitometry in the reflectance mode are the minimization of energy losses due to internal absorption or self-quenching due to the thickness of the silica gel bed and transmission losses through a glass plate [15,16].

Commercially available TLC plates of 250-μm bed thickness and 60-μm particle-size silica gel G gave reproducible data with respect to peak area measurement of varying concentrations of the benzodiazepines on the same chromatoplate and between chromatoplates. In addition, the background was more uniform than that found on soft gel plates (such as the Quantum TLC plates previously used [7]) resulting in less background noise and baseline drift.

The response of the TLC spectrophotodensitometer to each benzodiazepine was determined at 5-nm intervals from 210 nm to 350 nm, and is plotted in

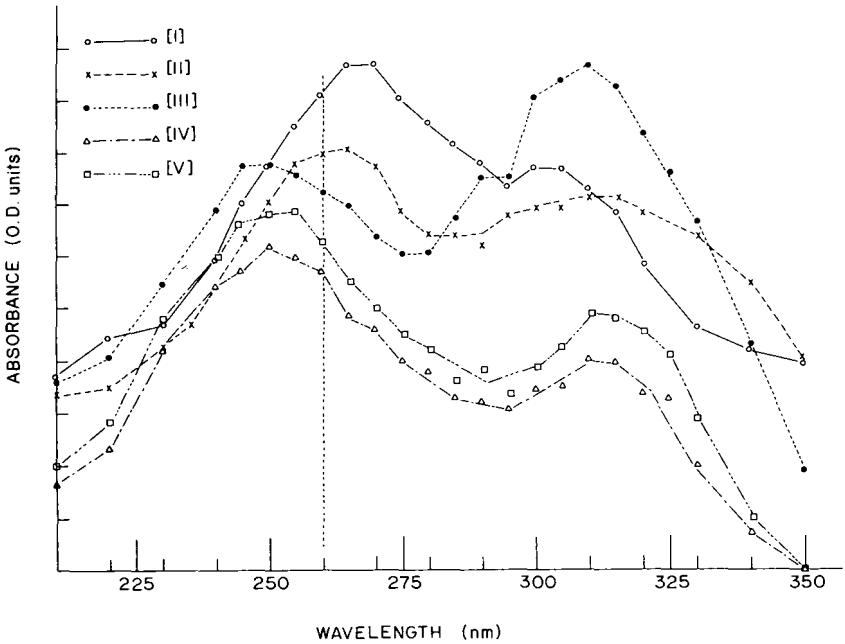

Fig. 3. UV absorption spectra of compounds I—V adsorbed on silica gel containing fluorescent indicator.

not be determined by automatic scanning because the reflected energy increased rapidly between 200 nm and 300 nm. This required continual re-setting of the baseline offset in both the single beam mode and in the double beam ratio mode. The optimal wavelength for the quantitation of the benzodiazepines was chosen to be 260 nm.

Although demoxepam is resolved from the other benzodiazepines using chloroform—acetone (1 : 1) as the developing solvent system ($R_F \approx 0.40$) (Fig. 3), a UV-absorbing impurity present in certain plasma samples gave erroneously high values for demoxepam by spectrophotodensitometry as compared to DPP analysis following elution of the same spot. Consequently, demoxepam is determined prior to TLC by selective extraction into 0.1 *N* NaOH, conversion to a quinazolinone derivative and quantitation by spectrofluorometry as described previously [1,3].

The recovery of chlordiazepoxide, N-desmethylchlordiazepoxide, diazepam and nordiazepam is 75 ± 10%, 72 ± 6%, 92 ± 8%, and 95 ± 5%, respectively with sensitivity limits in the range of 0.05 to 0.20 μg/ml by spectrophotodensitometry using a 1-ml plasma specimen per assay. The recovery of demoxepam is 95 ± 5% with a sensitivity limit by spectrofluorometry of 0.1 to 0.2 μg/ml using a 1-ml plasma specimen per assay.

The sensitivity of the spectrophotodensitometric method can be optimized for diazepam and nordiazepam by setting the excitation monochromator at 252 nm. In addition, a better separation may be obtained for diazepam ($R_F \approx 0.58$) and nordiazepam ($R_F \approx 0.47$) from the extracted lipid materials by using benzene—*n*-propanol—conc. ammonium hydroxide (80 : 20 : 1) as the devel-

oping solvent system. However, the resolution of chlordiazepoxide, N-desmethylchlordiazepoxide and demoxepam ($R_F \approx 0.38$, 0.24 and 0.28, respectively) is unsatisfactory. The sensitivity of the assay for diazepam and nordiazepam is not sufficient for the reliable determination of therapeutic levels following single or repeated administration of diazepam [21]* but it sufficient for the determination of blood levels of diazepam and nordiazepam resulting from overdosages of diazepam.

Spectrofluorodensitometry

Quantitation of drugs by fluorodensitometry in situ using either the intrinsic fluorescence of the compound or of a suitable fluorescent derivative is often a very useful means of increasing the sensitivity of the determination [18,19]. The tricyclic antidepressant drugs such as amitriptyline, nortriptyline and imipramine can be determined by spectrofluorodensitometry by spraying the chromatoplate with 10% perchloric acid, and heating the plate at 110° (oven) to generate the fluorescence [17—19]. The formation of fluorescent derivatives of drugs in situ at elevated temperatures by reaction with NH_4HCO_3 at 140—150° was also reported [22]. The qualitative detection of the benzodiazepines on ethanol-saturated TLC plates utilizing their intrinsic luminescence behavior at ambient and cryogenic temperature has been reported [23].

The benzodiazepines I—V were subjected to the above acidic and basic reactions reported for other compounds [18,19,22] and the chromatoplates were examined in the chromatogram analyzer using the fluorescence mode (a 0.500-in. aperture reducer and a No. 7-54 optical filter were inserted into the cylindrical illumination lens assembly. The emission or analyzer monochromator was attached to the analyzer leg, and a No. 3-73 optical filter was used with the 300—800 nm transmission lens in the analyzer leg without any reducers. A RCA 1P-21 photomultiplier was used in conjunction with 10-nm slits at the analyzer).

The fluorescent products formed under both acidic and basic reaction conditions had similar excitation (380 nm) and emission (475 nm) maxima suggesting the formation of the same product, i.e., quinazolines and quinazolinones which possess similar excitation and emission maxima [23].

Although chlordiazepoxide and its metabolites formed fluorescent products in situ under both acidic and basic reaction conditions, the sensitivity limits of detection were no better than that using direct UV spectrophotodensitometry (260 nm), hence fluorodensitometry did not offer any advantage over the former procedure.

Application of the method to biological specimens

Plasma samples from two subjects who had received single 30 mg oral doses of Librium and a third subject who received 10-mg oral doses of Librium at 0, 4, and 8 h (30 mg total) were assayed for intact chlordiazepoxide, by spectrophotodensitometry, by RIA [8] and by DPP [7] respectively. The concentrations of N-desmethylchlordiazepoxide and nordiazepam were also assayed by spectrophotodensitometry and by DPP. Demoxepam was assayed only by

Editor's note: Therapeutic levels of diazepam in man are reported to be between 100 and 200 ng/ml, following chronic oral administration of 10-mg doses.

spectrofluorometry. Comparison of the results obtained by each method (Tables I—III) gave satisfactory agreement for chlordiazepoxide and N-desmethylchlordiazepoxide. The concentration of N-desmethylchlordiazepoxide for subject No. 2 is below the limits of sensitivity either by spectrophotodensitometry or by DPP.

The 36-h sample of subject No. 3 had the only concentration of demoxepam (0.18 μg/ml) which was measurable by spectrofluorometry. All the other samples assayed gave readings below the sensitivity limits of the assay for this compound. Neither spectrophotodensitometry nor DPP detected the presence of nordiazepam.

The data presented here are in agreement with the pharmacokinetics and biopharmaceutics of chlordiazepoxide in man as reported by Boxenbaum et al. [24].

The joint determinations of chlordiazepoxide and its N-desmethyl metabolite were subjected to straight-line analysis [25]. The results are presented in Table IV. In comparing the TLC spectrophotodensitometric results to those of the other methods the fitted intercepts and slopes were not significantly different ($p \leqslant 0.05$) from 0 and 1 respectively, with high correlation coefficients in each case. Therefore, the plasma concentrations of chlordiazepoxide measured by either spectrophotodensitometry, RIA, or DPP are equivalent while the plasma concentrations of N-desmethylchlordiazepoxide measured by either spectrophotodensitometry or by DPP are also equivalent.

TABLE I

PLASMA LEVELS OF CHLORDIAZEPOXIDE AND ITS N-DESMETHYL METABOLITE FOLLOWING ORAL ADMINISTRATION OF A SINGLE 30-mg DOSE OF LIBRIUM TO SUBJECT No. 1

Nil = Below sensitivity limits, (⩽0.02 μg/ml for RIA; ⩽0.03—0.10 μg/ml for TLC—SPD; ⩽0.05—0.10 μg/ml for DPP); N.A. = not analyzed.

Time after	Chlordiazepoxide (μg/ml)			N-Desmethylchlordiazepoxide (μg/ml)	
dose (h)	Radio-immuno-assay	TLC—spectro-densito-metry	Differ-ential pulse polaro-graphy	Spectro-densito-metry	Differential pulse polarography
0.0	Nil	Nil	Nil	Nil	Nil
0.25	0.15	0.20	0.14	Nil	Nil
0.5	0.82	0.90	0.85	0.08	Nil
0.75	1.14	1.15	1.10	0.12	N.A.
1.0	1.22	1.22	1.30	0.18	N.A.
1.5	1.38	1.04	1.32	0.20	0.20
2	1.03	1.07	1.15	0.18	0.18
3	0.88	1.07	1.02	0.30	0.30
4	0.88	1.00	1.00	0.26	0.30
8	0.67	1.00	0.96	0.40	0.30
12	0.60	0.80	0.54	0.40	0.38
24	0.24	0.26	0.19	0.50	0.30
30	0.17	0.15	0.10	0.45	0.30
36	0.11	0.07	0.06	0.24	0.26
48	0.05	0.05	N.A.	0.23	0.19
72	Nil	Nil	Nil	0.13	0.12

TABLE II

PLASMA LEVELS OF CHLORDIAZEPOXIDE FOLLOWING ORAL ADMINISTRATION OF A SINGLE 30-mg DOSE OF LIBRIUM TO SUBJECT No. 2

Nil = Below sensitivity limits (≤ 0.02 μg/ml for RIA; ≤ 0.03—0.10 μg/ml for TLC—SPD; ≤ 0.05—0.10 μg/ml for DPP); N.A. = not analyzed

Time after dose (h)	Chlordiazepoxide (μg/ml)		
	Radioimmunoassay	TLC—spectrodensitometry	Differential pulse polarography
0.0	Nil	Nil	Nil
0.5	1.72	1.30	1.50
1.0	1.38	1.20	1.18
1.5	1.14	1.00	1.27
2.0	1.30	1.00	1.00
3.0	1.14	0.85	0.91
4.0	1.10	0.87	0.91
5.5	1.00	0.81	0.85
7.0	0.90	0.82	1.09
8	1.04	0.72	1.09
9.5	0.80	0.83	0.74
11	1.00	0.71	1.00
13	0.96	0.72	0.59
15	0.85	0.61	0.68
24	0.53	0.54	0.50
30	0.74	0.61	0.59
36	0.72	0.62	NA
48	0.62	0.43	0.41
72	0.26	0.30	NA

Plasma and serum samples from subjects suspected of ingesting an overdose of Valium were assayed for diazepam and nordiazepam by the spectrophotodensitometric assay (optimized for these compounds). Comparison of these results to those obtained either by DPP or GLC—ECD (Table V) gave satisfactory agreement.

CONCLUSIONS

The sensitivity and specificity of the spectrodensitometric assay renders it suitable either for monitoring plasma levels obtained following single or chronic oral administration of chlordiazepoxide hydrochloride, or as a rapid toxicological procedure in determining cases of overdosage of either chlordiazepoxide hydrochloride or diazepam. In addition, it is more rapid than any of the existing assay procedures [1,3,6—8,13]. The assay is non-destructive and the compounds may be eluted for further characterization. Alternatively, spray tests for the unambiguous identification of the compounds may be carried out on the plate [18,19,23].

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TABLE III

PLASMA LEVELS OF CHLORDIAZEPOXIDE AND ITS N-DESMETHYL METABOLITE FOLLOWING ORAL ADMINISTRATION OF A 10-mg DOSE OF LIBRIUM (3-times a day) AT 0,4, AND 8 h TO SUBJECT No. 3

Nil = Below sensitivity limits ($\leq$0.02 μg/ml for RIA; $\leq$0.03—0.10 μg/ml for TLC—SPD; $\leq$0.05—0.10 μg/ml for DPP).

Time (h)	Chlordiazepoxide (μg/ml)			N-Desmethylchlordiazepoxide (μg/ml)	
	Radio-immuno-assay	TLC—spectro-densito-metry	Differ-ential pulse polaro-graphy	TLC—spectro-densito-metry	Differential pulse polarography
0.0*	Nil	Nil	Nil	Nil	Nil
0.5	Nil	Nil	Nil	Nil	Nil
1.0	0.32	0.26	0.24	Nil	Nil
1.5	0.22	0.27	0.14	Nil	0.05
2	Nil	0.21	0.20	Nil	Nil
3	Nil	0.20	0.20	Nil	Nil
4*	Nil	0.21	0.19	0.04	0.05
5.5	0.40	0.42	0.33	0.08	0.05
7	0.64	0.39	0.40	0.10	0.05
8*	0.63	0.40	0.42	0.10	0.15
9.5	Nil	0.34	0.35	0.11	0.15
11	Nil	0.37	0.26	0.15	0.14
13	0.51	0.40	0.53	0.19	0.17
15	0.67	0.50	0.51	0.23	0.22
24	0.40	0.25	0.33	0.23	0.20
30	0.37	0.26	0.23	0.34	0.33
36	0.46	0.20	0.13	0.28	0.27
48	0.25	0.08	Nil	0.17	0.18
72	Nil	Nil	Nil	0.07	0.08

*Aministration of 10 mg of Librium

TABLE IV

RESULTS OF STRAIGHT-LINE ANALYSIS OF THE JOINT DETERMINATIONS

SPD = Spectrophotodensitometry; RIA = radioimmunoassay; DPP = differential pulse polarography.

Compound	Methods compared	Range of levels compared (μg/ml)	Intercept (μg/ml)	Slope	Correlation coefficient
Chlordiazepoxide	SPD versus RIA	0.05—1.72	0.081	1.00	0.914
Chlordiazepoxide	SPD versus DPP	0.05—1.72	0.061	1.11	0.957
N-Desmethylchlordiazepoxide	SPD versus DPP	0.04—0.50	0.022	0.786	0.897

TABLE V

PLASMA AND SERUM CONCENTRATIONS OF DIAZEPAM AND ITS N-DESMETHYL METABOLITE FOLLOWING SUSPECTED INGESTION OF OVERDOSES OF VALIUM

Case No.	Biological fluid	Diazepam (μg/ml)		N-Desmethyldiazepam (μg/ml)	
		Spectrophoto-densitometry	Level found previously	Spectrophoto-densitometry	Level found previously
528	Plasma	0.90	1.0*	0.2	0.29*
536	Serum	0.35	0.51**	0.85	0.67**
538 A	Plasma	1.80	2.01**	3.55	3.90**
538 B	Plasma	2.00	1.96**	3.75	3.30**
538 C	Plasma	1.45	1.41**	3.90	2,89**

* Differential pulse polarography.

** Electron-capture gas—liquid chromatography.

the drawings of the figures presented. Karl Bratin was a 1975 A.C.S. Summer Intern, from Clarkson College, Potsdam, N.Y.

REFERENCES

1 B.A. Koechlin and L. D'Arconte, Anal. Biochem., 5 (1963) 195.
2 B.A. Koechlin, M.A. Schwartz, G. Krol and W. Oberhansli, J. Pharmacol. Exp. Ther., 148 (1965) 399.
3 M.A. Schwartz and E. Postma, J. Pharm. Sci., 55 (1966) 1368.
4 M.A. Schwartz and E. Postma, J. Pharm. Sci., 61 (1972) 123.
5 W.R. Dixon, M.A. Brooks, E. Postma, M.R. Hackman, S. Spector, J.D. Moore and M.A. Schwartz, Clin. Pharmacol. Ther., 20 (1976) 450.
6 I.A. Zingales, J. Chromatogr., 61 (1971) 237.
7 M.R. Hackman, M.A. Brooks, J.A.F. de Silva and T.S. Ma, Anal. Chem., 46 (1974) 1975.
8 W.R. Dixon, J. Earley and E. Postma, J. Pharm. Sci., 64 (1975) 937.
9 M.A. Schwartz, B.A. Koechlin, E. Postma, S. Palmer and G. Krol, J. Pharmacol. Exp. Ther., 149 (1965) 423.
10 J.A.F. de Silva and C.V. Puglisi, Anal. Chem., 42 (1970) 1725.
11 J.A.F. de Silva, I. Bekersky, C.V. Puglisi, M.A. Brooks and R.E. Weinfeld, Anal. Chem., 48 (1976) 10.
12 E. Jacobsen, T.V. Jacobsen and T. Rojahn, Anal. Chim. Acta, 64 (1973) 473.
13 M.A. Brooks and J.A.F. de Silva, Talanta, 22 (1975) 849.
14 B. Peskar and S. Spector, J. Pharmacol. Exp. Ther., 186 (1973) 167.
15 E.J. Shellard (Editor), Quantitative Paper and Thin-Layer Chromatography, Academic Press, London, New York, 1968.
16 J.C. Touchstone (Editor), Quantitative Thin-Layer Chromatography, Wiley-Interscience, London, New York, 1973.
17 U. Breyer and K. Villumsen, Europ. J. Clin. Pharmacol., 9 (1976) 457.
18 D.B. Faber and W.A. Man in 't Veld, J. Chromatogr., 93 (1974) 238.
19 D.B. Faber, C. Mulder and W.A. Man in 't Veld, J. Chromatogr., 100 (1974) 55.
20 M. Gurkin and S. Cravitt, Amer. Lab., 4, No. 1 (1972) 54.
21 S.A. Kaplan, M.L. Jack, K. Alexander and R.E. Weinfeld, J. Pharm. Sci., 62 (1973) 1789.
22 R. Segura and A.M. Gotto, Jr., J. Chromatogr., 99 (1974) 643.
23 J.A.F. de Silva, N. Strojny and K. Stika, Anal. Chem., 48 (1976) 144.
24 H.G. Boxenbaum, K.A. Geitner, M.L. Jack, W.R. Dixon, H.E. Spiegel, J. Symington, R. Christian, J.D. Moore, L. Weissman and S.A. Kaplan, J. Pharmacokinet. Biopharm., 5 (1977) 3.
25 M. Kendall and S. Stuart, The Advanced Theory of Statistics, Vol. 2, Hafner, New York, 1961, pp. 397—405.

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CHROMBIO. 040

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF DIANHYDROGALACTITOL IN PLASMA BY DERIVATIZATION WITH SODIUM DIETHYLDITHIOCARBAMATE

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SUMMARY

A high-performance liquid chromatographic method is described for measuring submicrogram quantities of dianhydrogalactitol, a promising anti-neoplastic agent, in plasma. The drug is derivatized directly in plasma with sodium diethyldithiocarbamate to form a bis(dithiocarbamoyl) ester which absorbs UV light at 254 nm (a_m 17,000). The derivatized product is then extracted quantitatively into chloroform and separated by normal phase chromatography (μBondpak CN column). Dianhydrogalactitol concentration below 50 ng/ml of plasma can be detected in the eluent.

INTRODUCTION

Alkylating agents, consisting specifically of epoxides and nitrogen mustards, form a class of chemotherapeutic agents used in the control of cancer. The relatively high reactivity of such molecules toward nucleophiles and their lack of chromophoric intensity in the UV region limits the analytical methodology available for their determination at theurapeutic levels in biological samples. Capabilities for monitoring drug in various body fluid and tissue samples are necessary to evaluate efficiently new drugs and drug therapy.

One member of this group of anti-tumor agents is 1,2:5,6-dianhydrogalactitol (DAG), a hexitol diepoxide, currently in Phase I and II clinical evaluation. This agent is one of few active drugs capable of crossing the blood—brain barrier [1] and has shown potential utility in treatment of malignancies of the central nervous system.

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Initial pharmacokinetic studies of DAG distribution were carried out in mice [2] and rats [3]. Drug disposition was monitored radiochemically, by measuring total radioactivity in body fluid samples at timed intervals after administration of radio-labeled drug to test species. Although this method provided qualitative indications of drug distribution, its lack of specificity failed to distinguish among parent drug, metabolites and products formed as a result of non-enzymatic reaction of the epoxide ring with tissue nucleophiles.

More recently a gas chromatographic procedure was developed [4] for determination of submicrogram levels (sensitivity about 100 ng/ml) of DAG in biological fluids. It is based on extraction of the parent drug from potassium carbonate-saturated plasma with isopropanol, conversion to the corresponding *n*-butaneboronic ester and chromatography on a SE-30 column. One disadvantage of this method lies in the necessity for extracting the drug with very polar solvents, since undesired tissue components are co-extracted. Secondly, the method is somewhat time-consuming and requires considerable sample manipulation. Finally, the proclivity for DAG to covalently bind to blood components [2, 4] necessitates the rapid centrifugation of whole blood samples and storage of plasma at -40° prior to analysis.

An analytical method was sought in which chemical derivatization of DAG could be carried out directly in biological fluids to (1) stabilize the drug and prevent its loss due to binding with blood components after drawing of samples, (2) form a more hydrophobic derivative extractable into water-immiscible solvents, which are less likely to co-extract potential interfering species, and (3) form a derivative which strongly absorbs UV light at 254 or 280 nm. A high-performance liquid chromatographic (HPLC) method for DAG determination in plasma is described based on prior derivatization of drug with diethyldithiocarbamic acid, which specifically reacts at electrophilic sites.

EXPERIMENTAL

Apparatus

Chromatography was performed on a component system consisting of a Waters Assoc. (Milford, Mass., U.S.A.) Model 600-A solvent delivery system, Model U-6K injector and Model 440 dual channel absorbance detector operated at 254 nm. A 30 cm × 1/4-in. O.D. μBondpak CN column (Waters Assoc.) was used for all separations.

Materials

Crystalline dianhydrogalactitol (m.p. 98—100°) was obtained from the National Cancer Institute (Bethesda, Md., U.S.A.) and used without further purification. Sodium diethyldithiocarbamate (DDTC) was purchased from Sigma (St. Louis, Mo., U.S.A.). Fresh whole blood was obtained from healthy Beagle dogs. Dated human plasma was obtained from the Community Blood Center, Kansas City, Mo., U.S.A.

Derivatization

A 1-ml volume of plasma containing 0.05—50 μg of DAG was placed in a 15-ml centrifuge tube, to which were added 0.5 ml of 0.1 *M* potassium phosphate

buffer (pH 7.0), 0.5 ml of a 5% (w/v) aqueous solution of DDTC (prepared fresh daily), and 2 ml of water. The mixture was sealed with a PTFE-lined screwcap and allowed to stand at room temperature (22—25°) for 1 h.

Extraction

The plasma mixture was then extracted with 10 ml of chloroform for 3 min, and the system centrifuged at 1200 *g* for 5 min. The aqueous layer was discarded and the chloroform layer washed with 2—5-ml portions of a 33% (w/v) sodium chloride solution to remove protein distributed in the organic layer. An 8-ml aliquot of the washed chloroform solution was removed, evaporated to dryness at 40° and the residue dissolved in 200 μl of heptane—chloroform (7:3; v/v) solution.

Chromatography

In all runs, 10 μl of the solution containing derivatized DAG was injected. The mobile phase, heptane—chloroform (7:3) containing 1.2% acetic acid, was pumped through the column at a flow-rate of 2.5 ml/min.

Synthesis of 1,6-bis(diethyldithiocarbamoyl)-2,3,4,5-tetrahydroxyhexane

DAG (400 mg) and DDTC (1.0 g) were dissolved in 25 ml of 0.02 *M* phosphate buffer (pH 7.4). An immediate precipitate formed which was filtered after the mixture had been stirred for 1 h to yield 1 g (82%) of white solid (m.p. 145—147°). The product was established to be the bis adduct based on elemental analysis (calculated for $C_{16}H_{32}N_2O_4S_4$: C 43.24%, H 7.20%, N 6.31%; found: C 43.09%, H 7.15%, N 5.95%).

Synthesis of the bisacetonide derivative of 1,6-bis(diethyldithiocarbamoyl)-2,3,4,5-tetrahydroxyhexane [5]

1,6-Bis(diethyldithiocarbamoyl)-2,3,4,5-tetrahydroxyhexane (0.5 mmole; 222 mg) was dissolved in 5 ml of acetone, to which was added 2,2-dimethoxypropane (4 mmoles; 410 mg) and bis(*p*-nitrophenyl)phosphate (0.6 mmole; 204 mg). The mixture was stirred at room temperature for 1 h. A white precipitate formed which was collected by filtration to yield 260 mg (99%) of white solid (m.p. 119—121°). Analysis calculated for $C_{22}H_{40}N_2O_4S_4$: C 50.38%, H 7.60%, N 5.32%, found: C 50.28%, H 7.61%, N 5.28%.

RESULTS

Derivatization

DAG, like many chemotherapeutic alkylating agents, fails to absorb light above 210 nm. Therefore, development of a sensitive HPLC method for this compound which utilizes spectrophotometric monitoring of the effluent requires chemical derivatization of the drug with a chromophore-producing reagent prior to chromatography. DDTC was selected since it was found to react with DAG in aqueous, hydro-alcoholic or alcoholic solution to yield a product with significant UV absorbance at 254 nm (a_m = 17,000). The reaction was also found to take place directly in plasma containing 0.05 to 50 μg/ml of DAG, and therefore plasma samples could be derivatized without prior extrac-

tion of drug. A 5% solution of DDTC produced maximum reaction with this concentration range of DAG in 60 min, when the reaction was run at room temperature (22–25°) (Fig. 1). A less concentrated solution (1%) of DDTC produced a similar response, but reaction time needed to be increased significantly. Reaction rate could be accelerated significantly by heating the mixture, although maximum yield was not effected (i.e. at 80° and 50° reaction was complete in less than 10 min and 30 min, respectively). For convenience, reactions were carried out at room temperature with 5% reagent for 1 h. Since the DDTC–DAG adduct was stable in plasma for more than 6 h without measurable deterioration of the product, the isolation and work-up of the adduct from plasma samples was not critically time-dependent after reaction was complete.

Extraction

The plasma, containing DAG–DDTC adduct was extracted once with 2.5 volumes of chloroform. Quantitative extraction of adduct was observed. Diethyl ether, ethyl acetate, dichloromethane and 1,2-dichloroethane were also examined as potential extractants but failed to show extraction efficiency comparable to chloroform.

Chromatography

Fig. 2a shows a chromatogram of the extracted DAG–DDTC adduct using the analytical conditions outlined above. Elution time to the UV-detectable peak was 5.0 min, corresponding to an elution volume of 12.5 ml. No background interference was incurred at these retention times with drug-free plasma samples subjected to the assay (Fig. 2b).

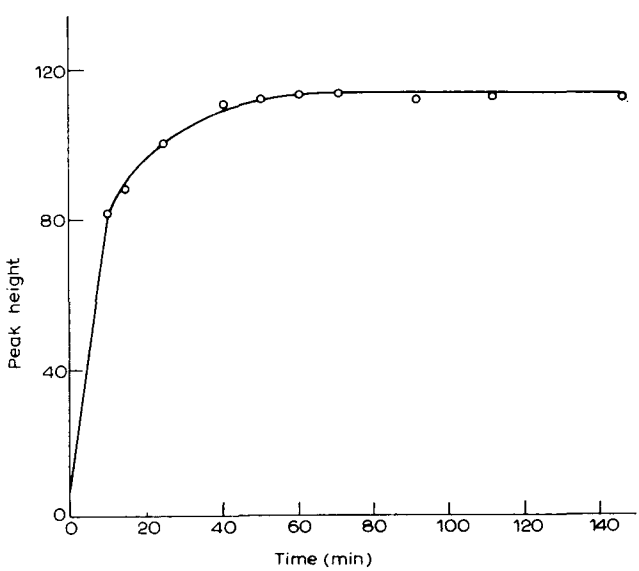


Fig. 1. Effect of reaction time on the conversion of DAG to its bis(diethyldithiocarbamoyl) ester by reaction of drug (10 μg) with 5% DDTC solution at 22°, as described in the text.

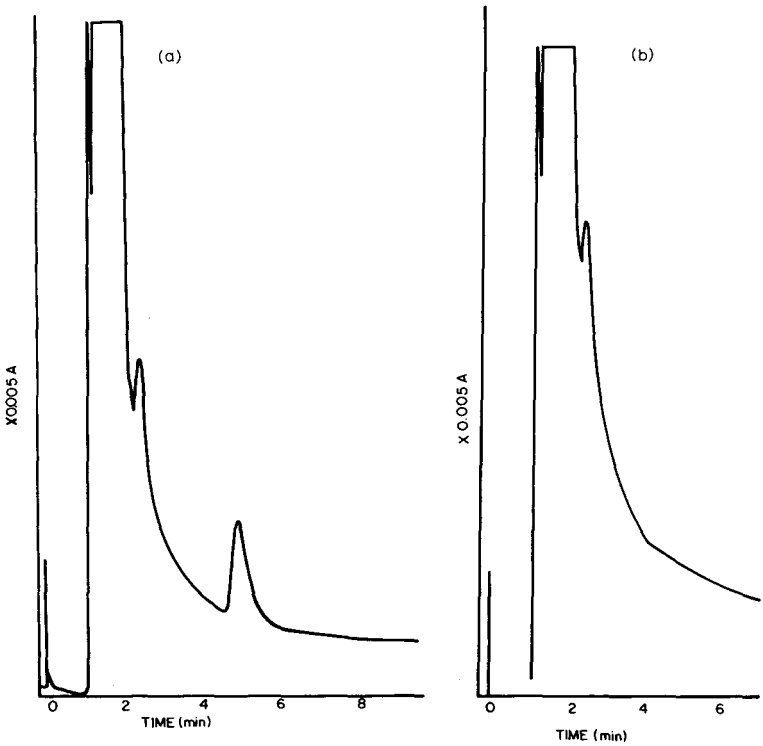


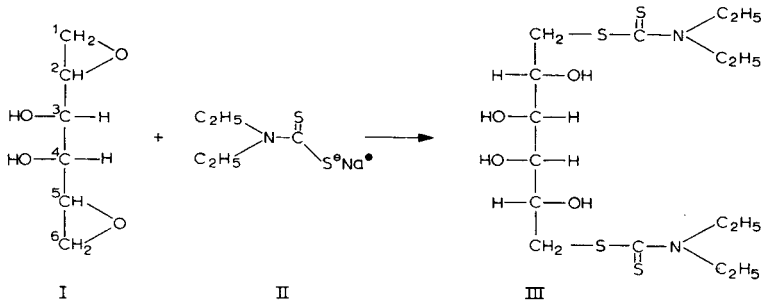
Fig. 2. Chromatograms of (a) the bis(diethyldithiocarbamoyl) ester of DAG obtained by carrying out the methodology described in the text on a 1-ml plasma sample containing 250 ng of DAG and (b) a drug-free plasma sample subjected to the assay.

Standard curves and sensitivity

A standard curve was prepared by carrying out the analysis on fourteen plasma samples to which had been added DAG at different concentrations ranging from 0.05 to 50 μg/ml. Over this concentration range, chromatogram peak heights were related linearly to DAG concentration. Linearity of response was determined by least-squares analysis of data points: slope 0.191, intercept -1.895, correlation coefficient 0.999. Detection limits were approximately 50 ng/ml of plasma, and the precision of the method 1—2% (as determined for duplicate samples).

Characterization of the derivatization reaction

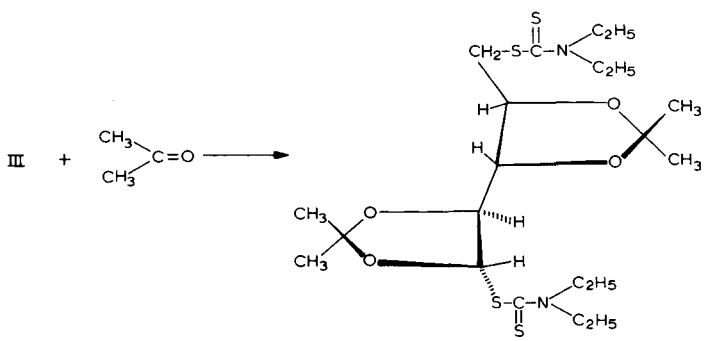
The derivatization reaction involved covalent addition of 2 moles of diethyldithiocarbamate (II) to the epoxides of DAG (I) to form an α-hydroxydithiocarbamoyl ester (III). The stoichiometry of the reaction, was confirmed by elemental analysis and from the mass spectrum of the adduct. Reaction can theoretically take place at either carbon of the oxirane skeleton, although in the absence of acid catalysis, it usually occurs at the least hindered carbon. The product was therefore assumed to be 1,6-bis(diethyldithiocarbamoyl)-2,3,4,5-tetrahydroxyhexane, III.



(1)

I II III

To verify that reaction of II with DAG occurs at the terminal carbons (C-1 and C-6) and exclude the possibility that nucleophilic attack takes place at C-2 or C-5, III was reacted with acetone. Under the conditions of the reaction, acetone reacts with hydroxyl groups on adjacent carbon atoms in the *threo*-configuration to gove fivemembered cyclic acetals [5]. Thus D-galactitol gives rise to only 2,3:4,5- and 2,3:5,6-isopropylidene derivatives. 1,3-Dihydroxy compounds (such as would be formed by reaction of II with DAG at C-2 or C-5) fail to react under these conditions to form dioxanes. The stoichiometry of the reaction with DAG was determined from elemental analysis to involve condensation of 2 moles of acetone per mole of III. The NMR spectrum of the product revealed the ratio of methyl to methylene and methine proton to be 24:16. The product of the reaction with acetone is therefore IV; thus confirming the structure of the DAG—DDTC adduct proposed in eqn. 1 as III.



(2)

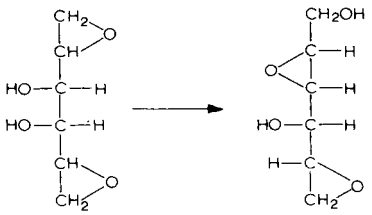
IV

HPLC analysis of the reaction mixture in which the acetonide is formed failed to reveal any underivatized DAG—DDTC adduct, indicating that the adduct is apparently pure III, and not a mixture of products generated by attack at combinations of C-1, C-2, C-5 and C-6. Also the absence of multiple peaks from the DAG—DDTC adduct, itself, lends support to the formation of the single product, III.

DISCUSSION

DAG can be chemically derivatized by reaction at either the hydroxyl or

epoxide functions. In either case, derivatization should enhance the stability of the molecule by preventing intramolecular rearrangement of the parent (eqn. 3).



(3)

Reaction with hydroxyl groups would involve the use of an electrophilic derivatizing agent. Because of the large number of nucleophile-containing molecules normally present in plasma, derivatization could not be carried out directly in biological fluid. In addition, most of these reagents are sensitive to water. The reagent would be consumed in reaction with many different compounds, introducing potential interferences at all subsequent stages of analysis, as well as requiring the use of very large excesses of reagent. Although this problem might be somewhat circumvented by prior extraction of DAG into an organic solvent, its hydrophilic nature requires the use of highly polar extraction solvents. Such solvents are non-specific, in that co-extraction of undesired materials occurs, and the aforementioned interferences are still encountered.

Expoxides react with nucleophilic compounds. Unlike the electrophilic reagents, relatively few endogenous substances or drugs react rapidly with nucleophiles. Reaction can therefore be carried out directly in plasma without the reagent being consumed in potentially-interfering side reactions with other substances. Accordingly, DAG was derivatized by reacting the epoxide with a suitable nucleophile directly in plasma. In addition, destruction of the epoxide ring in plasma by the derivatizing agent may protect DAG from covalent binding to blood components [2, 4].

Although mercaptides were initially examined as potential derivatizing agents because of their high nucleophilicity, their proclivity for facile oxidation to disulfides makes their use as clinical reagents impractical. Sulfur-containing nucleophiles other than mercaptans have not been exploited as analytical reagents. Dithiocarbamates were selected as derivatizing agents since they retain this high nucleophilicity, are often water soluble and are much less susceptible to oxidation. In addition, the aliphatic derivatives absorb UV light maximally at 254 and 283 nm, which is compatible with HPLC detectors. The reagent should be prepared freshly each day, but is relatively stable above pH 6. Below this pH, the molecule decomposes with evolution of CS_2 [6, 7]. The reagent will form chloroform-extractable chelates with heavy metals [7, 8], but these did not interfere with the assay.

Dithiocarbamic acids appear to be useful reagents for derivatization of alkylating agents prior to their HPLC analysis, derivatization taking place directly in plasma. In the case of DAG, a bis(dithiocarbamoyl) ester is formed, increasing the hydrophobicity of the molecule, so that it can be extracted into chloroform. The parent drug cannot be extracted into water-immiscible solvents, but must be extracted from salt-saturated solution with isopropanol [4], which presents a number of disadvantages as an extractant.

Although reactions of nucleophiles with epoxides potentially yield mixtures of addition products, derivatization of DAG with DDTC gave a single product formed by nucleophilic attack at the terminal carbons of DAG. Therefore, the concentration of DAG could be related to the integrated intensity of a single chromatographic peak, simplifying analysis and enhancing the sensitivity of the method. Early in the investigation, *p*-nitrobenzylpyridine was studied as a potential derivatizing agent. However, reaction with DAG produced a mixture of products, apparently resulting from indiscriminate attack of the pyridine nitrogen at C-1, C-2, C-5 and C-6. Reaction at C-2 or C-5 creates new asymmetric centers in the molecule so that diastereomeric pairs are generated. All of these products are resolved by HPLC, so that DAG appears in the chromatogram as a sequence of 6—8 peaks.

In conclusion, a HPLC method for clinical analysis of DAG is described, based on initial derivatization of the drug directly in plasma with DDTC. The general applicability of this derivatization sequence to the analysis of alkylating agents in biological samples is under investigation.

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REFERENCES

1 R.I. Geran, G.F. Congleton, L.E. Dudeck, B.J. Abbott and J.L. Gargus, Cancer Chemother. Rep., Part 2, 4 (1974) 53.
2 L. Institóris, É. Dzurillay and G. Pethes, Z. Krebsforsch., 79 (1973) 49.
3 V.A. Levin, M.A. Freeman-Dove and C.E. Maroten, J. Nat. Cancer Inst., 56 (1976) 535.
4 T. Kimura, L.A. Sternson and T. Higuchi, Clin. Chem., 22 (1976) 1639.
5 J.A. Mills, Advan. Carbohyd. Chem., 10 (1955) 1.
6 P. Zuman and R. Zahradnik, Z. Phys. Chem., 208 (1957) 135.
7 G.D. Thorn and R.A. Ludwig, The Dithiocarbamates and Related Compounds, Elsevier, Amsterdam, 1962, p. 43.
8 G. Eckert, Z. Anal. Chem., 155 (1957) 23.

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Biomedical Applications

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SELECTIVE ANALYSIS FOR ADENOSINE USING REVERSED-PHASE HIGH-PRESSURE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-pressure liquid chromatographic procedure for the selective determination of adenosine in the presence of other nucleic acid components is reported. Reversed-phase microparticle columns and an isocratic elution mode of dilute potassium dihydrophosphate and anhydrous methanol were used. The analysis is specific for adenosine and is achieved in less than 10 min. An example of the use of this analysis in a biomedical study is reported.

INTRODUCTION

The analysis of nucleotides in blood is now being carried out routinely by high-pressure liquid chromatography (HPLC) [1—7], and work is in progress to determine alterations caused by various disease states of free nucleotide concentrations in physiological fluids and cell extracts [8—11]. Less attention has been paid to nucleoside levels in cells. However, recent investigations have focused attention on nucleosides in cells [12,13,39,40], and especially on adenosine concentrations which have been found to be important in cardiac disease and certain birth defects. Adenosine is one of the physiological regulators of coronary blood flow and it has been observed that there is an increase in intracellular adenosine levels in cardiac hypertrophy and after brief periods of myocardial ischemia and hypoxia [14, 15]. It has also been found that excess adenosine in cultured mammalian cells is toxic [16] and it has been postulated that severe combined immunological defects in children, in some instances, are caused by an accumulation of adenosine which is a result of an adenosine deaminase deficiency [17]. In addition, although adenosine has potential for use as a preservative in blood storage [18] and in the treatment of the Lesch Nyhan syndrome, its use has been limited because of possible toxic effects.

Research on problems involving adenosine has been hampered by the lack of an adequate analytical method which permits the sensitive and quantitative analysis of adenosine in the presence of other UV-absorbing compounds present in cellular extracts. Therefore, the objective of this research was to develop

a rapid procedure for the selective determination of adenosine. Since HPLC is uniquely suited for nucleotide and nucleoside analyses [19—37] various modes of this technique were investigated for use in an adenosine assay. It was found that the reversed-phase partition mode provided the required efficiency and selectivity using totally porous, chemically bonded microparticle packings.

EXPERIMENTAL

Chemicals

The potassium dihydrophosphate was from Mallinckrodt (St. Louis, Mo., U.S.A.), reagent-grade quality, and was used without further purification. The methanol was reagent-grade, anhydrous. The nucleoside standards: Cytidine (Cyd), Uridine (Urd), Thymidine (dThyd), Adenosine (Ado), Guanosine (Guo), Inosine (Ino) and Xanosine (Xao), their nucleotides and their bases, were from Sigma (St. Louis, Mo., U.S.A.).

Apparatus

A liquid chromatograph (Waters Assoc., Milford, Mass., U.S.A.), Model ALC 202 with a double-beam micro-UV-detector operating at a fixed wavelength of 254 nm was used. The instrument was equipped with dual pumps and solvent programmer. For the Ado analysis however, only a single M6000 pump was used in the isocratic mode. The column (30 cm × 4 mm) was packed with μBondapak C_{18} (Waters Assoc.). Integration was done electronically using a Hewlett-Packard Model 3380A integrator.

Procedures

The distilled water used in preparing the eluents was first filtered through a Millipore filter, type HA (pore size, 0.45 μm). The pH of the buffer solutions was adjusted with H_3PO_4 to 5.8, prior to the addition of methanol. In order to dissolve the nucleosides, the standards were made up in 0.007 *F* KH_2PO_4 (pH 7.8). After the standards were dissolved, the pH was adjusted to 5.8 and the solution brought up to volume.

Operating conditions

The operating conditions were optimized to obtain a selective analysis for Ado in the presence of other naturally occurring nucleosides, nucleotides and their bases. The eluent used was a solution of anhydrous methanol—0.007 *F* KH_2PO_4, pH 5.8 (10:90 v/v). The temperature was ambient. The flow-rate was 2.0 ml/min, which produced a pressure drop of approximately 1500 p.s.i. Injections of the samples were via the Waters' U6K injector (a modified sample-loop type of injector). Hamilton syringes (10, 25 or 100 μl) were used to inject the sample volumes.

Cell extraction procedures

The extraction procedure used for the blood samples is that described by Khym [38]. In this procedure, 1 ml of the sample was denatured by the addition of 2 ml of cold trichloroacetic acid (6% by weight). After mixing vigorously using a vortex mixer, the sample was centrifuged for 3 min at 3600 *g*, and

filtered through a Millipore filter. A 1-ml aliquot of this solution was added to 1 ml of tri-N-octylamine—freon 113(1,1,2-trichlorotrifluoroethane) (0.5 *M*). After mixing 3—4 min and centrifuging for 3 min at 3600 *g*, the top layer was withdrawn and stored at −4°.

To determine the efficiency of recovery, the extraction procedure was carried out on 5 samples of serum to which known quantities of Ado were added. Recovery of Ado averaged 97%.

RESULTS

Separation of adenosine

Fig. 1A shows the resolution of Ado from 6 other nucleosides (Guo, Ino, Xao, Cyd, Urd, dThyd). These 6 nucleosides eluted within 5 min while the Ado peak is completely resolved. In the chromatogram of Fig. 1B, Ado and the other 6 nucleosides were co-injected with 5 nanomoles each of the bases of these 7 nucleosides. In Fig. 1C, 5 nanomoles of each of the mono-, di- and triphosphate nucleotides of these 7 nucleosides were co-injected with both the nucleosides and bases. These nucleic acid components, which may be found in blood extracts, did not interfere with the Ado peak.

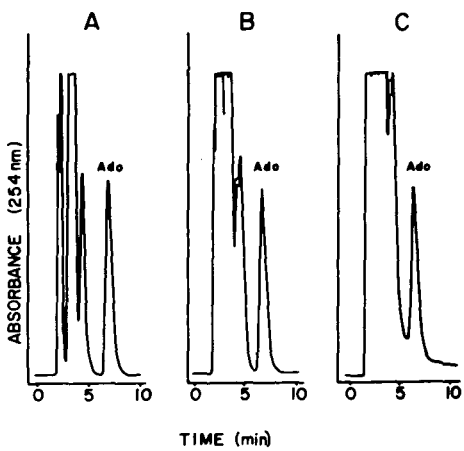

Fig. 1. (A) Separation of 5 nanomoles each of the 7 nucleosides cytidine, uridine, thymidine, guanosine, inosine, xanthosine and adenosine (Ado). (B). Co-injection of 7 nucleosides with 5 nanomoles each of the bases of these 7 nucleosides. (C) Separation of Ado from 5 nanomoles each of the nucleosides, the bases and the mono-, di- and tri-phosphate nucleotides of the 7 nucleosides. Column packing, μBondapak C_{18}; temperature, ambient; detector sensitivity, 0.02 a.u.f.s.; integrator setting 32. Eluent, anhyd. methanol—0.007 *F* KH_2PO_4 (pH 5.8) (10:90). Flow-rate, 2,0 ml/min.

Sensitivity and reproducibility

The lower limit of detection for Ado in a given chromatographic system will depend upon the detector sensitivity, noise levels and column efficiency. In the systems used for this study, 50 picomoles of Ado was readily detected (Fig. 2A). The peak shape remained constant up to 100 nanomoles (Fig. 2B). Excellent reproducibility of retention times and area was obtained. The relative

standard deviation of the retention time for 5 consecutive injections of standard solutions of Ado averaged 0.13%, while the peak area precision averaged 0.46%.

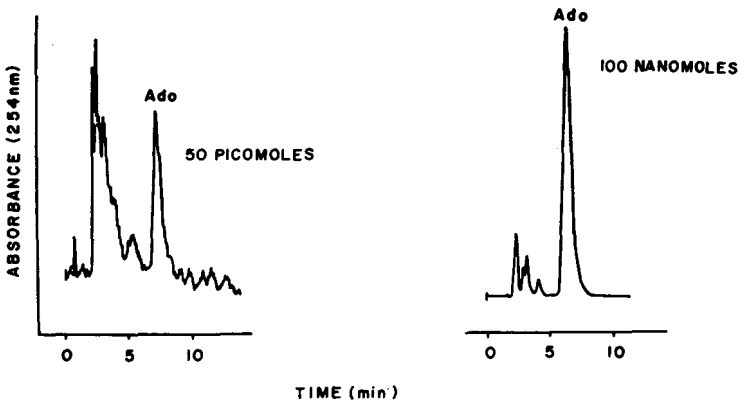

Fig. 2. (A) 5.0×10^{-11} moles (50 picomoles) of (Ado). Integrator setting, 1. (B) 1.0×10^{-7} moles (100 nanomoles) of Ado. Integrator setting, 512; further conditions as in Fig. 1.

Applications

Working in conjunction with Dr. Hilaire Meuwissen of Albany Medical College of Union University, we studied blood samples from a patient suffering from an adenosine deaminase deficiency with severe immunological defects along with normal control subjects. No free Ado was detected in normal human whole blood, erythrocytes or serum. However, in 3 serum samples taken at different time periods from the patient with the enzyme deficiency, 45 and 55 picomoles of Ado were readily detected. Two of these serum samples (Nos. 7 and 8) are shown in Fig. 3. Sample No. 10 of Fig. 3 shows a comparable serum sample of a control subject in which no Ado is found.

For positive identification of the peak with the retention time of Ado in the biological samples two techniques were used: standard addition and the enzymatic peak-shift. For the standard addition method, 50 picomoles of Ado were co-injected with the cell sample (Fig. 4B). In the enzymatic peak-shift technique, adenosine deaminase, which catalyzes the conversion of Ado to Ino, was used (Fig. 4C). With both standard solutions and cell extracts, the Ado was quantitatively converted to Ino. In addition to identifying the Ado, this technique showed that the Ado eluted free of any other UV-absorbing compound in serum; thus accurate quantitation of the Ado could be achieved with no errors caused by co-eluting compounds.

DISCUSSION

This analysis of adenosine using reversed-phase HPLC offers advantages over methods previously used. Rapid analyses can be achieved at ambient temperatures with sensitivity, selectivity and efficiency. The results are quantitative and other UV-absorbing constituents in cellular extracts do not interfere with

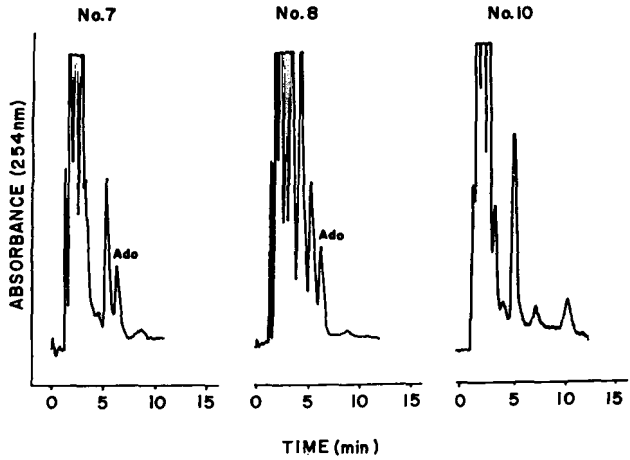

Fig. 3. Samples Nos. 7 and 8, 50 µl of serum extract from two patients suffering from adenosine deaminase deficiency. 45 and 55 picomoles are contained under the adenosine peak of sample numbers 7 and 8 respectively. Sample No. 10 shows the injection of 50 µl of serum extract from a control patient. Integrator setting, 2; further chromatographic conditions as in Fig. 1.

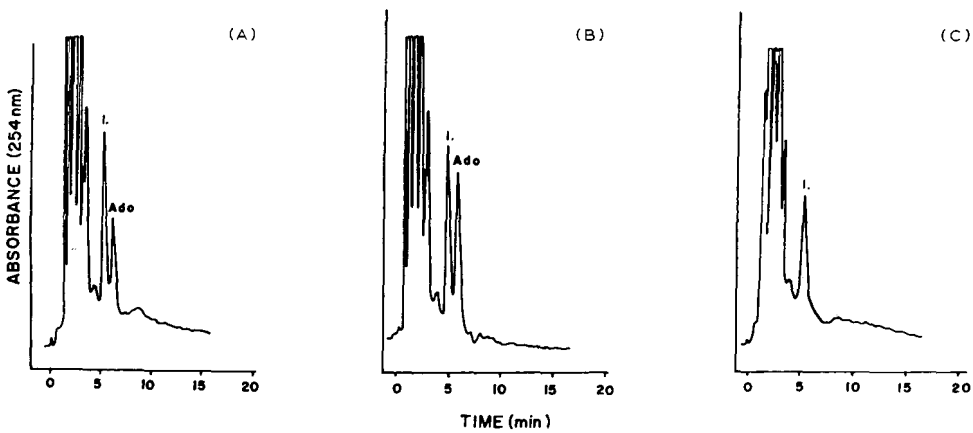

Fig. 4. Identification of Ado.(A) 50 µl of serum extract from an adenosine deaminase patient. (B) Co-injection of 50 picomoles of Ado with the serum extract. (C) Original serum extract after reaction with the enzyme, adenosine deaminase. Conditions as in Fig. 3; Peak 1, not identified.

the adenosine analysis. Minimal sample preparation is required prior to chromatography. Since the isocratic elution mode was used, no re-equilibration time was required between analyses. Thus the overall analysis time was shorter than the time necessary for an analysis in which gradient elution is used. In additon, with isocratic elution, it is easier to automate the procedure and therefore this method has potential for use in the clinical laboratory with a large number of samples. Furthermore, because the eluent is composed of a very weak salt solution and methanol, the solvent can readily be removed after fractions are

collected. Using the enzymic peak-shift together with retention times and the standard addition method, the adenosine peak can be positively identified. The enzyme used in this peak-shift, adenosine deaminase, is commercially available and is inexpensive. We found that the reversed-phase chemically bonded microparticle columns were very stable and the chromatograms highly reproducible over a period of time. Columns have been used continuously in our laboratory for 6 months with no loss in efficiency. However, if the retention time or the resolution of adenosine is found to shift with time, it is possible that organics from the eluent or the samples are being partitioned into the stationary phase. In such cases, the column can be cleaned with methanol or some moderately polar solvent. It should be noted that retention characteristics may vary slightly from batch to batch or from different manufacturers. Thus the conditions for this analysis may be used as a guide-line, but conditions should be optimized for each column to obtain the best analysis.

Although the results presented here were achieved using a Waters μBondapak C_{18} column, similar results were obtained in our laboratory using Whatman Partisil 10-ODS and DuPont Zorbax™ ODS columns on a DuPont Model 830 liquid chromatograph.

ACKNOWLEDGEMENTS

The authors thank Waters Assoc. for the use of the Waters ALC 202 liquid chromatograph, the DuPont Company for the use of the DuPont 830 instrument, Dr. Hilaire Meuwissen of Albany Medical College of Union University for the serum samples from the adenosine deaminase deficient patient, Anté Krstulovic for the work with the enzymatic peak-shift technique and Dorothy Morancy and Roberta Caldwell for their assistance with this manuscript.

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REFERENCES

1 C.G. Horvath, B.A. Preis and S.R. Lipsky, Anal. Chem., 39 (1967) 1422.
2 P.R. Brown, J. Chromatogr., 52 (1970) 257.
3 P.R. Brown, High Pressure Liquid Chromatography, Biochemical and Biomedical Applications, Academic Press, New York, 1973.
4 P.R. Brown, R.E. Parks, Jr. and J. Herod, Clin. Chem., 19 (1973) 919.
5 E.M. Scholar, P.R. Brown, R.E. Parks, Jr. and P. Calabresi, Blood, 41 (1973) 927.
6 P.R. Brown and R.E. Parks, Jr., Anal. Chem., 44 (1973) 1072.
7 R.E. Parks, Jr. and P.R. Brown, Biochemistry, 12 (1973) 3294.
8 C.D. Scott, Clin. Chem., 14 (1968) 521.
9 C.D. Scott, J. Attril and N.G. Anderson, Proc. Soc. Exp. Biol. Med., 125 (1967) 181.
10 C.A. Burtis and K.S. Warren, Clin. Chem., 14 (1968) 290.
11 C.A. Burtis, J. Chromatogr., 52 (1970) 97.
12 J.E. Mrochek, D.D. Chilcote and S.R. Dinsmore, Liquid Chromatographic Analysis of Urinary Nucleosides in Normal and Malignant States, American Chemical Society Meeting, August, 1973.
13 C.W. Gehrke, R.W. Zumwalt, K.C. Kuo and T.P. Walker, Symposium on Chromatographic Analysis of Biologically Important Compounds, American Chemical Society Meeting, August, 1975.
14 R.M. Berne and R. Rubio, Circ. Res., Suppl. III, 34 & 35 (1974) 109.

15 R. Rubio and R.M. Berne, Circ. Res., 25 (1969) 407.
16 K. Ishii and H. Green, J. Cell Sci., 13 (1973) 1.
17 H. Green, in H.J. Meuwissen et al. (editors), Combined Immunodeficiency Disease and Adenosine Deaminase Deficiency, Academic Press, New York, 1975.
18 C. Bishop and D.M. Surgenor, The Red Blood Cell, Academic Press, New York, 1964, pp. 173—175.
19 C.G. Horvath and S.R. Lipsky, Anal. Chem., 41 (1969) 1227.
20 P.R. Brown, S. Bobick and F.L. Hanley, J. Chromatogr., 99 (1974) 587.
21 N.G. Anderson, Anal. Biochem., 4 (1962) 269.
22 N.G. Anderson, J.G. Green, M.L. Barber and L.C. Ladd, Anal. Biochem., 6 (1963) 153.
23 E.M. Scholar, P.R. Brown and R.E. Parks, Jr., Cancer Res., 32 (1972) 259.
24 P.R. Brown, R.P. Agarwal, J. Gell and R.E. Parks, Jr., Compr. Biochem. Physiol., 43 (1972) 891.
25 M. Uziel, C.K. Koh and W.E. Cohn, Anal. Biochem., 25 (1968) 77.
26 R.P. Singhall and W.E. Cohn, Anal. Biochem., 45 (1972) 585.
27 W.E. Cohn, Science, 109 (1949) 377.
28 P. Reichard and B. Estbarn, Acta Chem. Scand., 4 (1950) 1047.
29 C.F. Crampton, F.R. Fraenkel, A.M. Benson and A. Wade, Anal. Biochem., 1 (1960) 249.
30 M. Uziel and C. Koh, J. Chromatogr., 59 (1971) 188.
31 F. Murakami, S. Rokushika and H. Hatano, J. Chromatogr., 53 (1970) 584.
32 C.D. Scott, R.L. Jolley and W.F. Johnson, Amer. J. Clin. Pathol., 53 (1970) 701.
33 J.E. Mrochek, W.C. Butts, W.C. Rainey and C.A. Burtis, Clin. Chem., 17 (1971) 72.
34 C.D. Scott, D.D. Chilcote and N.E. Lee, Anal. Chem., 44 (1972) 85.
35 F.R. Blattner and H.P. Erickson, Anal. Biochem., 18 (1967) 220.
36 H.J. Breter and R.K. Zahn, Anal. Biochem., 54 (1973) 346.
37 M. Carrara and G. Bernerdi, Biochim. Biophys. Acta, 155 (1968) 1.
38 J.X. Khym, Clin. Chem., 21 (1975) 1245.
39 D.C. Tormey, T.P. Waalkes, D. Ahmann, C.W. Gehrke, R.W. Zumwalt, J. Snyder and H. Hansen, Cancer, 35 (1975) 1095.
40 T.P. Waalkes, C.W. Gehrke, R.W. Zumwalt, S.Y. Chang, D.B. Lakings, D.C. Tormey, D.L. Ahmann and C.G. Moertel, Cancer, 36 (1975).

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Biomedical Applications

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RAPID SEPARATION OF URINARY ACIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Over a hundred acidic urinary constituents were separated within 30 min by using 5-μm octadecyl-silica columns and gradient elution with increasing acetonitrile concentration in dilute aqueous phosphoric acid solution at 70°. The column effluent was monitored with a UV detector at 280 nm or with a fluorescence detector at 260 nm excitation and 340 nm emission wavelengths. The high sensitivity and speed of analysis, the excellent reproducibility and adequate resolution obtained suggest that this technique may be useful to obtain metabolic profiles in routine clinical work.

INTRODUCTION

In the last decade various chromatographic techniques were developed to separate a large number of body fluid constituents concurrently. Most success has been attained by the use of gas chromatography to obtain metabolic profiles of various groups of analytically related metabolites [1—3] or fingerprints of volatile substances [4, 5] present in urine or other physiological fluids. The identification of the individual compounds is greatly facilitated by the tandem operation of the gas chromatograph with the mass spectrometer [6—8]. Gas chromatography, however, is limited by the requirement that the sample components be sufficiently volatile and the analysis of polar compounds necessitates an elaborate derivatization step prior to the chromatographic separation.

In order to overcome this shortcoming, Scott and collaborators [9, 10] employed ion-exchange chromatography and succeeded by using columns packed with anion-exchange resin to analyze over a hundred UV-absorbing components in human urine. The originally excessive time of separation could be drastically reduced from 24 to 14 h with the use of coupled anion- and cation-exchange columns [11]. Although the technique offers a powerful tool for the study of metabolic disorders, the analysis time is prohibitively

long to afford the routine application of ion-exchange chromatography for such multicomponent separations in the clinical field.

Our recent study [12] has demonstrated that polar biological substances can be rapidly separated by high-performance liquid chromatography on a non-polar stationary phase such as octadecyl-silica with aqueous eluents. In this type of chromatography, which is often referred to as "reversed-phase" chromatography, solute retention is governed by hydrophobic interactions with the hydrocarbonaceous functions of the bonded stationary phase [13]. Evidently, the magnitude of the hydrophobic interactions between closely related solutes and the non-polar stationary phase are sufficiently large and different to obtain satisfactory retardation and specificity even with biological compounds which are considered polar [14]. The practical merits of this technique stem from its relative simplicity as well as high reproducibility and efficiency with respect to that of ion-exchange chromatography under comparable conditions. Gradient elution with increasing concentration of a suitable organic solvent in the eluent facilitates the separation of a wide variety of substances on non-polar bonded phases in a convenient and reproducible fashion.

The advantages of this type of chromatography prompted us to investigate the separation of urinary constituents on the basis of their "hydrophobicity". In view of recent advances in urinary metabolic profiles of organic acids [15—17] for diagnostic purposes, our interest was focussed on the development of a rapid chromatographic method for the separation of substances present in the extract of acidified urine. The results illustrate that solvophobic chromatography [13] is a rapid and powerful method for the separation of a large number of biological substances present in physiological fluids.

EXPERIMENTAL

Apparatus

A Perkin-Elmer Model 601 high-pressure liquid chromatograph with an LC 55 variable wavelength UV detector (Perkin-Elmer, Norwalk, Conn., U.S.A.) and a Schoeffel FS-970 fluorescence detector (Schoeffel, Westwood, N.J. U.S.A.) was used in the gradient elution mode. The sample was introduced by using a Siemens high-pressure injection syringe with a 10-μl needle (ES Industries, Marlton, N.J., U.S.A.).

In all experiments a flow-rate of 2.0 ml/min and a concave gradient at curvature setting 55 were the most effective. The starting eluent was 0.1 *M* phosphate buffer, pH 2.1 (reagent grade H_3PO_4 and KH_2PO_4, Fisher, Pittsburgh, Pa., U.S.A.) and acetonitrile or methanol (Burdick and Jackson, Muskegon, Mich., U.S.A.) were used as gradient formers. The gradient run, at a 50-min setting for 100%, was terminated after approximately 30 min, when the organic solvent concentration reached about 35—40% (v/v). Subsequently the column was re-equilibrated with the aqueous eluent at 2.0 ml/min in 15 min. The column temperature was maintained at 70°.

It is noted that the use of syringe pumps for gradient elution with water—methanol and water—acetonitrile mixtures over 40% (v/v) can result in poor reproducibility due to changes in the viscosity and compressibility of the eluent with the solvent composition as discussed by Martin et al. [18] and

Abbott et al. [19]. We found that with the instrument used in this study good reproducibility of the retention values was obtained over the full solvent composition range when the retrace-gradient setting was used for re-equilibration. The results were also satisfactory when after completion of elution the shut-off valve of the pump for the organic solvent was closed and the pressure was raised to the starting value prior to re-equilibration.

Columns

In the course of this investigation various home-made and commercial columns were evaluated. All columns employed were made of 25 cm × 4.6 mm I.D. × 6.4 mm O.D. No. 316 stainless-steel tubing with zero dead-volume fittings. Octadecyl-silica was prepared from 10- and 5-μm Partisil (Whatman, Clifton, N.J., U.S.A.) with octadecyltrichlorosilane (Aldrich, Milwaukee, Wisc. U.S.A.) according to the literature [20]. Columns were packed with a home-made instrument equipped with a 50,000-p.s.i. Haskel reciprocating pump (Haskel Engineering & Supply Co., Burbank, Calif., U.S.A.) by using the isopycnic slurry method at 10,000 p.s.i. It has been found, that columns packed with 5-μm octadecyl-silica containing 16—18% (w/w) carbon yield the best separation under our conditions. Since recently such columns became commercially available, the results presented here were obtained with 5-μm LiChrosorb ODS columns of the above dimensions (Rainin Instruments, Boston, Mass., U.S.A.).

Samples

Urine samples have been obtained from Dr. P. Jatlow, School of Medicine, Yale University. A 5-ml volume of urine was saturated with NaCl, centrifuged and the pH of the supernatant was adjusted to 13 with 1 *M* NaOH by using a Model 26 pH meter (The London Co., Cleveland, Ohio, U.S.A.). First, it was extracted with 10 ml of ethylacetate (ACS grade; Mallinckrodt, St. Louis, Mo., U.S.A.) at 30° in a water-bath shaker (Eberbach, Ann Arbor, Mich., U.S.A.) for 15 min in order to remove neutral and basic organic compounds. After phase separation the pH of the aqueous phase was adjusted with concentrated HCl to pH 1 and the organic acids were extracted with another 10 ml of ethyl acetate as described above. The extract was then evaporated to dryness in a nitrogen stream and stored in the refrigerator. Prior to analysis the residue was dissolved in 0.5 ml of 1 *M* HCl.

The organic acids have been supplied by Sigma (St. Louis, Mo., U.S.A.) and by Aldrich.

RESULTS AND DISCUSSION

Our goal was to demonstrate that many low-molecular-weight constituents of physiological fluids can be faster and more efficiently separated by recently introduced microparticular non-polar stationary phases than by ion-exchange resins employed conventionally. Preliminary studies with urine samples have been carried out by monitoring the column effluent of our chromatographic system with the UV detector at 210 nm as well as with the fluorometer at different excitation and emission wavelengths. The results suggested, that a further improvement of the separation efficiency and the method of detection

could yield chromatograms of urine with over 500 resolved peaks.

Since the number of urinary constituents [21] exceeds the peak capacity of the present system it behooved us to limit the number of sample components by using an extraction procedure prior to the chromatographic separation on the one hand and by setting the wavelength of the UV detector to 280 nm on the other. The extraction of acified urine with ethyl acetate has long been used for the isolation of the less hydrophilic urinary constituents with good analytical recovery [22]. As most substances having aromatic moieties strongly absorb light at 280 nm the method presented here is eminently suitable to obtain chromatographic profiles of urinary aromatic acids.

With octadecyl-silica columns best results have been obtained, when the hydro—organic eluent is buffered in the neighbourhood of pH 2. In our experience, phosphoric acid buffer is particularly suitable for this purpose [12]. The use of low pH is required to retard the more hydrophilic acids on the nonpolar stationary phase. Earlier studies from our laboratory demonstrated that the capacity factors of unionized aromatic acids are 3 to 5 times higher than those of their conjugated bases [14]. The buffer not only maintains the eluent pH constant, thus enhances reproducibility, but also facilitates the rapid establishment of protonic equilibria both in the eluent and on the stationary phase [23]. It has been observed that asymmetric peaks are obtained in the absence of adequate buffering capacity, when both the acid solute and its conjugated base are present in the eluent.

Gradient elution with increasing organic solvent concentration in the eluent is also essential to obtain the peak capacity required for such multicomponent separations [24]. Although both acetonitrile and methanol yield suitable eluent gradients, the use of water—acetonitrile mixtures is preferred because the strong association between methanol and water results in dramatical changes in the physical properties of the solvent such as viscosity and density with changing solvent composition and can impair the reproducibility of the results.

A typical chromatogram of urinary aromatic acids as obtained with the UV detector at 280 nm is shown in Fig. 1. It is seen that over a hundred substances can be separated within 30 min. The identification of the individual peaks remains a major problem and would require the use of the mass spectrometer or some other method. We separated twelve aromatic acids of urinary significance under the same conditions and the chromatogram is shown in Fig. 2. Their retention values have been used for the tentative identification of the corresponding mandelic, phenylacetic, indoleacetic and cinnamic acid derivatives. The chromatogram of the urine extract spiked with these acids in Fig. 3 shows that the retention times of the marker peaks are the same as those of the pure substances in Fig. 2. This also supports our observation that the method is highly reproducible as long as the column properties remain unchanged (see below).

According to our experience the sensitivity of the UV detector can be increased by a factor of one hundred under the conditions used in these studies before the baseline noise interferes with data evaluation. Experiments with varying amounts of added aromatic acids such as *p*-hydroxymandelic acid or homovanillic acid showed, that the peak heights are linearly dependent on the

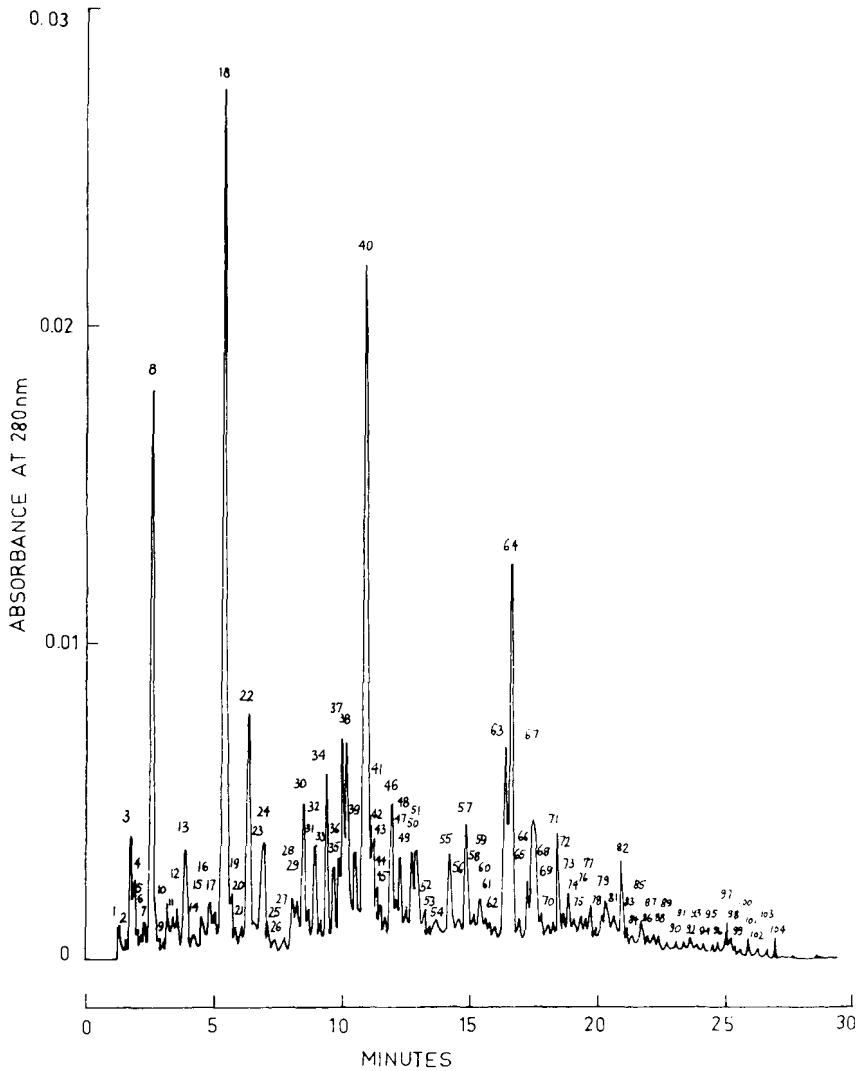

Fig. 1. Chromatogram of acidified urine extract. Column: 5-μm octadecyl-silica, 25 cm × 4.6 mm I.D.; temperature, 70°; flow-rate, 2.0 ml/min. Gradient elution from 0.1 *M* phosphate buffer, pH 2.1, with acetonitrile to about 40% (v/v) organic solvent concentration. Sample size, 10 μl containing the extract of 100 μl of urine.

urinary concentration of these components in the range of 0—200 mg/l.

The use of sensitive fluorescence detectors offers an alternative to obtain urinary profiles as shown in Fig. 4. Some of the peaks have been tentatively identified by the retention values of added marker substances. In general, the metabolites of tryptophane and many of the acids containing hydroxyphenyl moieties have been found to have strong fluorescence under the conditions employed. As the oxidation products of catecholamines very strongly fluoresce and they can efficiently be separated by reversed-phase chromatography [12], this method combined with a simple pre-column oxidation procedure [25] may offer an opportunity for the quantitative analysis of

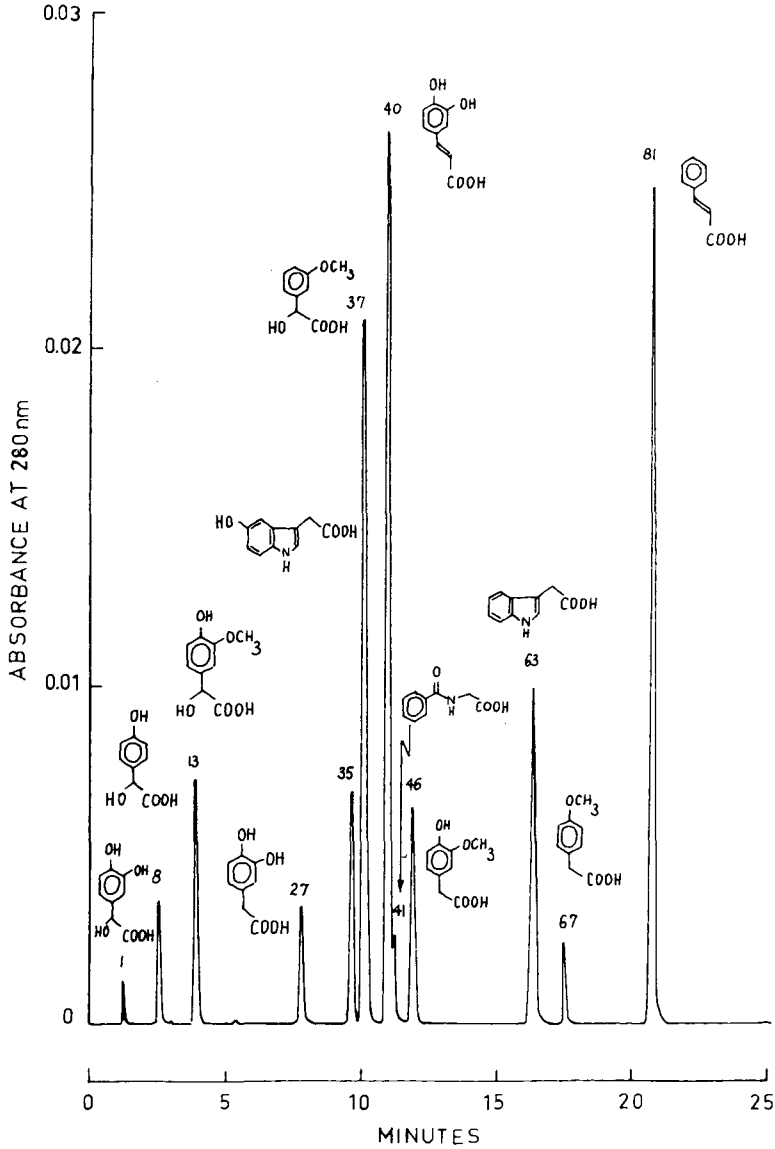

Fig. 2. Chromatogram of aromatic acids. Column and elution conditions as in Fig. 1. The peak numbers correspond to those shown in Figs. 1 and 3. Sample, 10 μl of an aqueous solution containing 1 μg of each compound.

urinary catecholamine derivatives at the femtomol level. When the effluent is monitored by the fluorescence detector at 260 nm excitation and 340 nm emission wavelength, 1-hydroxy-2-naphthoic acid is an appropriate internal standard.

It has been observed, that the elution pattern of the urinary acids can change with sample storage. Therefore, the reproducibility of the results may depend upon the strict adherence to an exact protocol for the handling of the urine samples. According to our experience, the dry extract can be stored in

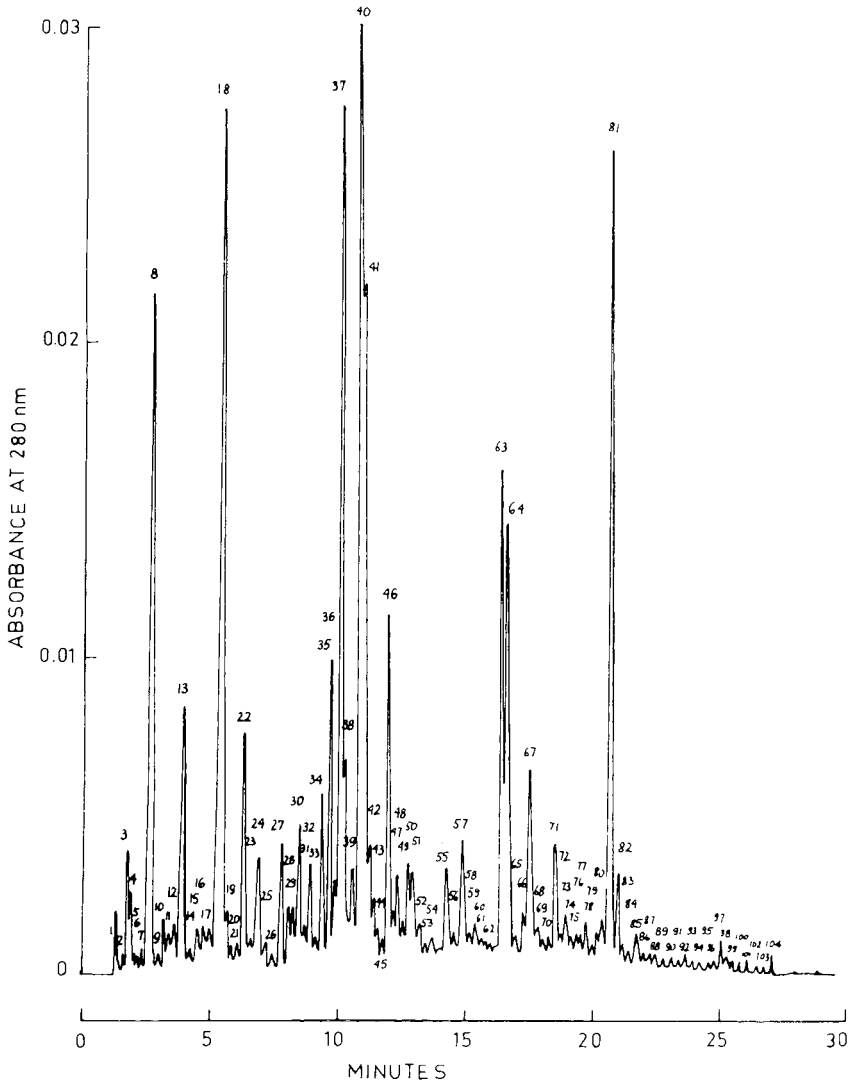

Fig. 3. Chromatogram of acidified urine extract with the added marker compounds shown in Fig. 2. Column and elution conditions as in Fig. 1.

the refrigerator over two months without changes in the chromatographic profile. It is noted, that the present method is eminently suited to follow chemical changes in the urine, which is exposed to the atmosphere at room temperature. At present little is known about these changes, which may alter the composition of the urine prior to the chromatographic analysis.

The retention times showed a slow decrease upon extensive use of a given column. This phenomenon is attributed to the slow hydrolysis of the octadecylsiloxane moiety on the surface of the stationary phase at 70°. Under such condition the useful life of a column packed with 5-μm octadecyl-silica containing 16—17% (w/w) carbon is approximately 300 h. We found, however, that the column can be easily rejuvenated by an in situ treatment with a dilute solution of octadecyltrichlorosilane in toluene [26].

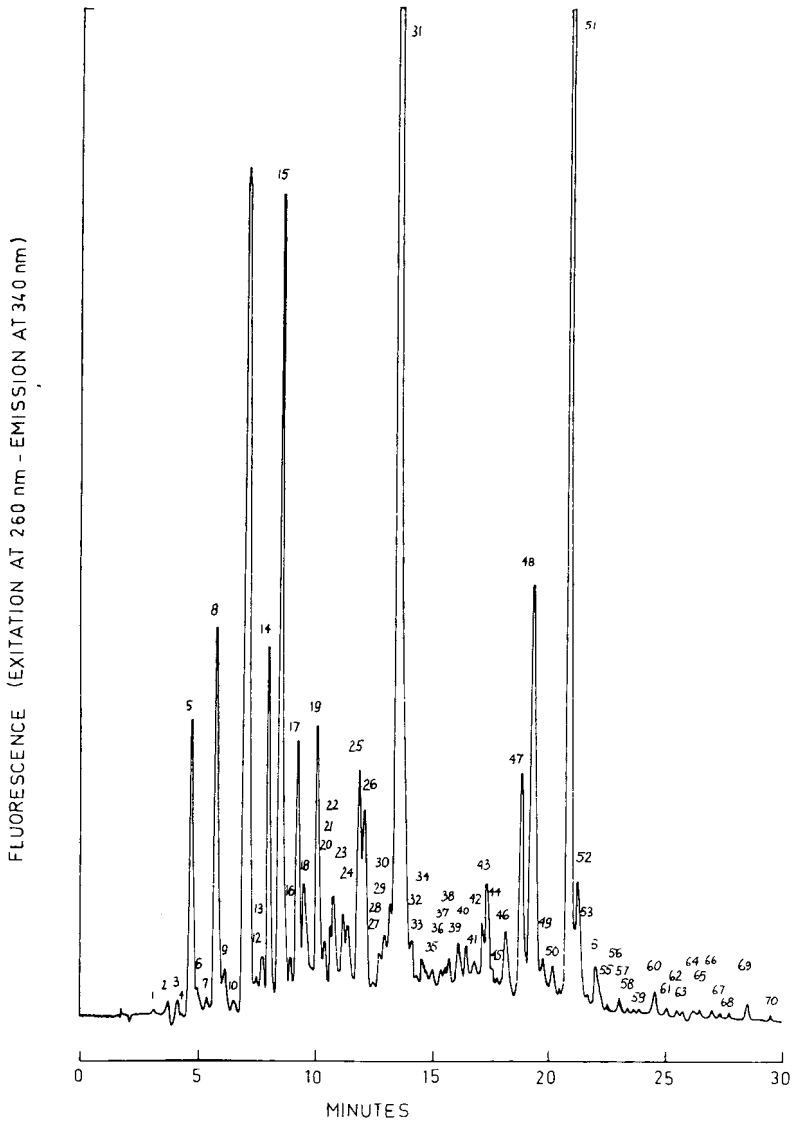

Fig. 4. Chromatogram of urine extract under conditions similar to those given in Fig. 1, but after the column was used for about 300 h. The fluorescence detector was set for 260 nm excitation and 340 nm emission wavelengths. The sensitivity and the time constant were 1 μA and 0.5 sec, respectively.

The thermodynamic basis of retention in this type of chromatography is, albeit very complex, fairly well understood [13, 14]. As a rule of thumb, the capacity factor increases with the non-polar surface area of the solutes, but polar and in particular ionized groups, which strongly interact with the aqeous eluent, reduce solute retention. The increase in eluent strength with increasing organic solvent concentration is mainly attributed to the reduction of the effective surface tension of the eluent. As seen in Fig. 2 the elution order of the

solutes corresponds to the qualitative statement regarding the relationship between the molecular structure and retention.

The clinical significance of new "high-resolution" techniques for the analysis of urinary constituents has been well recognized [27—29]. There is ample evidence, that many of the substances may have pathological importance. Gas chromatographic profiling and concomitant mass spectrometric analysis have been particularly useful for the characterization of inborn errors of metabolism [30]. About 50 diseases have already been found to give rise to high concentration of acidic metabolites in urine and for instance the excess amount of certain acidic metabolites has been related to such diseases as isovaleric aciduria, maple-syrup urine disease and glutaric aciduria [31—33]. The present technique could serve as a rapid method for the establishment of the relationship between the urinary level of individual aromatic acids and certain disease states. It is noted, that many of these acids are produced by the abnormal metabolism of phenylalanine [34]. The extinction coefficient of aliphatic acids, with the exception of keto acids, is usually very low at 280 nm, therefore, their analysis has to be carried out at a detector wavelength of 215 nm and below. The retention times of the short-chain aliphatic acids is relatively small under the conditions used and their separation may require lower column temperature and isocratic elution. Preliminary results indicate that closely related keto and hydroxy- acids, which cannot be separated by gas chromatography with packed columns [32], can be resolved by the present technique due to their different hydrophobicities.

Recently Chalmers et al. [22, 35, 36] have investigated urinary acids in man and established the quantitative ranges and frequency distribution patterns of excretion for a number of acids in a normal population. An important finding of these studies is that whereas the excretion patterns of acids widely differ from individual to individual, even extreme dietary alterations produce only small changes in the individual excretion patterns under normal conditions. Consequently, changes in the urinary acid profile of an individual may be used for early diagnosis of certain diseases.

High-pressure liquid chromatography appears to be an efficient tool for the rapid separation of the components of the physiological fluids. It can be used either for the determination of the physiological level of individual constituents having established clinical significance [37—39] or to obtain metabolic profiles by chromatographic fingerprinting. In each case it would be desirable to obtain positive identification of the peaks of interest and it is hoped, that the tandem operation of the mass spectrometer with the liquid chromatograph becomes practical to carry out this task. The present limitations notwithstanding, liquid chromatography has many advantages over gas chromatography and in view of the recent improvements of efficiency and sensitivity it can qualify as one of the "new high-resolution analytical systems, that are capable of separating and quantifying many of the individual constituents of a physiological sample" envisioned by Scott and Melville [21] and due to the high speed of analysis it "may be useful in the clinical laboratory for in-depth analysis".

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REFERENCES

1 C.E. Dagleish, E.C. Horning and M.G. Horning, Biochem. J., 101 (1966) 792.
2 E.C. Horning and M.G. Horning, Clin. Chem., 17 (1971) 802.
3 E. Jellum, O. Stokke and L. Eldjarn, Scand. J. Clin. Lab. Invest., 27 (1971) 273.
4 A. Zlatkis and H.M. Lieblich, Clin. Chem., 17 (1971) 592.
5 A. Zlatkis and F. Andrawes, J. Chromatogr., 112 (1975) 533.
6 F. Hutterer, J. Roboz, L. Sarkozi, A. Ruhig and P. Bacehin, Clin. Chem., 17 (1971) 789.
7 E. Jellum, O. Stokke and L. Eldjarn, Anal. Chem., 45 (1973) 1099.
8 E. Jellum, P. Størseth, J. Alexander, P. Helland, O. Stokke and E. Teig, J. Chromatogr., 126 (1976) 487.
9 C.D. Scott, Clin Chem., 14 (1968) 521.
10 C.D. Scott, W.F. Johnson and V.W. Walker, Anal. Biochem., 32 (1969) 182.
11 C.D. Scott, D.D. Chilcote and N.E. Lee, Anal. Chem., 44 (1972) 85.
12 I. Molnár and C. Horváth, Clin. Chem., 22 (1976) 1497.
13 C. Horváth, W. Melander and I. Molnár, J. Chromatogr., 125 (1976) 129.
14 C. Horváth, W. Melander and I. Molnár, Anal. Chem., 49 (1977) 142.
15 D.A. Mamer, J.C. Crawhall and K.B. Hammond, The Identification of Endogenous Urinary Metabolites by Gas Chromatography — Mass Spectrometry; A Collection of Mass Spectrometric Data, B.F. Stolinsky Research Lab., Univ. of Colorado, Medical Center, Denver, Colo., 1972.
16 E. Jellum, O. Stokke and L. Eldjarn, Clin. Chem., 18 (1972) 800.
17 T.A. Witten, S.P. Levine, J.O. King and S.P. Markey, Clin. Chem., 19 (1973) 586.
18 M. Martin, G. Blu, C. Eon and G. Guiochon, J. Chromatogr., 112 (1975) 399.
19 S.R. Abbott, J.R. Berg, P. Achener and R.L. Stevenson, J. Chromatogr., 126 (1976) 421.
20 D.G.I. Kingston and B.B. Gerhart, J. Chromatogr., 116 (1976) 182.
21 C.D. Scott and R.S. Melville, Clin. Chem., 26 (1970) 623.
22 A.M. Lawson, R.A. Chalmers and R.W.E. Watts, Clin. Chem., 22 (1976) 1283.
23 J.M. Engrasser and C. Horváth, Physiol. Chem. Phys., 6 (1974) 541.
24 C. Horváth and S.R. Lipsky, Anal. Chem., 39 (1967) 1893.
25 Y. Dalmaz and L. Peyrin, J. Chromatogr., 116 (1976) 379.
26 I. Molnár and C. Horváth, in preparation.
27 J.W. Rosevear, K.J. Pfatt and E.A. Moffitt, Clin. Chem., 17 (1971) 72.
28 C.C. Sweeley, N.D. Young, J.F. Holland and S.C. Gates, J. Chromatogr., 99 (1974) 507.
29 R.W.E. Watts, R.A. Chalmers and A.M. Lawson, Lancet, i(1975) 368.
30 J. Stern and C. Toothill (Editors), Organic Acidurias; Proc. 9th Symp., Soc. Study of Inborn Errors of Metabolism, Churchill Livingstone, Edinburgh, London, 1972.
31 S. Landaas, Clin. Chim. Acta, 54 (1974) 39.
32 C. Jakobs, E. Solem, J. Ek, K. Halvorsen and E. Jellum, J. Chromatogr., 143 (1977) 31.
33 S.I. Goodman, S.P. Markey, P.G. Moe, B.S. Miles and C.C. Teng, Biochem. Med., 12 (1975) 12.
34 R.A. Chalmers and S.M. Lawson, Chem. Brit., 11 (1975) 29.
35 R.A. Chalmers, M.J.R. Healy, A.M. Lawson and R.W.E. Watts, Clin. Chem., 22 (1976) 1288.
36 R.A. Chalmers, M.J.R. Healy, A.M. Lawson, J.T. Hart and R.W.E. Watts, Clin. Chem., 22 (1976) 1292.
37 I. Molnár, P. Jatlow and C. Horváth, in preparation.
38 A.P. Grahes and B.L. Karger, Clin. Chem., 22 (1976) 184.
39 U. Langenbeck, A. Hoinowski, K. Mantel and H.-U. Möhring, J. Chromatogr., 143 (1977) 39.

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Biomedical Applications

CHROMBIO. 046

Note

Gas-phase analytical methods in the characterization of ^{2}H- and ^{3}H-labeled 7α-hydroxy-5,11-diketotetranorprostane-1,16-dioic acid

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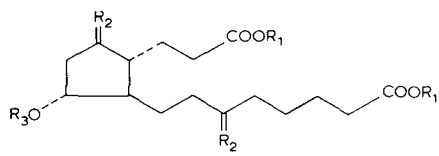
Gas-phase analytical methods [1] [i.e., gas—liquid chromatography (GLC)-Based separations combined with "normal" detectors, radioactivity monitors, and mass spectrometers] are employed extensively in drug metabolism and related studies. The use of radioisotopes in combination with gas—liquid radiochromatography (GLRC) in such research is well established [2, 3]. Stable-isotope labeling provides internal standards for GLC—mass spectrometry (MS)-based assays [4, 5] and characteristic isotope clusters for MS recognition of compounds as being drug or substrate-related [6, 7]. We now wish to report our use of these methods to characterize ^{2}H (deuterium)- and ^{3}H (tritium)-labeled 7α-hydroxy-5,11-diketotetranorprostane-1,16-dioic acid (I)



I R_1, R_3 = H; R_2 = O
II R_1 = CH_3; R_2 = NOC^2H_3; R_3 = H
III R_1 = CH_3; R_2 = $NOCH_3$; R_3 = $Si(CH_3)_3$

Hamberg [8] and Seyberth et al. [9] have employed the bis-($^{2}H_3$-methyloxime),bis-methyl ester of 7α-hydroxy-5,11-diketotetranorprostane-1,16-dioic acid (II)* as the internal standard in a GLC—MS assay to demonstrate the effect of certain drugs upon human urinary excretion of I, the major human urinary metabolite of prostaglandins E_1 and E_2. In using II as the internal standard it is introduced after endogenous I has been partially purified and derivatized to its bis-methyloxime,bis-methyl ester. We have sought to obtain labeled I in which the ^{2}H and ^{3}H are both present in the prostaglandin metabolite per se, so that the internal standard can be added to the urine sample at

*The underivatized compound, labeled with ^{3}H in the 2-position, is prepared biosynthetically from tritiated 15-keto-prostaglandin E_0 (15-keto-PGE_0) [8, 9].

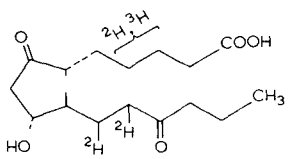
the beginning of the assay, thus compensating for yield losses incurred in the entire analysis. The present paper describes the use of several gas-phase analytical methods in characterizing ^{2}H,^{3}H-labeled I prepared by two different means.

EXPERIMENTAL

GLRC was carried out using a Barber-Colman Model 5000 instrument in which the column effluent is split between a flame ionization detector and a combustion tube (CuO—steel-wool at 800°) connected to a proportional counter. GLC—MS data were obtained using an LKB Model 9000 instrument with the following settings: source temperature, 270° electron energy, 70 eV accelerating voltage, 3.5 kV; trap current, 60 μA. Derivatization was carried out as previously reported [9].

RESULTS AND DISCUSSION

The first approach to producing the ^{2}H, ^{3}H-labeled internal standard involving its biosynthesis by rabbits from ^{2}H, ^{3}H-labeled 15-keto-PGE_0* (see below) employed the dosing and isolation procedures reported by Seyberth et al. [9]. However, the second reversed-phase partition chromatographic step used by these authors was omitted, as it is carried out on the esterified (diazomethane) isolate and we wished to obtain the free acid.



By the very nature of its method of isolation ^{2}H,^{3}H-labeled I must be less pure than if it had been esterified and carried through the complete isolation procedure [9]. As radioactivity is used as the measure of the quantity of internal standard added to an aliquot of urine, the mass added can only be a maximum. Further, as a result of the side-chain degradation the specific activity of the biosynthesized metabolite must be assumed to be one-half that of the tritiated precursor, and this approximation also results in the introduction of an additional inaccuracy. Finally, because of the extent and nature of the ^{2}H labeling, the internal standard (in its derivatized form) exhibits a signal at *m/e* 365 (see below), the ion monitored for quantitation of the endogenous metabolite in human urine.

The partial mass spectrum of ^{2}H,^{3}H-labeled 15-keto-PGE_0 as its bis-methyloxime,bis-methyl ester trimethylsilyl (TSM) ether is presented in Fig. 1. The most intense signals of the isotopic clusters associated with the M-31 (loss of OCH_3) and M-(31 + 90) (loss of OCH_3 + TMSOH) ions, *m/e* 470 and 380, respectively, indicate that the compound contains three ^{2}H per mole, as the corresponding signals for the unlabeled compound are found at *m/e* 467 and 377.

*Prepared by Rosegay [10].

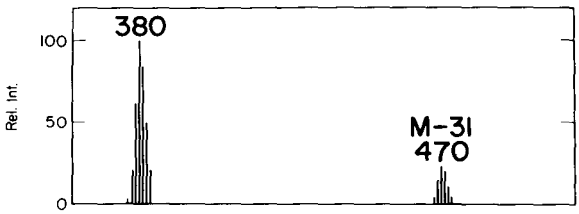


Fig. 1. Partial mass spectrum of ^2H, ^3H-labeled 15-keto-PGE_0 as its bis-methyloxime,bis-methyl ester, TMS ether.

The isolate from the rabbit urine was processed through the assay procedure [9] and the final thin-layer chromatographic (TLC) zone containing the bis-methyloxime,bis-methyl ester of the metabolite eluted and trimethylsilylated. Analysis of this derivatized radioactive material by GLRC gave the results shown in Fig. 2b and c. A radioactive component was observed which possessed the same retention time as the C_{24} fatty acid methyl ester (Fig. 2a) on a non-polar dimethyl polysiloxane stationary phase; this is the GLC behavior reported by Hamberg and Samuelson [11] for derivatized I from human urine. GLC—MS of this radioactive component from rabbit urine demonstrated that its mass spectrum was identical, except for the isotope clusters, to that published [11] for the human metabolite. The partial mass spectrum of this radioactive compound is given in Fig. 3. Whereas the M-31 and base peak M-(31 + 90) ions for derivatized unlabeled metabolite are found at *m/e* 455 and 365, respectively [11], the analogous ions for the radioactive rabbit metabolite are found at *m/e* 457 and 367. The isotope cluster patterns further characterize the compound as being derived from the ^2H,^3H-labeled 15-keto-PGE_0. That the metabolite contains two ^2H per mole rather than three is expected, for a significant loss of deuterium (and tritium) must result from the metabolic transformation.

Taub et al. [12] recently reported the synthesis of I, and this work has been extended to the preparation of the ^2H,^3H-labeled I [13]. GLC—MS analysis of this compound as its bis-methyloxime,bis-methyl ester, TMS ether (III) gave the results presented in Figs. 4 (GLC) and 5 (MS). The two major GLC peaks are the *syn* and *anti* isomers, and the partial mass spectrum of the major isomer indicates that it contains seven ^2H per mole, with no signal at *m/e* 365. GLRC analysis of the trimethylsilylated TLC zone eluate from a urine sample spiked with this internal standard and carried through the assay procedure gave the results shown in Fig. 6b; the radioactive component possesses, as required, the same retention behaviour as the C_{24} fatty acid methyl ester. The isotope clusters of this isolated internal standard were the same as those shown in Fig. 5, demonstrating that no isotopic exchange occurred during the assay procedure.

A 20-ml aliquot of urine from a normal adult male was spiked with ^2H,^3H-I just prior to initiation of the assay procedure [9]; partway through the assay the bis-($^2H_3$-methyloxime),bis-methyl ester of synthetically prepared I [12] was also introduced as per the approach of Seyberth et al. [9]. Calculations based on the ^2H,^3H-I internal standard [monitoring of ions of *m/e* 365 (endogenous I) and 372 (internal standard)] gave a value of 0.18 μg I per 20 ml, whereas the value obtained based on the other internal standard [monitoring of ions of *m/e* 365 and 368 (internal standard)] was 0.13 μg I per 20 ml.

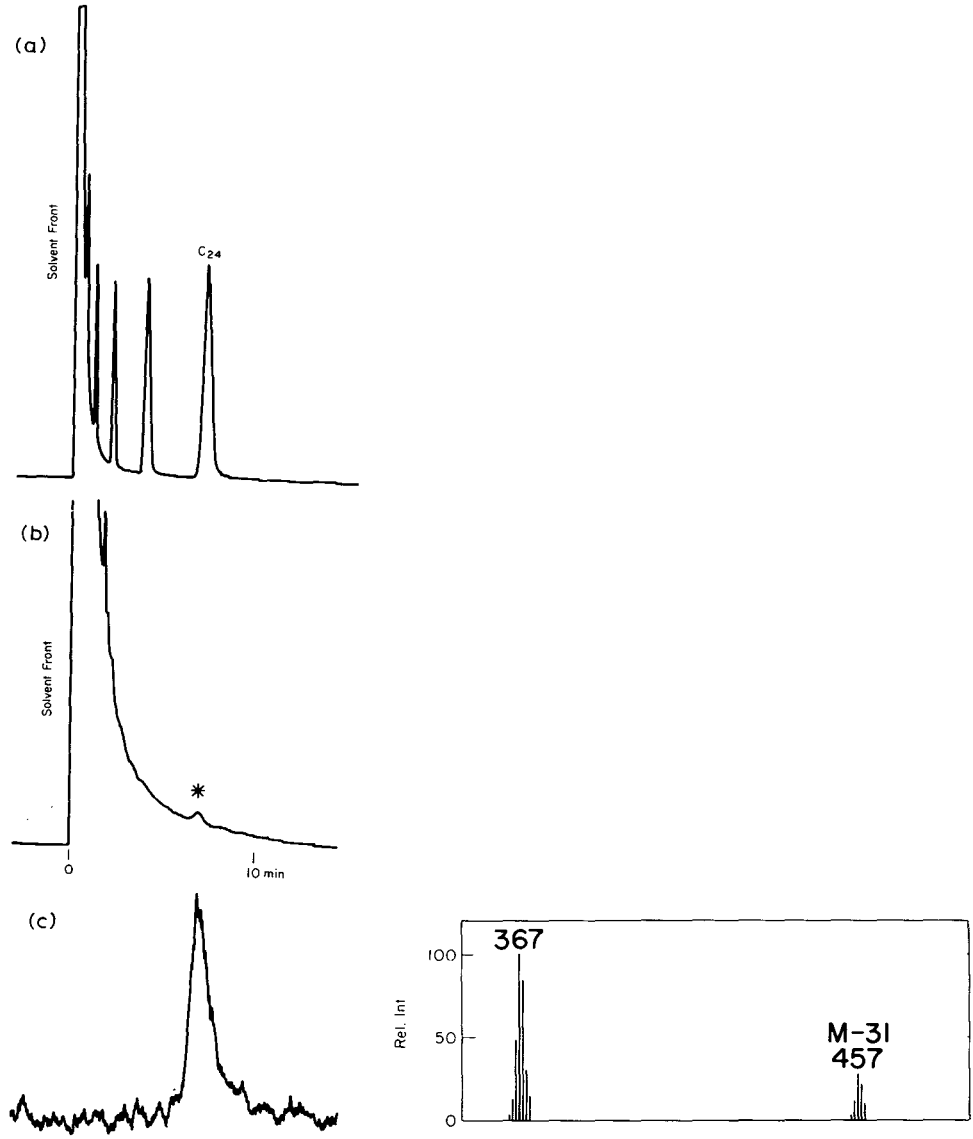


Fig. 2. (a) Gas chromatogram resulting from analysis of mixture of long-chain fatty acid methyl esters (C_{16}—C_{24}). Column conditions: 6 ft. × 4 mm I.D. glass U-tube packed with 1.0% OV-1 dimethylpolysiloxane stationary phase on 80—100 mesh acid-washed and silanized Gas-Chrom P, 220°; carrier gas flow-rate, 60 ml/min. (b) and (c) Simultaneous flame ionization and radioacitivity monitor detection records resulting from analysis of derivatized (bis-methyloxime,bis-methyl ester, TMS ether) rabbit metabolite of $^{2}H,^{3}H$-labeled 15-keto-PGE_0. Column conditions as for (a).

Fig. 3. Partial mass spectrum of the radioactive compound described in Fig. 2c.

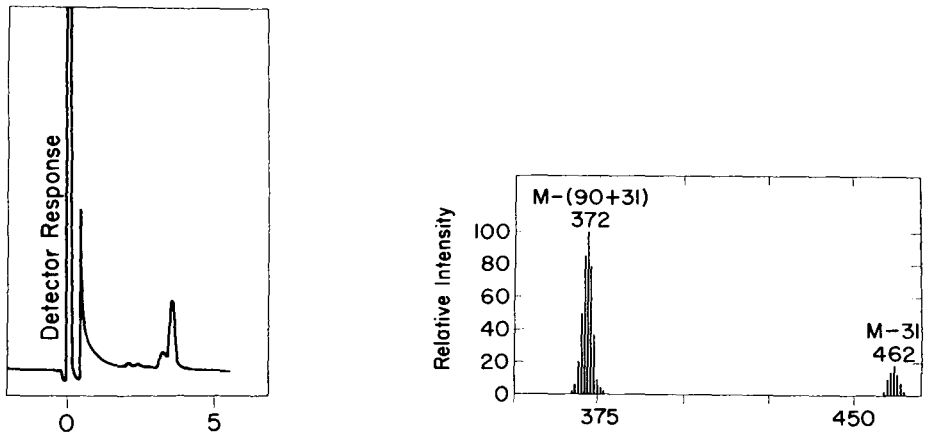


Fig. 4. Gas chromatogram resulting from GLC—MS analysis of synthetic ^{2}H, ^{3}H-labeled I (bis-methyloxime,bis-methyl ester, TMS ether). Column conditions: 5 ft. × 3 mm I.D. spiral glass tube packed with 1.5% SE-30 dimethyl polysiloxane stationary phase on acid-washed and silanized Gas-Chrom P, 218° ; carrier gas flow-rate, 30 ml/min.

Fig. 5. Partial mass spectrum of the major component in the sample described in Fig. 4.

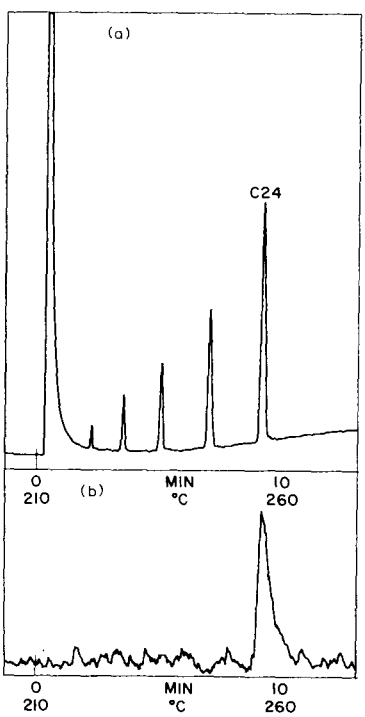


Fig. 6. (a) Gas chromatogram (flame ionization detection) resulting from analysis of mixture of long-chain fatty acid methyl esters (C_{16}—C_{24}). Column as described in Fig. 2; temperature programmed from 210° to 260° at 5°/min. (b) Radioactivity monitoring detection record resulting from analysis of a trimethylsilylated TLC zone eluate from a urine sample spiked with the synthetic ^{2}H,^{3}H-labeled I and carried through the assay procedure.

TABLE I

URINARY OUTPUT OF 7α-HYDROXY-5,11-DIKETOTETRANORPROSTANE-1,16-DIOIC ACID IN SUBJECTS TREATED WITH DIFLUNISAL*

Subject	Urinary output (μg per 24 h)		
	Control	2nd Day on run	5th Day on run
A	6.5	3.0	3.9
B	21.2	4.7	2.9
C	12.6	3.6	3.5

*375 mg twice a day.

The synthetically prepared $^{2}H,^{3}H$-I internal standard has been employed in a study to demonstrate the effect of the new analgesic diflunisal (2′,4′-difluoro-4-hydroxy-1′,1-diphenyl-3-carboxylic acid) upon the urinary excretion of I in normal male subjects [14]. The data in Table I demonstrate that administration of this drug resulted in a marked reduction in urinary output of the PG metabolite, presumably reflecting inhibition of PGE_1 and PGE_2 biosynthesis.

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We thank Drs. A. Rosegay and D. Taub for the labeled prostaglandins, Drs. H.W. Seyberth, J.A. Oates (Vanderbilt University School of Medicine) and M. Hamberg (Karolinska Institute) for their many helpful suggestions concerning the dosing and isolation procedures in connection with biosynthesis of the rabbit metabolite, Ms. K.F. Hooke for expert animal work, and Drs. S.L. Steelman, C.T. Smit Sibinga (University of Groningen), P. Schultz (University of Geneva) and K.P. Tempero for providing the samples of human urine from the diflunisal study.

REFERENCES

1 M.G. Horning, J. Nowlin, K. Lertratanangkoon, R.N. Stillwell, W.G. Stillwell and R.M. Hill, Clin. Chem., 19 (1973) 845.
2 N.R. Trenner, O.C. Speth, V.B. Gruber and W.J.A. VandenHeuvel, J. Chromatogr., 71 (1972) 415.
3 F.F. Sun and J.E. Stafford, Biochim. Biophys. Acta, 369 (1974) 95.
4 T.E. Gaffney, C.-G. Hammar, B. Holmstedt and R.E. McMahon, Anal. Chem., 43(1971) 307.
5 M.G. Horning, W.G. Stillwell, J. Nowlin, K. Lertratanangkoon, D. Carroll, I. Dzidic, R.N. Stillwell, E.C. Horning and R.M. Hill, J. Chromatogr., 91 (1974) 413.
6 W.E. Braselton, Jr., J.C. Orr and L.L. Engel, Anal. Biochem., 53 (1973) 64.
7 D.R. Knapp, N.H. Holcombe, S.A. Krueger and P.J. Privitera, Drug Metab. Dispos., 4 (1976) 164.
8 M. Hamberg, Biochem. Biophys. Res. Commun., 49 (1972) 720.
9 H.W. Seyberth, B.J. Sweetman, J.C. Frolich and J.A. Oates, Prostaglandins, 11 (1976) 381.
10 A. Rosegay, unpublished results.
11 M. Hamberg and B. Samuelson, J. Amer. Chem. Soc., 91 (1969) 2177.
12 D. Taub, Z.S. Zelawski and N.L. Wendler, Tetrahedron Lett., (1975) 3667.
13 A. Rosegay and D. Taub, Prostaglandins, 12 (1976) 785.
14 S.L. Steelman, C.T. Smit Sibinga, P. Schulz, W.J.A. VandenHeuvel and K.F. Tempero, paper presented at XIII Int. Conf. Int. Med., Helsinki, August, 1976.

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Biomedical Applications

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Note

Liquid chromatography assay for 3,4-dihydroxyphenylacetic acid in urine

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In support of our research on tyrosine metabolism, we have been developing a series of specific assay procedures for the various basic, neutral, and acidic metabolites in body fluids and tissue homogenates. The present brief report on urinary 3,4-dihydroxyphenylacetic acid (DOPAC) is intended to complement published work on 3-methoxy-4-hydroxyphenylacetic acid (homovanillic acid, HVA) [1] and the catecholamines [2]. DOPAC and HVA are the major catabolites from the catecholamine dopamine (DA) and as such both must be measured in studies of total DA production.

DOPAC can be measured by condensation with ethylene diamine and subsequent monitoring of the fluorescent product. This approach has been used by several authors following isolation by various extraction procedures combined with column chromatography or electrophoresis [3—5]. A more recent method employs gas chromatography with electron-capture detection [6]. After isolation of the DOPAC by adsorption chromatography, a volatile derivative is formed by reaction with pentafluoropropionic anhydride.

In this paper we describe a method for the measurement of urinary DOPAC based on liquid chromatography with electrochemical detection. DOPAC is isolated from acidified urine by ethyl acetate extraction and adsorption onto alumina, and is then quantitated by liquid chromatography with electrochemical detection.

MATERIALS AND METHODS

Reagents

The following reagents were used: 6 *M* hydrochloric acid; phosphate buffer, pH 7.4 (1.18 g potassium monobasic phosphate and 8.16 g dibasic phosphate

heptahydrate dissolved in 1 l of distilled water); 0.05 *M* Tris(trihydroxymethylaminomethane) buffer, pH 8.5 (2.21 g Tris hydrochloride and 4.36 g of the free base dissolved in 1 l of distilled water); 0.1 *M* acetate buffer, pH 4.7 (mobile phase); creatinine standard solution (1 mg/ml in distilled water); 1% picric acid in distilled water; 10% (w/v) sodium hydroxide; alumina (acid-washed Woelm W 200 neutral alumina according to the method of Anton and Sayre [7]); and standard urine pool, which is prepared as follows: Combine urine collected from healthy humans. Acidify to pH 2 with 6 *M* HCl and store 20-ml aliquots at −35° in glass scintillation vials. Determine the concentration of DOPAC in the urine pool by the method of standard additions. Analyze the urine pool with and without added DOPAC. Plot the peak height versus amount of DOPAC added and extrapolate to zero peak height to obtain the concentration of DOPAC in the urine pool.

Apparatus

The liquid chromatographic system was a commercial unit based on our earlier work [8,9] using a thin-layer amperometric detector (Bioanalytical Systems, Model LC-2A). DuPont Zipax anion exchange resin was dry packed in a 50 cm × 2.1 mm I.D. glass column (Altex Scientific, Model 251-02). The mobile phase was pumped at a flow-rate of 0.4 ml/min. The detector potential was set at +0.60 V versus an Ag/AgCl reference electrode. Samples were injected using a pneumatically actuated non-metallic 20-μl slider valve (Laboratory Data Control, Model CSV-20).

Procedure

Acidify urine to pH 2 with 6 *M* HCl upon collection and store frozen at −35° prior to analysis. Place 4 ml of urine (pH 2) in a 12-ml glass centrifuge tube, saturate with NaCl (ca. 0.6 g NaCl) and add 4 ml of ethyl acetate. Shake for 10 min on a reciprocal shaker, centrifuge briefly and transfer the ethyl acetate to a 6-in. culture tube using a disposable Pasteur pipet. Repeat the extraction with two additional 4-ml volumes of ethyl acetate. Dry the combined ethyl acetate layers over anhydrous sodium sulfate (ca. 1.5 g). Transfer the ethyl acetate to an acid-washed 12-ml centrifuge tube. Wash the residual sodium sulfate with about 1 ml of ethyl acetate and combine with the original extract.

Concentrate the extract to a volume of about 2 ml under a stream of nitrogen at 25°. Transfer the solvent to a 5-ml conical screw cap vial (Pierce, Reacti-Vial). Wash the centrifuge tube with about 1 ml ethyl acetate and combine with the ethyl acetate concentrate. Evaporate to dryness at room temperature using a stream of nitrogen and add 3 ml of 0.05 *M* pH 8.5 Tris buffer to the residue. Immediately add ca. 70 mg of aluminum oxide and shake on a reciprocal shaker for 15 min. The same quantity of alumina must be added to each vial in order to maintain a reproducible recovery. Allow the alumina to settle, then aspirate the buffer with a fine glass capillary. Wash the alumina twice with pH 7.4 phosphate buffer and once with water, mixing the alumina well each time. Carefully aspirate the final wash to near dryness. Cover the vial with aluminum foil and place a narrow (ca. 5 mm) filter paper wick through a slot in the foil extending into the wet alumina. Place the vials in a vacuum oven and dry in vacuo at 40° for 10 min. If care is taken in the final

aspiration step, then drying the alumina is not necessary. The small amount of water remaining after the final wash does not significantly influence the precision or accuracy of the method.

Elute the DOPAC from the alumina by the addition of 1 ml 1 *M* acetic acid to each vial. Shake the vials on a vortex mixer for 20 sec and allow the alumina to settle. Inject 20 μl of the eluent onto the chromatographic column using the 20-μl slider valve. Measure the peak height of DOPAC and determine the DOPAC concentration by comparison with the peak height obtained for the standard urine pool. Fig. 1 illustrates a representative chromatogram obtained from healthy individuals.

Levels of DOPAC are usually reported relative to the creatinine concentration. Creatinine is determined by the standard picric acid spectrophotometric method [10].

RESULTS AND DISCUSSION

DOPAC was determined in 15 urine samples collected from ten apparently healthy males, ranging from 21 to 30 years of age. DOPAC levels ranged from 0.39 to 1.05 μg per mg creatinine, which is in general agreement with other investigators. The absolute recovery of DOPAC was 48 ± 5.0% relative standard deviation and was found to be constant over a concentration range of at least 1 to 7 μg per ml of urine. The relative standard deviation of the method was found to be ±4.3%. The detector response current was measured from 1 to 100 ng injected and the linear calibration in nA versus ng DOPAC

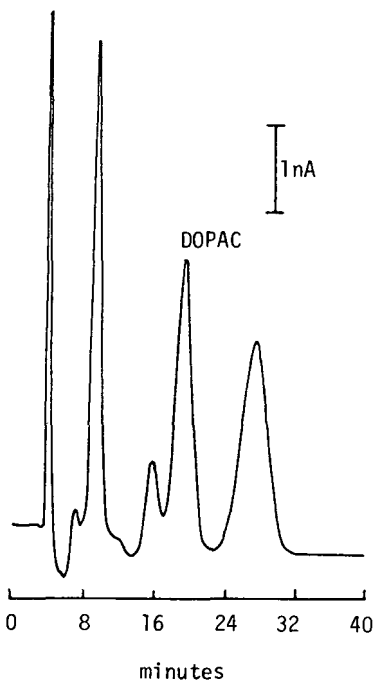

Fig. 1. Chromatogram of DOPAC isolated from a urine pool collected from healthy individuals.

is described by the following equation:

Current (nA) = (1.63 ± 0.004) (ng DOPAC)—(0.54 ± 0.06)

The detection limit for DOPAC in an aqueous standard was found to be 100 pg.

The present method has advantages of selectivity and sensitivity compared with other solution phase methods. The selectivity of the assay is derived from the combination of four steps, liquid—liquid extraction, liquid—solid extraction, liquid chromatography and electrochemical detection. The initial ethyl acetate extraction removes a majority of the aromatic acids from urine. Only those acids containing a catechol moiety are adsorbed onto the alumina and are then separated by high-performance liquid chromatography. Finally only those compounds oxidizing at +0.6 V versus Ag/AgCl or less are detected. In addition the high-performance liquid chromatographic approach does not require derivatization as do all gas—liquid chromatographic methods.

TABLE I

RETENTION OF CATECHOL ACIDS

Acid	Retention time (min)
3,4-Dihydroxymandelic	13.2
DOPAC	17.0
Protocatechuic	20.8
3,4-Dihydroxy-phenylpropionic	24.1
Gallic	35.0
Caffeic	67.1
Chlorogenic	∞

Several potential interferences have been examined and found to be adequately resolved from DOPAC by the DuPont Zipax (see Table I). Those compounds which elute from the column close to DOPAC are normally found in urine in much lower concentrations and therefore present no problem. If desired, better resolution at the expense of time can be accomplished by a decrease in the mobile phase ionic strength.

REFERENCES

1 L.J. Felice and P.T. Kissinger, Anal. Chem., 48 (1976) 794.
2 P.T. Kissinger, R.M. Riggin, R.L. Alcorn and L.-D. Rau, Biochem. Med., 13 (1975) 299.
3 B.D. Drujan, N. Alvarez and J.M. Diaz Borges, Anal. Biochem., 15 (1966) 8.
4 F. Eichhorn, A. Rutenberg and E. Kott, Clin. Chem., 17 (1971) 296.
5 F.S. Messiha, E. Bakustis and V. Frankos, Clin. Chim. Acta, 45 (1973) 159.
6 M.W. Weg, C.R.J. Ruthven, B.L. Goodwin and M. Sandler, Clin. Chim. Acta, 59 (1975) 249.
7 A.H. Anton and D.F. Sayre, J. Pharmacol. Exp. Ther., 138 (1962) 360.
8 R.M. Riggin, A.L. Schmidt and P.T. Kissinger, J. Pharm. Sci., 64 (1975) 680.
9 R.E. Shoup and P.T. Kissinger, Chem. Instrum., 7 (1976) 171.
10 B.L. Oser (Editor), Hawk's Physiological Chemistry, McGraw-Hill, New York, 14th ed., 1965, p. 1040.

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Note

Immunoelectrophoretic analysis of human meconium antigens of non-plasma origin

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Meconium is the first stool passed after birth and represents the accumulation of intestinal contents during foetal life. The main constituent of meconium is the mucilaginous ground substance which is rich in blood group substances [1,2] but other macromolecules such as serum proteins [3] and enzymes of pancreatic and intestinal origin have also been characterized [4, 5]. Knowledge of the distribution of the meconium components may be of value in increasing our understanding of the biochemistry of the foetal gut and perhaps of more immediate importance, be of use when developing methods of screening for diseases of gut metabolism in the neonate [6]. Immunochemical methods such as electroimmunoassay and immunoelectrophoresis provide rapid and simple ways for the characterization and determination of macromolecules in biological fluids and may be adapted to the routine analysis of such components, an important criterion in a screening procedure. However, until now, the analysis of meconium by such methods has been limited to the study of its serum protein component [4, 7]. I wish to report here the results of a cross immunoelectrophoretic analysis of meconium antigens of non-plasma origin.

EXPERIMENTAL

Meconium specimens and preparation of water soluble extract

Meconium specimens were collected during the course of a routine screening programme from 50 healthy neonates born at full term. All specimens were lyophilized, bulked and stored at −20° before use. A water-soluble extracts was prepared as follows: 200 mg of the bulked, dried material was suspended in 10 ml of saline with the aid of a Potter's tissue homogeniser and the resultant sus-

pension spun at 84,000 *g* in a Beckman SW50 rotor (r_{av} = 8.3 cm) for 30 min at 4°. The clear supernatant was collected and used in the subsequent electrophoretic analysis.

Immunoelectrophoretic analysis

Antisera to lactoferrin and secretory IgA were prepared in rabbits as previously described [8]. A similar procedure was used to raise antiserum against meconium proteins using a 1% suspension of meconium as an antigen source. After adsorption of the antiserum with gluteraldehyde-insoluble human serum [8] to remove antibodies to the serum proteins (SP), it was designated anti-meconium (—SP) serum. Antisera to human trypsin and chymotrypsin were obtained from Miles Labs. (Slough, Great Britain). Antiserum to carcino-embryonic antigen was obtained from DAKO-Immunoglobulins (Copenhagen, Denmark). Antiserum to human blood group A was a gift from the Welsh Transfusion Centre.

Quantitative cross immuno-electrophoresis of the meconium extract was carried out against the appropriate antisera as previously described [8]. After electrophoresis, the washed, dried plates were stained for protein with Coomassie blue [8], for carbohydrate by the periodic acid —NADI (naphthol-phenylenediamine) reaction [9] and for alkaline phosphatase, esterase, tryptic and chymotryptic activities by the methods described by Uriel [9]. Specific antiserum was used to identify arcs by the intermediate gel method [10]. The specific antiserum was incorporated into an intermediate gel interposed between the first dimension gel containing the separated antigens and the second dimension gel containing polyvalent antiserum (reference gel).

Gel chromatography

A 5-ml aliquot of the 2% meconium saline extract was separated on a column of Sepharose CL-4B (2.5 × 90 cm) with 0.05 *M* Tris—0.09 *M* acetic acid, pH 7.3, containing 0.3 *M* sodium chloride. The eluent was monitored at 280 nm and 5-ml fractions were collected. The elution profiles of the individual meconium antigens were then determined using the polyvalent antiserum with a "fused rocket" technique [11] and as the column had been previously calibrated with human serum proteins of known molecular weight, an approximate estimation of the molecular sizes of the major meconium antigens was made.

Inhibition of haemagglutination

Inhibition of haemagglutination was tested as follows: 50 μl of 2% washed group A red cells were mixed with 50 μl of the test solution and 50 μl of anti-A serum and agglutination assessed after standing for 30 min at room temperature. Serial dilution of the 2% meconium extract and chromatography fractions were studied for inhibition activity.

RESULTS

Cross immunoelectrophoresis of 2% meconium extract against anti-meconium (—SP) developed 7 arcs designated Mec I to Mec VII (Fig. 1). The Mec I arc had a distinct peak but all the arcs were elongated suggesting each com-

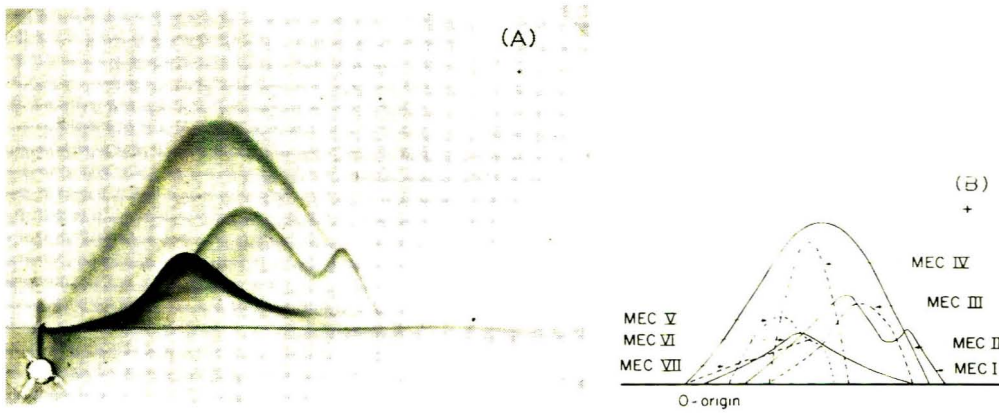

Fig. 1. Quantitative cross immunoelectrophoresis of meconium extract against anti-meconium serum adsorbed with human serum: antiserum (—SP). (A) 2.0% meconium extract against anti-meconium (—SP), immunoelectropherogram was stained with Coomassie blue. (B) Line diagram of meconium extract against anti-meconium (—SP). Mec III arc was due to carcinoembryonic antigen and Mec IV was due to alkaline phosphatase.

ponent had some degree of electrophoretic heterogeneity. Mec I, Mec II, Mec Vi and Mec VII stained for carbohydrate; Mec IV had alkaline phosphatase activity and the ascending anodal part of the Mec VI had chymotrypsin activity. None of the arcs had tryptic or carboxylesterase activity. Identification of antigens with specific antiserum was carried out using intermediate gel cross immunoelectrophoresis. An arc developed in the intermediate gel which contained antiserum to CEA (carcino-embryonic antigen) while the Mec III arc was absent from the polyvalent pattern that developed in the corresponding reference gel. No precipitation arcs developed in the intermediate gel when antiserum to chymotrypsin, trypsin, lactoferrin, secretory IgA or blood group substance A was used and there were no significant changes in the polyvalent patterns. A number of "high titre" anti-A sera were used but in no instance was it possible to develop a precipitation arc against the meconium extract although a meconium extract concentration of 0.02% could inhibit the haemagglutination of group A cells by all the anti-A sera tested. It was concluded that the anti-A activity titre was too low to be used in this immunoelectrophoretic system.

An approximate estimation of molecular weights of the unidentified meconium antigens was made by gel chromatography on a previously calibrated column of Sepharose CL-4B. Mec I had a molecular weight of about 100,000. Mec II was eluted in the void volume suggesting a molecular weight of 1,000,000—2,000,000. Mec VI had a molecular weight of about 500,000 and Mec VII demonstrated pronounced molecular polydispersity between 100,000 to 1,000,000. The Mec V arc was too faint and it was not possible to determine its elution profile. The inclusion of 6 *M* urea into the chromatography buffer had no effect on the elution profiles of the meconium antigens. Inhibition of haemagglutination activity was found throughout the column eluent although there was a peak of inhibitory activity in the void volume.

DISCUSSION

Seven meconium antigens of non-plasma origin have been detected, two of which have been identified as carcinoembryonic antigen (Mec III) and alkaline phosphatase (Mec IV). Some tentative conclusion concerning the nature of the other antigens can also be made. Mec I, Mec II and Mec VI were glycoproteins with molecular weights of approximately 100,000; 1,000,000—2,000,000 and 500,000 respectively. None of the antigens were proteins commonly associated with exocrine secretions, lactoferrin and secretory piece or secretory IgA, nor were they pancreatic proteinases. Although part of the Mec VI arc had chymotryptic activity, its molecular size and its negative reaction with antiserum to chymotrypsin precluded the possibility that Mec VI was chymotrypsin. The fact that the Mec VI arc did not also have tryptic activity implied that the antigen had some specificity towards chymotrypsin adsorption. Although no precipitation arcs developed with the anti-A sera, the marked inhibition of haemagglutination by the meconium extract leaves the possibility that one or more of the antigen arcs were due to blood group substances.

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REFERENCES

1 D. J. Buchanan and S. Rapport, J. Biol. Chem., 192 (1951) 251.
2 R. H. Côté and J. P. Valet, Biochem. J., 153 (1976) 63.
3 A. Deroches, D. Galerne, J. Baudon and G. Dastugue, J. Gynec. Obstet. Biol. Repr., 3 (1974) 543.
4 E. Eggermont, Biol. Neonate, 10 (1966) 266.
5 I. Antonowicz, S. Ishida and H. Shwachman, Pediatrics, 56 (1975) 782.
6 H.C. Ryley, in J.A. Dodge (Editor), Topics in Paediatric Gastroenterology, Pitman Medical, Tunbridge Wells, 1976, pp. 14—25.
7 H.C. Ryley, L. Neale, T.D. Brogan and P.T. Bray, Arch. Dis. Childhood, 49 (1974) 901.
8 H.C. Ryley, Biochim. Biophys. Acta, 271 (1972) 300.
9 J. Uriel, in C.A. Williams and M.W. Chase (Editors), Immunology and Immunochemistry, Vol. 3, Academic Press, London, New York, 1971, pp. 294—321.
10 N.H. Axelsen, in N.N. Axelsen, J. Krøll and B. Weeke (Editors), A Manual of Quantitative Immunoelectrophoresis, Universitetsforlaget, Oslo, Bergen, Tromsø, 1973, pp. 71—77.
11 P.J. Svendsen, in N.H. Axelsen, J. Krøll and B. Weeke (Editors), A Manual of Quantitative Immunoelectrophoresis, Universitetsforlaget, Oslo, Bergen, Tromsø, 1973, pp. 69—70.

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Note

Quantitation of amitriptyline and nortriptyline in human serum

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Amitriptyline and nortriptyline, its N-desmethyl metabolite, are widely used in the treatment of depression. Large individual differences in steady-state plasma concentrations of these drugs were observed in patients receiving the same dose, and several studies indicate an optimum clinical response to these tricyclic antidepressants may be associated with a relatively narrow range of steady-state plasma concentrations [1—4]. Safe and effective drug therapy can only be accomplished by monitoring amitriptyline and nortriptyline concentrations in serum; hence, there is need for methods which can be performed routinely in clinical chemistry laboratories.

Numerous methods for the determination of tricyclic antidepressants have been reported, however, none appears to have gained widespread routine use due to complexity or lack of specificity and sensitivity. Hammer and Brodie [5] assayed desmethylimiprimine and nortriptyline by Labelling these secondary amines with [^{3}H]acetic anhydride; however, this method is not applicable to measurement of tertiary amines including amitriptyline. Hucker and Stauffer [6] and Braithwaite and Widdop [7] used gas—liquid chromatography (GLC) with flame ionization detection to measure amitriptyline and the N-trifluoroacetyl derivative of nortriptyline. These methods, however, require large volumes of serum, are subject to interference from endogenous compounds, and are not readily applicable to routine monitoring. Borgå and Garle [8] reported an electron-capture–gas chromatographic procedure for the N-heptafluorobutyric acid derivative of nortriptyline. Similarly, Walle and Ehrsson [9] developed a very sensitive electron-capture detection method for this derivative of nortriptyline. Neither of these procedures, however is applicable to the determination of amitriptyline.

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Recently a method for measuring various tricyclic antidepressants by means of gas chromatography—mass fragmentography has been reported [10]. Although this method is specific, it requires a large volume of serum (4 ml) and the instrument is expensive and not yet widely available in clinical chemistry laboratories.

Recently several methods using GLC with nitrogen sensitive detection have been described for the quantitative determination of tricyclic antidepressants. Gifford and co-workers [11] used an alkali flame ionization detector and a unique backflush system to determine various tricyclic antidepressants without need for derivatization. Jorgensen [12] determined amitriptyline and the N-acetyl derivative of nortriptyline, however, it is our experience that the latter compound chromatographs poorly. Bailey and Jatlow [13] recently described a method for amitriptyline and nortriptyline which did not require derivatization of the secondary amine, but used 3 ml of serum.

We present a GLC method utilizing nitrogen sensitive detection for the simultaneous determination of amitriptyline and nortriptyline as the N-trifluoroacetyl derivative which has been developed for routine use in our Therapeutic Drug Monitoring Laboratory. Although formation of this derivative requires an additional step in the procedure, the resultant compound has much better chromatographic characteristics; hence, sensitivity of the method is improved and the analytical procedure is less susceptible to changes in column conditioning.

MATERIALS AND METHODS

Chemicals

Acetone, ethyl acetate, hexane and isopropanol, all nanograde, were obtained from Mallinckrodt (St. Louis, Mo., U.S.A.); boric acid, sodium hydroxide, and sulfuric acid were obtained from J.T. Baker (Phillipsburg, N.J., U.S.A.); heptane (chromatoquality) was obtained from Matheson, Coleman & Bell, (East Rutherford, N.J., U.S.A.); potassium chloride was obtained from Fisher Scientific (Fairlawn, N.J., U.S.A.). Amitriptyline HCl was provided by Merck, Sharp & Dohme Research Lab. (West Point, Pa., U.S.A.); doxepin HCl was provided by Pfizer Pharmaceutical (Brooklyn, N.Y., U.S.A.); maprotiline HCl was provided by Ciba-Geigy (Basle, Switzerland); nortriptyline HCl was provided by Eli Lilly (Indianapolis, Ind., U.S.A.); tri(butoxyethyl)phosphate was obtained from ICN Life Sciences Group (Plainview, N.Y., U.S.A.) and trifluoroacetic anhydride (99+%) was obtained from Aldrich (Milwaukee, Wisc., U.S.A.).

Reagents

Prepare stock solutions (100 μg/ml) of amitriptyline (AMI), nortriptyline (NOR), doxepin (DOX) and maprotiline (MAPRO) by dissolving 10 mg of each compound in methanol to a volume of 100 ml. Prepare working solutions (1 μg/ml) of AMI and NOR by diluting 1 ml of their respective stock solutions to 100 ml with methanol, prepare an internal standard working solution (40

ng/ml) by evaporating to dryness 400 μl of DOX and MAPRO stock solution and dissolving the residue in heptane—isopropanol (99:1) to a volume of 1 l. Prepare a pH 10 borate buffer by dissolving 24.7 g of boric acid, 29.8 g of potassium chloride and 14.1 g of sodium hydroxide in distilled water to a volume of 1 l [14]. Prepare 0.1 *N* sulfuric acid by diluting 2.8 ml of concentrated sulfuric acid with distilled water to a volume of 1 l. Prepare 1 *N* sodium hydroxide by dissolving 40 g of sodium hydroxide in distilled water to a volume of 1 l.

Preparation and extraction of standards, controls and patient samples

Prepare serum standards (0, 50, 100, 200 and 300 ng/ml) by adding appropriate amounts (0, 50, 100, 200 and 300 μl) of AMI and NOR working solutions to 16 × 125 mm screw-top culture tubes and evaporating them to dryness under nitrogen at room temperature. Add 1.0 ml of pooled serum to each standard and 1.0 ml of control and patient serum to their respective 16 × 125 mm screw-top culture tubes. To each sample, add 1.0 ml of pH 10 borate buffer and vortex briefly. Add 7.0 ml of internal standard working solution to each tube using a Repipette from Lab. Industries (Berkeley, Calif., U.S.A.) and close with PTFE-lined screw caps.

Extract the samples by shaking on an Eberbach shaker for 15 min; then centrifuge for 10 min at 2000 rpm. Transfer the heptane layer (top) to clean tubes, containing 1.3 ml of 0.1 *N* sulfuric acid. Cap, extract as above for 10 min and centrifuge for 10 min.

Aspirate the organic layer (top), add 500 μl of 1 *N* sodium hydroxide to the aqueous layer and vortex each briefly. Add 5.0 ml of hexane to each, extract and centrifuge the samples as described above.

Transfer the hexane (top) layer to 5.0 ml mini-vials (Applied Science Labs., State College, Pa., U.S.A.) and evaporate the hexane to dryness (being careful not to overdry) under nitrogen at room temperature. To each residue, add 100 μl of ethyl acetate and vortex briefly. Add 30 μl of trifluoroacetic anhydride to each vial and cap them with aluminium foil. Incubate the vials in a heating block at 50—55° for 20 min. Allow the samples to cool to room temperature, then evaporate them to dryness under nitrogen at room temperature, again being careful not to overdry.

Prior to analysis, add 20 μl of acetone to the residue, vortex for 20 sec and inject 4.0 μl into the gas chromatograph.

Gas—liquid chromatography

A Model 3920 Perkin-Elmer dual-column gas chromatograph with dual nitrogen—phosphorus detectors and an all-glass receiver system (Perkin-Elmer, Norwalk, Conn., U.S.A.) was used. A Hitachi—Perkin-Elmer Model 165 recorder was operated at 1 mV full scale and a chart speed of 10 mm/min. The glass columns were 138 cm × 2 mm I.D., configured for the all-glass version utilizing heated on-column injectors. The column packing was 3% OV-17 on 100—120 mesh Gas-Chrom Q (Applied Science Labs).

The injection port temperature was 230°, the detector temperature was

270°, and the oven temperature was programmed from 220—275° at 8°/min. The carrier gas (nitrogen) flow-rate was 35 ml/min; air, 100 ml/min; and hydrogen, 4 ml/min.

The detector was operated in the nitrogen/phosphorus mode with the jet potential control in position 3. After flow-rates of the support gases were set, the potentiometer controlling the bead temperature was increased from zero until the recorder deflection caused by background was 15% full-scale with the electrometer attenuation set at × 10.

RESULTS AND DISCUSSION

In preliminary studies we prepared several derivatives of the desmethyl compounds nortriptyline and maprotiline. Of the compounds studied, the monotrifluoroacetyl derivatives of nortriptyline and its internal standard maprotiline exhibited chromatographic peaks which were not subject to interference by substances normally encountered in serum. Similarly, the tertiary amines, amitriptyline and doxepin, which are not derivatized were well resolved from each other. We selected doxepin and maprotiline as internal standards for amitriptyline and nortriptyline respectively because these compounds would not normally be administered concurrently with these drugs.

Representative chromatograms of serum extracted and derivatized according to our procedure are presented in Fig. 1. Chromatogram A with the internal standards omitted was obtained from the serum of a normal individual not receiving amitriptyline or nortriptyline. Chromatogram B was obtained with normal serum and the internal standards included in the extraction procedure. Chromatogram C was obtained from the serum of a normal individual to which amitriptyline and nortriptyline (100 and 150 ng/ml, respectively) were added. Chromatogram D was obtained from the serum of an individual receiving amitriptyline at a dose of 150 mg daily. The resultant levels were 121.0 ng/ml amitryptiline and 65.0 ng/ml nortriptyline. The drugs and their internal standards are well separated from each other and are free from interference from endogenous serum substances. The observed differences in retention times between amitriptyline and doxepin, and between nortriptyline and maprotiline were 0.35 min and 1.1. min, respectively.

A typical standard curve was obtained with this procedure. A linear relation between the ratio of peak height of drugs to internal standard, and the concentration of each drug in serum over a range of 0—300 ng/ml was found. The sensitivity, which was calculated from the minimum detectable amount at twice the noise level under normal operating conditions, was determined to be 5 ng/ml for both amitriptyline and nortriptyline when the sample volume was 1 ml.

The recovery of amitriptyline, doxepin, nortriptyline and maprotiline from serum over a range of 0—300 ng/ml is presented in Table I. The recoveries were determined in the following manner: 1 ml of drug-free serum was added to separate tubes containing 50, 100, 200 and 300 ng of each drug. These samples were extracted as described above; however, all volume transfers were quantitated. A second group of non-extracted standards containing the same amount

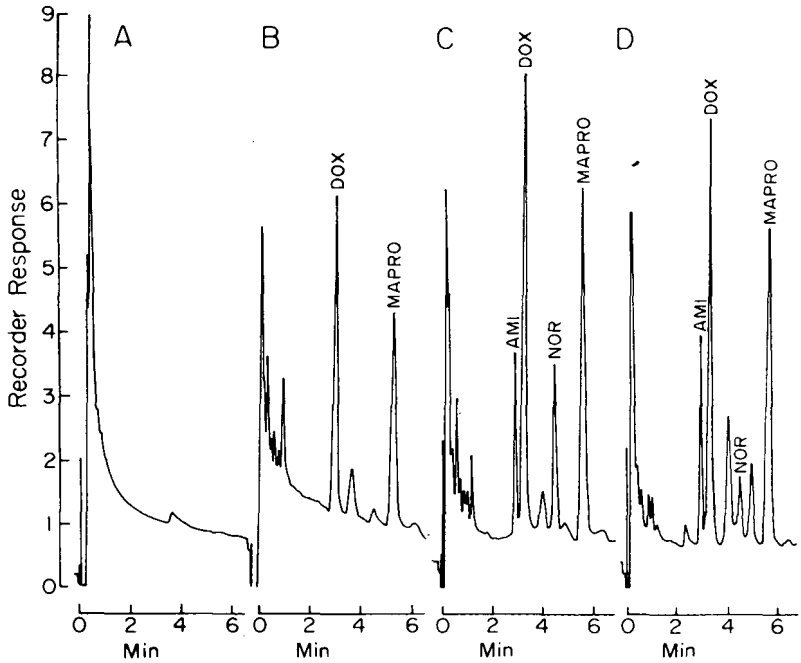

Fig. 1. Chromatograms obtained by the analysis of 1 ml of normal serum. Temperature was programmed from 220—275°, at 8°/min. Column packing was 3% OV-17 on 100—120 mesh Gas-Chrom Q. AMI = amitriptyline, NOR = nortriptyline, DOX = doxepin, MAPRO = maprotiline. (A) Drug-free serum, internal standards omitted; (B) serum to which doxepin and maprotiline (300 ng/ml) were added; the internal standards were included in the extraction; (C) serum to which amitriptyline (100 ng/ml) and nortriptyline (150 ng/ml) were added, internal standards included; (D) serum obtained from a patient receiving 150 mg amitriptyline daily; concentrations of amitriptyline and nortriptyline were determined to be 121 ng/ml and 65 ng/ml, respectively.

TABLE I

ABSOLUTE RECOVERIES OF AMITRIPTYLINE, DOXEPIN, NORTRIPTYLINE AND MAPROTILINE

Each value is the result of a single determination at the concentration indicated.

Drug (ng/ml)	Recovery (%)			
	AMI	DOX	NOR	MAPRO
50	87.8	89.2	69.1	85.1
100	81.2	92.8	68.1	80.0
200	91.0	99.1	78.1	86.4
300	98.7	85.0	85.5	91.8
Mean	86.9	91.5	75.2	85.8

of the drugs was prepared. All samples were derivatized as described in the methods section and exactly 4.0 μl of each sample was chromatographed. The peak heights of the extracted samples were then corrected for solvent transfers. Absolute recoveries of the compounds were calculated by comparing the ratio of the corrected peak height of the extracted samples to that of the non-extracted standards. The average recoveries of these compounds were: amitriptyline, 86.9%; doxepin, 91.5%; nortriptyline, 75.2%; and maprotiline 85.8%.

The within-run coefficient of variation of our assay at a serum concentration of 100 ng/ml amitriptyline and 150 ng/ml nortriptyline was 2.1% and 2.5% (n=12) respectively. Over a period of four months the between-run precision of this assay at these concentrations was 3.0% for amitriptyline and 3.8% for nortriptyline (n=20).

Fig. 2 illustrates chromatogram A obtained from a sample collected in a B-D vacutainer (Becton-Dickinson, Rutherford, N.J., U.S.A.) and chromatogram B, from a sample collected in a J-Vac (Jelco Laboratory, Raritan, N.J., U.S.A.) evacuated blood collection tube, each from a patient not receiving amitriptyline or nortriptyline therapy. Chromatogram C was obtained from a patient receiving nortriptyline, whose blood was collected in a B-D vacutainer. In contrast to the chromatograms obtained from the samples collected in the B-D vacutainers, the chromatogram of the sample collected in the J-Vac tube does not exhibit a large peak with the retention of 3.7 min which interferes with the

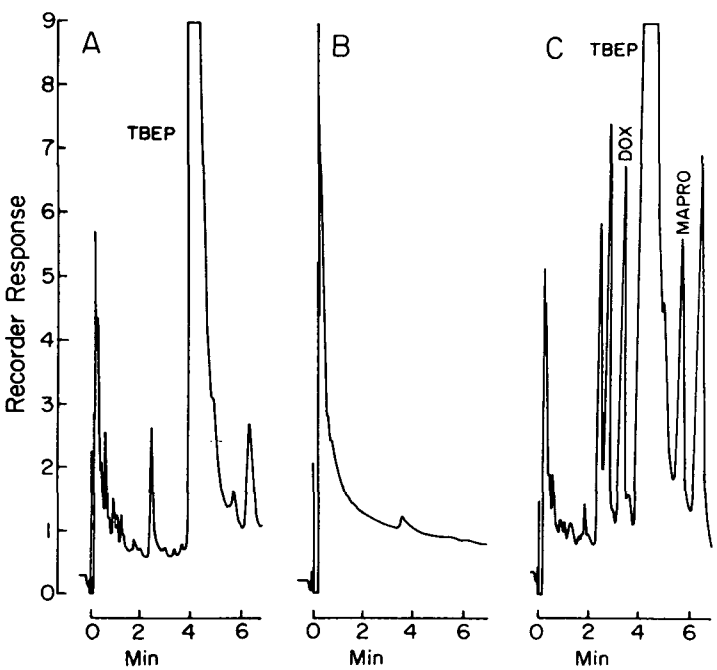

Fig. 2. Chromatograms obtained by the analysis of 1 ml of serum. (A), serum collected in a B-D vacutainer and (B), serum collected in a J-Vac evacuated blood collection tube, each obtained from patients not receiving amitriptyline or nortriptyline drug therapy; (C), 1 ml of serum collected in a B-D vacutainer from a patient receiving nortriptyline. TBEP = tri-2-butoxyethylphosphate.

quantitation of nortriptyline. This peak has been tentatively identified as tri-2-butoxyethyl-phosphate, a plasticizer used in the manufacture of the rubber B-D vacutainer stoppers. Authentic tri-2-butoxyethylphosphate had the same retention time as the interfering compound.

We have analyzed serum from patients receiving therapeutic doses of other drugs which are often administered concurrently with tricyclic antidepressants and have not encountered any chromatographic interference with our procedure. Although imiprimine and its N-desmethyl metabolite had the same retention times as doxepin and nortriptyline, respectively, it is highly unlikely that these compounds would be given concomitantly with amitriptyline or nortriptyline.

REFERENCES

1 M. Asberg, Clin. Pharmacol. Ther., 16 (1974) 215.
2 R.A. Braithwaite, R. Goulding, G. Theano, J. Bailey and A. Coppen, Lancet, i (1972) 1297.
3 P. Kragh-Sorenson, M. Asberg and C. Eggert-Hansen, Lancet, i (1973) 113.
4 V.E. Ziegler, B. Co., J. Taylor, P. Clayton and J. Biggs, Clin. Pharmacol. Ther., 19 (1976) 795.
5 W.M. Hammer and B. Brodie, J. Pharmacol. Exp. Ther., 157 (1967) 503.
6 H.B. Hucker and S.C. Stauffer, J. Pharm. Sci., 63 (1974) 296.
7 R.A. Braithwaite and B. Widdop, Clin. Chim. Acta, 35 (1971) 461.
8 O. Borgå and M. Garle, J. Chromatogr., 68 (1972) 77.
9 T. Walle and H. Ehrsson, Acta Pharm. Suecica, 8 (1971) 27.
10 J.T. Biggs, W. Holland, S. Chang, P. Hipps and W. Sherman, J. Pharm. Sci., 65 (1976) 261.
11 A. Gifford, P. Turner and C.M.B. Pare, J. Chromatogr., 105 (1975) 107.
12 A. Jorgensen, Acta Pharmacol., 36 (1975) 79.
13 D.N. Bailey and P. Jatlow, Clin. Chem., 22 (1976) 277.
14 U.P. Geiger, T. Rajagopalan and W. Reiss, J. Chromatogr., 114 (1975) 167.

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Book Review

Advances in mass spectrometry in biochemistry and medicine, Vol. 1, (Proceedings of the 2nd International Symposium, Milan, June, 1974), edited by A. Frigerio and N. Castagnoli, Spectrum Publications/Halsted (Wiley), New York, 1976, XXI + 586 pp., price £ 27.20, US$ 46.40, ISBN 0-470-28121-9.

The 51 papers in this volume constitute the Proceedings of the 2nd International Symposium of Mass Spectrometry in Biochemistry and Medicine, held at the Mario Negri Institute for Pharmacological Research, Milan, in June, 1974. This meeting was one of an annual series organised by Dr. A. Frigerio. In a succinct preface, Dr. E.C. Horning reaffirms the importance of analytical methodology in the advance of research.

More than half of the contributions are based on combined gas chromatography—mass spectrometry (GC—MS): the utility of this technique in a wide variety of metabolic studies is particularly well exemplified. Several papers include interesting correlations of fragmentation patterns with structural features. In many of the reports, emphasis is placed on the quantitative determination of drugs or natural metabolites at low levels in biological samples, by means of selected ion monitoring ("mass fragmentography"). In this technique, the use of deuterium-labelled analogues of the substrates as internal standards is well accepted. Other stable isotopes may be similarly applied, and in one instance a ^{14}C-labelled drug (> 80% ^{14}C) is used, the amounts introduced into the mass spectrometer being small enough to avoid serious hazard from radioactivity. It is noteworthy that the analyses described in this volume are effected largely with packed GC columns of short length and modest efficiency: open tubular columns, mentioned here only in one paper (Eyem), have recently come into more widespread use. The necessity for accurate determination of retention parameters is given proper weight in the account by Sweeley's group of computer-aided qualitative analysis of biological extracts of complex composition. The power of computerised GC—MS is demonstrated in other papers, e.g. by the detailed metabolic studies of deuterium-labelled steroids reported by J. Sjövall and co-workers. Effective work can be done without computer aid, as indicated by the investigations by Maume, Padieu and colleagues on steroid metabolism in cell cultures.

The important question of stereoselectivity in drug metabolism is most effectively studied by GC—MS: in one approach, use is made of "pseudo-racemates",

i.e. 1:1 mixtures of an optically-active compound with its deuterium-labelled enantiomer distinguishable by mass spectrometry (Castagnoli). Several papers contain valuable details of isolation procedures, and one may note renewed interest in ion-pair extraction (Cattabeni; L.E. Martin), in extraction with salt—solvent pairs (M.G. Horning), and in the acylation of amines in aqueous solution (Eyem).

Other applications of GC—MS include analyses of polycyclic hydrocarbons in air (Van Cauwenberghe), sequencing of polypeptides (Caprioli), pyrolysis GC—MS of suphanilamides (Przbylski) and studies of nucleoside derivatives (Gelpi; K.M. Baker).

Several papers deal with newer MS techniques, e.g. atmospheric pressure ionisation (Horning), negative chemical ionisation (Dougherty) and field desorption (Schulten; Morris; Games). The scope of quantitative field desorption MS in pharmacokinetics is explored (Maurer). Quantitative analyses based on the integrated ion current technique are described for amines in brain tissue (Boulton; Dolezalova).

To summarise, this volume contains a wealth of information on a range of techniques and applications centred on MS. Many of the papers are the fruits of long experience, while others reflect fresh approaches to the manifold problems that occur between the inception of a biochemical analytical scheme and the acquisition of the required data from the mass spectrometer or computer system. Much of the work included here has now been published in journals; however, this collection conveniently summarises a substantial moiety of the work on the title subject that was current in 1974. Recent developments have consolidated many of the trends represented in this Symposium.

Among a number of typographical errors should be noted some obscurity on pp. 119 and 135 (apparently due to omission of lines of text) and incorrect formulae on p. 126 (14) and p.158 (VII). The book is clearly printed and is provided with a subject index, as well as a list of the 143 contributors with their addresses.

Glasgow (Great Britain) C.J.W. BROOKS

Erratum

J. Chromatogr., 143 (1977) 289—297

Page 290, *Internal standard* section, 4th line, "21.0% $^2H_2$, 73.3% $^2H_1$" should read: "21.0% $^2H_1$, 73.3% $^2H_2$".

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1 A. T. James and A. J. P. Martin, *Biochem. J.*, 50 (1952) 679.
2 L. R. Snyder, *Principles of Adsorption Chromatography*, Marcel Dekker, New York, 1968, p. 201.
3 H. C. S. Wood and R. Wrigglesworth, in S. Coffey (Editor), *Rodd's Chemistry of Carbon Compounds, Vol. IV, Heterocyclic Compounds*, Part B, Elsevier, Amsterdam, Oxford, New York, 2nd ed., 1977, Ch. 11, p. 201.
4 E. C. Horning, J.-P. Thenot and M. G. Horning, in A. P. De Leenheer and R. R. Roncucci (Editors), *Proc. 1st Int. Symp. Quantitative Mass Spectrometry in Life Sciences, Ghent, June 16–18, 1976*, Elsevier, Amsterdam, Oxford, New York, 1977, p. 1.

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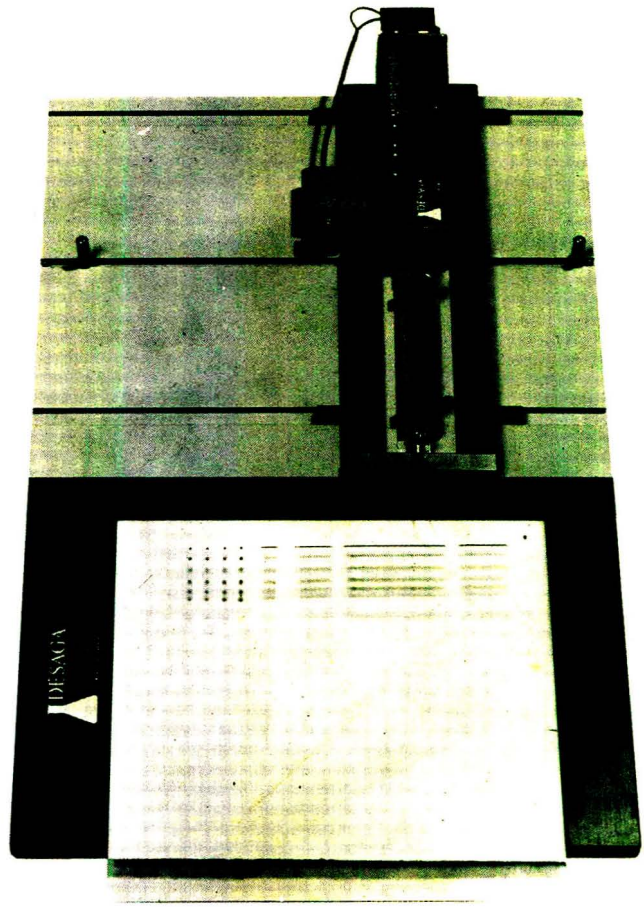

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