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JOURNAL OF CHROMATOGRAPHY BIOMEDICAL APPLICATIONS

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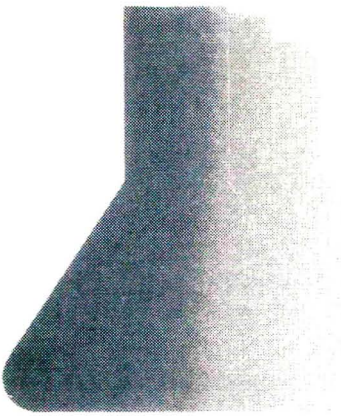

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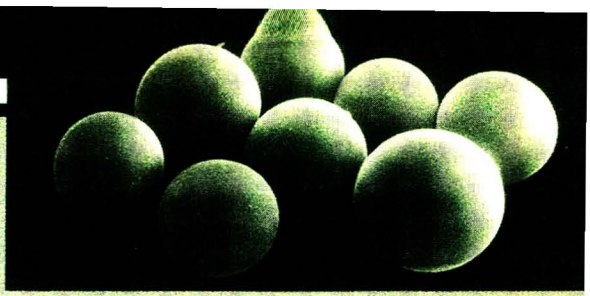

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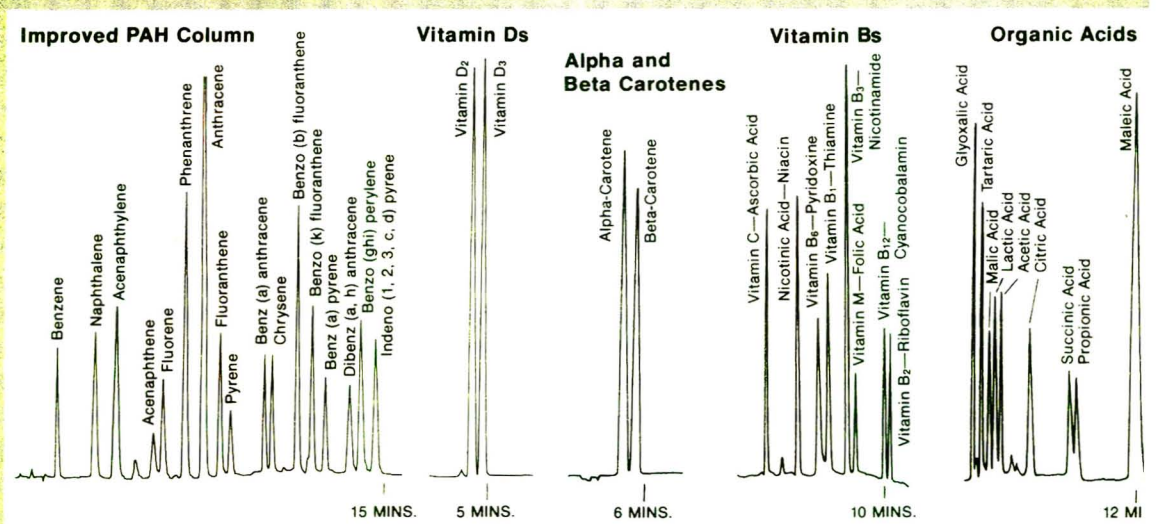

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Editorial

The increasing use of chromatographic methods in the biomedical field has resulted in such a growth in the number of manuscripts submitted to the Biomedical Applications Section of the Journal of Chromatography, that within the past seven years the number of papers published per year has increased by a factor of six. It is clear that it would be impossible for the Editorial Board members alone to evaluate all the manuscripts submitted and to review them from the viewpoint of the highly specialised topics involved. In order, therefore, to maintain the traditionally high standard of the papers appearing, it has been necessary to constitute an unofficial body of specialist referees. After seven years of cooperation we would like to express our deep gratitude to these coworkers for their efficient and generous help without which it would not be possible either to increase the quality of the papers published or to keep the publication time reasonably short. In this context our gratitude is particularly due to the following specialists:

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CHROMBIO. 1901

PREPARATION OF PENTAFLUOROBENZYL ESTERS OF ARACHIDONIC ACID LIPOXYGENASE METABOLITES

ANALYSIS BY GAS CHROMATOGRAPHY AND NEGATIVE-ION CHEMICAL IONIZATION MASS SPECTROMETRY*

ROBERT J. STRIFE and ROBERT C. MURPHY*

Department of Pharmacology, University of Colorado Medical School, Denver, CO 80262 (U.S.A.)

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

SUMMARY

5-Hydroxyeicosatetraenoic acid (5-HETE) and leukotriene B_4 have recently been shown to possess potent chemotactic and chemokinetic properties. Because of the very low concentrations found in certain biological systems, negative-ion chemical ionization mass spectrometry has been investigated as a potential assay method for detecting these compounds. A facile derivatization to form the pentafluorobenzyl esters, and clean up are reported for these compounds at the 15-ng level. Gas chromatographic properties, negative-ion chemical ionization mass spectra, and positive-ion electron impact spectra are reported for the pentafluorobenzyl ester, trimethylsilyl ether derivatives of 5-HETE and leukotriene B_4 isomers.

INTRODUCTION

The metabolism of arachidonic acid by lipoxygenase pathways has been appreciated for several years [1]; however, the description of the 5-lipoxygenase pathway, which in certain cells leads to the leukotrienes, has provided further insight into the complex biochemical events mediated by arachidonic acid metabolites [2]. The recent description of the potent chemotactic and chemokinetic properties of 5-hydroxyeicosatetraenoic acid (5-HETE) [3] and leukotriene B_4 (LTB_4) towards the polymorphonuclear leukocyte [4] has resulted in considerable interest in detecting and measuring

*Presented in part at the 31st annual meeting of the American Society for Mass Spectrometry and Allied Topics, Boston, MA, 1983.

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these compounds in biological samples. While bioassays for these chemotactic properties are reasonably sensitive, they lack specificity, quantitative accuracy, and are somewhat time-consuming. Recently, techniques based on high-performance liquid chromatographic (HPLC) methods using UV detection of the conjugated double bonds in these lipoxygenase metabolites have been described, which are sensitive from 1 to 10 ng injected on the HPLC column [5, 6]. A specific assay by gas chromatography—mass spectrometry (GC—MS) using a deuterated internal standard has been described for 5-HETE [7] as the methyl ester, trimethylsilyl ether derivative. Larrue et al. [8] have also reported a specific assay for monohydroxyeicosanoids using 12-hydroxystearic acid as internal standard and the methyl ester, trimethylsilyl derivatives. We report herein a facile procedure for the derivatization of monohydroxyeicosanoids and LTB_4 isomers into the pentafluorobenzyl (PFB) ester, trimethylsilyl ester species. These derivatives have excellent properties in negative-ion chemical ionization (CI) MS which should permit detection of these eicosanoids from biological extracts by selected-ion recording techniques.

MATERIALS AND METHODS

Instrumentation

The MS was performed on a VG Micromass 7070H mass spectrometer (Altrincham, U.K.) interfaced to a gas chromatograph (Hewlett-Packard Model 7625A, Palo Alto, CA, U.S.A.) modified for capillary column operation with a glass falling needle injector [9] (R.H. Allen, Boulder, CO, U.S.A.). Chromatography was carried out on a 5-μm DB-1 capillary column (J & W Scientific, Rancho Cordova, CA, U.S.A.) threaded directly into the ion source of the mass spectrometer. The ion source conditions were 4 kV accelerating potential, electron energy 70 eV (electron impact, EI) or 60 eV (negative-ion CI) with methane gas pressure adjusted to give maximum signal for the PFB esters. An ion source exit slit of 0.0372 mm was constructed for negative-ion CI operation. HPLC was carried out with a 5-μm Nucleosil C_{18} column (250 mm $\times$ 4.6 mm) using methanol—water—acetic acid (67:33:0.02), adjusted to pH 5.7 with concentrated ammonium hydroxide. Silica gel thin-layer plates (LK-6D, Whatman, Clifton Park, NJ, U.S.A.) were scanned for radioactivity using a Nuclear Chicago Actigraph III (Des Plains, IL, U.S.A.). Solvents were distilled in glass or HPLC grade (Fisher, Springfield, NJ, U.S.A.) and all reagents used were of the highest quality commercially available.

Glassware was routinely cleaned in chromic acid, rinsed thoroughly with doubly distilled water, and where indicated, silylated for 20 min at room temperature with 10% dimethyldichlorosilane in toluene, followed by a methanol wash.

Eicosanoids

12-Hydroxyeicosatetraenoic acid (12-HETE) was prepared from platelets by the method of Sun [10]. Racemic leukotriene A_4 (LTA_4) methyl ester was a kind gift from Dr. J. Rokach (Merck-Frosst, Montreal, Canada); [^{3}H] LTA_4 and [^{3}H] 12-HETE were obtained from New England Nuclear (Boston, MA, U.S.A.). The Δ^6-*trans*-LTB_4 isomers (5,12-di-HETEs) were synthesized by

hydrolysis of LTA_4 methyl ester (350 μg) for 5 min at room temperature in 1 ml of tetrahydrofuran–0.01 *M* hydrochloric acid (1:1). Conversion to the free acid was accomplished by alkaline hydrolysis (tetrahydrofuran—0.2 *M* lithium hydroxide, 1:1) for 1 h at room temperature. The yield was 91% based on UV absorbance. The synthesis of labeled Δ^6-*trans*-LTB_4 was essentially the same, but started with 300 ng of LTA_4 methyl ester and 5 μCi of [^{3}H] LTA_4 methyl ester. This synthesis was carried out in silylated glassware. The synthetic products were purified by reversed-phase HPLC [5] and the diastereoisomeric pairs were collected separately.

Derivatization

Straight-chain fatty acids (C_{19}—C_{23}) were converted to their PFB esters essentially by the method of Min et al. [11], modified by Blair et al. [12] with 30 μl of acetonitrile, 10 μl of 35% PFB bromide in acetonitrile and diisopropylethylamine (10 μl). Most excess reagent was removed by evaporation under a stream of nitrogen. Any underivatized free fatty acids were methylated by diazomethane treatment for 10 min in diethyl ether—methylene chloride. After evaporation the remaining residue was dissolved in 100 μl of methylene chloride, and applied to a short column of silicic acid (0.5 g Silicar CC-4, Mallinckrodt, St. Louis, MO, U.S.A.) in a disposable pipet. The saturated fatty acid derivatives were quantitatively eluted with 2 ml of methylene chloride. The solvents were rapidly concentrated at 40°C by a stream of nitrogen. Reaction times and temperature using the derivatizing reagents were altered as described below.

The eicosanoids were reacted with 35% PFB bromide in acetonitrile (10 μl), diisopropylethylamine (10 μl), and 30 μl of acetonitrile for 10 min at ambient temperature (23—25°C). Clean up of the PFB ester was carried out using column chromatography (Silicar CC-4) after dissolving the dried residue with 100 μl of 50% ethyl acetate in methylene chloride. The monohydroxy PFB esters were eluted with 2-ml volumes of various solvents as described in Table I.

TABLE I

ELUTION OF TRITIUM-LABELED 12-HETE (15 ng) FROM SILICIC ACID AFTER REACTION WITH PFB BROMIDE AT ROOM TEMPERATURE

Percent ethyl acetate*	Percent total radioactivity eluted from silicic acid**,*** (cpm)	
	Free acid	Reaction
0	3.1	69.8
5	1.2	17.9
10	5.4	3.8
15	29.6	2.4
25	40.1	2.6
50	15.8	2.4
100	4.8	1.1

*In methylene chloride, 2 ml per fraction.
**Sample was dissolved in ethyl acetate—methylene chloride (1:1), 100 μl and added to the short silicic acid column.
***Total recovery based on counting of standard aliquots = 90%.

The extent of derivatization of [^{3}H]12-HETE (15 ng carrier) was further evaluated by thin-layer chromatographic (TLC) separation of the combined methylene chloride and 5% ethyl acetate eluates (Table I) using hexane—ethyl acetate—acetic acid (1:1:0.05) as developing solvent. The Δ^6-*trans*-LTB_4 esters were isolated by further step elution of the Silicar CC-4 column with 2 ml each of 10%, 20% and 30% ethyl acetate in methylene chloride. Trimethylsilylation was carried out by reaction of the dried eluates with 50 μl of bis(trimethylsilyl)trifluoroacetamide—acetonitrile (1:1) at 60°C for 15 min.

RESULTS

After derivatization of the mixture of C_{19}—C_{23} straight-chain fatty acids with PFB bromide at elevated temperatures (40°C) by the method of Blair et al. [12], a brown, viscous residue was always obtained. When the fatty acid mixture was derivatized with PFB bromide in acetonitrile at room temperature for 10 min, no such residue was obtained. The extent of the derivatization under these mild conditions was evaluated by subsequent methylation of any remaining free fatty acids before GC analysis. As seen in Fig. 1, the room temperature derivatization of the fatty acids was approximately 90% complete after 2 min. In data not shown, room temperature derivatization for 10 min resulted in greater than 95% conversion of the PFB esters.

The room temperature derivatization condition and use of diisopropylethylamine was evaluated for PFB ester formation of hydroxyeicosanoids.

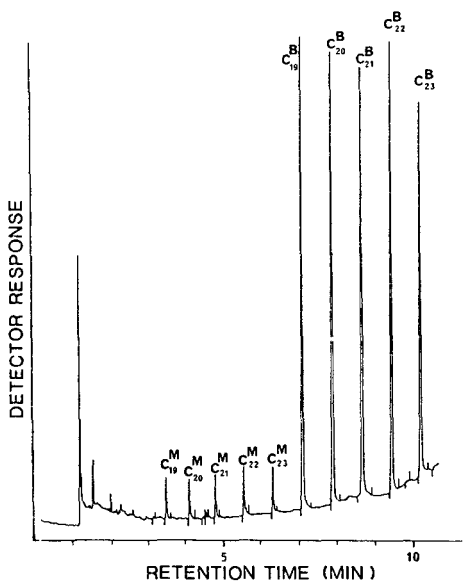

Fig. 1. Capillary gas chromatogram of methyl (M) and pentafluorobenzyl (B) esters, after reaction of straight-chain free fatty acids (5 μg) with PFB bromide, 2 min at room temperature, followed by diazomethane treatment. Column 30 m, DB-1, flame ionization detector, temperature program 220°C up at 8°C/min. Carrier gas helium at approximately 1.5 ml/min at 200°C (0.5% of sample on column).

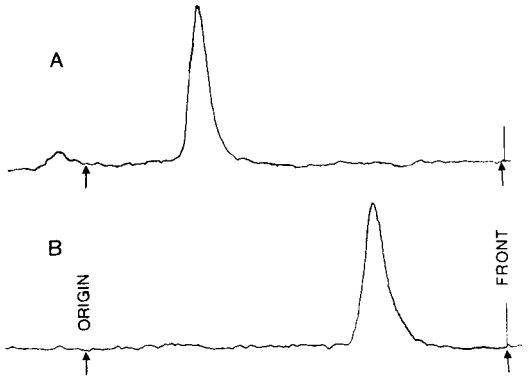

Fig. 2. Radiochromatograms of the TLC separation of standard aliquots of (A) 12-HETE, (B) 12-HETE after reaction with PFB bromide for 10 min and CC-4 mini-column clean up. Standard aliquots from a stock solution contain 15 ng 12-HETE with 0.1 μCi [^{3}H]12-HETE. The developing solvent was hexane—ethyl acetate—acetic acid (1:1:0.005).

Since [^{3}H]12-HETE was readily available, it was used as a model compound to evaluate PFB ester formation, and elution properties of HETE-PFB esters on CC-4. Likewise, [^{3}H]Δ^6-*trans*-LTB_4 isomers were readily synthesized from commercially available [^{3}H]LTA_4 methyl ester and served as a model for LTB_4. The extent of reaction was followed by column chromatography and TLC. Fig. 2 shows the TLC analysis of [^{3}H]12-HETE (15 ng) and that after treatment with PFB bromide at room temperature for 10 min and CC-4 clean up. Approximately 90% of the 12-HETE was recovered as a single, less polar compound (Fig. 2B) which was identified by MS following trimethylsilylation as the TMS ether, PFB ester of 12-HETE. The non-reactivity of the hydroxyls towards PFB bromide under these conditions was further verified by the failure of tricosanol to undergo any reaction with PFB bromide (data not shown).

Derivatization of the Δ^6-*trans*-LTB_4 isomers with PFB bromide at room temperature for 10 min was also studied. Analysis of reaction products using [^{3}H]Δ^6-*trans*-LTB_4 showed that at the 15-ng level these dihydroxyeicosanoic acids were consistently esterified and recovered 76—85% based upon elution of

TABLE II

GC RETENTION CHARACTERISTICS OF PFB ESTER, TMS ETHER EICOSANOIDS AND METHYL ESTER, TMS ETHER EICOSANOIDS

	PFB*	Me
5-HETE	21.0	21.3
12-HETE	21.0	21.3
Δ^6-*trans*-LTB_4	24.7	24.8
LTB_4		23.6
5,6-di-HETE	24.2	23.9

*Equivalent chain length of corresponding ester derivative, Me = methyl ester, PFB = pentafluorobenzyl ester. Determined by capillary GC (5-m DB-1 column).

radioactivity from the CC-4 column. When microgram amounts were derivatized, quantitation by UV spectroscopy (λ_{max} 270 nm, ϵ 50,000) gave identical results for derivatization and recovery. The GC retention characteristics of these compounds are summarized in Table II.

Mass spectrometry

Fig. 3 is the positive-ion EI spectrum of the PFB derivative of tricosanoic acid. A molecular ion of low abundance was typically seen for all these straight-chain esters (Fig. 3, *m/z* 534). The most abundant ion in all cases under EI conditions was *m/z* 181 corresponding to a PFB cation, possibly expanded to a tropylium structure. All straight-chain fatty acids studied had two ions at high mass due to the loss of $C_7H_2F_5$ and a further loss of water from the carboxyl cation. This was confirmed by high-resolution MS of PFB-tricosanoic acid (Fig. 3). A series of hydrocarbon ions (*m/z* 43, 57, 71, 85 and 97) constituted the major ion current under EI conditions.

The positive-ion EI mass spectra of the TMS ether, PFB ester of 5-HETE and Δ^6-*trans*-LTB_4 are summarized in Table III. The most abundant ions observed from these molecules (*m/z* 73 and 181) corresponded to the moieties introduced by derivatization, rather than being structurally characteristic for

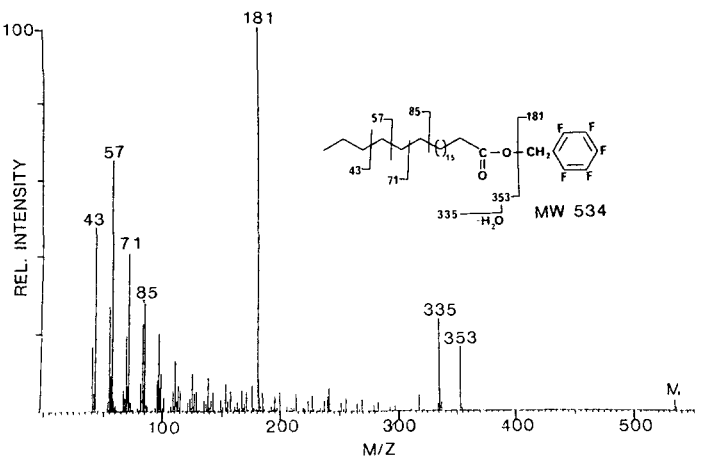

Fig. 3. Positive-ion EI (70 eV) mass spectrum of PFB tricosanoate.

TABLE III

SIGNIFICANT IONS IN THE POSITIVE ION EI MASS SPECTRA (70 eV) OF 5-HETE AND Δ^6-*TRANS*-LTB_4 AS THE TMS ETHER PFB ESTER

5-HETE	572(1), 482(1), 421(7), 369(3), 305(8), 215(4), 190(8), 181(42), 150(20), 129(16), 117(13), 105(17), 91(20), 81(32), 79(34), 75(50), 73(100)
Δ^6-*Trans*-LTB_4	645(1), 549(13), 459(12), 433(3), 407(2), 395(7), 369(12), 283(4), 217(21), 191(16), 181(41), 171(5), 167(5), 129(65), 103(7), 91(5), 75(35), 73(100)

the eicosanoid. However the 5-HETE-PFB, TMS did yield molecular ions and ions from the loss of trimethylsilanol (M—90) in low abundance. Information concerning the position of the hydroxyl substitution on the arachidonic acid backbone is provided by two ions resulting from α-cleavage to the trimethylsilyl ether moiety. In 5-HETE these ions are seen at *m/z* 305 and 369; in Δ^6-*trans*-LTB_4 similar fragmentations are observed at *m/z* 549 and 369. The origins of the abundant ions at *m/z* 129 and 217 have been previously described [13].

Negative-ion CI mass spectra of each of the PFB esters in this study yielded abundant ions due to the loss of the PFB radical to give a stabilized carboxylate anion. Fig. 4 shows the negative-ion CI mass spectrum of the derivatized tricosanoic acid. This loss is characteristic for all molecules studied thus far [11, 12, 14]. The TMS ether, PFB ester derivative of 5-HETE (Fig. 5) and 12-HETE (data not shown) also yielded an abundant ion for further loss of the elements of trimethylsilanol (90 daltons) to produce *m/z* 301. The negative-ion CI mass spectra of 5- and 12-monohydroxyeicosanoids were

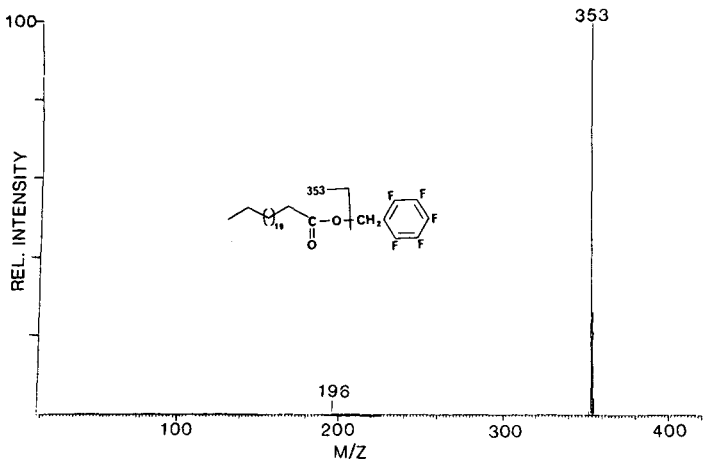

Fig. 4. Negative-ion CI (CH_4) mass spectrum of PFB tricosanoate.

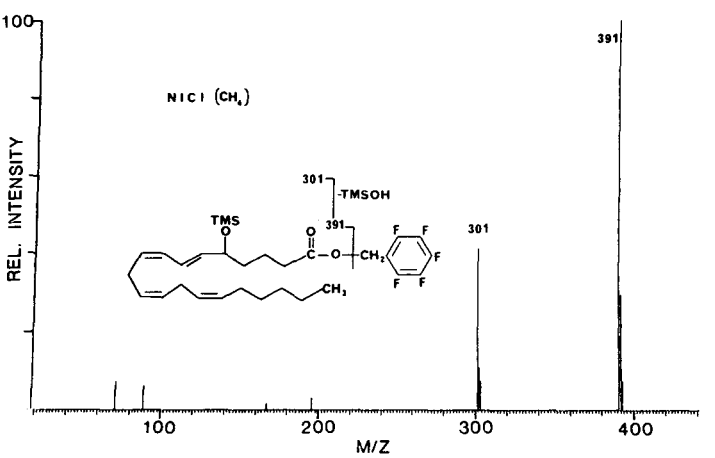

Fig. 5. Negative-ion CI (CH_4) mass spectrum of 5-HETE, PFB ester, TMS ether.

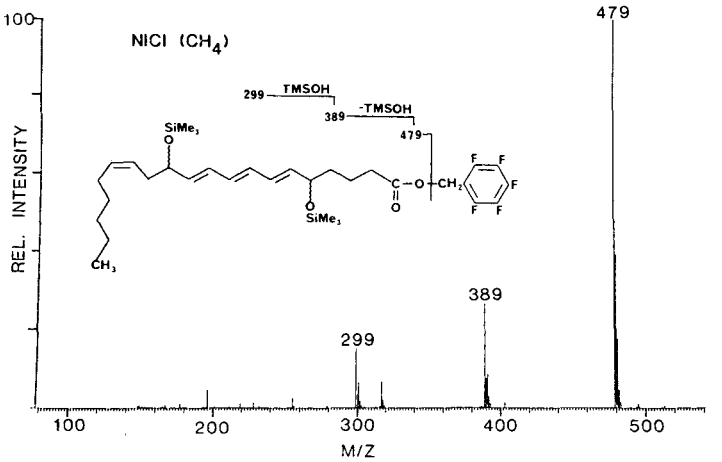

Fig. 6. Negative-ion CI (CH_4) mass spectrum of Δ^6-*trans*-LTB_4, PFB ester, di-TMS ether.

essentially indistinguishable from each other and these compounds had identical GC retention times.

The derivatized Δ^6-*trans*-LTB_4 fragmented under CI conditions to yield the negative-ion mass spectrum in Fig. 6. The most abundant ion corresponded to loss of the PFB radical moiety and formation of the carboxylate anion (*m/z* 479). Two sequential losses of trimethylsilanol yielded the next most abundant ions (*m/z* 389 and 299). In all molecules studied the ion currents carried by the carboxylate anion (M—181) were quite high being 75%, 43%, and 29% of the total ionization for the fatty acids, mono- and dihydroxyeicosanoids, respectively. Negative-ion production under CI conditions was approximately 20% greater than positive-ion production optimized for electron impact. Comparing the abundance of structurally significant ions at high mass (e.g. *m/z* 369 from Table III and *m/z* 391 from Fig. 5 for derivatized 5-HETE) and their percentages of total ionization suggests that a 130-fold increase in sensitivity can be achieved by utilizing negative-ion CI of these derivatives.

DISCUSSION

The previous procedures for the derivatization of carboxylic acids with PFB bromide have employed relatively high temperatures and long reaction times [12, 14]. As a consequence, substantial residues (often yellow-brown) from the reagents themselves are encountered which to some extent limit the total quantity which can be injected onto the gas chromatograph. Many different conditions have been used to effect this esterification [12, 14, 15—21]. Kawahara [15, 16] was perhaps the first to use this derivative for analytical purposes to enhance the measurement of mercaptans, phenols and acids by GC with electron-capture detection (ECD). However, it was not until 1973 that Wickramasinghe and co-workers [17, 18] reported useful conditions for utilizing this derivative to analyze nanogram amounts of prostaglandins. In a systematic fashion, optimal conditions were evolved for prostaglandin $F2_\alpha$ to maximize signal while minimizing background in GC—ECD analysis. Waddell

et al. [14] have recently reported reaction times as long as 30 min at 40°C and at large excess of catalytic amine. The esterification yield obtained under the various conditions at the nanogram level has not been clearly established in any of these reports. In general, 15% at most of the final derivatized sample [2, 4] and as little as 0.1% [3], is actually introduced into the analytical system. In cases where a substantial amount of analyte is isolated, total sample introduction is not important. However, when the amount of isolable material is small, as with leukotrienes which are present in physiological solutions, it becomes important to introduce as much of the biological extract as possible, when the signal measured is not background limited.

We do not feel that the less than quantitative recovery of these derivatives is due to loss of radioactive impurities. Labelled starting materials were analyzed by HPLC and TLC. The average recoveries of 80% and greater for the various derivatized products were quite suitable in light of the small quantities being handled. LTB_4 and its isomers are also susceptible to oxidative destruction, some of the loss may result from this.

The EI mass spectra of the PFB ester, TMS ethers of the eicosanoids are typified by abundant ion currents corresponding to the TMS (m/z 73) and PFB (m/z 181) moieties. Much less abundant but structurally significant ions were present at high mass, and the position of the hydroxyl group was suggested by ions due to α-cleavage of the TMS ether moiety.

Negative-ion CI mass spectra of the TMS ether, PFB ester derivatives of eicosanoids are typified by the large ion currents carried mainly by the resonance stabilized carboxylate anion [11, 12, 14]. The abundance of this ion was striking. Under negative-ion CI with other perfluoronated derivatives such as those containing perfluoroalkyl moieties, the majority of the ion current is carried by small fragments from the derivatizing moiety. One possible explanation of this phenomenon is that the PFB anion is not as stable as the carboxylate anion. The stability of the benzyl cation in positive-ion MS has been widely appreciated. Indeed there have been numerous studies of the gas phase structure of this species and its rearrangement to the more stable tropylium cation. In contrast, the number of π electrons in a tropylium anion would not fit the $(4n + 2)$ π electron rule and it would also be anti-aromatic [22]. Benzyl anions are not stabilized by such structures. In part this may provide a rationale for the absence of such a species in these mass spectra. Nevertheless, the structurally significant carboxylate anions are produced in high yield with little subsequent fragmentation, which should enable sensitive detection and quantitation of mono- and dihydroxyeicosanoids using stable isotope dilution and GC—MS techniques.

ACKNOWLEDGEMENT

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SOLVENT OPTIMIZATION OF REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY FOR POLAR ADRENAL STEROIDS USING COMPUTER-PREDICTED RETENTIONS

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SUMMARY

Computer-aided optimization of a mobile phase has been applied to the isocratic reversed-phase separation of ten polar adrenocortical steroids, including aldosterone and reduced metabolites of cortisol and cortisone. A method based on a seven-step procedure for calculation of the Chromatographic Optimization Function (COF) has been used. Logarithmically transformed retention indices were used for computing multiple polynomial regressions for the retention times of compounds as a function of solvent composition, with the resultant COF as a dependent parameter used for selection of the better mobile phase. Peak cross-overs and overlaps are accommodated in this method and the maximum acceptable analysis time factor is incorporated as well as weighting factors for priority separations. The utility of this procedure for complex mixtures of closely eluting compounds is discussed with respect to the Overlapping Resolution Map method and with the COF method of Glajch and Kirkland as used for automated optimization. Its application to aldosterone-containing samples from human adrenocortical tumours is illustrated.

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TABLE I

SYSTEMIC NAMES, TRIVIAL NAMES AND ABBREVIATIONS OF STEROIDS USED

No.	Abbreviation	Trivial name	Systematic name
1	17-isoAldo	Iso-aldosterone	11β,21-Dihydroxy-18-al-(17α)-4-pregnene-3,20-dione
2	18OH-A	18-Hydroxy-11-dehydrocorticosterone	18,21-Dihydroxy-4-pregnene-3,11,20-trione
3	Aldo	Aldosterone	11β,21-Dihydroxy-18-al-4-pregnene-3,20-dione
4	20α-DHE	20α-Dihydrocortisone	17α,20α,21-Trihydroxy-4-pregnene-3,11-dione
5	20α-DHF	20α-Dihydrocortisol	11β,17α,20α,21-Tetrahydroxy-4-pregnen-3-one
6	20β-DHE	20β-Dihydrocortisone	17α,20β,21-Trihydroxy-4-pregnene-3,11-dione
7	18OH-B	18-Hydroxycorticosterone	11β,18,21-Trihydroxy-4-pregnene-3,20-dione
8	E	Cortisone	17α,21-Dihydroxy-4-pregnene-3,11,20-trione
9	20β-DHF	20β-Dihydrocortisol	11β,17α,20β,21-Tetrahydroxy-4-pregnen-3-one
10	F	Cortisol	11β,17α,21-Trihydroxy-4-pregnene-3,20-dione

INTRODUCTION

The complete separation of complex naturally occurring mixtures of steroid hormones poses several problems due to the wide range of polarities encountered, and their tendency to cluster in groups of similar polarity, composed of steroids generated by a number of alternative metabolic pathways. An example in point is aldosterone and its congeners, including various 18-hydroxylated steroids. The separation of these using both reversed-phase and normal-phase high-performance liquid chromatography (HPLC) with binary solvent systems has been described [1]. The application of these systems to biological samples containing small quantities of aldosterone has, however, revealed a further requirement to separate aldosterone and 18OH-B from several UV-absorbing metabolites of cortisol and cortisone, a requirement that is not satisfied by the binary solvent systems.

In our original study of steroid hormone separation by HPLC we used gradient elution with binary solvent systems to optimize the separation of typical steroid mixtures corresponding to adrenal and testicular hormones. A dioxane—water gradient was, for example, selected for resolving polar adrenal steroids including mineralocorticoids such as aldosterone [2]. It became apparent from further studies that individual ODS-type packings with different levels of residual accessible silanol groups could show considerable specific selectivities for steroids [3]. The selectivity patterns of a number of such packings have been documented [4] and selected non-maximum coverage packings have been utilized in the separation of certain complex steroid metabolite mixtures [5].

Standardization of bonded-phase technology [6] has, however, increasingly limited the opportunity to exploit mixed mode chromatography for difficult separations. As an alternative strategy we have, therefore, now explored the use of computerized optimization of three and four solvent mobile phases. The general value of such systems for complex separations has long been appreciated [7,8], and systematic statistical procedures for mobile-phase optimization have recently been developed. In this study the general methods described by Glajch and co-workers [9,10] have been modified to take into account the behaviour of the steroids encountered in our biological samples which includes cross-overs using different binary solvents. A satisfactorily optimized mobile phase has been defined using the modified procedure, and its use is illustrated by application to aldosterone-containing samples from human adrenocortical tumours.

MATERIALS AND METHODS

Steroid standards were obtained from Steraloids (Croydon, U.K.), Ikapharm (Ramat-Gan, Israel) and the Medical Research Council Steroid Reference Collection (by courtesy of Professor D. Kirk). Their systematic and trivial names, together with the abbreviations used in this study are given in Table I. Samples of human adrenocortical tumours were obtained at surgery. Freshly disaggregated cell suspensions prepared therefrom were incubated in tissue culture medium (10^6 cells per ml) and the supernatants were stored at −20°C. When

required, aliquots were thawed and extracted as described previously [4] except that ethyl acetate rather than dichloromethane was used, because of the polarities of the compounds involved. For identification of cortisol metabolites, fresh cell suspensions were incubated with [^{3}H-1,2,6,7]cortisol (5 μCi, special activity 90 Ci/mmol, Amersham International, U.K.) for 24 h.

Separations were carried out isocratically on 150 × 5 mm I.D. or 250 × 5 mm I.D. stainless-steel columns slurry packed with ODS-Hypersil (Shandon Southern, Runcorn, U.K.). Chromatographic conditions were controlled using a Spectraphysics SP8000 chromatograph and steroids were eluted at a solvent flow-rate of 1 ml/min at 45°C and detected with a Schoeffel FS770 variable-wavelength spectrophotometer at 240 nm. Organic solvents were obtained from Rathburn Chemicals (Walkerburn, U.K.) and single glass-distilled water was prepared from Milli-Q low-conductivity feedstock. Computations were carried out with an 8K microcomputer (Commodore PET series 2001).

RESULTS AND DISCUSSION

In a preliminary study we examined the feasibility of separating all the steroids in Table I using reversed-phase HPLC with various binary mobile phases. Their retention times were established with four such systems based on different organic solvents compatible with UV detection of 4-en-3-one steroids (240 nm). A maximum-coverage end-capped C_{18}-type packing (ODS-Hypersil) was used. The binary mixtures consisted respectively of 35% methanol, 20% dioxane, 20% acetonitrile, and 12% tetrahydrofuran in water. These organic modifier concentrations were selected because they were of approximately equal solvent strengths in respect of the range of compounds studied giving retention times of 19 ± 1 min for the first steroid eluted, 17-iso-aldosterone using a 25-cm column (Fig. 1). Under the isocratic conditions used this solvent strength was empirically deemed the best practical compromise between resolution and analysis time, and did not significantly impair the accuracy or sensitivity of determinations of cortisol, the least polar compound under investigation, and a major component of adrenal tissue samples. However, none of these binary systems afforded a satisfactory separation of aldosterone and 18OH-B and all of the other polar steroids. Examples of incompletely resolved or co-chromatographing compounds were observed in each case (Fig. 1).

The primary requirement was to separate and measure aldosterone without interference from other, unrelated, steroids (Table I) together with the resolution of the 18-hydroxysteroid congeners of aldosterone, 18OH-B and 18OH-A. These objectives, and the cross-overs noted on the different binary systems (Fig. 1), dictated the approach that was taken to computer-aided optimization of the mobile phase.

Most of the strategies for optimization of solvent composition in HPLC are based on a formula [11] which defines the three independent factors that affect resolution, viz. selectivity, efficiency and retention:

$$R_s = 1/4\,(\alpha-1)\cdot\sqrt{N}\cdot k'/(k'+1) \quad (1)$$

where R_s = resolution factor, N = plate number, k' = solute capacity factor, and α = selectivity factor (k_2/k_1).

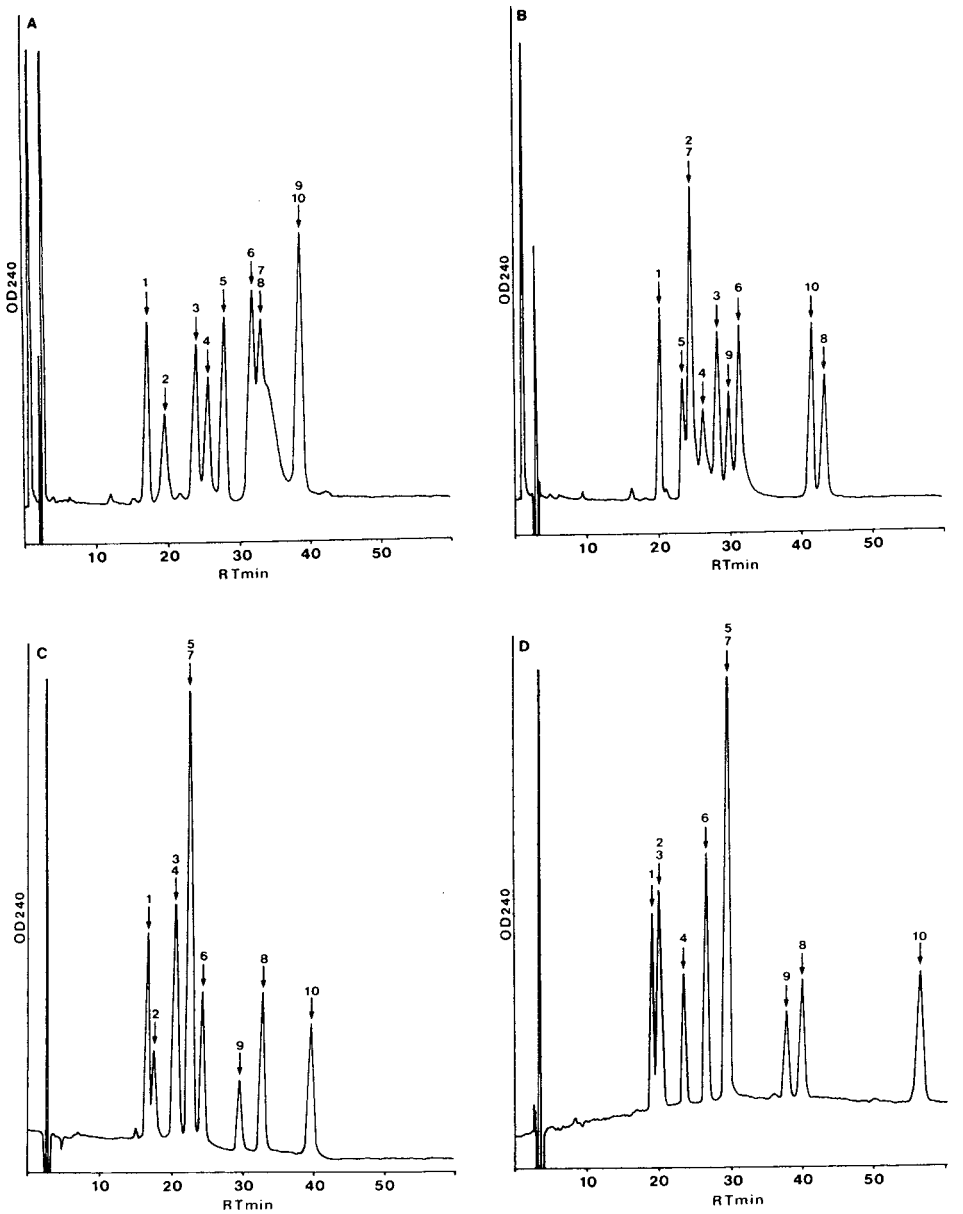

Fig. 1. Isocratic separation of steroids in Table I with binary solvent mixtures. The mobile phase was (A) 35% methanol, (B) 20% acetonitrile, (C) 20% dioxane and (D) 12% tetrahydrofuran, all in water. All separations were carried out with a solvent flow-rate of 1 ml/min at 45°C, using a 250 x 5 mm I.D. ODS-Hypersil column.

Glajch et al. [9] have proposed two methods to optimize complex separations, the Chromatographic Optimization Function (COF), and Overlapping Resolution Mapping (ORM), both based on a seven-step simplex procedure (Fig. 2). The COF is based on a peak resolution parameter and is a modification

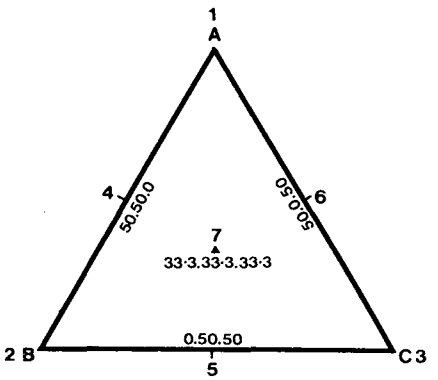

Fig. 2. Simplex design for ternary solvent optimization. This is the seven-point method used by Glajch et al. [9] modified from the ten-point design described by Snee [15].

of the Chromatographic Response Function (CRF) [12]. Unlike the latter it includes a weighting factor for pairs of interest [10]. The COF is calculated on the basis of the formula:

$$\text{COF} = \sum_{i=1}^{k} A_i \ln \frac{R_i}{R_{id}} + B\,(t_m - t_l) \qquad (2)$$

where R_i = resolution between ith pair, R_{id} = desired resolution between that pair, t_l = actual time of analysis, t_m = maximum acceptable analysis time, and A_i and B are arbitrary weighting factors (A may be different for each pair). If $R_i > R_{id}$ then $R_i = R_{id}$; if $t_l < t_m$ then $t_l = t_m$.

Glajch's procedure [9] calculates the COF for each of the seven chromatograms defined in Fig. 2 and derives the best polynomial regression for the four variables, i.e. COF and the amounts of the three solvents. The method was originally designed to optimize the mobile phase without specific identification of individual peaks and is suited for application to a completely automated system. If, however, there is a cross-over or an overlap between any of the peaks, a more complex procedure than that originally described is required [9]. The extension of the method to accommodate cross-overs was deemed cumbersome by Glajch et al. [9] and a procedure based on resolution contour maps for each pair of compounds was, therefore, devised by these workers.

The ORM system [9,10] is based on a graphical representation of the expected overlap between every pair of peaks with four-solvent mobile phases. It is again derived from data generated by the seven experiments denoted in Fig. 2 and is expressed in the form of domains (solvent selectivity areas) superimposed one upon another within the bi-dimensional space triangle (Fig. 2) defined by the three limiting binary compositions. (Such limiting mixtures are sometimes referred to as pseudocomponents, here they can be termed pseudo-solvents.) The ORM can accommodate peak cross-overs and it was considered a significant improvement over the original COF method by Glajch et al. [9]. However, the resulting map only defines the limits of an area corresponding to a range of mobile phases which result in no overlapping, i.e. resolution of all the peaks under consideration; thus, in the original published form [9]

it does not generate a truly unique optimized solution, although recent modifications encompassed in the SENTINEL (DuPont) procedure have rectified this problem. The total analysis time on the other hand is only optimized in the sense that a maximum value is determined by the overall solvent strength selected, and there is no further systematic, interactive optimization of analysis time. The major disadvantage of the ORM is that if it is applied to a large number of compounds with similar retention times it is probable that a complete solution will not result and the overlay intersection of all the solvent selectivity areas will completely cover the overlapping resolution map. In such cases a partial solution can sometimes be obtained by excluding solvent-selectivity areas corresponding to pairs of minor importance. In the present study, however, application of the original ORM method to all ten compounds (Table I) necessitated exclusion of the major compound of interest, aldosterone, in order to generate a solution because priority weighting factors are not available with this technique, or with SENTINEL. An alternative procedure based on a modification of the COF method was, therefore, chosen, with retention data from the seven chromatograms defined in Fig. 2.

In order to minimize bias to the statistical calculations various transformations of the absolute retention values were calculated and their effects on the multiple polynomial regressions examined. Transformations studied included the relative retention time (RT_n/RT_0) in respect of an internal standard, the logarithm of the absolute retention time and the relative logarithm of retention times ($\ln RT_n/\ln RT_0$). 17-Iso-aldosterone was used as the internal standard for calculation of relative retention times and their logarithmic transformations because it showed no cross-overs with other steroids, and a similar retention time with each of the binary systems (Fig. 1). The relative logarithm of retention times gave a ten-fold reduction in the probability of deviation from the calculated regression line when compared with absolute retention values, and was also an improvement on linear retention indices; it was therefore used in all subsequent calculations.

Glajch et al. [9] use a statistical procedure in which the COF is a dependent variable of solvent composition of the mobile phase (solvents A, B, C vs. COF). In reality, however, it is the relationship between retention times and solvent composition that reflects the real chromatographic situation. We have, therefore, calculated all the relative logarithmically transformed retention times, as a function of solvent composition (solvents A, B, C vs. $\ln RT_0/\ln RT_n$). To obtain this result we do not calculate a single polynomial, as in Glajch's procedure [9], but derive instead a separate polynomial regression for the logarithmic retention index of each compound in relation to the internal standard (17-iso-aldosterone). Multiple polynomial regressions (6th degree incomplete) are derived by our program without preselection of the form of the equation. In this case the COF is simply a dependent parameter of the relative retention times calculated according to eqn. 2, and its values used solely to select the better composition. In our first attempt to define an optimized four-solvent mobile phase, appropriately transformed retention data were used from the seven basic chromatograms generated with methanol, dioxane, acetonitrile and water. This combination of solvents, however, did not generate a satisfactory solution to the chromatographic problem when the optimized mobile phase

was calculated and applied to the standards in Table I (data not shown). The results nevertheless provided a good test of the ability of our program to predict the retention times and values of COF and a good correlation between expected and observed values was obtained for the ten compounds ($p < 0.05$).

The data generated with methanol, dioxane, acetonitrile and water also showed that the modified COF procedure used here gives a better fit of predicted-to-actual values when applied to those compounds in Fig. 1 without

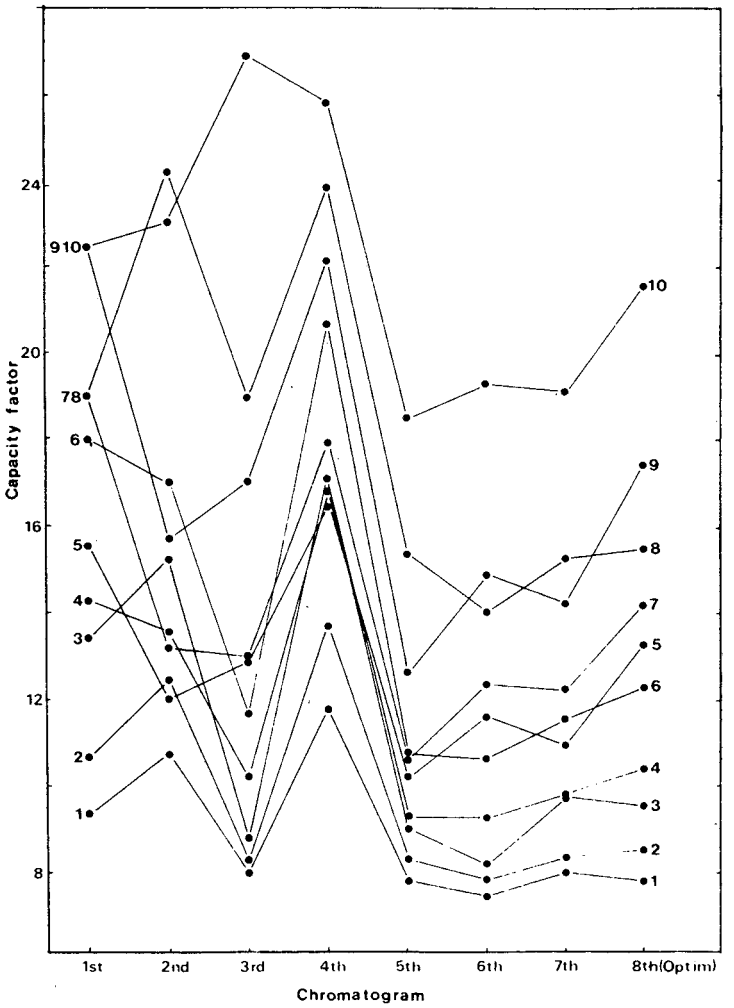

Fig. 3. Solvent selectivity data for steroids in Table I, obtained for the seven different mobile phases denoted by Fig. 2. The 1st, 2nd and 3rd chromatograms correspond to pseudo-solvents A, B and C and comprise, respectively, the binary mixtures methanol—water (35:65), acetonitrile—water (20:80), and tetrahydrofuran—water (12:88). The mobile phases for chromatograms 4, 5 and 6 were three-component mixtures denoted by the corresponding points on Fig. 2, and comprising the mixtures A—B, B—C and C—A; the 7th was obtained with a four-component mixture (A—B—C). The 8th chromatogram illustrates the actual retentions observed using the optimized mobile phase methanol—tetrahydrofuran—water (22.4:4.3:73.3). All separations were carried out with a solvent flow-rate of 1 ml/min at 45°C, using a 150 × 5 mm I.D. ODS-Hypersil column.

cross-overs, to which the original COF method of Glajch et al. [9] (as distinct from their ORM procedure) can also be applied. Thus, values of $p < 0.01$ were obtained for solvents (A, B, C) vs. $\ln RT_0/\ln RT_n$ compared with $p = 0.05$ for solvents (A, B, C) vs. COF. Furthermore, this method will cope with overlaps and cross-overs, unlike, for example, the CRF-based method of Berridge for unattended optimization [13].

Despite observed differences between dioxane—water, methanol—water and acetonitrile—water binary mixtures (Fig. 1), the failure of the computed ternary system for these particular solvents is not altogether surprising. Better resolution is to be expected when the three organic solvents that are selected are well separated in respect of their proton acceptor, proton donor and dipole interaction parameters [14]. Dioxane and acetonitrile both belong to Group VI as defined by Snyder [14], methanol to group II and tetrahydrofuran to group III. These considerations have been extensively discussed by Glajch et al. [9].

A second set of experiments was therefore carried out to provide the data for optimizing the mobile phase in respect of methanol, tetrahydrofuran, acetonitrile and water, as illustrated in Fig. 2. These three organic modifiers are well separated in terms of their solubility parameters and have been recommended as generally preferred solvents for reversed-phase ternary systems [10]. Results are shown in Fig. 3, and the initial values of COF calculated therefrom in Table II. The desired resolution (R_{id}) was set at 1 min and a maximum analysis time (t_m) of 22 min was chosen (see eqn. 2), based on the performance of the columns used in these experiments. The goodness of fit of the model was controlled by the F-ratio, as discussed by Snee [15], calculated for each compound prior to the insertion of the weighting factors and computation of the optimized mobile phase yielding the maximum COF value. This was done by sequential calculation of the COF for all possible combinations, initially at 4% steps in pseudosolvents A, B and C (Fig. 2) followed by 1% steps, once appropriate weighting factors had been defined, and their effects on the predicted separations determined.

The optimum calculated mobile phase under these conditions was methanol—tetrahydrofuran—water (22.4 : 4.3 : 73.3) corresponding to pseudo-

TABLE II

VALUES OF COF IN SEVEN BASIC CHROMATOGRAMS DENOTED BY FIG. 2 FOR STEROIDS IN TABLE I, RESULTS OF WHICH ARE ILLUSTRATED IN FIG. 3

Chromatogram	COF *
1	−11.26
2	−4.59
3	−8.77
4	−6.67
5	−6.49
6	−1.92
7	−3.88

*Computed with weighting factor A set at 1 for all compounds, a desired separation factor of 1 min and a maximum analysis time of 22 min (see eqn. 2); weighting factor B was 0.1.

TABLE III

EXPECTED AND OBTAINED RELATIVE RETENTION TIMES AND COF VALUES* FOR POLAR STEROIDS USING OPTIMIZED TERNARY MOBILE PHASE**

	Compound***										COF
	1	2	3	4	5	6	7	8	9	10	
RT_n/RT_1 (expected)	1.0	1.06	1.16	1.25	1.51	1.44	1.62	1.77	1.92	2.30	−0.85
RT_n/RT_1 (obtained)	1.0	1.07	1.18	1.28	1.59	1.48	1.69	1.84	2.0	2.11	−0.50

*Calculated as in Table II.
**Methanol—tetrahydrofuran—water (22.4 : 4.3 : 73.3).
***See Table I.

solvent A—B—C (64 : 0 : 36) (Fig. 2), giving a predicted COF of −0.85. When this system was used to separate the steroids in Table I not only was the predicted retention order fulfilled, but observed retentions were close to predicted values (Table III), giving an effective COF value of −0.50. A value of

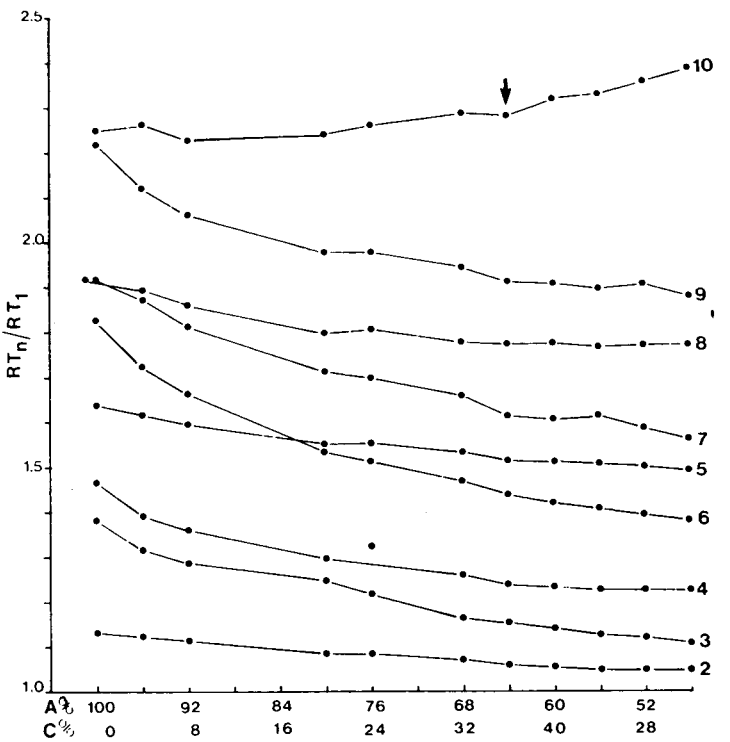

Fig. 4. Computer-predicted retentions for selected three-component mobile-phase mixtures (methanol—tetrahydrofuran—water). The input data were from chromatograms 1—7 in Fig. 3. A denotes methanol—water (35 : 65), C tetrahydrofuran—water (12 : 88). The arrow indicates the retentions predicted for the mobile phase identified by the program as optimal, corresponding to A—B—C (64 : 0 : 36).

0.1 was assigned to weighting factor B. As is evident from Fig. 3 there was also a significant reduction in total analysis time, reflecting the fact that a full version of eqn. 2 was used. This is important because any reduction in total analysis time without compromising resolution, over and above that simply achieved by prior solvent strength selection in respect of the pseudosolvents A, B and C, results in improved precision in quantitative analysis when using isocratic conditions. Berridge [13] has used a version of the CRF formula that involves a t_m component but his TERNOPT programme does not accommodate peak overlaps or cross-overs, being designed for fully automated optimization. As mentioned above the CRF procedure does not include priority weightings for compounds of specific importance.

The separations predicted and achieved under optimized conditions using our programme can be best visualized by reference to Fig. 4. By inserting a variety of weighting factors (A_i) for priority separations of compounds 1–10 into the computation we have used the program to generate predicted retentions for a large number of three- and four-solvent mobile phases. Those corre-

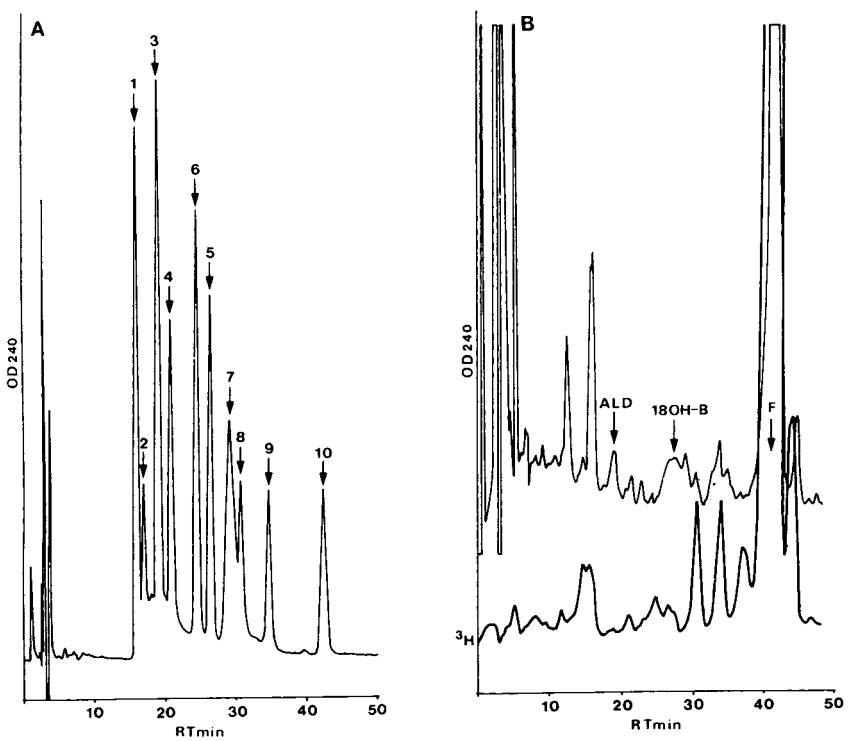

Fig. 5. Separation of steroid standards (A) and a sample (B) using the optimized ternary mobile phase. The identity of cortisol and cortisone metabolites in the sample from a cell suspension of human adrenocortical tumour cells is demonstrated by the concurrent profile of [^{3}H]radiometabolites generated from [^{3}H]cortisol. Chromatographic conditions comprised a mobile phase of methanol–tetrahydrofuran–water (22.4 : 4.3 : 73.3) at a flow-rate of 1 ml/min at 45°C with a 250 × 5 mm I.D. ODS-Hypersil column. See Table I for key to identity of all steroid standards.

sponding to the three-component methanol tetrahydrofuran water mixtures on either side of the optimum conditions have been plotted in Fig. 4. They demonstrate the capability of the program to control the predicted retentions in a systematic manner as well as illustrating the manner in which the optimized separation differs from its neighbours.

As a final test of its utility, the optimized ternary mobile phase was applied to an aldosterone-containing sample from a human adrenocortical tumour. The results are illustrated in Fig. 5 and demonstrate the solution to our original problem, viz., the separation of aldosterone and 18OH-B from UV-absorbing metabolites of cortisol and cortisone.

This separation is not, of course, necessarily the best obtainable under any isocratic conditions, but simply the best in relation to the original choice of solvents comprising the mobile phase and the choice of the ratio between water and each organic solvent that defines the mobile phase at 1, 2 and 3 in Fig. 2, i.e. pseudosolvents A, B and C. The range of potential solvent mixtures explored in this study can best be visualized as a plane intersecting a pyramid (Fig. 6). The results obtained do not, therefore, rule out a better isocratic solution with a different choice of A, B and C, particularly as the addition of an organic solvent to water may change its bulk properties with effects on retention that are not intuitively obvious [9].

To select a completely optimized isocratic mobile phase without preselection

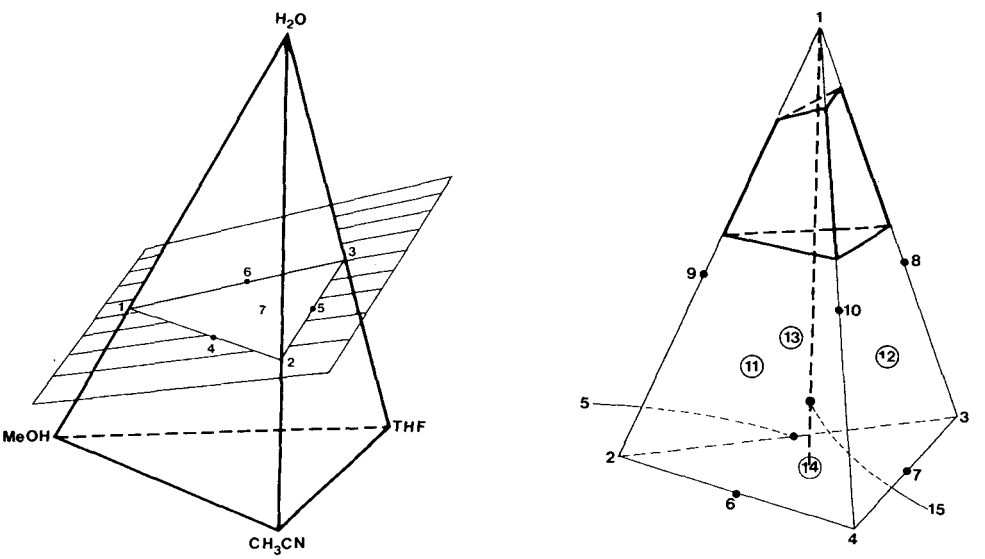

Fig. 6. Three-dimensional representation of the range of mobile-phase mixtures available with four solvents. The conditions searched by computer-aided statistical methods in the present study correspond to a plane intersecting the tetrahedron, as illustrated.

Fig. 7. Statistical design for searching the complete range of mobile phases afforded by four solvents. The numbers indicate individual mobile phases for which retention data for all compounds must be generated. In isocratic reversed-phase separations, some water-rich and water-poor phases can be eliminated; a twelve-point search design is then required to control the behaviour of compounds within the mobile-phase conditions represented by the resultant truncated pyramid.

of solvent strength, as distinct from optimizing gradient elution conditions, it is, therefore, necessary in principle to search the full quaternary system represented in three-dimensional space by a tetrahedron (Fig. 7). The seven-point statistical approach illustrated in Figs. 2 and 6 must consequently be modified into a more complex one. To search the full range of four solvent phases, such as, for example, might be employed in a normal-phase separation, a fifteen-step design is necessary because no preselected equation is used for calculation of polynomial regressions (Fig. 7). Given the nature of the solvents available for a normal-phase separation and their widely different solubility parameters [10, 14], it might be expected that this would be likely to give a more effective separation of a complex group of compounds of similar polarity such as we have been concerned with in this study. Antle [16] has described the use of ORM and COF type procedures to optimize normal-phase separation of simple steroid hormone mixtures. The computational COF method was not precisely described but was probably of the form described by Glajch et al. [9]; better results were, however, obtained with the ORM method and by visual optimization, reflecting to some extent the limitations of the original COF method.

However, despite these advantages of normal- as opposed to reversed-phase systems, it must be borne in mind that the separations illustrated here are only one facet of a more general analytical problem that simultaneously involves other groups of natural steroids of widely different polarities. Although flow-programming can be used to facilitate the elution of individual strongly retained compounds, for the general problem gradient elution still probably provides the best solution [2]. For this purpose normal-phase systems present certain practical problems due, in part, to difficulties in ensuring reproducibility of re-equilibration. If, on the other hand, isocratic reversed-phase systems are chosen the tetrahedron in Fig. 6 can be searched by a simplified procedure. As one component of the system is inevitably water, the parts of the tetrahedron corresponding to extremely water-rich and water-poor phases can be eliminated as they will lead to solutions with unacceptably long and short analysis times, respectively. The resultant truncated pyramid requires a total of twelve experiments to generate the requisite data for statistical optimization. The seven-step method described here required a total calculation time of approximately 1 h to search all combinations of mobile phases with ten compounds and 4% steps between A, B and C once the retention values for the seven chromatograms had been inserted and the multiple polynomial regressions calculated and controlled for each compound, a step which itself required approximately 1 h. It took 14 h to identify the optimum conditions when 1% steps were programmed, corresponding to a precision of ±0.1—0.7% in the actual organic modifier and water concentrations defined. While the computation time and memory capacity required to run a twelve- or fifteen-step programme are obviously considerably greater, they are nevertheless, within the scope of the current generation of personal computers. Further aspects of the interactive program which we have outlined here and which we have termed the Chromatographic Optimization Coefficient (COC) procedure, are detailed elsewhere [17]. These include the development of a program for full quaternary reversed-phase isocratic optimization. The same statistical approach can be used for optimizing gradient elution with advantages

of enhanced precision in prediction of retention times compared with the ORM-based seven-step, isoselective multisolvent gradient elution procedure (IMGE) and the semi-empirical step-selective multisolvent gradient elution (SMGE) procedure developed by Kirkland and Glajch [18,19]. Ultimately, a fully systematic procedure for multisolvent gradient elution (i.e. without constraints on the relative proportions of the different organic modifiers and water) should be possible.

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CHROMBIO. 1910

SIMULTANEOUS MEASUREMENT OF TYROSINE, TRYPTOPHAN AND RELATED MONOAMINES FOR DETERMINATION OF NEUROTRANSMITTER TURNOVER IN DISCRETE RAT BRAIN REGIONS BY LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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SUMMARY

Concomitant measurement of monoamine neurotransmitter turnover in discrete rat brain areas with the use of radiolabeled amino acid precursors permits simultaneous evaluation of interacting transmitter systems. [^{3}H]Tyrosine and [^{3}H]tryptophan were administered via indwelling catheters to unrestrained rats. Content and specific activity of norepinephrine, dopamine, 5-hydroxytryptamine, and the metabolites dihydroxyphenylacetic acid, homovanillic acid, and 5-hydroxyindoleacetic acid in addition to tyrosine and tryptophan were quantified by liquid chromatography with electrochemical detection and liquid scintillation counting. The method employs a simple extraction procedure without prior cleanup for chromatography. Neurotransmitter turnover rates that incorporated tyrosine- or tryptophan-specific activities were found to be two to four times greater than those that did not include them.

INTRODUCTION

Recent efforts to correlate behavioral observations in rodents with neurochemical findings have been most successful when data have been evaluated in terms of central nervous system (CNS) neurotransmitter interactions and relatively simple neuronal circuits [1—5]. In many instances these interactions have involved the monoamine neurotransmitters norepinephrine (NE), dopamine (DA), and 5-hydroxytryptamine (5-HT). Behavioral, neurochemical,

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and anatomical evidence support the view of the existence of functional interactions between neuronal elements of the monoamines. For example, considerable evidence has accumulated that 5-HT neurons provide inhibitory influences to dopaminergic neuronal activity [1, 6, 7]. The function of these biogenic amines depends closely on their distribution in particular brain areas. Therefore, evaluation of specific neurotransmitter roles requires their assessment in discrete neuroanatomical regions or specific nuclei.

It is not surprising that methods for the determination of monoamines in brain have been the focus of many investigators for a number of years. Several analytical methods for their separation, detection, and quantitation have been employed. Spectrophotofluorometric procedures have been widely used to assay the biogenic amines [8, 9], but require either the isolation of each individual compound or the use of compound-specific methods of fluorescence development and in some instances lack adequate sensitivity. For these reasons fluorometry has not proven conducive to the simultaneous estimate of a large number of compounds. Gas chromatography—mass spectrometry while offering identification of compounds with excellent selectivity and limits of detection requires equipment that is not routinely available and utilizes relatively complex derivatization techniques [10, 11]. Radioenzymatic methods rely on enzymatic labeling of the catechol or indole nucleus with subsequent isolation followed by counting of radioactivity [12, 13]. This methodology, although highly sensitive, requires multiple-sample handling steps, considerable technical skill, and does not permit the simultaneous measurement of the precursors and metabolites of the biogenic amines under study.

TABLE I

MAJOR PUBLISHED REPORTS OF METHODS MEASURING BIOGENIC AMINE NEUROTRANSMITTERS, PRECURSORS, AND METABOLITES IN BRAIN

Compounds analyzed	References
Single compounds	
NE	16
5-HT	17, 18
MHPG	19
Catecholamines/metabolites	
EPI, NE, DA	20
NE, DA	21—25
DA and metabolites	26—28
NE, DA and metabolites	29, 30
Indoleamine/metabolites	
5-HT and metabolites	31, 32
5-HT and metabolites + TRP	33, 34
Multiple neurotransmitters/metabolites/precursors	
DOPA, 5-HTP	35
DA, 5-HT	36, 37
NE, DA, 5-HT	38
MHPG, DOPAC, HVA, 5-HIAA	39
DA, 5-HT and metabolites	40, 41
NE, DA, 5-HT and metabolites	42—45
NE, DA, 5-HT and metabolites + TRP	46
NE, DA, 5-HT and metabolites + TYR, TRP	47, 48

The utility of determining picomole quantities of the neurotransmitters, their precursors, and metabolites by the use of reversed-phase liquid chromatography with electrochemical detection has been clearly established within a relatively short time since its potential and specificity were first recognized by Adams [14] and applied by Kissinger et al. [15] as a rapid, inexpensive and highly sensitive procedure for the assay of these compounds. Table I lists the most important published reports using this mode of detection in brain tissue and the compounds quantified [16—48]. As can be seen, numerous investigators have described procedures to measure various combinations of the catecholamines, indoleamines, and their respective metabolites. However, few papers [47, 48] have described the simultaneous determination of the two amino acid precursors, tyrosine (TYR) and tryptophan (TRP), concomitantly with these other compounds.

Measurements of steady-state tissue content of NE, DA, 5-HT and their metabolites in specific brain areas provide limited insight into changes occurring within these neuronal systems. The determination of neurotransmitter turnover has proven to be a more meaningful and useful estimate of neuronal activity. Various methods of assessing monoamine turnover have been extensively employed in recent years. These non-radiometric techniques have included measurement of: (1) the rate of accumulation of L-dihydroxyphenylalanine (DOPA) or 5-hydroxytryptophan (5-HTP) after decarboxylase inhibition [49, 50]; (2) the rate of decrease in DA or NE concentration following tyrosine hydroxylase inhibition with α-methylparatyrosine [51]; (3) the rate of 5-HT accumulation following inhibition of monoamine oxidase [52, 53]; (4) the concentration of dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), or 5-hydroxyindoleacetic acid (5-HIAA) at steady state or following inhibition of formation (by monoamine oxidase inhibitors) or transport (by probenecid) [52, 54—58]. Nevertheless, these methods have considerable drawbacks. First, techniques inducing neurotransmitter changes beyond the range of normal concentrations produce abnormal behavioral states in the animal. This eliminates any possibility of relating neurochemical findings in this situation to ongoing behavior observed in a test paradigm. Second, methods resulting in the accumulation or decline of neurotransmitters, precursors, or metabolites produce an abnormal neurochemical environment by altering normal biochemical feedback and regulatory processes. This seriously hinders attempts to investigate neurotransmitter interactions. Third, changes observed in metabolite concentrations can result from more than one synaptic mechanism, and consequently they provide information of limited value. The radiolabeled turnover procedure described in this report is not susceptible to these criticisms, and provides data that can be evaluated in terms of behavioral correlates and the interactions of different neuronal systems. Radiolabeled turnover can be determined by applying steady-state kinetics to the decline of transmitter specific activity over time utilizing a single-compartment model [59]. Moreover, accuracy requires that the specific activity of the precursor amino acids be included in the determinations also [59].

This report describes a procedure by which content and radioactivity of the monoamine neurotransmitters and metabolites can be measured simultaneously with the precursors TYR and TRP within small tissue samples (< 10 mg) for accurate and precise assessments of monoamine turnover.

EXPERIMENTAL

Animals

The male Long—Evans hooded rats (450—500 g) used in this work were progeny of rats originally obtained from Charles River (Wilmington, MA, U.S.A.) and bred in-house. The animals were housed in stainless-steel wire cages within a temperature (22 ± 1°C), light (12:12 cycle commencing at 07.00 hours), and humidity (50%) controlled environment ventilated with filtered air. NIH-07 laboratory chow (Ziegler Bros., Gardners, PA, U.S.A.) and distilled water were provided ad libitum.

Surgical preparation of animals

Administration of the radiolabeled precursor amino acids TYR and TRP was via a chronic indwelling jugular catheter. Application of the tracer dose in this manner requires no anesthesia and avoids stress, both of which influence neuronal activity, while enhancing the precision of the administered doses as compared to manual restraint and intravenous injection. Animals were surgically implanted with catheters as described originally by Weeks [60] and modified by Lane et al. [61]. Briefly, the animals were anesthetized with pentobarbital, an opening made on the ventral neck, and a small polyvinyl chloride catheter (Tygon®, 0.01 in. I.D.) inserted into the external facial vein and run approximately 25 mm toward the heart. The catheter was anchored with surgical silk and run under the skin to a point above the scapula where it exited through a small polyethylene harness. The catheter was filled with heparinized saline and enclosed in a small brass shimstock box attached to the harness. At least ten days were allowed for the animals to recover from the surgery and become accustomed to the cannula harness attached to their backs. Function of the catheter was checked by injection of thiopental 2—3 days before sacrifice.

Precursor injection

On the day of sacrifice L[^{3}H]2,6-TYR (specific activity, 30 Ci/mmol) and L[^{3}H(G)]tryptophan (specific activity, 4.5 Ci/mmol) (New England Nuclear, Boston, MA, U.S.A.) were dried under a stream of nitrogen and redissolved in saline so that 100 μl of the mixture contained 1.0 mCi of [^{3}H]TYR and 0.5 mCi of [^{3}H]TRP. Animals were injected with 100 μl of the mixture (33 and 110 nmol of amino acid, respectively) between 09.00 and 11.00 hours and were decapitated either 60, 90, 120, or 180 min later (n = 3 to 4 animals per time point).

Tissue dissection and extraction

Brains were quickly removed, rinsed in isotonic saline (4°C), and dissected on ice essentially according to the method of Heffner et al. [62] using a modified slicing apparatus. Brain regions were quickly frozen on dry ice, weighed, and stored at −70°C until extraction.

Individual brain regions were homogenized in ground-glass tissue grinders (1.0 ml volume; Radnoti Glass Technology, Monrovia, CA, U.S.A.; 10 ml volume; Kontes, Evanston, IL, U.S.A.) and extracted essentially according to

Co et al. [45] with 25 volumes of ice-cold 1.0 *M* formic acid—acetone (15:85, v/v) containing epinine (N-methyldopamine) as internal standard. Aliquots (300 μl) were taken from each homogenate and centrifuged at 1000 *g* for 10 min at 4°C (Sorvall RC2-B, DuPont Instruments, Newtown, CT, U.S.A.). Supernatants were extracted with 1.0 ml cold heptane—chloroform (8:1, v/v), the organic layer discarded, and the aqueous layer dried under nitrogen and stored at −20°C until analysis (< 1 week). Samples were thawed and redissolved in mobile phase (see below) prior to injection of 20 μl into the liquid chromatograph.

Apparatus

The chromatographic system consisted of a 25 cm × 4.6 mm I.D. Biophase-ODS 5-μm C_{18} column (Bioanalytical Systems, West Lafayette, IN, U.S.A.), a PM-11 Milton-Roy minipump (State College, PA, U.S.A.), and a Model 7125 syringe-loading rotary injection valve (Rheodyne, Cotati, CA, U.S.A.). A pre-column 2-μm filter (Rheodyne) minimized the accumulation of particulate matter on the analytical column. The signal from a TL-8A glassy carbon detector cell was amplified by a Model LC-3 amperometric controller (Bioanalytical Systems) with output to dual-chart recorders set at 20 and 200 nA full-scale deflections. Two recorders set at different amplification ranges were used since tissue concentrations of the compounds of interest often differ by more than an order of magnitude. The flow-rate was 1.5 ml/min with a back-pressure of 172 bars. Columns were continuously perfused with mobile phase at minimum flow-rates when not employed for actual tissue analyses.

Chromatography

The mobile phase consisted of 0.15 *M* monochloroacetic acid in triple glass-distilled water made 0.1 m*M* with disodium-EDTA and adjusted to pH 3.00 with 10 *M* sodium hydroxide. This solution was passed through 0.22-μm pore nitrocellulose filter supported by a coarse-grade fritted-glass funnel (Millipore, Bedford, MA, U.S.A.) and degassed under vacuum. Sodium octyl sulfate was added as the ion-pairing agent at 200 mg/l, and acetonitrile added to a final concentration of 7.3%. The solution was pumped at ambient temperature during chromatography.

Detector potential was maintained at +950 mV vs. Ag/AgCl reference electrode. These chromatographic conditions permitted the routine quantitation of TYR, TRP, NE, DA, and 5-HT as well as DOPAC, HVA, and 5-HIAA (Fig. 1).

Scintillation counting

All samples were spiked with ^{14}C-tracer amounts of NE, DA, 5-HT, TYR, and TRP (ca. 500 dpm each) to provide a measure of the accuracy of peak collection for these compounds for scintillation counting. Eluate corresponding to specific peaks was collected quantitatively from the detector in scintillation vials according to the deflection of the recorder pen. Volumes of 15 ml of Aquasol-2® (New England Nuclear) were added to individual chromatographic fractions, and the vials were placed in a Packard Tri-Carb 460 CD Liquid Scintillation System (Packard Instruments, Downers Grove, IL, U.S.A.) for

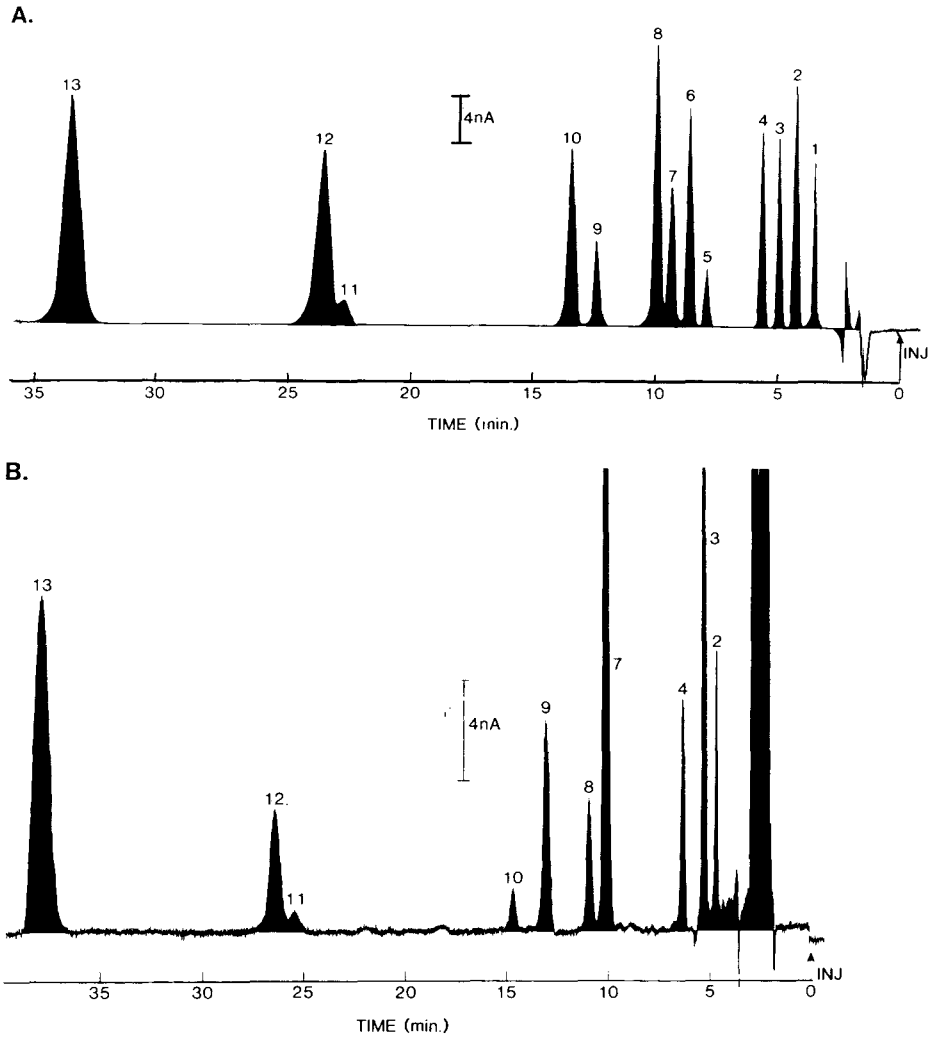

Fig. 1. Chromatograms of (A) 20 μl of a mixture of working standards containing 1 pmol/μl of NM and 3-MT, 8 pmol/μl of TYR and TRP, and 3 pmol/μl of all the others, and (B) 4.8 mg of tissue from nucleus accumbens showing quantified peaks. Off-scale deflections were measured on a second recorder set at 10% of the above sensitivity. Peaks: 1 = L-DOPA; 2 = NE; 3 = TYR; 4 = DOPAC; 5 = NM; 6 = 5-HTP; 7 = DA; 8 = 5-HIAA; 9 = EPN; 10 = HVA; 11 = 3-MT; 12 = 5-HT; 13 = TRP. See text for chromatographic conditions.

counting of radioactivity. Dual-label ($^3H/^{14}C$) efficiencies were computed by reference to double-quench correction curves developed from a set of external standards.

Chemicals

Standard mixtures were prepared from L-norepinephrine bitartrate (NE), L-tyrosine (TYR), dopamine hydrochloride (DA), DL-3,4-dihydroxyphenylalanine (DOPA), 3,4-dihydroxyphenylacetic acid (DOPAC), 4-hydroxy-3-

methoxyphenylacetic acid (HVA), 3-methoxytyramine hydrochloride (3-MT), L-tryptophan (TRP), 5-hydroxytryptamine creatinine sulfate (5-HT), 5-hydroxyindole-3-acetic acid (5-HIAA), 5-hydroxy-L-tryptophan (5-HTP), and deoxyepinephrine hydrochloride (epinine), all obtained from Sigma (St. Louis, MO, U.S.A.), and DL-normetanephrine (NM) from Calbiochem-Boehring (San Diego, CA, U.S.A.). Sodium octyl sulfate was obtained from Eastman Kodak (Rochester, NY, U.S.A.). All other reagents and solvents were of analytical grade and used without further purification.

Standards

Stock standard solutions were made 1 m*M* in 0.01 *M* hydrochloric acid for each compound and stored at 4°C (stable for at least six months). Mixtures of working standards were prepared from stocks with 0.01 *M* hydrochloric acid according to the relative detector responses generally expected in the tissue samples: TYR and TRP were diluted to 8.0 μM, and all other compounds including epinine were made 3.0 μM. Working standard solutions were stored at 4°C and freshly prepared each week.

A 20-μl aliquot of the mixture of working standards was routinely chromatographed at the beginning and end of each day. Additionally, a standard containing TYR alone (8 μM) was routinely run following every second chromatographic analysis.

The efficacy of the extraction procedure in the presence of tissue was evaluated by estimation of the recovery of the components of the working standard solution. Cerebellar tissue was homogenized as described above and three 300-μl aliquots removed. Two aliquots were spiked with 50 μl of the working standard solution and the third served as a tissue blank. The three samples were then carried through the rest of the procedure in conjunction with the individual tissue samples. Recoveries were determined for each compound from the difference in peak heights of the spiked and unspiked samples in comparison to the peak heights from 20 μl of the mixture of working standards injected directly into the chromatograph.

Data analysis

Concentrations of each compound were determined by comparing sample peak heights with peak heights obtained from the mixture of working standards. All measures of content were corrected by the epinine recovery value for each sample. All radioactivity measures were corrected by the epinine recovery value for the sample or the ^{14}C-recovery for the specific compound (if available), whichever was lower. Choice of the lower value is based on the rationale that accurate collection of eluent associated with a specific peak would contain the amount of radioactivity reflecting the epinine recovery value. However, human error in the manual collection of eluent representing a specific peak could lead to less than expected amounts of radioactivity due to incomplete collection. Therefore, employing the smaller of the two recovery values would compensate for this source of error. Concentrations were expressed as μg/g tissue weight and specific activities as dpm/nmol.

Neurotransmitter turnover was determined utilizing steady-state kinetics with the radiolabel assumed to disappear from a single open neuronal compart-

ment [59]. Neurotransmitter specific activities were used to determine a line on a log–linear plot across the sacrifice time points by computerized linear regression techniques. Turnover constants were then computed both with and without incorporation of the amino acid specific activities [45, 59].

RESULTS AND DISCUSSION

Voltammetric responses

The response of the electrochemical detector depends upon the applied voltage and the nature of the compound being oxidized as shown in Fig. 2. Catechols in comparison to the other compounds of interest were most readily sensed by the detector with current first noted at about +400 mV. Further increases in current response above +600 mV were small. The 3-methoxylated metabolites of the catecholamines were more difficult to oxidize with current initiated at an applied voltage of about +700 mV. This group of compounds reached a plateau in the voltammograms and displayed little additional response above +800 mV. The 5-hydroxyindoles differed from both these groups in their electrochemical response. Current was initiated at +450 mV and reached a plateau at about +700 mV before increasing again at +1000 mV. Similar voltammograms have been reported previously for 5-hydroxylated indoles [63]. TYR and TRP required higher applied voltages (+850—+900 mV) than the other compounds tested to initiate an electrode response. In addition, the current–potential curves for these amino acids did not plateau in the range of potentials tested. Other authors have reported $E_{1/2}$ values for TYR or TRP greater than +1000 mV [64].

An operating potential of +950 mV was set for all analyses to obtain

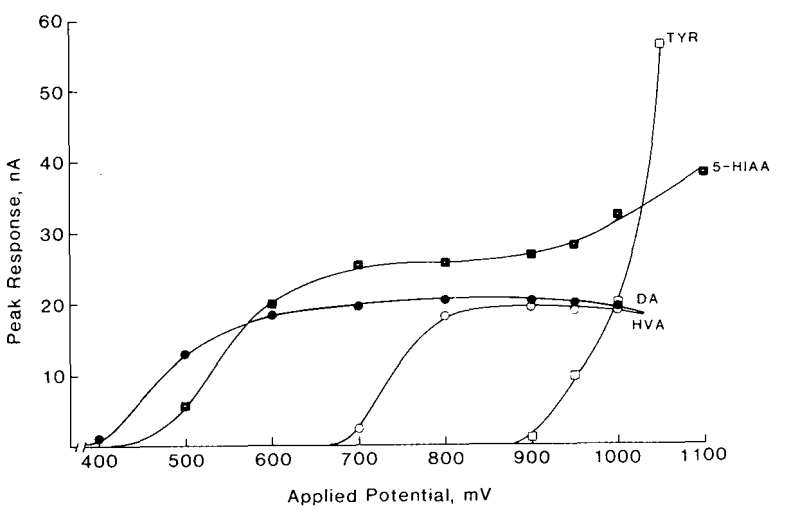

Fig. 2. Current–potential curves for standard compounds. DA is representative of catechol compounds (NE, DOPA, DOPAC), HVA represents the 3-methoxylated catecholamine metabolites (3-MT, NM), and 5-HIAA is indicative of the 5-hydroxyindoles (5-HT, 5-HTP). The curve for TRP is similar to that for TYR. All curves were generated from the current response to 80 pmol of each compound.

sufficient current responses for TYR and TRP. At this voltage the detector responses of all other compounds had attained plateaus. Higher potentials were impractical because of the background noise generated.

Linearity of detector response

The linearity of detector response for each compound of interest was confirmed by injecting amounts of 1—400 pmol into the chromatograph. Fig. 3 shows the resulting curves for representative compounds demonstrating linearity for amounts spanning more than two orders of magnitude. Correlation coefficients determined by regression analysis of the amount injected and the detector response were all greater than 0.996. The detection limits based on a signal-to-noise ratio of 2:1 ranged from 51 fmol for 5-HIAA to 6.5 pmol for DOPAC. The variation in repeated injections of the same amount of standard was less than ± 2%.

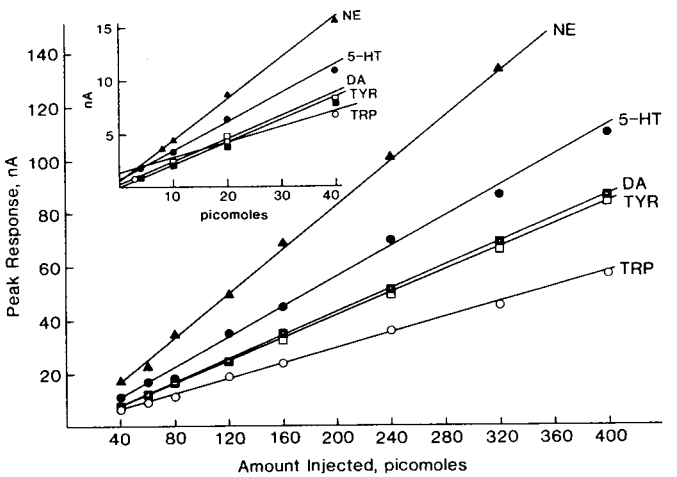

Fig. 3. Linearity of response curves for representative standard compounds (1—400 pmol of each compound in 20 μl) at an applied potential of +950 mV. The ratio of the slope of the standard curve for each compound relative to the slope of the standard curve for epinine was: NE = 2.05; TYR = 1.02; DOPAC = 2.08; DA = 1.05; HVA = 0.90; 5-HIAA = 2.32; 5-HT = 1.34; TRP = 0.68.

Chromatographic performance

The relatively large number of peaks in each chromatogram required a mobile phase which could be manipulated to maintain optimal resolution. Changes in pH altered the retention times of the acids in the chromatogram in a consistent manner without substantially affecting the amines. Increased mobile phase acidity increased retention of these compounds through increased protonation of the carboxyl groups and subsequent increased ion-pair formation with the octyl sulfate. The elution time of TRP (the last peak) was particularly sensitive to pH and effectively established the lower pH limit that could be utilized. Similarly, TYR resolution required a mobile phase of pH 3.00 since above pH 3.10 and below pH 2.95 it was eluted with neighboring peaks (Fig. 1). Changes in the acetonitrile component also altered the chromatography consistently. Increased organic solvent decreased the retention

times of all the compounds. In contrast, additional sodium octyl sulfate selectively increased the retention of the amines without substantially affecting the acids. The mobile phase constituents thus comprised a flexible and versatile system that could be altered as column performance warranted.

The separation of a mixture of standards is shown in Fig. 1A. Generally, the catechol compounds were eluted before the indoles and the acidic metabolites before the 3-methoxylated metabolites. Fig. 1B demonstrates the effectiveness of the chromatographic conditions for simultaneous determination of these compounds from nucleus accumbens.

Peaks in the chromatogram were identified by co-elution with authentic standards and by peak superimposition, that is, by addition of known amounts of standards to tissue samples and comparison of the increased peak heights at the corresponding retention times. DOPA, 5-HTP, and 4-hydroxy-3-methoxyphenylene glycol (MHPG) were not routinely identifiable in tissue samples. Only trace amounts of NM could be normally detected and only in certain brain regions. 3-MT was observable only in DA-rich areas such as nucleus accumbens and corpus striatum with levels apparently resulting largely from postmortem enzymatic degradation of DA [65].

Other potential chromatographic interferences were also checked. Epinephrine was eluted immediately after NE, octopamine immediately following DOPAC, and tyramine and tryptamine between HVA and 3-MT, but these peaks were not observed in tissue samples. Metanephrine, the 3-methoxylated metabolite of epinephrine, was eluted with DA but would not have been present in sufficient amounts in tissue to cause significant interference. The catecholamine metabolites vanillylmandelic acid, dihydroxymandelic acid, and dihydroxyphenylene glycol were eluted in the solvent front.

The occurrence of unknown peaks is a concern when unpurified brain extracts are being chromatographed. A few peaks of this nature were clearly identified in specific brain regions, but they were generally small and could not be characterized as monoamine-related. Moreover, these peaks contained no appreciable radioactivity. With the relatively high potential applied to the detector and the crude nature of the brain extracts it is possible that one or more of these peaks represented small TYR- or TRP-containing peptides.

External and internal standardization

The formic acid—acetone homogenization medium employed in these procedures extracts a large number of compounds of neurochemical interest [66] in a single unpurified fraction. However, the specificity of the electrochemical detector in combination with the high-efficiency column makes the use of a non-specific extraction procedure feasible.

The relatively high voltage applied and the large number of endogenous compounds oxidized resulted in a gradual decay in detector sensitivity over the course of a day. Consequently, a mixture of working standards was run immediately before the first and after the last tissue samples each working day. The results from analysis of this data indicated that slight changes in retention times (and peak heights) were also a factor. The mean difference in the peak heights for all compounds in the working standards mixture except TYR and TRP from the beginning to the end of the day was 3.0 ± 1.4% with no values

greater than ± 6—7%. The amino acid changes were greater. TRP declined on the average 14.4% and TYR 41.5% from the beginning to the end of the day. A TYR standard was therefore run after every second tissue sample throughout the day to more closely monitor this change. The basis for these latter changes in electrode response can be seen in the voltammogram in Fig. 2. Since neither amino acid has attained a plateau on the current—potential curve at +950 mV, relatively little fouling of the electrode would markedly depress the peak response. The changes in peak responses from beginning to the end of the day for all the working standards were assumed to occur linearly with respect to time, and external standard values relative to a specific tissue sample were interpolated accordingly.

The use of internal standards in quantitative analytical procedures is generally considered to be superior to direct calibration because it provides an inherent correction factor. Analyses of the monoamines by liquid chromatography with electrochemical detection have frequently relied upon dihydroxybenzylamine (DHBA) or other compounds such as N-methyl-5-hydroxytryptamine added to tissue samples before extraction and chromatographic separation. Such compounds mimic the chemical behavior of the catechols and/or indoles both in extraction and chromatographic characteristics. Hence, they effectively control for variable extraction or injection volumes and variations in detector performance within a day. In this work epinine was used as the internal standard.

Spiked and unspiked cerebellar homogenates were employed as tissue blanks to compare the recovery of epinine as internal standard to the recovery of added amounts of each compound to be measured. Epinine was chosen as internal standard because it is not detectable in brain tissue of normal animals

TABLE II

COMPARISON OF INTERNAL STANDARD RECOVERY WITH RECOVERY OF INDIVIDUAL COMPOUNDS IN THE PRESENCE OF TISSUE

Cerebellar homogenates were split and some of the replicates were spiked with known amounts of authentic standards of the compounds of interest. All replicates were then carried through the procedure (see Experimental) and the rates of epinine (EPN) recovery compared with the rates of recovery of the other compounds. The percent recovery values reported are the means ± S.D. with n = 16 for EPN and n = 25—26 for all other compounds. Ratios were computed by dividing the mean spike recovery for an individual compound by the mean EPN recovery.

Compound	Percent recovery	Ratio to EPN
EPN	84.6 ± 12.3	1.00
TYR	95.6 ± 16.0	1.13
NE	85.1 ± 10.4	1.01
DA	91.1 ± 12.6	1.08
DOPAC	93.1 ± 12.0	1.10
HVA	83.5 ± 10.6	0.99
TRP	87.3 ± 11.2	1.03
5-HT	86.7 ± 11.4	1.03
5-HIAA	92.0 ± 12.8	1.09

TABLE III

REGIONAL CONCENTRATIONS OF MONOAMINE PRECURSORS, NEUROTRANSMITTERS, AND METABOLITES IN RAT BRAIN

Brain regions were dissected as described in Experimental. Values represent means ± S.E.M. for 12—13 determinations expressed as μg/g tissue. No differences in content were observed among any of the time points after radiolabeled precursor administration. N.D. = not detectable.

Brain region	TYR	NE	DA	DOPAC	HVA	TRP	5-HT	5-HIAA
Nucleus accumbens	11.69 ± 0.77	1.03 ± 0.06	6.32 ± 0.20	1.36 ± 0.07	0.55 ± 0.03	4.77 ± 0.96	1.18 ± 0.02	0.68 ± 0.02
Corpus striatum	11.42 ± 0.76	0.29 ± 0.02	10.41 ± 0.03	1.41 ± 0.04	0.84 ± 0.04	4.79 ± 0.96	0.78 ± 0.02	0.71 ± 0.03
Hypothalamus	11.17 ± 0.83	2.26 ± 0.06	0.53 ± 0.02	0.11 ± 0.01	N.D.	4.40 ± 0.95	1.08 ± 0.03	0.60 ± 0.02
Hippocampus	10.95 ± 0.78	0.43 ± 0.03	0.21 ± 0.03	N.D.	N.D.	4.40 ± 0.89	0.54 ± 0.01	0.53 ± 0.02
Parietal cortex	10.04 ± 0.64	0.40 ± 0.02	0.56 ± 0.03	0.10 ± 0.01	0.10 ± 0.01	4.27 ± 0.82	0.46 ± 0.01	0.33 ± 0.01

TABLE IV

REGIONAL MONOAMINE NEUROTRANSMITTER TURNOVER RATES COMPUTED WITH AND WITHOUT INCLUSION OF TYR AND TRP SPECIFIC ACITIVITIES

NE, DA and 5-HT specific activities were utilized to compute turnover constants in two different ways: (1) (*HL*) the half-life of the decrease in neurotransmitter specific activity was derived from the decline portion of the curve; (2) (*AA*) TYR and TRP specific activities were included with the slope of the decline in neurotransmitter specific activity in the equation shown in Results and discussion. Turnover rate is the product of the turnover constant and the concentration of neurotransmitter. The turnover rates in this table are based upon 10—13 specific activity determinations with animals treated as described in Experimental. Values are expressed as μg/g tissue/h ± S.E.S. (standard error of the slope).

Brain region	NE		DA		5-HT	
	HL	*AA*	*HL*	*AA*	*HL*	*AA*
Nucleus accumbens	0.14 ± 0.10	0.69 ± 0.51	2.21 ± 0.30	6.12 ± 0.82	0.33 ± 0.07	1.09 ± 0.24
Corpus striatum	0.02 ± 0.02	0.08 ± 0.10	2.71 ± 0.42	9.16 ± 1.43	0.21 ± 0.06	0.73 ± 0.20
Hypothalamus	0.11 ± 0.26	2.84 ± 6.72	0.19 ± 0.05	0.53 ± 0.14	0.31 ± 0.07	0.95 ± 0.22
Hippocampus	0.04 ± 0.05	0.17 ± 0.21	0.07 ± 0.02	0.14 ± 0.04	0.16 ± 0.04	0.44 ± 0.12
Parietal cortex	0.10 ± 0.07	0.22 ± 0.15	0.19 ± 0.05	0.45 ± 0.13	0.13 ± 0.07	0.33 ± 0.17

and because it had a more acceptable retention time in the elution pattern than the more frequently employed DHBA. Table II gives the mean recovery of epinine in the unspiked tissue blanks and the average recoveries of the other compounds in the spiked homogenates. The ratio of the recoveries of the other compounds to the epinine recovery range from 0.99 to 1.13 and indicate that epinine is a valid internal standard in representing the chemical behavior of the precursors, neurotransmitters, and metabolites quantified in this assay.

Across more than 500 tissue samples analyzed in this laboratory the epinine recovery averaged 84.3 ± 8.0%. This value is higher than that of most other compounds used as internal standards employing similar methods (see Table I) and represents good reproducibility. These attributes ensue from the simple extraction procedure employed in which little loss of sample occurred through manipulations to separate or isolate individual compounds.

Recovery of radioactivity from collection of NE, DA, 5-HT, TYR, and TRP peaks was calculated by comparing the counts of the ^{14}C-labeled compound in an eluate fraction to the counts added in the initial ^{14}C-spike. When peak collection for a particular fraction was complete, the ^{14}C-radioactivity recovery was within 2—3% of the epinine recovery value. Background radioactivity was consistently low in both the ^{3}H- and ^{14}C-channels in the range of 12—15 dpm.

Monoamine turnover utilizing TYR and TRP

Determinations of precursor, neurotransmitter, and metabolite concentrations in representative brain regions are shown in Table III. These values are in excellent agreement with reported values for similarly dissected brain areas (e.g., refs. 42, 45, 66, 67).

Table IV gives the turnover rates for NE, DA, and 5-HT in the same brain areas calculated both with and without incorporation of simultaneous changes in TYR or TRP specific activities. For the turnover rates expressed without inclusion of the amino acid precursors, half-lives ($t_{1/2}$) were calculated for NE, DA, and 5-HT from the regression lines of the declines in specific activity in each brain region. The $t_{1/2}$ values were then substituted into the equation $k = \ln 2/t_{1/2}$ to derive a turnover constant. The product of this constant times the neurotransmitter concentration in that brain area equals the turnover rate.

The development of computational procedures to incorporate TYR or TRP specific activities into calculation of the turnover constant has been reported by Neff et al. [59]. These methods were derived to more closely simulate the results of an intravenous pulse injection of radiolabeled precursor. Incorporation of label into the neurotransmitter is initially rapid, but since a portion of the labeled amino acid recirculates in the bloodstream for some time, synthesis of [^{3}H]neurotransmitter continues at a decreasing rate. If this continued synthesis is not accounted for, then the computed turnover constant (as calculated for Table IV above) will be less than the true value. The amino acid specific activities were included in the calculation with the decline in neurotransmitter specific activity by the following equation:

$$k = \frac{2 \times M}{(AA_{t_1} + AA_{t_2} - NT_{t_1} - NT_{t_2})}$$

where k = turnover constant; M = slope of the regression line for neurotrans-

mitter specific activity; AA = TYR or TRP specific activity at time t; NT = neurotransmitter specific activity at time t. These values are also shown in Table IV. As can be seen, the inclusion of the TYR or TRP specific activities results in most cases in a two- to four-fold increase in neurotransmitter turnover rates. This magnitude of change has also been observed by Neff et al. [59].

Concentrations of biogenic amine metabolites are currently thought to provide appropriate indices of the amount of functional neurotransmitter released [54, 57], and their turnover rates have been reported [28, 56, 68]. The procedure described in this report can also provide data on tissue content and specific activity of these compounds. Since metabolite formation lags behind neurotransmitter synthesis, care should be taken that the data points used to determine the specific-activity regression lines for the metabolites are only those occurring after the attainment of peak-specific activity, that is, are on the declining portion of the specific-activity curve.

The described procedure has merit in that it permits the accurate and precise determination in discrete rat brain regions of the content and specific activity of monoamine-related substances from precursor amino acids through metabolites. Furthermore, the inclusion of the amino acid specific activities in the calculation of the neurotransmitter turnover rates produces a more accurate biochemical measure. Several studies have demonstrated the effects of brain TYR and TRP availability on the synthesis of their respective neurotransmitters [69—71]. The present technique combines ease of sample preparation, internal standardization, and sensitivity to fulfill the need for simultaneous determination of monoamine turnover for correlation with behavioral observations and for the study of transmitter interactions.

ACKNOWLEDGEMENTS

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REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND SEPARATION OF PEPTIDE HORMONES IN HUMAN TISSUES AND PLASMA WITH REFERENCE TO CHOLECYSTOKININS

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SUMMARY

A reversed-phase high-performance liquid chromatographic method for the separation of peptide hormones has been applied to the measurement of large and small molecular forms of cholecystokinins in biological tissues. The method was validated for samples of human plasma and intestinal tissues, and for porcine gut extracts. The two-stage chromatographic process used semi-preparative reversed-phase packing for initial sample preparation, followed by gradient elution on a Hypersil ODS-5 column. Peptides in the fmol—nmol range were separated reproducibly, and recovered quantitatively. The method has been applied to the purification of a novel biologically active CCK peptide from a porcine gut extract.

INTRODUCTION

Separation methods applicable to peptides include molecular exclusion, electrophoresis and ion-exchange chromatography [1]. Recently, reversed-phase high-performance liquid chromatography (HPLC) has been shown to produce good separation of various standard peptides [2—4]. The potential for high resolution coupled with rapid and reproducible separations using HPLC suggested that this technique should be suitable for the preparation and analysis of peptides from biological samples.

HPLC should have several advantages over conventional chromatography of peptides because, in addition to its high-resolving power, recoveries are very high and samples retain both immuno- and bioactivity [3]. Furthermore, the conditions used for analytical separation may be adapted to a preliminary preparative step permitting clean-up of plasma or tissue samples, removing most of the protein, salts and lipids, without resorting to conventional solvent

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extraction or protein precipitation steps when recoveries are often low due to problems with partition or co-precipitation.

We have investigated this approach for a family of peptide hormones found in gut and brain, the cholecystokinins (CCKs). The name cholecystokinin was given to the active principle in gut extracts that caused gallbladder contraction when injected into a second animal. This agent was first discovered in the 1920s and subsequently isolated and sequenced in the 1960s as a 33-amino acid peptide [5]. Subsequent studies have identified a series of CCK peptides in gut and brain of 4, 8, 12, 39 and 58 amino acids [6—10], all sharing a common carboxyl terminal amino acid sequence. Cholecystokinins have been difficult to assay reliably using conventional radioimmunoassays in both blood and tissues firstly because of this molecular heterogeneity which requires knowledge of the proportions of the various molecular forms to determine the biological significance of the levels and secondly because of the similarities of CCKs to a series of related peptides sharing the same C-terminal pentapeptide sequence, but with different biological effects — the gastrins [11].

Extraction and separation of the CCKs from each other and from gastrins, by HPLC, prior to radioimmunoassay of fractions using an antibody common to the C-terminal portion of these molecules, offer the potential of assaying each form of the peptide individually.

HPLC coupled to bio- and immunoassay procedures has been used in this study analytically to assay fmol amounts of CCK peptides in plasma and in intestinal tissues, and preparatively to prepare nmol amounts of pure peptides from a commercial gut extract.

EXPERIMENTAL

Reagents and materials

Acetonitrile (HPLC 'S' grade 50% transmission at 205 nm) was obtained from Rathburn Chemicals (Walkerburn, U.K.), sodium chloride and hydrochloric acid (AristaR grade) from BDH (Enfield, U.K.). Hypersil ODS, 5-μm packing (Shandon Southern, Runcorn, U.K.) and Lichroprep RP-18 (25—40 μm, Merck, Darmstadt, F.R.G.) were supplied by HPLC Technology (Cheshire, U.K.). Sep-Pak C_{18} cartridges were obtained from Waters Assoc. (Northwich, U.K.). All glassware was siliconised before use, with trimethylsilane (Repelcote, Hopkin and Williams, Romford, U.K.). Distilled water was prepared and stored in glass containers. Two stock solutions were used. Solution A was 0.155 mol/l sodium chloride, adjusted to pH 2.1 with hydrochloric acid. Solution B was acetonitrile—water (3:2). All solvents were filtered before use using a 0.45-μm Millipore filter (HAWP-aqueous; FHLP-organic, Millipore, Harrow, U.K.). Solvents were regularly degassed with helium, through 25-μm solvent inlet filters).

Radiolabelled $Na^{125}I$ was purchased from Amersham Radiochemicals (Amersham, U.K.). Chloramine T and sodium metabisulphite were obtained from BDH; Norit GSX charcoal and dextran C from Hopkin and Williams.

Pure peptides were used for column calibration: 99% pure CCK 33 and 39 were generous gifts from Dr. V. Mutt (Karolinska Institute, Stockholm, Sweden); sulphated and non-sulphated CCK 8 were gifts from Squibb, Wirral,

U.K. The synthetic decapeptide caerulein was generously supplied by Farmitalia, Milan, Italy. Gastrin 17-1 was obtained from Serono (London, U.K.) and gastrin 34 was a gift from Dr S. Bloom. The CCK 8 antiserum was a gift from Dr. V. Go (Mayo Clinic, Rochester, NY, U.S.A.).

Semi-preparative apparatus

LiChroprep RP-18 (25—40 μm) was slurry packed in 10 × 0.46 cm stainless-steel columns using 20-μm frits, according to the manufacturer's recommended procedure. After slurrying the silica in isopropanol the column was packed in methanol at 200 bar. Columns were equilibrated as follows: 50 ml acetonitrile; 50 ml of solution B; 25 ml each of solutions A and B (1:1), and finally 50 ml of solution A. Each column was used for four plasma samples (total 40 ml plasma). After elution of peptides from each sample, columns were washed with 50 ml solution of B and then equilibrated as above. An Eldex (Owens Polyscience, Macclesfield, U.K.) high-pressure single-piston pump was used to load plasma samples at 2 ml/min and elute peptides at 1 ml/min from these semi-preparative columns. Sep-Pak C_{18} cartridges were washed with 20 ml each of methanol, acetonitrile, solution B, 10 mmol/l hydrochloric acid and solution A before use. Each cartridge was used once only.

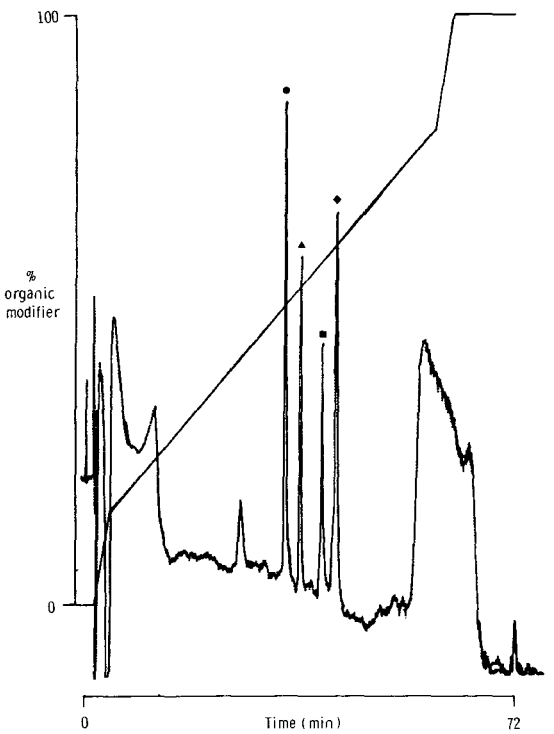

Fig. 1. Separation of mixture of CCK standards. (•) CCK 4 (Tetrin), (▲) caerulein (Ceruletide), (■) CCK 8 and (♦) CCK 33. 0.05 a.u.f.s.; 225 nm; chart-speed 2 mm/min; column, Hypersil ODS. Gradient betweeen 0.155 mol/l sodium chloride, pH 2.1 and acetonitrile—water (3:2).

Analytical apparatus

The high-performance liquid chromatograph (Applied Chromatography Systems, Luton, U.K.) was equipped with a microprocessor gradient controller, together with a Rheodyne sample injector (Model 7215) with a 2-ml loop, and a UV monitor (Cecil Instruments, Cambridge, U.K.). A dual-pen recorder (J.J. Lloyd, Southampton, U.K.) provided both a UV trace and a gradient profile. Data were collected and analysed on a Trilab microprocessor and printer–plotter (Trivector Systems, Sandy, U.K.). A reversed-phase octadecylsilane 5-μm support (Hypersil ODS) was packed in methanol at 350 bar using a slurry packer (Magnus 6050) in the recommended manner. Columns were stainless steel (316 grade), 10 × 0.46 cm and fitted with zero dead volume end fittings and 2-μm frits.

HPLC analyses were carried out at ambient temperature and monitored at 225 nm, where applicable. The flow-rate was 1 ml/min. A gradient was established between 0.155 mmol/l sodium chloride, pH 2.1 and acetonitrile–water (3:2) as described previously [4] and shown in Fig. 1.

Blood sample preparation

Human blood (20 ml) was collected into lithium heparin tubes containing 8000 IU aprotinin (trasylol, Bayer, Leverkusen, F.R.G.) to inhibit protease activity. After mixing, the blood was centrifuged at 2000 *g* for 10 min. Plasma was removed and stored in glass at −20° C until required. Prior to semi-preparative HPLC the plasma was diluted and centrifuged at 3000 *g* for 15 min to sediment cryoprecipitate. Plasma (10 ml) was diluted with 90 ml of solution A to render the viscosity suitable for pumping onto the column, and the ionic strength suitable for solute–column interaction.

Tissue sample preparation

Full-thickness sections of human jejunal, ileal and mid intestine were washed and stored at −20° C until required. Mucosal layers were separated from muscle layers by scraping. Two extraction procedures were used; a neutral extraction to preferentially extract the smaller molecular forms of cholecystokinin and an acid extraction which primarily extracts the larger forms [8].

Neutral extraction

Samples (ca. 100 mg) were weighed and plunged into 1 ml of boiling water for 2 min. Tissues were homogenised for 30 sec with a Potter-Elvejham homogeniser. After reboiling for a further 15 sec the samples were centrifuged at 2000 *g* for 10 min, and the resulting supernatant decanted.

Acid extraction

To ca. 100 mg tissue samples 1 ml of water was added and the samples boiled for 2 min. The extracts were adjusted to 0.5 mol/l acetic acid by the addition of 17.5 μl of glacial acetic acid and the tissues homogenised as above. The sample was left at 4° C for 30 min, then centrifuged and the supernatant decanted.

Semi-preparative chromatography

Diluted plasma supernatants were loaded onto a LiChroprep RP-18 column. This reversed-phase column packing has a high loading capacity for peptides in a protein-rich medium. The column was then washed with 10 ml of acidified saline (solution A) before eluting peptides with 10 ml of organic modifier (solution B). The sample was reduced to 50% of its original volume by removal of acetonitrile under nitrogen, 5 ml of solution A were added prior to the analytical HPLC step.

Tissue samples were processed through Sep-Pak C_{18} cartridges (with lower peptide:protein load capacity) in the same manner as described for plasma.

Radioimmunoassay

Column fractions or standards in acetonitrile—saline (400 μl), trasylol 1000 IU, veronal buffer 0.1 mol/l, pH 7.6, and antibody 1/40,000 were incubated in a total volume of 2 ml for three days, followed by addition of [^{125}I]CCK 8 (2000 cpm) and a further incubation of three days. The tracer used was non-sulphated CCK 8 iodinated by the chloramine T method and the mono-iodinated product separated from other reaction products by ion exchange [12]. The chromatography used was a gradient of 0.05 mol/l to 0.5 mol/l ammonium carbonate on a DE52 (1 × 10 cm) column (Whatman, Maidstone, U.K.). Separation of bound from free peptide was achieved using charcoal. Activated charcoal (40 g/l) equilibrated with dextran C (4 g/l) in phosphate-buffered saline and 500 μl of the mixture added to each tube. Tubes were centrifuged at 2000 *g* for 5 min and the supernatants decanted. Samples were counted in a gamma-counter and the percentage of tracer bound calculated for standards and samples. Initial binding was 50%, non-specific binding less than 3% and reproducibility within- and between-assay 10.1% and 14.2%, respectively. The detection limits for the assay were 12 fmol per tube for CCK 8, 24 fmol per tube for CCK 33 and 39 fmol per tube for gastrin 17, and for gastrin 34. The antibody had less than 3% molar cross-reactivity with CCK 4 and less than 0.1% cross-reactivity with other gut peptides.

Bioassay

CCK bioactivity was assayed using an in vivo guinea pig gallbladder contraction assay modified from that of Ljunberg [13]. Fasting guinea pigs (ca. 300 g) were anaesthetised with Hypnorm (1 ml/kg intramuscular, Janssen Pharmaceuticals, Lamberhurst, U.K.) and valium (0.5 ml/kg intra-peritoneal, Roche, Welwyn Garden City, U.K.), the abdomen opened, the gallbladder exposed and a silk ligature attached to its apex after the gallbladder bile had been removed by aspiration. The silk thread was attached to a counter-weighted beam, the other end of which was connected to a linearsyn pressure transducer. The transducer was in turn connected to an amplifier, containing smoothing circuits to damp out respiratory movements. By stabilising the baseline this modification allowed greater amplification of the gallbladder contraction signal and made the assay sensitive to less than 0.2 pmol CCK 8 or CCK 33 — a much greater sensitivity than previously described in in vivo bioassays. Standard peptides and samples lyophilised to remove acetonitrile and then reconstituted were assayed by measuring the amplitude of gallbladder contraction induced following

administration of samples through a jugular vein. The gallbladder contraction produced by a particular sample was independent of the intravenous sample size between 0.05 and 1.0 ml.

Validation

Estimation of peptide recovery. Extraction procedures for both plasma and tissue samples were assessed for recovery at various stages, using both fmol of radiolabelled non-sulphated CCK 8 and/or pmol amounts of sulphated CCK 8, non-sulphated CCK 8 and CCK 33. Recovery of iodinated tracer was assessed using gamma-counting and recovery of unlabelled peptides estimated by radioimmunoassay.

Semi-preparative stage. Approximately 15,000 cpm (0.5 fmol) of iodinated CCK 8 were added to 10 ml of human plasma. A portion of the sample (1 ml) was counted both before and after loading onto the Lichroprep RP-18 column. Likewise, 1 ml of the saline wash solution A and 1 ml of eluent solution B were retained for estimation of radioactivity. Similar studies were performed with iodinated CCK 8 in tissue samples. Peptides (4 pmol CCK 8 SO4 and 13 pmol CCK 33) were added to 10 ml of human plasma and treated as above. CCK was estimated using radioimmunoassay; stock solutions were used for comparison of pre- and post-extraction samples.

Analytical stage. Radiolabelled or standard unlabelled cholecystokinins were applied to the analytical HPLC. Fractions (1 ml) were collected for the duration of the chromatography and analysed using the gamma-counter or radioimmunoassay as appropriate.

Reproducibility. Microgram quantities of a single batch of HPLC-purified caerulein (Ceruletide, Farmitalia) were repeatedly chromatographed to determine the reproducibility of the analytical system.

RESULTS AND DISCUSSION

Previous studies [3] have shown the capabilities of HPLC in the separation of a wide variety of purified hormonal polypeptides. Recently other groups have used reversed-phase HPLC for the isolation and characterization of peptides from several biological matrices, viz. brain [14] neurosecretory system of the carp [15], leukaemia viruses [16], human [17] and bovine [18] pituitary tissues and salivary glands [19]. In each case there are preliminary extraction or purification steps unrelated to HPLC. In this study, in contrast, we have modified our analytical HPLC technique to provide a simple preparative step. Using this technique small forms of CCK peptide hormones have been assayed in biological fluids and tissues. We have shown the HPLC has several advantages over conventional chromatography including high resolution, virtually complete recovery of peptides (> 90%) which retained both bio- and immunoactivity, and a chromatography time of minutes rather than hours. Various detection systems could be used after HPLC; UV spectra where microgram quantities of peptides were available; bioassay for pmol amounts and radioimmunoassay where a sensitivity to fmol of peptide was required.

CCK 8 SO4 and CCK 33/39 in plasma and tissues were separated reproducibly on this system as shown by the radioimmunoassay profiles of

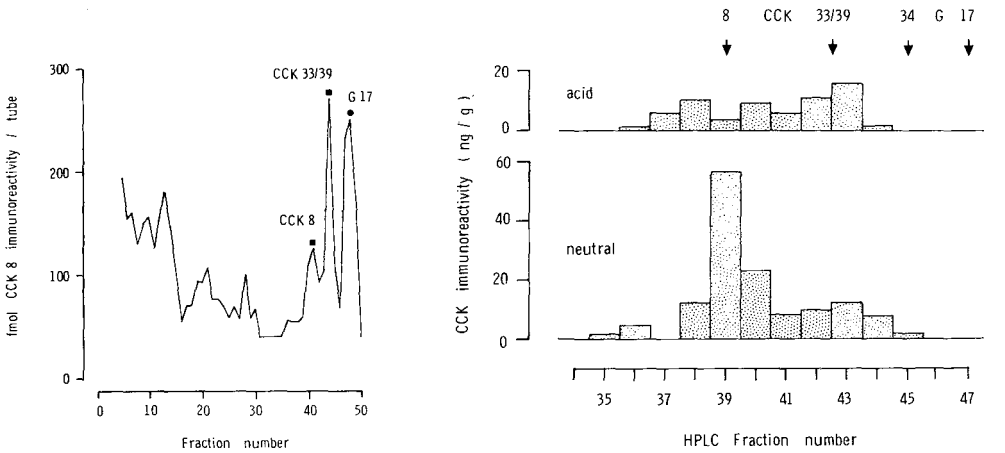

Fig. 2. Radioimmunoassay profile of a plasma sample fractionated into 1-ml fractions by HPLC. For reference the elution times of standard CCK 8, CCK 33/39 and gastrin 17 are shown.

Fig. 3. CCK immunoreactivity in acid and neutral extracts of jejunal mucosa separated by reversed-phase HPLC into 1-ml fractions. The elution times of pure standards CCK 8 and 33/39 peptides and gastrin 34 and gastrin 17 peptides are shown by arrows.

plasma (Fig. 2) and tissue (Fig. 3) sample. Indeed such a separation is a necessity if a non-specific antiserum which cross-reacts with the common C-terminal portion is used to assay forms of CCK. Gastrins were well separated from CCKs and from each other. In addition, other forms of immunoreactivity were present, particularly in plasma which were not biologically active CCKs or gastrins. These results parallel the separation of standard CCK peptides (Fig. 1) [4].

Purification of a novel biologically active CCK peptide was achieved using HPLC. Chromatography of a commercial gut extract (Boots Pancreozymin, Boots Pure Drug, Nottingham, U.K.) showed it to contain numerous peptides detected by UV absorbance (Fig. 4). Only some of these fractions, however, were likely to be CCKs as shown by the fact that only a few fractions contained bioactivity. In a more detailed analysis using column fractions both bio- and immunoactivity were noted in regions corresponding to CCK 8 and CCK 33/39 and in a region which did not co-chromatograph with these standards (Fig. 5). This column fraction (fraction 35) was subjected to gel chromatgraphy of a Sephadex G50SF matrix (Fig. 6) which again showed the CCK to be intermediate in molecular size between CCK 8 and CCK 33. These fractions from the Sephadex were then rechromatographed on reversed-phase HPLC. A single peak was detectable by UV (Fig. 6b) representing considerable purification of the original extract in a form suitable for determination of primary structure.

The HPLC system was validated with respect to recovery of peptides using both labelled and unlabelled peptides added to plasma and tissue samples. The recovery of [^{125}I]CCK 8 from plasma during the initial extraction procedure was 105 ± 10% S.D. (n = 6) and from the analytical column 98.5 ± 4% (n = 5). Recovery of CCK 8 S04 in this system was 95.6% (n = 3, range

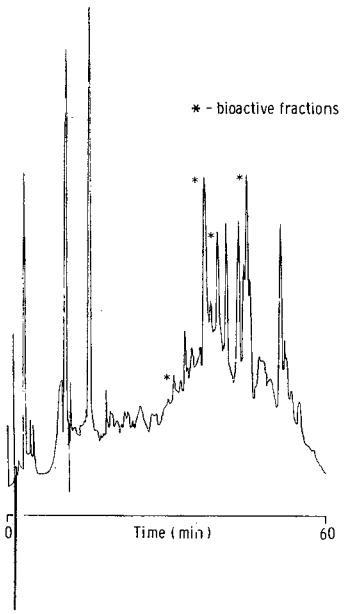

Fig. 4. High-performance liquid chromatogram of Boots Pancreozymin (a commercially available gut extract). Details as in Fig. 1. Asterisks represent bioactive fractions.

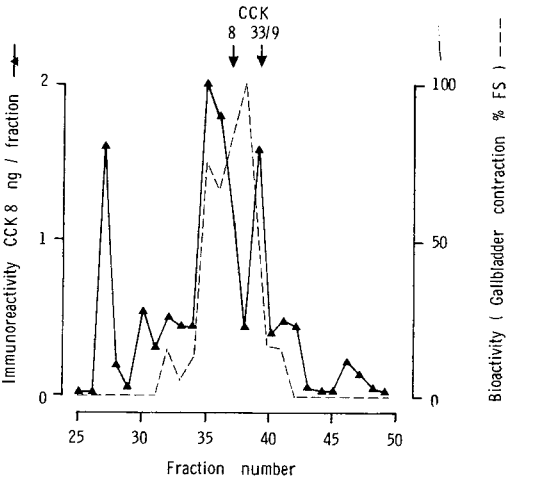

Fig. 5. CCK immunoreactivity (designated by ▲) and CCK bioactivity (— — —) in HPLC fractions from a gut extract of porcine upper intestinal mucosa. Elution times of standard CCK 8 and CCK 33/39 are designated by vertical arrows.

92—100%) after the initial extraction, and 96.0 ± 5% (n = 4) at the analytical step. Recovery of CCK 33 added to plasma and processed through both the preparative and analytical systems was 103.3 ± 11% (n = 4). Recovery of tracer from tissue homogenates was not significantly different from 100%. These results, although remarkable when compared with conventional chromatography of peptides, are in accordance with other methods for peptides separated by HPLC [3].

Although CCK 8 is insoluble in acid conditions, no losses of CCK 8 were observed during HPLC analysis at pH 2.1. Presumably this is because it is in intimate association with protein prior to column loading, is protected whilst on the column and only elutes when sufficient acetonitrile is present in the eludate to maintain its subsequent solubility.

The radioimmunoassay for carboxyl terminal of CCKs could be performed in the presence of acetonitrile and acid saline if two precautions were followed. Firstly the buffer used was of relatively high osmolality (0.1 mol/l) in order to maintain the pH of the assay tubes > 7.4. Secondly if the samples were not lyophilised to remove acetonitrile prior to assay then appropriate amounts of acetonitrile were added to standards. This is because acetonitrile has a slight non-specific inhibitory effect on antigen—antibody binding.

When acetonitrile was blown-off under nitrogen losses of labelled or unlabelled CCKs were insignificant until the volume was reduced by > 60%. Therefore, the volume was never reduced to less than 50% of the original.

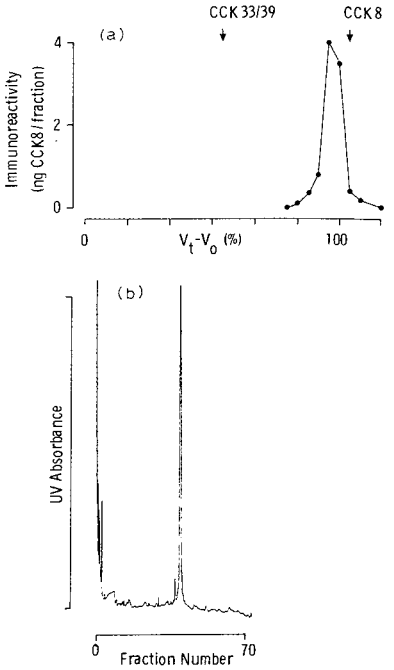

Fig. 6. (a) Gel chromatography (Sephadex G50 SF) of HPLC fraction 35 from a porcine gut extract possessing CCK immuno- and bioactivity of unknown molecular size. Intermediate form of CCK. The elution positions of CCK 33/39 and CCK 8 on Sephadex G50 SF are shown by arrows. (b) Rechromatography by HPLC of immunoreactive material from Sephadex G50 SF (intermediate form on CCK). Details as in Fig. 1.

Before the analytical HPLC the sample was made up to its original volume with solution A, to adjust pH and ionic strength to optimal conditions for interaction of solute with the analytical column packing. The small amounts of acetonitrile remaining did not preclude the peptides chromatographing satisfactorily on the analytical HPLC.

Using a pure synthetic analogue of CCK, caerulein, the coefficient of variation of retention time (min) observed was 0.017 (n = 7).

The method described here for peptide extraction from plasma and tissues has been validated for large and small forms of cholecystokinin using the analytical tool as a basis for semi-preparative conditions. In this system, CCKs are separated from each other and from gastrins, are recovered quantitatively and may thus be assayed. In principle, this system is applicable to extraction of many peptides from biological media providing the analytical step has been optimised for the peptides of interest.

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FLUOROMETRIC DETERMINATION OF TOTAL VITAMIN C IN WHOLE BLOOD BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH PRE-COLUMN DERIVATIZATION

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SUMMARY

A reliable and semi-automated high-performance liquid chromatographic (HPLC) method is described for the determination of total vitamin C in whole blood. After deproteinization of whole blood and enzymatic oxidation of *l*-ascorbic acid to dehydro-*l*-ascorbic acid, the latter is condensed with *o*-phenylenediamine to its quinoxaline derivative. This derivative is separated on a reversed-phase HPLC column and detected fluorometrically. Total vitamin C in whole blood can be determined in concentrations as low as 0.2 μmol/l.

Special attention was paid to the stability of vitamin C in whole blood and of its quinoxaline derivative in the extract. Results of our investigations showed that total vitamin C in whole blood is stable for eight days at −20°C, provided ethyleneglycol-bis-(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid and glutathione are immediately added to the blood sample. The quinoxaline derivative of vitamin C in the blood extract is stable for at least 24 h if stored in the dark at 4°C.

Routine vitamin C determinations can be carried out in a series of 100 samples within 48 h. The within-assay and between-assay coefficients of variation were 3.7% and 4.6%, respectively. The between-assay analytical recovery of *l*-ascorbic acid added to whole blood samples was 97.0 ± 7.0% (mean ± S.D.). Reference values of vitamin C in whole blood of normal healthy Dutch adults were found in the range 20—80 μmol/l.

INTRODUCTION

Vitamin C, a water-soluble vitamin, consists of *l*-ascorbic acid and its oxidized form dehydro-*l*-ascorbic acid. Both forms are equally biologically active [1]. Numerous methods have been described for the analysis of *l*-ascorbic acid and/or dehydro-*l*-ascorbic acid in various biological samples. These include the indicator-dye reduction method with dichlorophenolindophenol [2, 3], the ketone derivatization method with dinitrophenylhydrazine

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[3—5], an enzymatic method with ascorbic acid oxidase [6] and high-performance liquid chromatographic (HPLC) methods with electrochemical [7—9] and UV detection [10—12].

However, the indicator-dye reduction and ketone derivatization methods are not very specific and have the drawback that blank values have to be determined by chemical interference in the colour-inducing reaction. The enzymatic method as described lacks sensitivity for the analysis of the low concentrations of vitamin C observed in whole blood and is difficult to perform in large-scale routine analysis. HPLC methods with electrochemical detection allow only the determination of *l*-ascorbic acid, while UV measurement suffers from the very low absorbance of dehydro-*l*-ascorbic acid. Keating and Haddad [12] described an HPLC method with UV detection for the analysis of *l*-ascorbic acid and dehydro-*l*-ascorbic acid in foodstuffs. They enhanced the absorbance of the latter by pre-column derivatization with *o*-phenylenediamine (OPDA) to 3-(1,2-dihydroxyethyl)furo[3,4-*b*]quinoxaline-1-one (DFQ). However, their method has the drawback that *l*-ascorbic acid may be easily oxidized to dehydro-*l*-ascorbic acid during sample handling. This oxidation probably also occurs during deproteinization of blood when oxygen is released from oxyhaemoglobin [13]. Due to this oxidation and possible previous oxidation during blood collection, transport and storage, systematic errors may arise. Therefore, and since *l*-ascorbic acid and dehydro-*l*-ascorbic acid are equally biologically active, we developed a method for the analysis of total vitamin C in whole blood by incorporating an oxidation step in the procedure.

After enzymatic oxidation of *l*-ascorbic acid to dehydro-*l*-ascorbic acid, the latter, having insufficient UV absorbance, is condensed with OPDA to the highly fluorescent DFQ. This derivative is separated from interfering compounds on a reversed-phase HPLC column. Fluorometric detection then permits the determination of total vitamin C down to concentrations below those normally occurring in blood.

MATERIALS AND METHODS

Apparatus

HPLC was performed using a Gilson Model 302 constant-flow pump (Meyvis, Bergen op Zoom, The Netherlands), a Micromeritics 725 Autoinjector (CLI, Schijndel, The Netherlands), and a Kratos FS 950 Fluoromat fluorescence spectrophotometer (Kipp Analytica, Delft, The Netherlands) equipped with a mercury light source (type FSA 110), an excitation interference filter of 365 nm (type FSA 401) and an emission cut-off filter of 418 nm (type FSA 426).

A Knauer stainless-steel column (80 × 4.6 mm I.D.) was home-packed with ODS-Hypersil 3 μm (Shandon Southern Products, Astmoor, U.K., Cat. No. 580 × 24) by the balanced-density slurry technique using a Haskel pump type MCP 110 (Ammann Technik, Stuttgart, F.R.G.). Elution profiles were displayed on a Kipp BD-8 recorder (Kipp Analytica).

Reagents

EGTA—glutathione solution: A solution, containing 0.24 *M* ethyleneglycol-bis-(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA) and 0.20 *M*

glutathione, was prepared by dissolving 1.5 g of glutathione (Sigma, St. Louis, MO, U.S.A.) in 25 ml of double-distilled water and adjusting the pH to 6.5 with 2 *M* sodium hydroxide. In this solution 2.25 g of EGTA (Sigma) was dissolved and the pH was adjusted to 6.5 with 10 *M* sodium hydroxide. The EGTA—glutathione solution was stable for at least two months if stored in the dark at 4°C.

Ascorbate oxidase spatulae were obtained from Boehringer (Mannheim, F.R.G.). *o*-Phenylenediamine (OPDA) was from Merck (Darmstadt, F.R.G.).

The HPLC mobile phase containing 0.08 *M* potassium dihydrogen phosphate and 20% (v/v) methanol, pH 7.8, was flushed with a stream of helium gas for 10 min before use.

l-Ascorbic acid stock standard solution contained about 350 mg/l *l*-ascorbic acid (Merck) in 1% (w/v) metaphosphoric acid. This solution was stored in the dark at 4°C and was stable for at least fourteen days. The *l*-ascorbic acid working standard solution was prepared daily by diluting 2 ml of the stock standard solution with water to 100 ml.

Blood extraction and derivatization procedures

Blood was collected from veins directly into 5-ml plastic tubes containing 0.1 ml of EGTA—glutathione solution. One millilitre of whole blood was slowly transferred with thorough mixing using a vortex mixer to a 10-ml plastic tube containing 4 ml of 0.3 *M* trichloroacetic acid (TCA). The tube was allowed to stand for about 20 min in the dark at 4°C, mixing once after about 10 min. Thereafter the tube was centrifuged at 2000 *g* and 4°C for 10 min. A 1.5-ml aliquot of the supernatant was transferred to a 5-ml plastic tube. After adding 0.2 ml of 4.5 *M* sodium acetate buffer pH 6.2 and an ascorbic acid oxidase spatula, the tube was placed in a waterbath at 37°C for 5 min, mixing once after about 2 min. After removal of the enzyme-carrying spatula 0.25 ml of a freshly prepared 0.1% (w/v) OPDA solution was added. After mixing, the tube was wrapped with aluminium foil to screen it from daylight and placed in a waterbath at 37°C for 30 min. Thereafter the tube was stored in the dark at 4°C for HPLC analysis within 24 h. The centrifuge tube, containing the pellet and a residual 2 ml of supernatant, was stored in the dark at −20°C for repeated derivatization and HPLC analysis, if required, within one week.

The working standard solution was derivatized by adding 1 ml to a 10-ml tube containing 4 ml of 0.3 *M* TCA and 1.2 ml of 4.5 *M* sodium acetate buffer pH 6.2. After adding an enzyme spatula, *l*-ascorbic acid was oxidized by heating in a water-bath at 37°C for 5 min. After wrapping the tube with aluminium foil, the resulting dehydro-*l*-ascorbic acid was condensed to DFQ by adding 0.5 ml of OPDA solution and heating in a water-bath at 37°C for 30 min. The tube was stored in the dark for HPLC analysis within 24 h.

Chromatographic conditions

HPLC analysis of DFQ was carried out by injecting 20 µl of the derivatized sample extract onto the ODS-Hypersil column. The column was eluted isocratically with the mobile phase at a flow-rate of 1.0 ml/min. The effluent was monitored with the fluorescence spectrophotometer. The recorder was set at 10 mV full scale. Duration of the chromatographic run was about 10 min per sample.

Calculation

The vitamin C concentration of the sample was calculated from peak heights with the working standard solution as the reference. In routine analysis this solution was run before each series of five samples. Assuming that whole blood contains 85% of aqueous phase [14] and taking into account the volumes of sample, added 0.3 *M* TCA, the supernatant aliquot and volumes of added buffer and reagent solution, the concentration of vitamin C in the original blood sample was calculated by multiplying the concentration of vitamin C found by a factor of $\left(\frac{0.85+4.0}{1.0}\right) \times \left(\frac{1.5+0.20+0.25}{1.5}\right) = 6.3$.

Selection of fluorometric parameters

Under the chromatographic conditions employed, DFQ had its excitation maximum at 355 nm and its emission maximum at 425 nm. The light source and filters for the fluorescence detector were chosen in accordance to these wavelengths. Both the excitation maximum and the emission maximum of DFQ proved to be pH-independent over the range 3.5—8.5. pH 7.8 was chosen as a result of an optimization procedure for best HPLC separation.

Recovery test

The recovery of *l*-ascorbic acid added to whole blood was determined by analysing 1-ml portions of whole blood to which 20 μl of the *l*-ascorbic acid stock standard solution had been added. The original vitamin C was raised in this way by 39.0 μmol of *l*-ascorbic acid per litre.

RESULTS

Characteristics of the HPLC procedure

Typical elution profiles of a derivatized blood extract and the working standard solution are shown in Fig. 1. In blank experiments only a solvent peak was recorded. The HPLC procedure afforded an excellent separation of DFQ from other extract components.

As far as has been investigated the fluorescence response was linear from concentrations corresponding to 0.3 μmol/l up to about 170 μmol/l in the whole blood sample. Assuming a signal-to-noise ratio of 3, the detection limit of the vitamin C assay described in this paper corresponds to a concentration of vitamin C in whole blood of 0.2 μmol/l.

Efficiency of the extraction procedure

To investigate the efficiency of the extraction of vitamin C by the procedure described under Materials and methods, 1.0-ml aliquots of a series of five different whole blood samples were deproteinized in the presence of ^3H_2O (24×10^3 dpm). After centrifugation and removal of the supernatant the residual pellet was extracted twice more with 4 ml of 0.3 *M* TCA. The three supernatants of each sample were analysed for vitamin C and ^3H_2O. It was found that after the first extraction 10.3 ± 1.3% (mean ± S.D.) of the total amount of vitamin C and 9.9 ± 0.4% (mean ± S.D.) of the total amount of

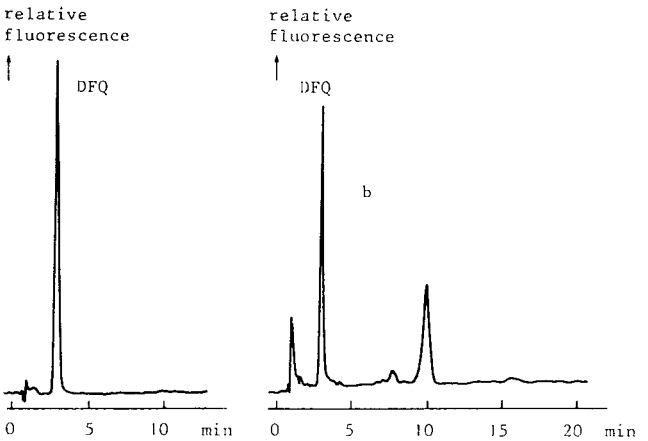

Fig. 1. Typical HPLC elution profiles of the working standard solution (a) and of a whole blood sample (b).

^3H_2O were still present in the pellet. From these results it was concluded that the efficiency of the extraction of vitamin C by the method described amounted to 100% for whole blood samples of 1 ml.

Derivatization

The minimum periods needed for complete oxidation of *l*-ascorbic acid to dehydro-*l*-ascorbic acid and for complete derivatization of the latter to DFQ were determined for whole blood samples as well as for the working standard solution. Portions (1 ml) of a pooled whole blood sample and of the working standard solution were analysed as described under Materials and methods, the reaction periods for the oxidation step being varied from 1 to 8 min in 1-min steps. Another eight 1-ml portions of the same pooled whole blood sample and of the working standard solution were analysed with reaction periods for the condensation reaction with OPDA ranging from 5 to 40 min in 5-min steps. All measurements were carried out in duplicate. The derivatized sample was injected onto the ODS-Hypersil column immediately after termination of the reaction period for the condensation reaction. For the working standard solution the minimum reaction periods to achieve maximum peak height of DFQ were 5 min and 25 min for the oxidation step and condensation reaction, respectively. For a whole blood sample these periods were 2 and 25 min, respectively. No decrease in peak height of DFQ was observed in tests with reaction periods of 8 min for oxidation and 40 min for derivatization as compared with the peak heights observed in tests with reaction periods of 5 and 25 min. The reaction periods of the assay were chosen in accordance with these findings.

Stability of DFQ

Derivatized extracts of a pooled whole blood sample were exposed to daylight at ambient temperature, or were stored in the dark at ambient temperature, 4°C or −20°C. After the storage period DFQ was determined in

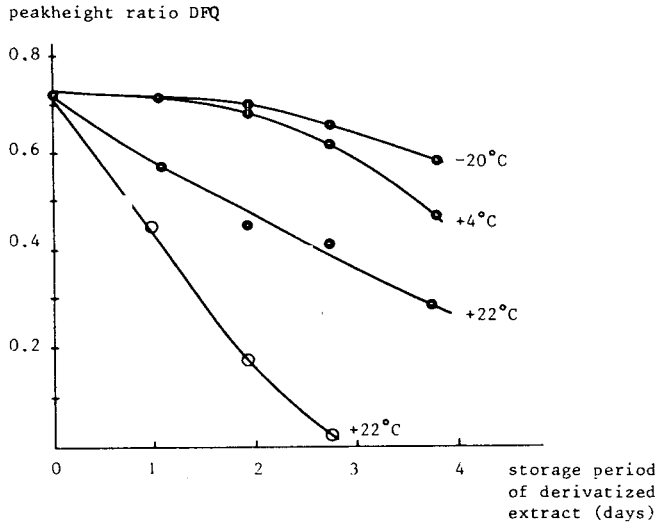

Fig. 2. Stability of DFQ from a whole blood sample under exposure to daylight (○) and in the dark (●). The ratio of the peak height of DFQ in the stored extract to that in a freshly prepared working standard solution is plotted against the storage period.

the extract by injecting 20 μl onto the ODS-Hypersil column. The peak height of DFQ was compared with the peak height of a freshly derivatized working standard solution. The results are shown in Fig. 2. As can be seen from this figure, DFQ deteriorates rapidly under daylight exposure whereas it is stable for at least one day if stored in the dark at 4° C or −20° C.

Stability of vitamin C in whole blood

In order to investigate the stability of vitamin C in whole blood after its collection in the EGTA—glutathione solution, 1-ml portions of a pooled whole

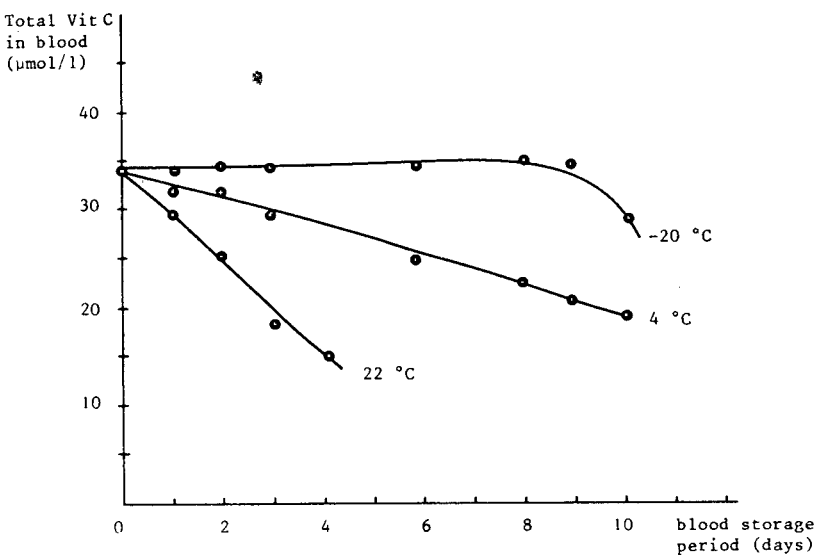

Fig. 3. Stability of vitamin C in human whole blood containing EGTA and glutathione.

blood sample were stored in the dark at ambient temperature, 4° C and —20° C and were analysed during consecutive days. The results are shown in Fig. 3. From this figure it can be concluded that whole blood samples for vitamin C analysis may be stored in the dark at —20° C for a period of eight days without appreciable loss of vitamin C.

Precision and recovery

In order to test the within-assay and between-assay precisions of the method, several 1-ml portions of the same whole blood sample with and without the addition of 39.0 μmol/l *l*-ascorbic acid were stored in the dark at —20° C and analysed for vitamin C eight times in succession and also in a series of eight consecutive days. The results are given in Table I. The coefficient of variation (C.V.) of the between-assay is somewhat higher than the C.V. of the within-assay, although both are below 5%. Table I also shows the recovery of *l*-ascorbic acid added to whole blood (97—98%).

TABLE I

PRECISION OF THE METHOD FOR DETERMINATION OF TOTAL VITAMIN C

	Within-assay precision		Between-assay precision	
	Whole blood	Recovery test*	Whole blood	Recovery test*
n	8	8	8	8
Mean	39.8 μmol/l	97.7%	39.2 μmol/l	97.0%
S.D.	1.5 μmol/l	6.3%	1.8 μmol/l	7.0%
C.V.	3.7%		4.6%	

*Recovery tests were performed as described in Materials and methods.

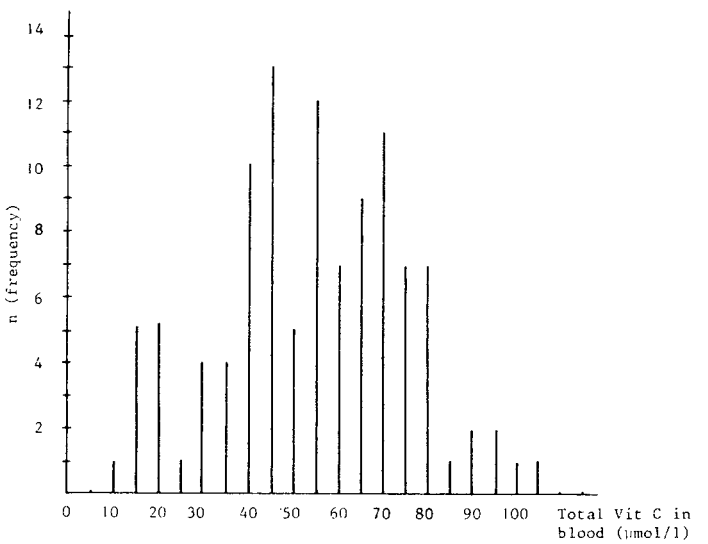

Fig. 4. Frequency distribution of the concentration of total vitamin C in whole blood samples of normal healthy Dutch adults (*n* = 108).

Establishing a reference range

From the analysis of vitamin C in whole blood samples of a group of 108 normal healthy Dutch adults (61 males and 47 females), a total range for vitamin C of 10—105 μmol/l was found with a mean value of 54 μmol/l. The frequency distribution of the vitamin C concentrations in these samples seems to be non-Gaussian (Fig. 4). For the assessment of a "normal" range, the distribution free method of Rümke and Bezemer [15] was used. By setting the limits of percentiles at 2.5% and 97.5% with a reliability of 95%, a reference range was obtained of 20—80 μmol/l. Accordingly, a concentration of vitamin C below 20 μmol/l would be regarded as below normal.

DISCUSSION

The procedure described in this paper provides a fast, sensitive and reliable method for the determination of total vitamin C in whole blood, i.e. the sum of the concentrations of *l*-ascorbic acid and dehydro-*l*-ascorbic acid. The method is suited for large-scale routine analysis. Precision and recovery are good. The linearity range and sensitivity permit the determination of total vitamin C in whole blood in concentrations far below and above normal. Systematic errors due to oxidation of *l*-ascorbic acid to dehydro-*l*-ascorbic acid during sample storage and handling are eliminated by incorporating this oxidation in the method. Blood samples containing EGTA and glutathione can be stored in the dark at −20°C for at least eight days without significant deterioration of vitamin C.

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CHROMBIO. 1880

DETERMINATION OF (ENDOGENOUS) VITAMIN K_1 IN HUMAN PLASMA BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING FLUOROMETRIC DETECTION AFTER POST-COLUMN ELECTROCHEMICAL REDUCTION

COMPARISON WITH ULTRAVIOLET, SINGLE AND DUAL ELECTROCHEMICAL DETECTION

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SUMMARY

A new method for the fluorometric detection of K_1 and K_2 vitamins using post-column electrochemical reduction is described. Dual electrochemical detection in the reduction/reoxidation mode for coulometric/coulometric as well as coulometric/amperometric detection appears to be more sensitive and selective towards the plasma background than simple reductive electrochemical detection, but fluorometric detection after coulometric reduction offers the best results. Combination of normal-phase chromatography and the described method is only possible if supporting electrolyte is added post-column, but leads to higher detection limits.

This highly sensitive method is applied to the determination of vitamin K_1 in human plasma samples.

INTRODUCTION

Vitamin K (Fig. 1) is an essential cofactor in a microsomal enzyme system that activates the precursors of the blood-clotting factors II, VII, IX and X. In order to obtain quantitative information about the role of vitamin K in haemostasis a sensitive method of analysis is required.

Several papers describing the analysis of low concentrations of vitamin K_1 in milk and plasma using high-performance liquid chromatography (HPLC) with UV detection have been published recently [1—4]. However, due to the

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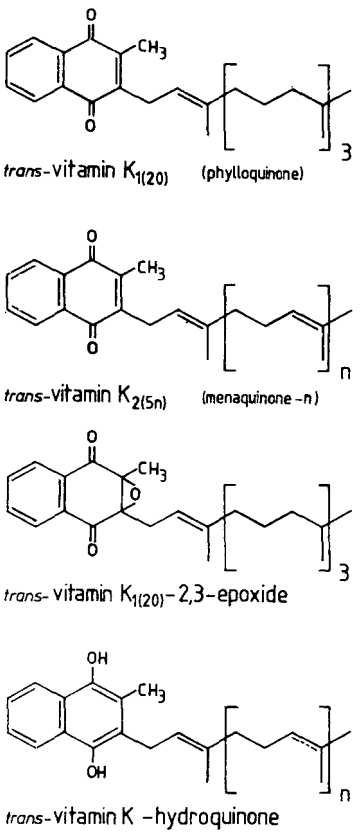


Fig. 1. Structural formulae of the K vitamins of interest.

unfavourable ratio of the amount of K_1 to the other lipids extracted from the biological material, an off-line multidimensional chromatographic system is required for the complete separation of K_1 from the interfering lipids. Such a multidimensional chromatography is not practical for routine analysis because two chromatographic systems are needed. Besides, eluent fractionation with extra sample handling as evaporation and second injection is necessary. Use of a more selective and sensitive detection could simplify the assay considerably.

Electrochemical detection of K_1 based on the amperometric reduction of the quinone has been applied to the determination of relatively high concentrations of K_1 in rat plasma [5, 6]. However, detection in the reductive mode appears to be non-selective towards the plasma background resulting in unfavourable limits of detection.

The selectivity of detection can be improved by reoxidizing the formed hydroquinone using a dual-electrode configuration [7]. This can be done with amperometric [8] as well as with coulometric detectors.

Although amperometric detectors generally offer low detection limits in the subnanogram range, one of the main drawbacks of dual amperometric detection is that only a small fraction of the analyte is reoxidized which results in loss of sensitivity. With coulometric detectors theoretically 100% of the analyte is converted. This method appeared to be applicable to deter-

mine K_1 levels after oral and intravenous administration, but endogenous levels (0.1—0.7 ng/ml [2], 0.9—7.8 ng/ml [4]) cannot be monitored. The combination of coulometric reduction with amperometric reoxidation appears to be more promising.

Generally, fluorometric detection is a sensitive and selective method. However, since the K vitamins do not possess native fluorescence, a reduction to the highly fluorescent hydroquinone is necessary. Methods based on chemical [9—11] and photochemical reduction [11] have been described. These methods require post-column addition of reagents which can cause problems concerning reproducibility in long-term routine analysis.

The aim of our work was to develop an on-line fluorometric detection method with sufficient selectivity towards the plasma background by applying a complete electrochemical, i.e. coulometric, reduction after a single chromatographic run. The method is applied to the determination of K_1 in human plasma and its capabilities are compared with single and dual electrochemical detection.

EXPERIMENTAL

Apparatus

The liquid chromatograph was constructed from commercially available and custom-made parts and consisted of a thermostatted glass eluent reservoir (293 K), a reciprocating plunger membrane pump (DMP 1515, Orlita, Giessen, F.R.G.) with a Bourdon-type damping system, a manometer, an injection device (Model U6K, Waters Assoc., Milford, MA, U.S.A.) and a thermostatted column (stainless-steel precision-bore tubing, 100 × 3.0 mm I.D.). A fixed-wavelength detector (Model 440, Waters Assoc.) operating at 254 nm, an amperometric electrochemical detection system (E.D.T., London, U.K.), a dual coulometric detection system (Model 5100-A, Coulochem, E.S.A., Bedford, MA, U.S.A.) and a fluorometric detection system of the double-monochromator type (SFM-23LC, Kontron, Zurich, Switzerland) were used for detection.

Chromatograms were recorded on flat-bed recorders (BD 8, Kipp & Zn., Delft, The Netherlands). Columns were packed by means of an air amplifier booster pump (DSTV-122, Haskel, Burbank, CA, U.S.A.) according to a procedure described elsewhere [12].

Chemicals and materials

Vitamins K_1, K_1 epoxide, $K_{2(20)}$ and $K_{2(30)}$ were kindly donated by Hoffmann-La Roche (Mijdrecht, The Netherlands). Organic solvents were of analytical grade (J.T. Baker, Phillipsburg, NJ, U.S.A.). Water was purified by means of a Milli-Q Water Purification System (Millipore, Bedford, MA, U.S.A.). Hypersil-MOS 5 μm (Shandon, Cheshire, U.K.) was purchased from Ahrin (The Hague, The Netherlands).

Procedures

Chromatography. Capacity ratios were calculated from the retention times of the compounds and of an unretained compound, for which potassium

periodate was used. The theoretical plate height was calculated from the retention time and half the peak width at 0.6 of the peak height. The detection limits are based on a signal-to-noise ratio of 3.

Removal of oxygen from the mobile phase. Oxygen was removed from the mobile phase by continuously bubbling through nitrogen that was made oxygen-free by means of a pyrogallol solution and that was presaturated with the mobile phase. No attempts were made to remove oxygen from the samples.

Preparation of the plasma samples. Blood samples were taken before and after oral administration of vitamin K_1 to a healthy volunteer. The samples were collected in heparinized tubes and centrifuged at 1000 *g* for 5 min. The plasma samples were stored at 243°K until analysis.

Isolation of K vitamins from plasma. To 1 ml of plasma in a brown conical flask 50 ng of $K_{2(30)}$ were added as an internal standard. Then 1 ml of a sodium chloride solution (0.9%, w/w) was added. The proteins were denaturated by mixing with 3 ml of isopropanol. After addition of 10 ml of *n*-hexane the stoppered tubes were placed on a vertically rotating disk for 1 h at 20 rpm. After centrifugation for 10 min at 1000 *g* the upper layer was removed and evaporated at reduced pressure at ambient temperature. The residue was dissolved in 1 ml of methanol. Aliquots of 50 µl were injected onto the column. The samples were shielded from light.

RESULTS AND DISCUSSION

Chromatographic phase system

The K vitamins are strongly hydrophobic compounds only differing in carbon chain length and number of double bonds. These compounds can be separated by normal-phase as well as by reversed-phase chromatography. In this study we focussed our main attention on the reversed-phase systems because of their compatibility with electrochemical detection and reaction systems. The modifier content has to be very high (90—100%) in order to

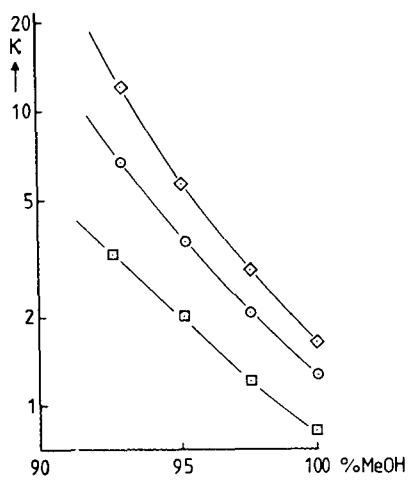


Fig. 2. Log—log plot of the relationship between the capacity ratios of vitamins K_1 (○), $K_{2(20)}$ (□) and $K_{2(30)}$ (◇) and the methanol content of the mobile phase.

achieve reasonably small capacity ratios and consequently short analysis times. Both methanol and acetonitrile can be used as modifier, but we prefer methanol for the reasons outlined below.

The relation between the capacity ratios of K_1, $K_{2(20)}$ and $K_{2(30)}$ and the methanol content of the mobile phase is given as a double-logarithmic plot in Fig. 2.

The phase system was optimized with respect to the selectivity between the K vitamins and co-extracted plasma constituents. A methanol content of 92.5% offers the best results, while 95% can be used for higher concentrations of K vitamins, e.g. after administration of K vitamins. This phase system appeared to be stable for several months, showing sufficient efficiencies (theoretical plate number = 2500—3000 on 10-cm columns at a flow-rate of 1 ml/min) to obtain adequate detection limits.

Electrochemical detection

The influence of the composition of the mobile phase, the concentration of the supporting electrolyte and the pH on the reduction and reoxidation signals was investigated for the coulometric detection system. If oxygen is not removed from the mobile phase, the background current of the reductive electrode is raised to such an extent that no reduction or oxidation signals can be observed. Removal of oxygen by bubbling through nitrogen proved to be satisfactory.

The influence of the electrolyte concentration on the reduction and oxidation signals is shown in Fig. 3a and b. Without sodium perchlorate no response is obtained. At very low concentrations of electrolyte the response increases with the concentration of the electrolyte. Although the addition of 0.1% of

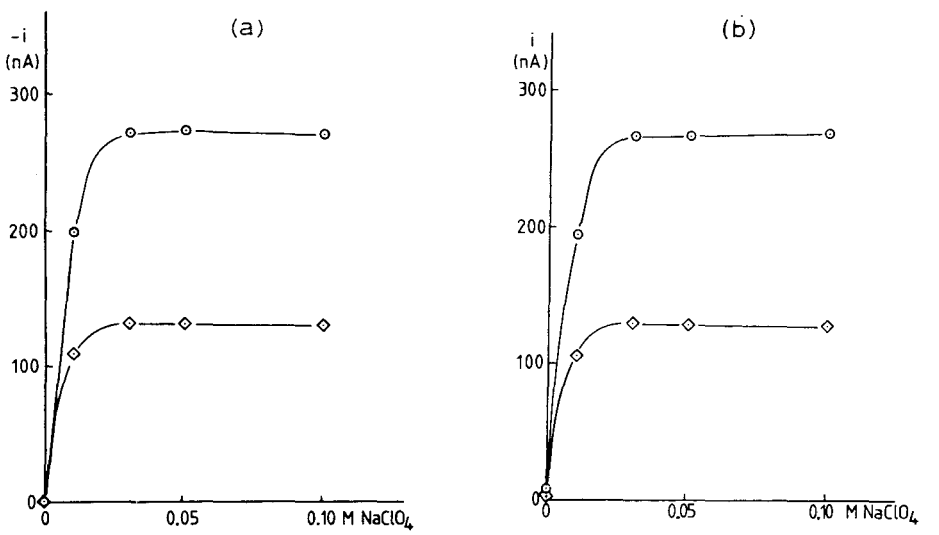


Fig. 3. (a) Influence of the concentration of $NaClO_4$ on the reduction signals. Conditions: stationary phase, Hypersil-MOS (5 μm); mobile phase, 5% water in methanol; flow-rate, 1.0 ml/min; applied potential, —400 mV. (○), K_1 (50 ng); (◇), $K_{2(30)}$ (35 ng). (b) Influence of the concentration of $NaClO_4$ on the oxidation signals. Conditions as for Fig. 3a, applied potential +400 mV.

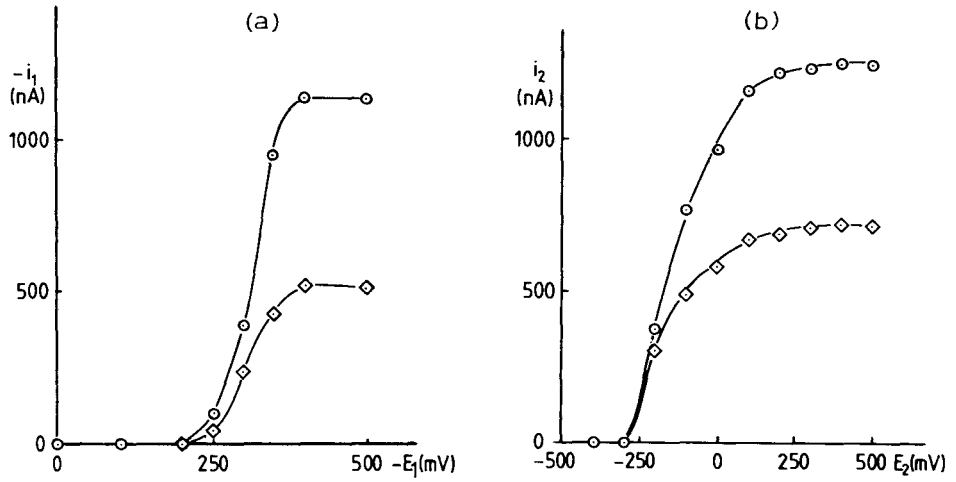


Fig. 4. (a) Relation between the reduction current and the applied potential. Conditions as for Fig. 3a, mobile phase containing 0.03 *M* $NaClO_4$. Potential applied to electrode 2, +400 mV. (b) Relation between oxidation current and potential applied to cell 2 for K_1 (○) and $K_{2(30)}$ (◇). Conditions: stationary phase, Hypersil-MOS (5 μm); mobile phase, 5% water in methanol, containing 0.03 *M* $NaClO_4$. Flow-rate 1.0 ml/min; potential applied to cell 1, —400 mV.

perchloric acid results in lower absolute reduction and oxidation potentials, a concentration of 0.03 *M* of sodium perchlorate appeared to be optimal with respect to the signal-to-noise ratio.

In comparison with methanol—water mixtures, the baseline noise levels with acetonitrile—water mixtures are increased, while the response is about the same resulting in inferior detection limits.

Typical relations between reduction and oxidation currents and the applied potentials are shown in Fig. 4a and b. In long-term use the relations between current and potential tend to change to a small extent, probably due to pollution of the electrodes. Regeneration appeared to be possible by changing the polarities of the potentials of the electrodes overnight. In a phase system consisting of 7.5% water in methanol with 0.03 *M* sodium perchlorate the detection limit of K_1 is about 250 pg in the reductive mode and about 150 pg in the reoxidation mode.

The yield of conversion is determined by injecting known amounts of the K vitamin and measuring the number of coulombs involved in the conversion. For the reduction of K_1 the yield of conversion to the hydroquinone amounts to at least 99.6%, which is confirmed by the signal of the second electrode, having the same potential as the first electrode (—400 mV), where no peaks were observed.

Coulometric detectors convert electrochemically active compounds with absolute half-wave potentials even higher than those of the compounds of interest, resulting in a decreased selectivity of detection in comparison with amperometric detection. Therefore we combined the coulometric cells, applied in the reduction mode, with an amperometric cell in the oxidation mode. Although with this combination the noise level appeared to be increased to a

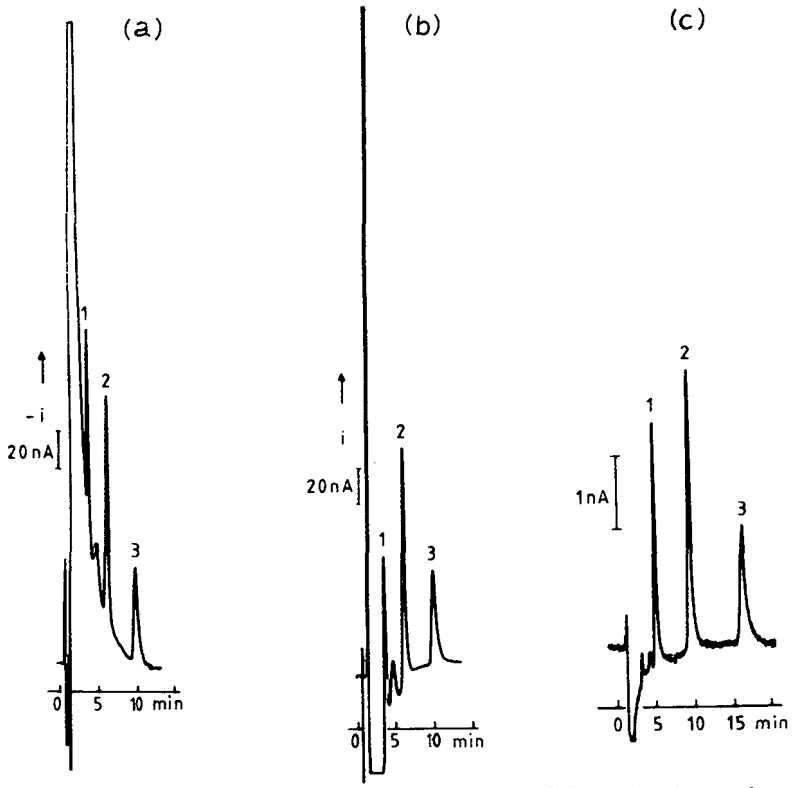


Fig. 5. Comparison of the discrimination power of detection by coulometric reduction (a), coulometric reoxidation (b) and amperometric reoxidation (c). Conditions: stationary phase, Hypersil-MOS (5 μm); mobile phase, 5% water in methanol, containing 0.03 *M* $NaClO_4$, flow-rate, 1.0 ml/min. Applied potentials: (a) —400 mV, (b) +400 mV, (c) cells 1 and 2 —400 mV, amperometric cell +300 mV vs. Ag/AgCl. 1 = $K_{2(20)}$ (50 ng), 2 = K_1 (50 ng), 3 = $K_{2(30)}$ (35 ng).

small extent resulting in a detection limit of 280 pg, this combination has to be preferred to the coulometric detection because of the better selectivity of detection when determining K_1 in plasma, as is shown in Fig. 5a—c.

Fluorometric detection

The influence of parameters such as solvent type, modifier content, sodium perchlorate concentration, addition of perchloric acid, solvent flow-rate and the choice of excitation and emission wavelengths, was investigated with the UV detector placed between the analytical column and the electrochemical detector, now used as a post-column reactor. Comparison of the UV signals and the fluorescence signals makes it possible to observe the net changes in fluorescence yield.

The absence of oxygen is essential for the fluorometric detection of K vitamins. If oxygen is present, either the K vitamins are not reduced and consequently no fluorescence is observed, or the fluorescence is completely quenched. The influence of the flow-rate on the fluorescence signal was very small, which means that the K vitamins are reduced quantitatively at the normally used flow-rates (0.4—1.5 ml/min).

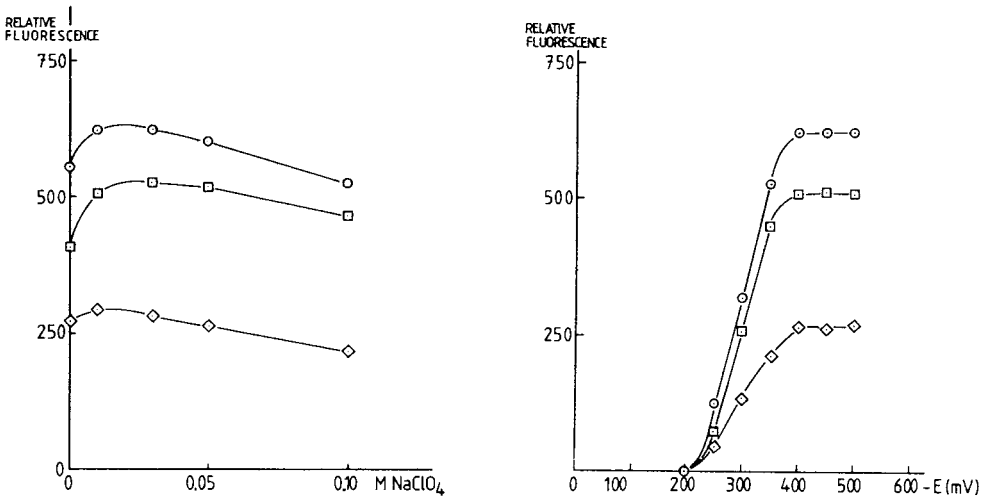


Fig. 6. Influence of the concentration of $NaClO_4$ in the mobile phase on the fluorescence signals of K_1 (○), $K_{2(20)}$ (□) and $K_{2(30)}$ (◇). Conditions: stationary phase Hypersil-MOS (5 μm); mobile phase 7.5% water in methanol; flow-rate, 1.0 ml/min; applied potentials −400 mV. λ_{ex} = 320 nm, λ_{em} = 420 nm.

Fig. 7. Relation between the fluorescence signals and the potentials applied to the electrodes for K_1 (○), $K_{2(20)}$ (□) and $K_{2(30)}$ (◇). Chromatographic conditions as for Fig. 6, mobile phase containing 0.03 *M* $NaClO_4$.

The optimal wavelengths appeared to be 320 nm and 420 nm for excitation and emission, respectively.

The sodium perchlorate concentration in the mobile phase hardly influences the fluorescence as is shown in Fig. 6. Even without supporting electrolyte in the mobile phase a considerable fluorescence is obtained. At higher perchlorate concentrations the capacity ratio is increased resulting in lower maximal outlet concentrations and consequently lower fluorescence signals. In Fig. 7 the relation between the fluorescence and the potential applied to the working electrodes of the coulometric cells is given. For K_1 and K_2 the reduction is complete with both cells operating at −400 mV.

Upon the addition of 0.1% perchloric acid complete reduction takes place at lower absolute potentials (−200 mV). However, the fluorescence signal is decreased to nearly 50%. Use of acetonitrile instead of methanol, which has the advantage of a lower pressure drop over the column and a slightly better efficiency, resulted in an increase of the noise level and so in a detection limit of about 150 pg. Using a mobile phase consisting of 5% water in methanol with 0.03 *M* of sodium perchlorate, the detection limit of K_1 is about 25 pg, and with 7.5% water about 50 pg, which is superior to UV detection (100 and 150 pg, respectively). Besides, the selectivity of this detection mode is much better than for UV detection, as is demonstrated in Fig. 8a and b.

Reduction of K_1 epoxide requires a much lower potential than reduction of K_1 and K_2 vitamins. With potentials as low as −1 V fluorescence was observed. Further lowering of the potentials results in an increased fluorescence. However, complete reduction could not be accomplished since at these

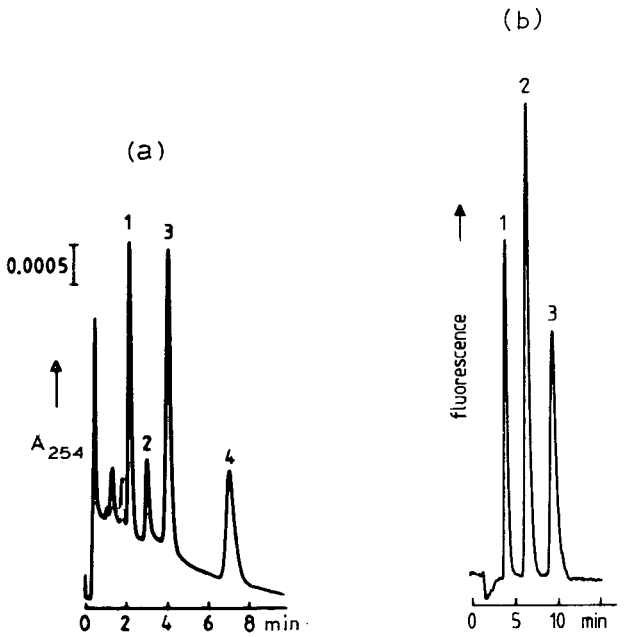


Fig. 8. Comparison of sensitivity and selectivity of UV detection at 254 nm (a) and fluorescence detection (b). Conditions as for Fig. 7. (a) 1 = $K_{2(20)}$ (50 ng), 2 = K_1 epoxide (50 ng), 3 = K_1 (50 ng), 4 = $K_{2(30)}$ (35 ng). (b) 1 = $K_{2(20)}$ (10 ng), 2 = K_1 (10 ng), 3 = $K_{2(30)}$ (7 ng).

potentials the electrochemical reaction system was electronically overloaded, meaning that the selected potential cannot be realized. The problem might be overcome by using a potentiostat with a higher capacity. The present method is less suited for the determination of small concentrations of K_1 epoxide.

Fluorometric detection after normal-phase chromatography

Since fluorometric detection of K vitamins after reversed-phase chromatography is possible even when no supporting electrolyte is added to the mobile phase, we also investigated the possibility of coulometric reduction and fluorometric detection in normal-phase systems.

A normal-phase system consisting of LiChrosorb Si 60 as stationary phase and 1% diisopropyl ether in *n*-hexane as the mobile phase combines good selectivities and sufficient retention to minimize peak broadening and consequently to optimize detection limits. However, such a system does not allow the direct use of the coulometric reaction system. It is not possible to dissolve sufficient supporting electrolyte, e.g. lithium perchlorate, to apply well-defined potentials. Therefore an electrolyte solution consisting of 0.1 *M* sodium perchlorate in methanol with 10% ethanol was added post-column to the mobile phase in a ratio of 7:3 according to a method used for the amperometric detection of *cis*- and *trans*-K_1 in a normal-phase system [6].

Although fluorometric detection appeared to be possible, the detection limits were much higher than with reversed-phase systems. The detection limit of K_1 amounted to about 5 ng, which, compared with 25 pg for reversed-phase

chromatography, cannot be explained only by the dilution due to the addition of supporting electrolyte solution. In fact the quantum efficiency for K_1 is much smaller in an apolar medium such as hexane than in a polar medium such as methanol, which was demonstrated by reducing a solution of K_1 with lithium boron hydride and measuring the fluorescence with a spectrofluorometer.

Isolation of K_1 from plasma

K_1 can be isolated from plasma by liquid—liquid extraction using an alcohol to liberate the lipids from the lipoprotein complex, followed by extraction with an apolar solvent such as *n*-hexane, diethyl ether or dichloromethane. The described method appeared to be the easiest and most reliable of all possible methods to extract K_1 from plasma.

The residue after evaporation has to be redissolved in at least 1 ml of methanol, since it is not possible to dissolve the lipids completely in smaller volumes. In the latter case lipid droplets will be formed in which a part of the amount of K_1 will dissolve preferentially. The recovery of K_1 when 1 ml of methanol is used, is 98%, while with 0.2 ml of methanol the recovery amounts to only about 30%.

These problems do not exist when using the described normal-phase system. The residue can be dissolved completely in 0.2 ml of *n*-hexane. Unfortunately the sensitivity and selectivity of the normal-phase system with UV detection are not adequate for accurate determination of the low endogenous levels.

The recovery of K_1 isolated from plasma was determined by extracting plasma samples spiked with amounts of K_1 in the range 1—1000 ng/ml. The peak areas were compared with those obtained by direct injection of the same amounts of K_1 dissolved in methanol or hexane. The recovery appeared to be 98.0 ± 2.2%.

Quantification

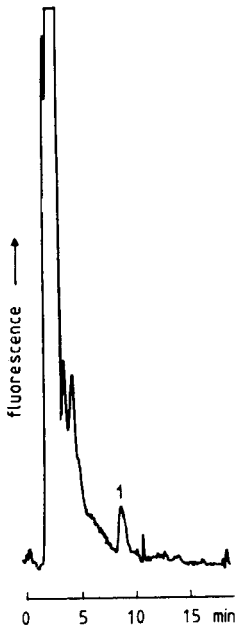
The linearity and precision of the determination of K_1 by HPLC with fluorometric detection, preceded by liquid—liquid extraction, were investigated by determining human plasma samples spiked with known amounts of K_1 varying from 1 to 1000 ng/ml.

The linearity, characterized by the correlation coefficient, amounts to 0.99998.

Because of the presence of endogenous K_1 in human plasma, the blank value was determined by the method of standard addition. The within-day and day-to-day coefficients of variation amount to 2.1% (n = 5) and 3.2% (n = 10), respectively.

Application to plasma samples

The developed method has been applied to the determination of K_1 in human plasma. Fig. 9 shows the chromatogram of an extract of a plasma sample taken from a healthy fasting volunteer. It is clearly demonstrated that the endogenous concentration corresponding to 2.3 ng/ml can be determined, even without any clean-up. It should be noted that, due to the large volume used to redissolve the sample, the injected amount is only 115 pg.



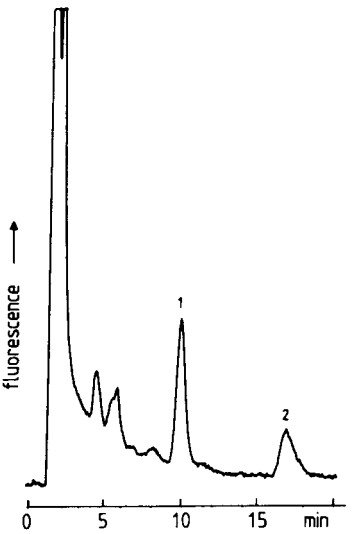


Fig. 9. Chromatogram of an extract of plasma of a healthy volunteer. 1 = phylloquinone (K_1). Conditions: see Fig. 8.

Fig. 10. Chromatogram of an extract of plasma of a healthy volunteer after oral administration of 10 mg of phylloquinone. Blood sample was taken 6 h after administration. 1 = Phylloquinone (K_1), 2 = $K_{2(30)}$. Conditions: see Fig. 8, except for flow-rate = 0.9 ml/min.

Fig. 10 shows a chromatogram of an extract of a plasma sample taken from a healthy volunteer, 6 h after oral administration of 10 mg of K_1.

The method is directly applicable to the determination of K_1 in human plasma after administration. The determination of endogenous levels is also possible, although a further clean-up of the extract by fractionating the plasma lipids will increase the reliability of the method, especially when small changes in the endogenous level have to be observed.

The method is being applied to a pharmacokinetic study of K_1 and to the determination of K_1 in vegetables.

CONCLUSIONS

A relatively simple method based on improvement of the detection for the determination of low concentrations of K vitamins in biological specimens is described. Post-column coulometric reduction combined with either fluorometric detection or amperometric reoxidation allows the determination of endogenous levels of vitamin K_1. Fluorometric detection has the lowest detection limit (25 pg), coulometric reoxidation shows a better detection limit (150 pg) than amperometric reoxidation (280 pg), but the selectivity of the latter mode is more favourable.

Combination of normal-phase chromatography with the described method is possible but more complicated and is inferior to reversed-phase chromatography.

Further research will be devoted to the optimization of the sample pretreatment in order to increase the recovery, and will be reported in due course.

ACKNOWLEDGEMENTS

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GAS CHROMATOGRAPHIC—MASS SPECTROMETRIC DETERMINATION OF AMITRIPTYLINE AND ITS MAJOR METABOLITES IN HUMAN SERUM

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SUMMARY

A gas chromatographic—electron-impact ionization mass spectrometric method has been developed for the determination of amitriptyline (AMT) and its metabolites, nortriptyline (NT), 10-hydroxyamitriptyline (10-OH-AMT) and 10-hydroxynortriptyline (10-OH-NT) in human serum. The lower limit of detection was 2 ng/ml for all compounds except 5 ng/ml for 10-OH-NT. The calibration curves for AMT and 10-OH-AMT were linear up to 100 ng/ml, and up to 200 ng/ml for NT and 10-OH-NT. The accuracy of the assay in terms of coefficient of variation was less than 7%. The extraction efficiency was almost quantitative for all compounds except 60% for 10-OH-NT.

Using this method, human serum samples which had been collected after oral administration of a single 50-mg dose of AMT were analyzed. Ratios of the conjugation of each metabolite were estimated, including AMT.

INTRODUCTION

The tricyclic antidepressant amitriptyline (AMT) is mainly metabolized to nortriptyline (NT) via N-demethylation, and their hydroxy metabolites by oxidation at position 10 [1—3]; the structures of these compounds are shown in Fig. 1. As minor metabolites, desmethylnortriptyline and amitriptyline N-oxide have been identified in human urine. The *trans* and *cis* enantiomers of the 10-hydroxylated metabolite, which are essentially equipotent to the parent compound in terms of blockade of norepinephrine uptake [4], can be analyzed stereoselectively using high-performance liquid chromatography [5—8]. The hydroxy metabolites have been demonstrated to be conjugated in considerable quantities in human urine or plasma [1, 2, 9]. The presence of the N-glucuronide of AMT in human urine was suggested by Breyer-Pfaff et al. [2],

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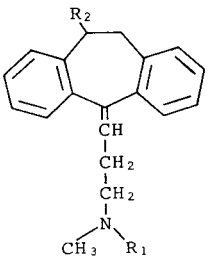


	R_1	R_2
I	CH_3	H
II	H	H
III	CH_3	OH
IV	H	OH

Fig. 1. Structures of amitriptyline (I), nortriptyline (II), 10-hydroxyamitriptyline (III) and 10-hydroxynortriptyline (IV).

but no information has been reported about conjugation of AMT in human plasma.

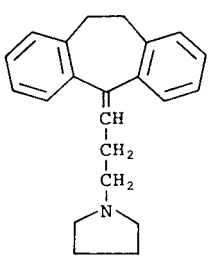
Although various methods have been available for the determination of AMT and its metabolites in biological fluids, including thin-layer chromatography [3, 10], gas chromatography [11, 12], high-performance liquid chromatography [5—8, 13—15] and gas chromatography—mass spectrometry (GC—MS) [9, 16—18], the GC—MS method is the most suitable for the separative determination because of its high sensitivity and specificity. A GC—electron-impact (EI) MS method has been described by Alván et al. [17] for the measurement of NT and 10-OH-NT and by Biggs et al. [18] for tricyclic antidepressants including AMT and NT. Garland et al. [9] have published a GC—chemical ionization (CI) MS method for the quantitation of AMT and its metabolites in plasma. The method was not readily applied because the standard curves of hydroxy metabolites were not linear due to use of monodeuterated analogues as internal standards.

Therefore, we have developed a more convenient method for the analysis of AMT and its major metabolites in serum using GC—EI-MS, and then applied the procedure to evaluate the conjugation ratios of these compounds.

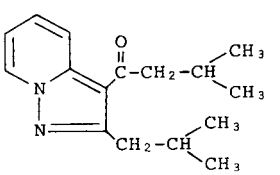
EXPERIMENTAL

Materials

Hydrochlorides of AMT and NT, *cis* and *trans* isomers of 10-OH-AMT and 10-OH-NT, and IS-1 (Fig. 2) were all gifts from H. Lundbeck, Denmark. IS-2 was synthesized according to a method described elsewhere [19]. Analytical reagent grade *n*-hexane and benzene were distilled in a glass still before use.



IS—1



IS—2

Fig. 2. Structures of internal standards.

Distilled water was passed through Amberlite MB-3. Other reagents and solvents were used as received. β-Glucuronidase—arylsulphatase was purchased from Boehringer Mannheim (Mannheim, F.R.G.).

Apparatus

A JEOL Model JMS-D300 mass spectrometer was employed in conjunction with the manufacturer's Model JGC-20K gas chromatograph and JMA-3100 mass data analysis system. Aliquots of each standard compound were injected into the GC—MS system after trifluoroacetylation, and then EI ionization mass spectra were recorded under the following conditions. A glass column, 1 m × 2 mm I.D., was packed with 2% OV-17 on 80—100 mesh Chromosorb W HP (Gasukuro Kogyo, Tokyo, Japan). Oven and injection port temperatures were 235°C and 250°C, respectively. Helium was used as a carrier gas, maintaining a column head pressure to 0.9 kg/cm^2. The MS conditions were: ionization current, 300 μA; ion source temperature, 200°C; separator temperature, 280°C; EI mode. For selected-ion monitoring, the mass spectrometer was set to monitor *m/z* 58 for AMT and 10-OH-AMT and *m/z* 84 for IS-1 at an ionization voltage of 24 eV, and *m/z* 232 for NT, 230 for 10-OH-NT and 201 for IS-2 at 70 eV. Other GC—MS conditions were the same as those described above, except that mass fragmentograms were recorded on a Rikadenki Model R-14 four-pen recorder.

Extraction

To a 1-ml aliquot of standard or unknown serum sample were added 200 μl of aqueous solution containing IS-1 (1.5 μg free base per ml), 200 μl of IS-2 in *n*-hexane (0.4 μg/ml) and 0.1 ml of 5 *M* NaOH. The serum was then extracted with 6 ml of *n*-hexane by shaking for 10 min in a mechanical shaker. After centrifuging at 1300 *g* for 10 min, 5 ml of the *n*-hexane layer were evaporated to dryness. To the residue were added 100 μl of 0.1% triethylamine in *n*-hexane and of trifluoroacetic anhydride. The mixture was reacted at 50°C for 10 min. After the removal of solvent by evaporation, the residue was vortexed with 0.1 ml of benzene and 0.1 ml of distilled water followed by centrifugation. An aliquot of the resultant benzene layer was injected into the GC—MS system.

For the determination of total quantity including conjugate, serum samples were treated with 1 *M* acetate buffer solution (pH 5.2) containing 0.1 U of β-glucuronidase and 0.05 U of arylsulphatase at 37°C for 16 h prior to the above extraction.

Quantitation

Quantitation of AMT and 10-OH-AMT was separately performed from that of NT and 10-OH-NT. All determinations were carried out by calculating a peak height ratio to internal standard.

Serum specimens

Blood samples were withdrawn from one male healthy volunteer, who had received a tablet of AMT (50 mg, Tryptanol®, Merck-Banyu, Japan) with a glass of water. Serum samples were prepared as usual and then kept at −20°C until assayed.

RESULTS AND DISCUSSION

Mass spectra of AMT, trifluoroacetyl (TFA)-NT, TFA-10-OH-AMT, TFA-10-OH-NT, IS-1 and IS-2 are presented in Fig. 3. Both AMT and TFA-10-OH-AMT gave one major fragment ion *m/z* 58 $[CH_2N(CH_3)_2]^+$, while TFA-NT and TFA-10-OH-NT showed several fragment-ion peaks with *m/z* 232 and 230 $[M-(NCH_3COCF_3, H)]$ as base peak, respectively. IS-1 gave one major fragment ion, *m/z* 84 $[CH_2N\square]^+$, whereas IS-2 gave several fragment ions in addition to the base peak ion *m/z* 201 $[M-CH_2CH(CH_3)_2]^+$. In selected-ion monitoring, these base peak ions were monitored.

Under the GC—MS conditions employed here, a slight peak separation was observed between enantiomers of each hydroxy metabolite, but the retention

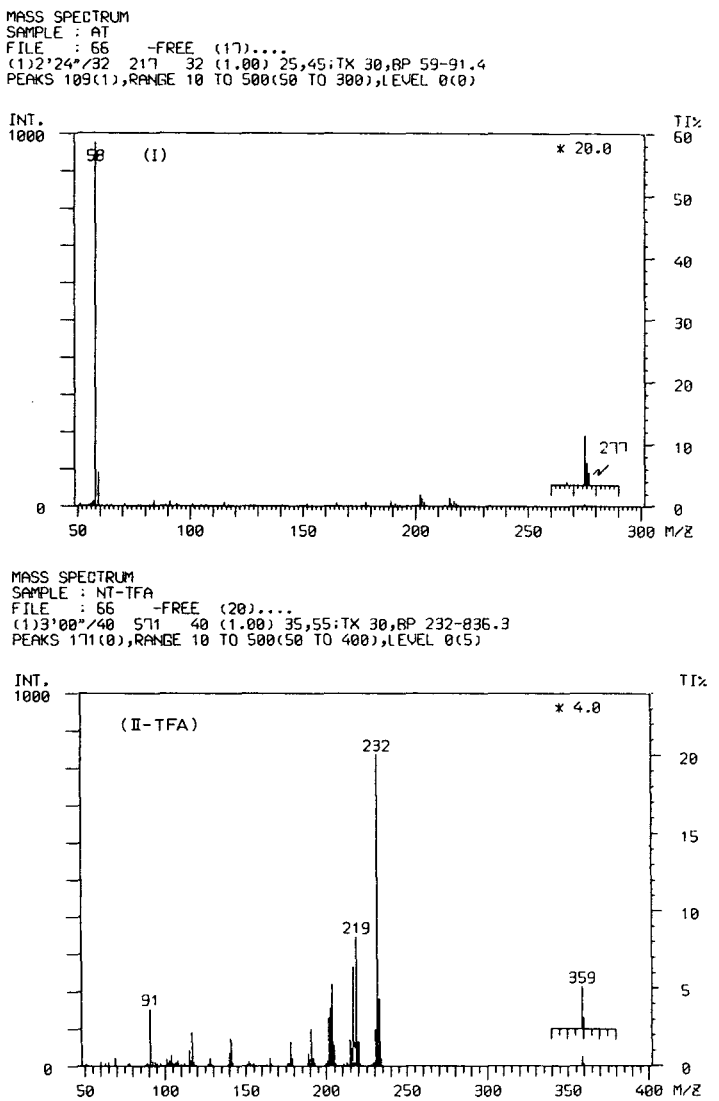
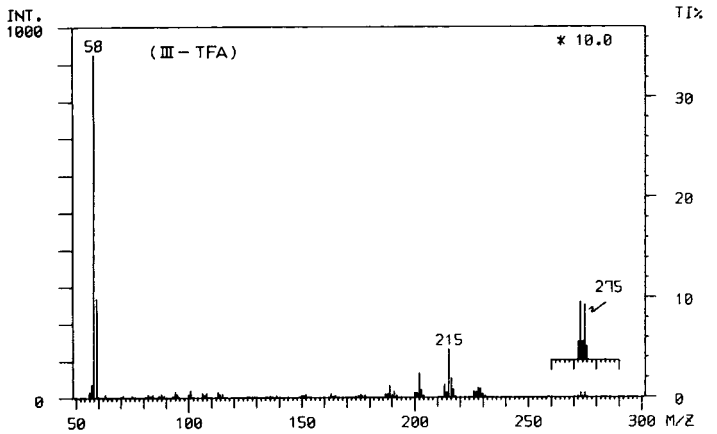
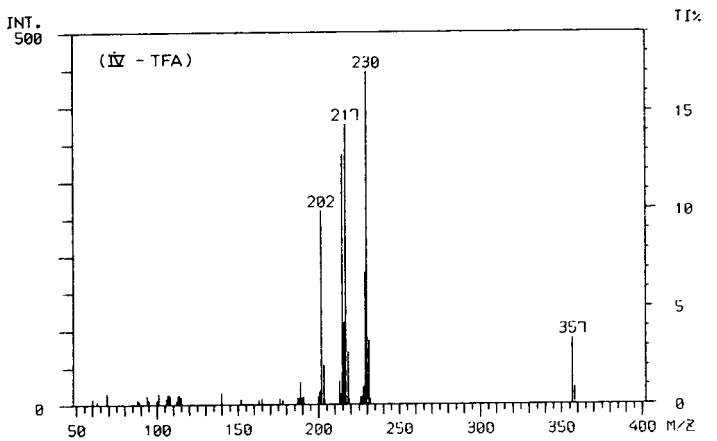


Fig. 3.

MASS SPECTRUM
SAMPLE : 10-OH-AT-TFA
FILE : 66 -FREE (18)....
(1)3'09"/42 367 42 (1.00) 35,50;TX 30,BP 59-268.9
PEAKS 139(1),RANGE 10 TO 500(50 TO 300),LEVEL 0(3)



MASS SPECTRUM
SAMPLE : 10-OH-NT-TFA
FILE : 66 -FREE (21)....
(2)3'27"/46 392 46 (1.00) 40,60;TX 30,BP 230-447.7
PEAKS 118(0),RANGE 10 TO 500(50 TO 400),LEVEL 0(5)



MASS SPECTRUM
SAMPLE : IS-1
FILE : 66 -FREE (23)....
(2)2'51"/38 247 38 (1.00) 30,45;TX 30,BP 85-99.0
PEAKS 141(1),RANGE 10 TO 500(50 TO 350),LEVEL 0(1)

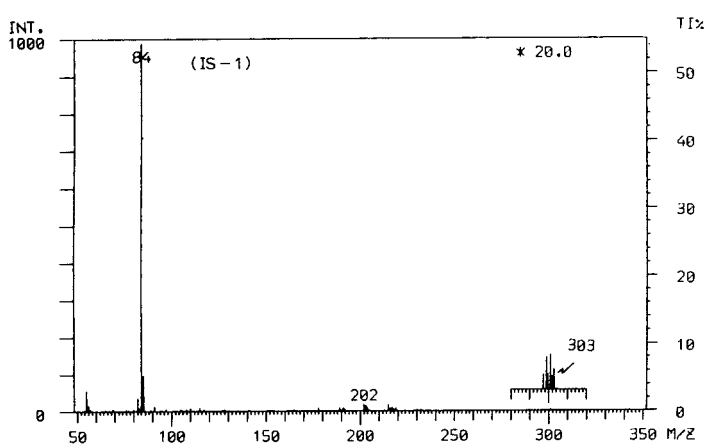


Fig. 3.

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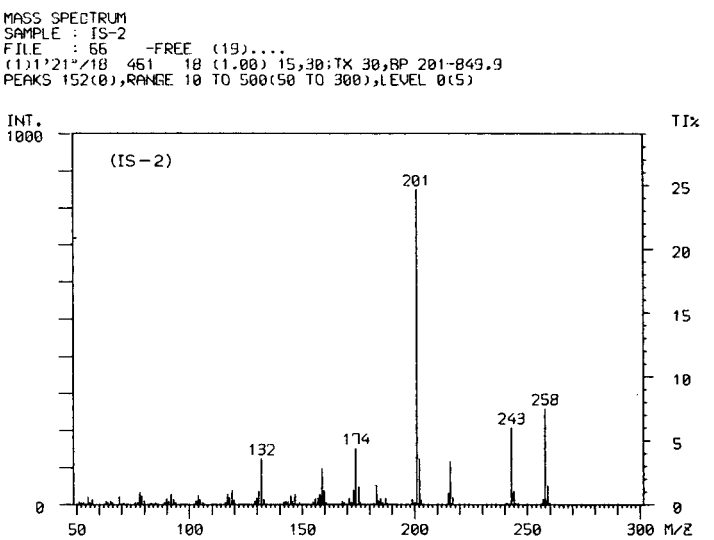

Fig. 3. Mass spectra of amitriptyline (I), nortriptyline (II), 10-hydroxyamitriptyline (III), 10-hydroxynortriptyline (IV), IS-1 and IS-2. II, III, IV are trifluoroacetyl derivatives.

times and mass spectra were identical after trifluoroacetylation. This was due to the conversion from the stereospecific hydroxy compound to a non-chiral compound by this reaction, as illustrated in Fig. 4; i.e. the stereospecific hydroxy compounds were dehydrated to the corresponding 10,11-dienes, which were confirmed by the mass spectra. Kraak and Bijster [6] demonstrated the presence of the *trans* form of each hydroxy metabolite in human serum, and Suckow and Cooper [7] suggested the presence of both configurations in human serum using high-performance liquid chromatography. As apparent from the above evidence, our method was not so stereospecific that it gave the total quantity of the two isomers.

Typical mass fragmentograms for the measurement of AMT and 10-OH-AMT, and of NT and 10-OH-NT are presented in Fig. 5A and Fig. 5B, respectively. Mass fragmentograms obtained from blank serum (Fig. 5a) indicated that the determination was not disturbed by human serum. Similar mass fragmentograms were obtained even after the incubation of blank serum with

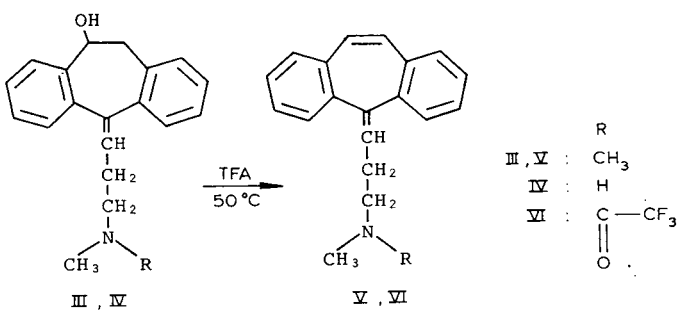

Fig. 4. Trifluoroacetyl derivatives of 10-hydroxyamitriptyline (III) and 10-hydroxynortriptyline (IV). V = III-TFA, VI = IV-TFA.

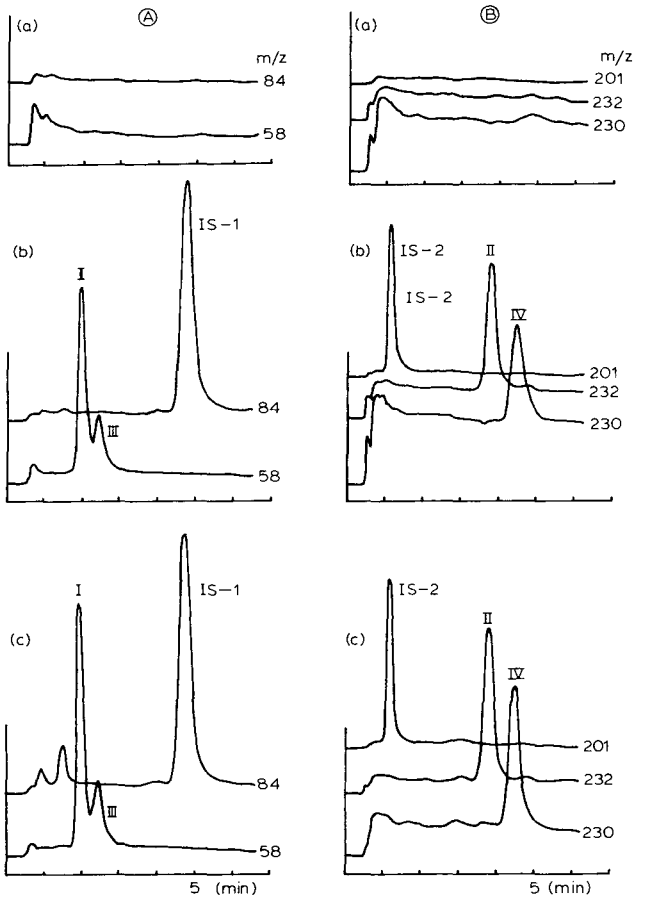

Fig. 5. Mass fragmentograms for measurement of (A) amitriptyline (I) and 10-hydroxy-amitriptyline (III), and (B) nortriptyline (II) and 10-hydroxynortriptyline (IV). (a) Blank serum, (b) blank serum spiked with standard compounds, 50 ng (c) serum treated with amitriptyline.

β-glucuronidase—arylsulphatase. Fig. 5b and c are mass fragmentograms obtained from serum spiked with authentic compound and internal standard, and serum sample obtained after drug administration, respectively.

Dynamic range, accuracy and recovery in the determination of each compound are summarized in Table I. Linear calibration curves were obtained over the dynamic range. To check the accuracy and precision of the method, four serum samples spiked with authentic samples were analyzed at two concentration levels, 10 and 100 ng/ml. Coefficients of variation at these levels were less than 7%. This result indicates that the method is accurate and precise. To determine recovery, *n*-hexane extracts of blank serum containing internal standard alone were spiked with authentic sample and then treated according to the above method. The slope was compared with that found with serum samples containing authentic sample and internal standard. As shown in Table I, the overall percentage recovery of AMT, 10-OH-AMT and NT was greater than 90% and that of 10-OH-NT was about 60%. These recoveries are better

TABLE I

DYNAMIC RANGE, COEFFICIENT OF VARIATION (C.V.) AND RECOVERY OF AMITRIPTYLINE (I), NORTRIPTYLINE (II), 10-HYDROXYAMITRIPTYLINE (III) AND 10-HYDROXYNORTRIPTYLINE (IV)

Compound	Dynamic range (ng/ml)	C.V. (%)		Recovery (%)
		10 ng/ml	100 ng/ml	
I	2—100	2.8	2.3	100
II	2—200	2.2	3.4	96
III	2—100	4.6	5.2	94
IV	5—200	6.9	3.1	58

TABLE II

SERUM CONCENTRATIONS OF AMITRIPTYLINE (I), NORTRIPTYLINE (II), 10-HYDROXYAMITRIPTYLINE (III) AND 10-HYDROXYNORTRIPTYLINE (IV) AFTER ORAL ADMINISTRATION OF AN AMITRIPTYLINE PREPARATION (TRYPTANOL, 50 mg) TO A HUMAN SUBJECT

Results are expressed in ng/ml.

Compound		Time (h)						
		2	4	6	12	15	24	32
I	Free	23	23	14	8	13	7	3
	Conjugated	18	9	18	11	3	23	3
II	Free	7	10	8	11	7	9	4
	Conjugated	nd*	nd	nd	nd	nd	nd	nd
III	Free	15	15	4	nd	5	nd	nd
	Conjugated	91	169	108	93	31	21	18
IV	Free	15	18	14	26	18	16	11
	Conjugated	8	26	32	28	23	16	5

*nd = not determined.

than those of methods published previously. For example, Garland et al. [9] had to add a substantial amount of chlorpromazine to the plasma sample prior to extraction in order to increase extraction efficiency. Even so, their recovery ranged from 36 to 69%. On the other hand, our method did not require any addition of such a carrier substance. The assay limits were 2 ng/ml for AMT, NT and 10-OH-AMT, and 5 ng/ml for 10-OH-NT when a 1-ml aliquot of serum was extracted. These sensitivities are inferior to those of the GC—CI-MS method [9], but superior to those of high-performance liquid chromatography [7]. It has also been recognized that GC—CI-MS [9] gives a lower sensitivity for 10-OH-NT than for other metabolites.

The serum concentrations of unconjugated and conjugated AMT, NT, 10-OH-AMT and 10-OH-NT after a single oral dose of 50 mg of AMT, are presented in Table II. Quantities of conjugated form were calculated as the difference between total and free quantities. The total quantities were obtained

from samples which had been treated with β-glucuronidase—arylsulphatase before extraction. AMT was conjugated to a level of half the total amount during the period of measurement. The conditions of enzymatic hydrolysis were checked with three levels of enzymes (0.104 and 0.052 U, 0.208 and 0.104 U, and 0.520 and 0.260 U of β-glucuronidase and of arylsulphatase, respectively) using urine samples of a patient treated with AMT. As shown in Fig. 6, the lowest level of enzyme employed in this study was sufficient for hydrolysis of conjugates of two hydroxy metabolites, but the enzyme level was not good enough for conjugates of AMT and NT. Therefore, the values for the conjugation of AMT and NT presented in Table II were underestimated. The type of conjugation of AMT in serum has not been identified. It is probable that the type is N-glucuronide because conjugated AMT in serum was hydrolyzed to intact AMT by treatment with β-glucuronidase—arylsulphatase as shown by this study, and because the N-glucuronide of AMT was suggested to be present in human urine by Breyer-Pfaff et al. [2]. Such a quaternary ammonium N-glucuronide has been identified as a unique metabolite of cyproheptadine [20] and tripelennamine [21] in human urine. Axelrod et al. [22] reported that N-glucuronide resisted hydrolysis with β-glucuronidase, which was rather non-enzymatic. The resistance to β-glucuronidase was also confirmed in hydrolysis of conjugate of AMT, but its hydrolysis was enzymatic as shown by the dependence of the hydrolysis rate on enzyme concentration. A first-order kinetic analysis of Fig. 6 indicated that over 80% of AMT was conjugated in human urine. The conjugation of NT in serum was not detected, although the conjugation of NT was suggested in urine as shown in Fig. 6. It is

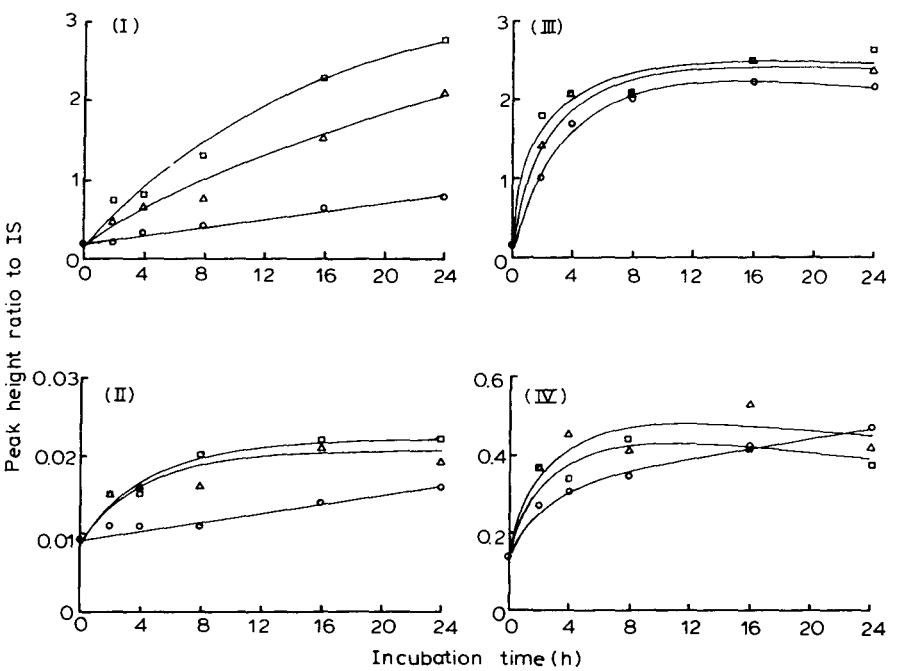

Fig. 6. Releasing profiles of amitriptyline (I), nortriptyline (II), 10-hydroxyamitriptyline (III) and 10-hydroxynortriptyline (IV) after incubation of urine of a patient (dosed with amitriptyline) with 0.104 and 0.052 U (○), 0.208 and 0.104 U (△), and 0.520 and 0.260 U (□) of β-glucuronidase and arylsulphatase, respectively.

considered that secondary amine is more easily conjugated than tertiary amine, but to our knowledge it has not been described that NT is conjugated in serum. In contrast to NT, 10-OH-AMT was almost completely conjugated, though the free component could be detected within a few hours after dosing. This extensive conjugation is in agreement with results obtained in similar studies by other workers [9], who found that more than 85% of 10-OH-AMT was conjugated. About 60% of total 10-OH-NT was conjugated, which was consistent with data obtained by other workers. Kragh-Sørensen et al. [23] showed that 51—85% of 10-OH-NT was conjugated in human plasma after oral administration of NT. From the data of Garland et al. [9] it was estimated that the conjugation ratio of 10-OH-NT ranged from 56 to 84% in human plasma after oral administration of AMT.

It is well known that there are wide inter-patient variations in the serum concentration of AMT. Many studies have been devoted to establishing the relationship between serum concentrations of AMT and/or NT and clinical response, but the data are still conflicting. In order to clarify these questions it would be important to measure not only intact drug but also its metabolites, because the hydroxy metabolites have pharmacological activities comparable with AMT, and the biological fate of the drug depends on individual metabolic activity.

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COMBINED GAS CHROMATOGRAPHIC—MASS SPECTROMETRIC PROCEDURE FOR THE MEASUREMENT OF CAPTOPRIL AND SULFUR-CONJUGATED METABOLITES OF CAPTOPRIL IN PLASMA AND URINE

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SUMMARY

A gas chromatographic—mass spectrometric method is described for the simultaneous measurement of the novel anti-hypertensive drug captopril, and the following metabolites: captopril disulfide dimer, S-methyl captopril, and S-methyl captopril sulfone. With this method all derivatives can be chromatographed using conventional gas chromatography of hexafluoroisopropyl esters in one temperature-programmed run and these can then be quantitated using selected-ion monitoring techniques.

Using urine or plasma, captopril, S-methyl captopril and the disulfide dimer of captopril in concentrations as low as 1 ng/ml, 10 ng/ml and 25 ng/ml, respectively can be detected. The reproducibility of the method is satisfactory both within-assay and inter-assay.

This technique has demonstrated that the pattern of urinary excretion of these compounds in both man and rat was similar. Excretion of unchanged captopril, S-methyl captopril and the disulfide dimer over 6 h in man given captopril (50 or 100 mg) chronically was 18.3%, 0.97% and 3.06%, respectively. Corresponding excretion of these three compounds in the rat following a single 10 mg/kg dose was 18.3%, 2.69% and 1.8%, respectively. A possible sulfone oxidation product of S-methyl captopril was not detected in the urine of either man or rat.

INTRODUCTION

The angiotensin-converting enzyme inhibitor captopril {1-[(2D)-3-mercapto-2-methyl-1-oxopropyl]-L-proline} is a thiol-containing dipeptide based on proline [1]. This drug is now being widely used as an antihypertensive drug [2]. Its metabolism has been studied in both man [3, 4] and rat [5, 6] and has been shown to occur entirely by conjugation of the sulfur of captopril either as thiomethyl derivatives or disulfide conjugates with other thiol compounds

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such as cysteine, glutathione and even proteins. Methods used to quantitate captopril and these metabolites have relied largely on thin-layer chromatographic techniques [4] with radioactive captopril or high-performance liquid chromatographic (HPLC) methods [7—10], and gas—liquid chromatographic [11] and gas chromatographic—mass spectrometric (GC—MS) methods [12, 13] have also been reported for the measurement of captopril, and in some instances, the disulfide dimer of captopril [8, 12, 13]. GC methods have relied on the use of N-ethylmaleimide to derivatize the sulfur of captopril in blood at the time of collection to prevent formation of oxidized captopril species [11—13]. Derivatization of the carboxyl group of captopril has been accomplished using hexafluoroisopropanol esters [11, 13] or methyl esters [12]. The most sensitive and specific method appears to be GC—MS using selected-ion monitoring (SIM) [12, 13]. Recent attention has focused on an S-methyl metabolite of captopril as a possible inactivation product of captopril metabolism whose formation may be time-dependent [3]. We present in this paper a specific GC–MS method for measuring S-methyl captopril and the sulfone oxidation product of S-methyl captopril in addition to captopril and its dimer in plasma and urine. Data are also presented to show the excretion of these compounds in rat and human urine.

EXPERIMENTAL

Reagents and chemicals

N-Ethylmaleimide (NEM, Sigma), perfluorobutyric anhydride (HFB, Pierce), 1,1,1,3,3,3-hexafluoroisopropanol (HFI) dimethyl sulfate (Fluka, puriss grade), (4*R*)-3-((2*S*)-3-mercapto-2-methylpropanoyl)-4-thiazolidine carboxylic acid (YS-980, Santen Pharmaceutical), captopril and captopril disulfide dimer (Squibb) were used as obtained. All other chemicals were analytical reagent grade. Ethyl acetate (Merck) was purified over alumina and distilled prior to use.

GC conditions

The stationary phase was 3% OV-101 coated on 100—120 Chromosorb W AW DMCS packed into a 2 m × 2 mm I.D. glass column. Helium (flow-rate 30 ml/min) was the carrier gas. Injector and detector zone temperatures were 280°C. Temperature program conditions were: initial temperature 150°C, initial hold time 1 min, rate of rise 10°C/min, final temperature 290°C.

MS conditions

A Finnigan Model 4021 gas chromatograph—mass spectrometer equipped with an INCOS data system was used for the instrumental analyses. The ion source temperature was 250°C (corrected) and the electron energy was 70 eV for electron impact mass spectrometry (EIMS). When positive-ion chemical ionization mass spectrometry (CIMS) was used, isobutane (Matheson) was the reagent gas at a source pressure of 0.3 torr and source temperature of 150°C (corrected). Extractor voltage settings were 4 V for EIMS and 10 V for CIMS and these were varied by the data system as function of mass to compensate

for mass discrimination of the quadrapole mass filter. The filament current was kept constant at 0.25 mA.

Sample preparation

Urine or plasma (100 μl—1 ml), 1 ml of potassium phosphate buffer, pH 7.4 (0.1 *M*), 10 μl of YS-980 (1 mg/ml solution in acetone) and 100 μl of N-ethylmaleimide (NEM) (10 mg/ml solution in water) were added successively. After 10 min reaction time excess NEM was removed by extraction with 5 ml of purified ethyl acetate. The aqueous phase was then acidified with 500 μl of hydrochloric acid (2 *M*), saturated with sodium chloride (ca. 2 g) and finally extracted with 10 ml of purified ethyl acetate. The ethyl acetate (8 ml) was transferred to a clean reaction tube and evaporated to dryness under nitrogen. Traces of water were azeotroped out with dichloromethane and the dry extract reacted with 50 μl of both hexafluoroisopropanol (HFI) and perfluorobutyric anhydride (HFB). HFB was present as a transesterification agent to facilitate esterifications with HFI. Derivatization proceeded for 15 min at 60°C before finally removing all reagents by evaporation under nitrogen. The residue was reconstituted into dry purified ethyl acetate (100 μl).

Synthesis of S-methyl captopril

Captopril (100 mg) was dissolved in 20 ml barium hydroxide solution (0.2 *M*) and a 10% molar excess of dimethyl sulfate was then gradually added with regular mixing at ambient temperature. After 15 min the solution was acidified with sulfuric acid (2 *M*) and centrifuged to separate out the insoluble barium sulfate. The supernatant was saturated with sodium chloride and extracted with 3 × 10-ml volumes of purified ethyl acetate. The organic layer was then transferred to a clean tube and evaporated to dryness under nitrogen. The remaining oil was dissolved in a small volume of distilled water, neutralized with dilute sodium hydroxide (0.1 *M*) and lyophilized. The residue was treated with dichloromethane (1 ml) to azeotrope out any moisture and lyophilized. This last procedure was repeated twice and the hydroscopic crystals (sodium salt) obtained were stored in a vacuum desiccator. EI mass spectra of the HFI ester gave *m/z* 381 (M^+), *m/z* 366 $(M—CH_3)^+$, *m/z* 334 $(M—SCH_3)^+$ and *m/z* 264 (proline moiety)$^+$ (Fig. 1a). CI mass spectra (isobutane) gave the expected ion $(MH)^+$ at *m/z* 382 (Fig. 2a). Thin-layer chromatography (TLC) (silica) with benzene—acetic acid (3:1) gave a single spot, R_F 0.53.

Synthesis of sulfone of S-methyl captopril

S-methyl captopril (sodium salt) (10 mg) prepared as described above was dissolved in 2 ml of Tris buffer, pH 10 (100 m*M*) and treated with an excess of hydrogen peroxide (30%, v/v, 200 μl).

After 30 min the reaction mixture was acidified with 2 *M* hydrochloric acid, saturated with sodium chloride and extracted with 10 ml purified ethyl acetate. The ethyl acetate was evaporated to dryness under dry nitrogen to yield an oily residue which was not contaminated with S-methyl captopril as judged by GC—MS. EI mass spectra of the HFI ester gave *m/z* 413 $(M)^+$, *m/z* 334 $(M—CH_3 \cdot SO_2)^+$, *m/z* 264 (proline moiety)$^+$, and *m/z* 149 $(CH_3 \cdot SO_2 \cdot CH_2 \cdot CH(CH_3) \cdot CO)^+$ (Fig. 1b). CI mass spectra (isobutane) gave the expected ion

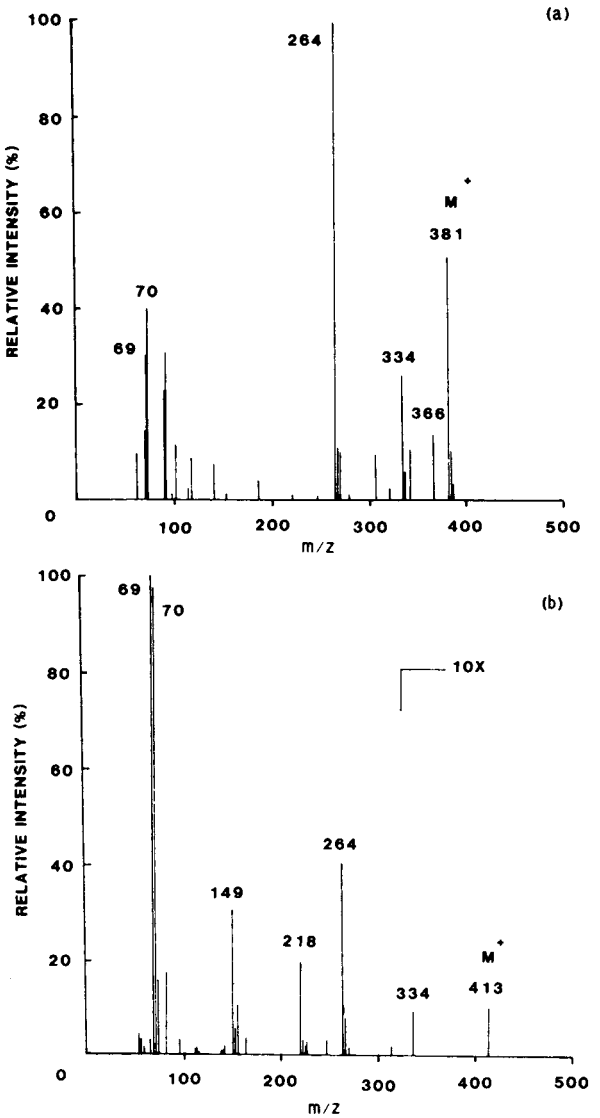

Fig. 1. EI mass spectra of (a) S-methyl captopril HFI ester and (b) S-methyl captopril sulfone HFI ester.

$(MH)^+$ at *m/z* 414 (Fig. 2b). TLC (silica) with benzene—acetic acid (3:1) gave a single spot, R_F 0.30.

Reproducibility studies

Reproducibility studies were performed for captopril, the dimer and S-methyl captopril by doing replicate analyses of urine samples which had been spiked with a known amount of pure compound. These samples were assayed both within a run and after several runs over a period of one month.

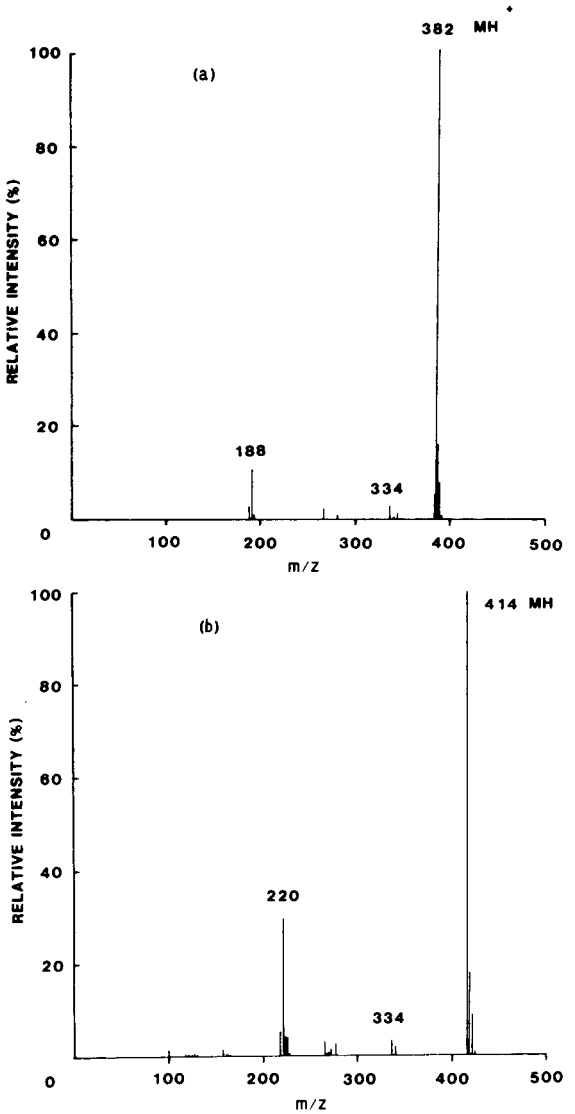

Fig. 2. Isobutane CI mass spectra of (a) S-methyl captopril HFI ester, and (b) S-methyl captopril sulfone HFI ester.

Quantitation

Blank urine (100 μl) or plasma (1 ml) were spiked with various amounts of captopril (10 ng to 10 μg), S-methyl captopril (25 ng to 2 μg), captopril disulfide dimer (25 ng to 5 μg) and treated as for the unknown samples. Quantitation was achieved using SIM of the fragment ions at *m/z* 264, 366, 334 and 282. Peak areas were evaluated using the INCOS data system. The areas of the peaks from ions *m/z* 264, 366 and 334 were expressed as a ratio of the area of the internal standard (YS-980) peak (*m/z* 282 ion). These ratios

were then related to those obtained from unknown samples containing the same amount of YS-980.

Urine sampling procedures

Eight patients (mean age 57.4, range 53—60 years) who had a history of essential hypertension with normal renal function and who were already receiving captopril (50—100 mg p.o., t.i.d.) as part of a comprehensive pharmacokinetic study of this drug were investigated. Patients had been on captopril for at least two weeks prior to collection of urine for this study. The bladders were emptied immediately prior to the dose and urine was collected over a 6-h period following the dose of captopril. Urine was immediately treated with NEM (10 mg/ml urine) on voiding to alkylate the sulfhydryl group and prevent spontaneous oxidation of captopril to disulfides [4]. Urine was then frozen in aliquots until assayed.

Rats of either sex (mean weight 274 g, range 250—325 g) were housed in metabolic cages and captopril (10 mg/kg) was given to rats by gavage of a fresh aqueous solution (1 mg/ml). Urine was collected over a period of 24 h into tubes containing 50 mg NEM. Urine was subsequently aliquoted out and frozen.

RESULTS AND DISCUSSION

The HFI esters of S-methyl captopril and captopril chromatographed readily on a packed column eluting at 3.4 min and 9.8 min, respectively. Furthermore, these same conditions also facilitated chromatography of the disulfide dimer of captopril which eluted at 12.6 min (Fig. 3a). Chromatography of a urinary extract of a patient receiving captopril (50 mg) chronically using SIM-MS analysis shows peaks at 3.4 min, 9.8 min and 12.6 min (Fig. 3b), which by comparison of both EI and CI (isobutane) mass spectra of authentic standards confirmed the presence of S-methyl captopril, captopril and the disulfide dimer.

Similarly extracts of rat urine (see Methods) gave an almost identical profile to that obtained from human urine with only the S-methylcaptopril, captopril and the disulfide dimer evident. There were some very small unknown peaks in both rat and human urinary extracts eluting between 4 and 8 min which were also present in control urine extracts devoid of captopril. It was evident, however, that if urine was stored at room temperature for even relatively short periods (1 h) or subjected to more than one thawing an extra peak sometimes appeared in the chromatogram at 5.2 min and levels of S-methyl captopril and captopril fell. This peak was identified as the sulfone (S-dioxide) of S-methyl captopril by its EI and CI mass spectra and by comparison with authentic sulfone prepared from the oxidation of S-methyl captopril (see Methods) which gave identical retention times and mass spectra to the unknown peak (Figs. 1b and 2b). Although this compound was well extracted from salt-saturated urine (recovery approximately 50%) there did not appear to be significant amounts in either fresh rat or human urine obtained over 24 h after captopril administration.

Captopril, the dimer of captopril and the sulfone of S-methyl captopril gave

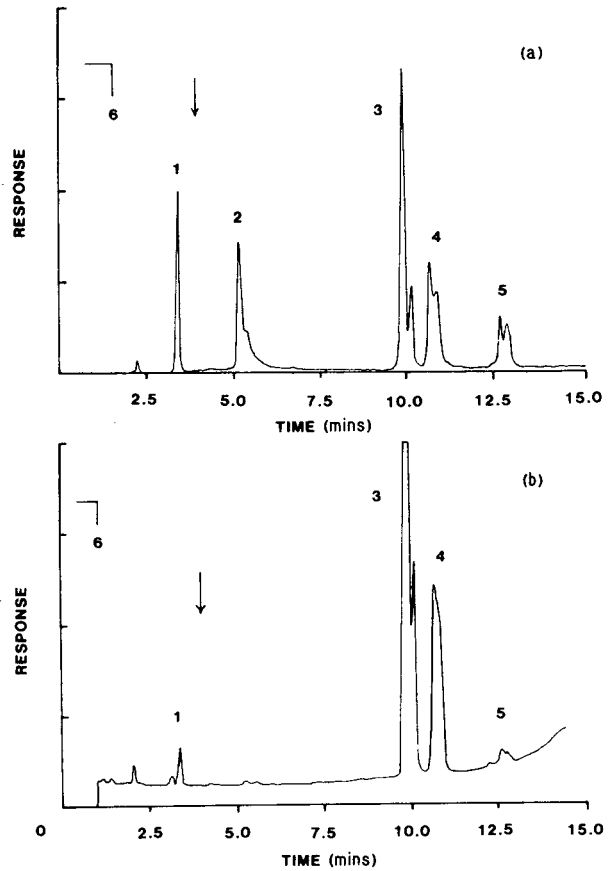

Fig. 3. Traces from EIMS SIM analysis of the HFI esters of (a) aqueous extract of S-methyl captopril (1), S-methyl captopril sulfone (2), captopril-NEM (3), the internal standard YS-980-NEM (4), and captopril disulfide dimer (5): and (b) urinary extract of a patient receiving captopril (50 mg p.o. t.i.d.) showing excretion of S-methyl captopril (1), captopril (3), and the disulfide dimer (5). The arrow denotes possible elution time of a potential sulfoxide metabolite of S-methyl captopril. Event 6 denotes time during which helium effluent was vacuum diverted away from the MS source.

split chromatographic peaks. The presence and amount of these split peaks were not altered by removing peroxide impurities in the ethyl acetate by elution through alumina. Both EI and CI (isobutane) GC—MS gave identical mass spectra of all portions of the split peaks suggesting that the composition of the split peaks was uniform, and probably, one compound. This phenomenon also occurs in HPLC analysis [7, 9, 14] and has been attributed to inter- and intra-molecular bonding of captopril [15]. Epimerization at the C-2 position of the side chain by derivatization is another possibility contributing to split peaks, however, this is unlikely since this phenomenon also occurs with HPLC analysis where derivatization was not used and where chromatography was performed at ambient temperature [14]. The possibility of a mixture of stereoisomers contributing to the dual peaks cannot entirely be ruled out although product literature from Squibb suggests a pure DL stereo-

isomer of captopril is used. This splitting of the peak did not, however, affect reproducibility when peak areas were used for quantitation. Analyses of urinary extracts were greatly simplified and detection limits vastly improved by using SIM analysis of the ions at *m/z* 264, 334 and 366 together with the ion at *m/z* 282 which was the most intense ion of the YS-980 EI mass spectrum (thiazolidine moiety). Columns were conditioned daily with two 1-μl injections of bis-trimethyltrifluoro acetamide (BSTFA) and two 1-μl injections of 1 μg of derivatized captopril. The use of HFI ester of captopril [11, 13] and the use of NEM for derivatizing the thiol of captopril in blood at the time of collection have previously been described [11—13]. We have used a combination of these two treatment procedures similar to that described by Matsuki and co-workers [11, 13] with SIM analysis [13] for measurement of captopril and metabolites in plasma or urine. The use of a close structural analogue of captopril (YS-980) in this method is an improvement over existing GC procedures for measuring free captopril which have either used a captopril derivative which did not undergo S-alkylation with NEM in the method [11, 13] or a deuterated analogue of captopril whose fragment ions interfere with quantitation of captopril at low concentrations [12]. In addition, complicated and time-consuming extraction steps previously reported have been considerably abbreviated allowing shorter sample preparation time, hence shorter analysis time.

All compounds that were quantitated exhibited linear calibration curves from the limit of detection to at least 10 μg for captopril and disulfide dimer and 3 μg for S-methyl captopril. All curves passed through the origin. Coordinates for these curves were; captopril ($y = 0.265x + 0.0009$), S-methyl captopril ($y = 0.045x - 0.0003$) and disulfide dimer ($y = 0.041x + 0.002$).

Reproducibility studies for both compounds were evaluated by performing replicate assays of control urine samples containing different amounts of both compounds (Table I). Within-assay variability for captopril was 4.6% at 270 ng/ml and 2.7% at 3.7 μg/ml (Table I). Corresponding values for S-methyl captopril were 2.1% at 250 ng/ml and 2.5% at 1.1 μg/ml and for the dimer 12.6% at 2 μg/ml.

Inter-assay variability of these same samples was evaluated over a period of four weeks ($n = 7$). Coefficients of variation for captopril were 9.1% at 270 ng/ml and 8.7% at 2.7 μg/ml (Table I). Similarly, S-methyl captopril gave variabilities of 8.3% at 250 ng/ml and 6.5% at 1.1 μg/ml and 15.1% for the dimer at 2 μg/ml. Detection limits for captopril, S-methyl captopril and the dimer using the method described were 1 ng/ml, 10 ng/ml and 25 ng/ml re-

TABLE I

ASSAY PARAMETERS FOR MEASUREMENT OF CAPTOPRIL, S-METHYL CAPTOPRIL AND DISULFIDE DIMER IN URINE

Parameter	Captopril	S-methyl captopril	Disulfide dimer
Detection limit (ng/ml)	1	10	25
Reproducibility within-day (%)	4.6 (270 ng/ml) 2.7 (3.7 μg/ml)	2.1 (250 ng/ml) 2.5 (1.1 μg/ml)	12.6 (2 μg/ml)
Reproducibility day-to-day (%)	9.1 (270 ng/ml) 8.7 (2.7 μg/ml)	8.3 (250 ng/ml) 6.5 (1.1 μg/ml)	15.1 (2 μg/ml)

TABLE II

EXCRETION OF CAPTOPRIL, S-METHYL CAPTOPRIL AND THE DISULFIDE DIMER OF CAPTOPRIL IN URINE OF MAN AND RATS

Dose administered by gavage of an aqueous solution.

Metabolite	Rat (10 mg/kg)*				Man**	
	0—6 h	*n*	6—24 h	*n*	0—6 h	*n*
Captopril	18.3 ± 9.5	5	2.8 ± 1.6	5	18.3 ± 4.2	6
Captopril disulfide dimer	1.80 ± 0.29	4	0.76 ± 0.70	5	3.06 ± 1.26	8
S-Methyl captopril	2.69 ± 0.88	5	1.71 ± 1.10	5	0.97 ± 0.14	5

*All values expressed as a percentage of dose; mean ± standard error.
**Patients on captopril chronically, 50- or 100-mg dose.

spectively (Table I). However, the detection limits described were more than adequate for urine and plasma samples encountered. Recoveries of S-methyl captopril and captopril were greater than 80% and 50% for the dimer and sulfone of S-methyl captopril. Similar recoveries and variability estimates were found for plasma samples.

The levels of captopril and the metabolites in urinary extracts of both man and rat are shown in Table II. The 24-h urinary excretion of free captopril in rat was a sizeable proportion (21.1%) of the dose administered and it was the same (18.3%) in the 0—6 h period in both rat and man.

Previous studies in both man [4] and rat [16] have shown that only about 50—60% of a captopril dose is absorbed hence the excretion of unchanged captopril in this study would increase by approximately a factor of two. Excretion of free captopril in man over 6 h assuming the bioavailability was 50% would therefore approach 36% of the dose. However, the values reported here are lower than previously reported using radiolabelled captopril and TLC separation procedures which have shown 37% excretion of unchanged captopril in man following a 100-mg dose after 8 h [4] and 47% in the rat [6]. These differences in excretion of free captopril may reflect the increased specificity of our GC—MS assay methodology over TLC—radiometric procedures and this is supported by a recent GC—MS study which showed that 20.9% of a 10 mg/kg dose of captopril is excreted as unchanged captopril in the rat over 24 h [13]. The disulfide dimer of captopril was present in both human and rat urine, albeit only a small proportion of the captopril administered. In two of the eight human urine samples no dimer was detected whilst one subject excreted almost 10% of this metabolite suggesting a large individual variability in dimerization of captopril. Urinary excretion of the dimer in the rat was significantly less than in man ($p < 0.005$) (Table II).

The presence of the disulfide dimer of captopril in urine was not unexpected since this material forms spontaneously from aqueous solutions of captopril and may in addition also form enzymatically, probably through the mixed function oxidase system [17]. Incubation of rat serum or human plasma with captopril has been shown to result in about 10% formation of dimer over 30

min [5]. In view of the ease of formation of the dimer the levels encountered in this study were surprisingly low, in agreement with a previous study in man [4] using TLC procedures. The excretion in the rat however was three-fold lower than in a previous study [6]. The small excretion of dimer may reflect the action of reductases similar to those present for oxidized glutathione [18, 19]. Moreover, the lower but consistent values found in the present study are more likely to reflect real excretion data rather than problems in assay methodologies. The suggestion that only a small amount of captopril oxidation to the dimer occurs is supported by the lack of S-methyl captopril oxidation products. Although the sulfone is extractable into ethyl acetate from salt-saturated urine neither rat nor human urine contained measureable amounts. Large variations in excretion of dimer may occur if urine is not properly stored or captopril is not completely alkylated to prevent spontaneous dimer formation and this may in part explain the previously reported higher level in the rat [6].

Spontaneous formation of the oxidation products of S-methyl captopril was observed when old thawed urines were reanalysed again suggesting that storage is critical. This may be an explanation for the formation of large amounts of the apparent sulfoxide of S-methyl captopril (3%) in the rat using a previously described radiolabelled procedure [6].

In the present studies, the urinary excretion of S-methyl captopril was evident in both man and rat and was about three-fold higher ($p < 0.005$) in rats when urine was collected over a 6-h period. It is not clear at this stage whether this reflects differences in the doses given to the two species, or to different clearances, or a lower degree of S-methylation in humans.

CONCLUSIONS

In summary, a simple, sensitive and specific GC—MS method for quantitation and identification of S-methyl captopril, S-methyl captopril sulfone, captopril and the disulfide dimer in biological fluids is presented. Data are presented to show excretion values for those metabolites in human and rat urine after pharmacological doses of captopril. In the present study S-oxide formation of S-methyl captopril, as the sulfone was not observed to be a significant pathway of captopril elimination in both man and rat. The formation of other oxidized polar disulfide conjugates of captopril could account for other routes of captopril elimination and at present we are examining these.

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CHROMBIO. 1907

DETERMINATION OF THE TWO MONONITRATE METABOLITES OF ISOSORBIDE DINITRATE IN HUMAN PLASMA AND URINE BY GAS CHROMATOGRAPHY WITH ELECTRON-CAPTURE DETECTION

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SUMMARY

This paper describes a sensitive method for the determination of 2-isosorbide mononitrate and 5-isosorbide mononitrate as metabolites of isosorbide dinitrate at concentrations down to 2 ng/ml of 2-isosorbide mononitrate in both plasma and urine, and 5 ng/ml and 10 ng/ml of 5-isosorbide mononitrate in plasma and urine, respectively.

The two mononitrate metabolites are extracted at basic pH into ethyl acetate, which is then evaporated to dryness. The residue is dissolved in a basic aqueous solution, which is washed with heptane and then re-extracted into ethyl acetate. The metabolites are quantitated by gas chromatography, using a ^{63}Ni electron-capture detector. Conjugates of 2- and 5-isosorbide mononitrate are determined in urine after enzymatic hydrolysis.

INTRODUCTION

Isosorbide dinitrate (ISDN) is an organic nitrate vasodilator. Very low plasma levels are reached after oral administration to man, and ISDN is detectable below its limit of quantitation in the 12-24 h urine only [1]. A glutathione-dependent enzyme system, glutathione S-transferase, catalyses the denitration of ISDN during its passage through the liver to give two isosorbide mononitrates (ISMN): 2-ISMN and 5-ISMN [2].

In a study of the urinary excretion of ISDN in the rat, Rosseel and Bogaert [3] found (without quantitation) 5-ISMN to be the main metabolite, 2-ISMN being excreted in much smaller amounts; both mononitrates were largely excreted in conjugated form. Concentrations of ^{14}C-labelled ISDN, 2-ISMN, and 5-ISMN in blood were measured radiometrically after thin-layer chromatography [4]; the limit of accurate measurement of ISDN and both metabolites

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was 2 ng/ml, but 9 ml of blood were needed. Using this method to study the biotransformation of ISDN after oral administration of 5 mg of [^{14}C] ISDN to human subjects, Down et al. [5] showed that up to 13% was excreted as free and conjugated 5-ISMN and 1% as free 2-ISMN. Some gas chromatographic (GC) methods have been published [6—8], but they either lack sensitivity [6], or require a large sample volume [6, 7]. Rosseel and Bogaert [8] described a GC method with electron-capture detection (ECD) using a capillary column for the simultaneous determination of ISDN down to 0.5 ng/ml, and 2-ISMN and 5-ISMN down to, respectively, 2 ng/ml and 20 ng/ml only, from 2 ml of plasma.

In a recent paper, Geigenberger et al. [9] determined simultaneously ISDN, 2-ISMN and 5-ISMN in serum and not in urine by GC—ECD using benzene as extraction solvent.

A rapid and sensitive method of assaying unchanged ISDN in plasma and urine has recently been developed in our laboratories [1]. Using GC—ECD, this method can measure concentrations down to 0.5 ng/ml. The extraction conditions elaborated for ISDN, however, were not convenient for 2-ISMN and 5-ISMN, and the method therefore did not appear practicable for the simultaneous assay of ISDN and the two mononitrate metabolites in a single run due to their different polarity.

This present paper describes a procedure which permits the quantitative GC determination of free 2-ISMN and 5-ISMN in human plasma and urine. The conjugated metabolites can also be assayed in urine after enzymatic hydrolysis. The limits of quantitation are 2 ng of 2-ISMN per ml of plasma and urine, 5 ng of 5-ISMN per ml of plasma, and 10 ng of 5-ISMN per ml of urine.

EXPERIMENTAL

Chemicals and reagents

The two mononitrate metabolites (2-ISMN and 5-ISMN) of ISDN were supplied by Sanol (Monheim, F.R.G.). The solvents and reagents used are all of analytical grade: ethyl acetate (Nanograde 3427; Mallinckrodt, St. Louis, MO, U.S.A.), *n*-heptane (Uvasol 4366; Merck, Darmstadt, F.R.G.), sodium hydroxide (Titrisol Merck 9956), potassium carbonate (Merck 4928, used to alkalinize plasma or urine).

The pH 5 buffer is prepared with 14.8 ml of 0.2 *M* acetic acid solution and 35.2 ml of 0.2 *M* sodium acetate solution, which are diluted to 100 ml with water. β-Glucuronidase (Sigma, St. Louis, MO, U.S.A.) is bacterial β-glucuronidase containing about 61.5 units/g.

Calibration solutions

The methanolic solutions of the two metabolites contain 2—500 ng of 2-ISMN and 5-ISMN per 25 μl.

Equipment

All the glassware (flasks, glass tubes) is pretreated to prevent adsorption. It is immersed in toluene containing hexamethyldisilazane, trimethylchlorosilane and pyridine (1%, v/v, each) for 15 min and rinsed with methanol. The treat-

ment is repeated every month. Between treatments, the glassware is cleaned as usual and rinsed with methanol.

A Hewlett-Packard Model 5713 A gas chromatograph equipped with a Hewlett-Packard electron-capture detector (Model 18 713 A) is used. The thermoregulators of the detector and the injection port have been modified to permit continuous adjustment of the temperature. The peak areas are given by a Hewlett-Packard computing integrator (Model 3388 A). The column is operated at 123°C with argon—methane (90:10) at a flow-rate of 75 ml/min; the injector temperature is 162°C and the detector is set at 205°C. The glass column (1 m × 2 mm I.D.) is washed [10], and packed with 3% QF-1 on Gas-Chrom Q (80—100 mesh) (Supelco, Bellefonte, PA, U.S.A.). The filled column is flushed with the carrier gas at a flow-rate of 75 ml/min and heated to 150°C at a rate of 1°C/min. The column temperature is held overnight at 150°C and increased to 210°C for conditioning by injection of 200 µl of Silyl 8 (Pierce, Rockford, IL, U.S.A.) in fractions of 10 µl. After this treatment, the column is held at 210°C for 45 min and it is ready for use at 123°C.

Enzymatic hydrolysis of 2-ISMN and 5-ISMN conjugates in urine

One millilitre of urine, 1 ml of acetate buffer (pH 5) and approximately 10 mg of β-glucuronidase are heated for 16 h at 37°C. The extraction procedure is then performed as described below for the free mononitrate metabolites.

Extraction of free 2-ISMN and 5-ISMN from plasma and urine

One millilitre of plasma or urine is introduced into a 5-ml glass centrifuge tube with approximately 100 mg of potassium carbonate and shaken on a Vortex mixer for 1 min; then 3 ml of ethyl acetate are added. The tube is stoppered and shaken mechanically for 10 min at 250 rpm in an Infors shaker, then centrifuged for 3 min at 2500 *g*.

An aliquot of the ethyl acetate phase is transferred to another 5-ml tube and taken to dryness under a stream of nitrogen at room temperature. Evaporation must be performed very carefully and stopped just as the tube reaches dryness.

One millilitre of 2 *M* sodium hydroxide and 2 ml of heptane are added to the dry residue, and the tube is then shaken mechanically for 10 min at 250 rpm and centrifuged. The heptane phase is separated and discarded. Then 1 ml of ethyl acetate is added to the sodium hydroxide phase, shaken for 10 min at 250 rpm and centrifuged. An aliquot of the ethyl acetate phase is transferred into another tube and carefully taken to dryness, as mentioned above; 150 µl ethyl acetate are added to the dry residue and the tube is shaken on a Vortex mixer.

Gas chromatography

A 3-µl portion of the ethyl acetate solution is injected into the gas chromatograph. The content of 2-ISMN and 5-ISMN is calculated from the peak areas by reference to the two respective calibration curves. These curves are obtained by extraction of plasma or urine spiked with increasing amounts of 2-ISMN and 5-ISMN (from 2 to 500 ng/ml). Standard samples for the calibration curves are extracted and plotted every day.

Study in man

A healthy male subject, who had been advised to take no drugs during the 14 days preceding the experiment and none besides ISDN throughout the duration of the study, received 5 mg of ISDN as one Isoket® tablet. Blood samples were collected before and 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 8, 12 and 24 h after the administration of the drug, and centrifuged. Plasma was removed and stored at −20° C until analysis. Urine was collected during the following time intervals: 0—4, 4—8, 8—12, 12—24, 24—48, 48—72, 72—96 and 96—104 h. The volume was measured and an aliquot was stored at −20° C.

RESULTS AND DISCUSSION

Extraction procedure

No internal standard was used. Attempts to use 1,2-dinitrobenzene and 2,3-dinitrotoluene were unsuccessful: the extraction conditions for 2-ISMN and 5-ISMN are different from those required for these two compounds. The chromatographic conditions mentioned above do not permit the use of isomannide mononitrate, which has the same retention time as 5-ISMN.

Hydrolysis of conjugated 2-ISMN and 5-ISMN in urine

Preliminary experiments were carried out to determine the best conditions for the hydrolysis of the 2-ISMN and 5-ISMN conjugates in human urine. Stability tests showed that 2-ISMN and 5-ISMN are stable in urine at 37° C for 48 h.

Samples of the same urine fraction, containing the conjugates of 2-ISMN and 5-ISMN, from a volunteer treated with ISDN were subjected to enzymatic hydrolysis. The reaction was studied in aliquots of this urine incubated with 10 mg of β-glucuronidase and acetate buffer (pH 5) for 1, 2, 3, 4, 16 and 24 h at 37° C.

Fig. 1 shows that 5-ISMN is less easily hydrolyzed than 2-ISMN; the maximum yield of 5-ISMN is obtained after incubation for 16 h whereas that of 2-ISMN is reached after 4 h.

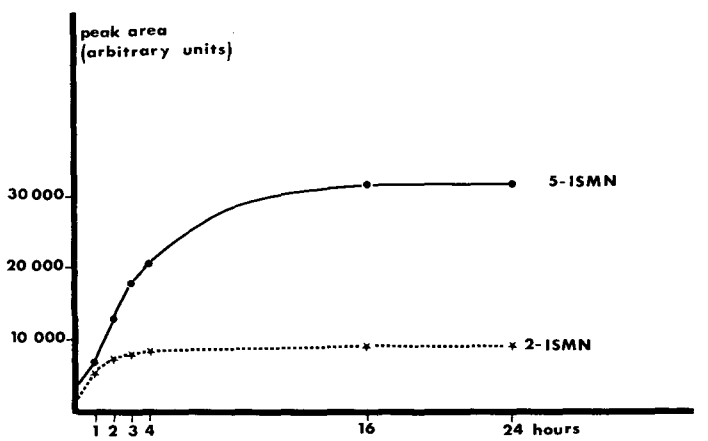

Fig. 1. Effect of incubation time on hydrolysis of 2-ISMN and 5-ISMN conjugates.

Identical results were found after a further 24-h incubation during which fresh enzyme (10 mg) was added five times.

Incubation with β-glucuronidase—arylsulphatase did not modify the hydrolysis. It may be concluded that the conjugates of 2-ISMN and 5-ISMN are glucuronides.

Within-day precision

The within-day precision of the method was checked by determining six plasma and urine samples spiked with different concentrations of 2-ISMN and

TABLE I

WITHIN-DAY PRECISION OF THE ASSAY AND RECOVERY OF 2-ISOSORBIDE MONONITRATE IN SPIKED PLASMA AND URINE SAMPLES

Amount added (ng/ml)	In plasma			In urine		
	Mean amount found (ng/ml) (n = 6)	Standard deviation (±)	Recovery (%)	Mean amount found (ng/ml) (n = 6)	Standard deviation (±)	Recovery (%)
2	1.97	0.09	98.5	2.0	0.40	100.0
5	4.8	0.79	96.0	5.4	0.59	108.0
10	9.2	0.73	92.0	9.8	0.43	98.0
50	51	5.8	102.0	51	2.2	102.0
100	99	7.2	99.0	97	4.5	97.0
200	201	7.0	100.5	—	—	—
500	497	41.4	99.4	466	26.1	93.2
Mean			98.2			99.7
C.V. (%)			3.4			5.0

TABLE II

WITHIN-DAY PRECISION AND RECOVERY OF 5-ISOSORBIDE MONONITRATE IN SPIKED PLASMA AND URINE SAMPLES

Amount added (ng/ml)	In plasma			In urine		
	Mean amount found (ng/ml) (n = 6)	Standard deviation (±)	Recovery (%)	Mean amount found (ng/ml) (n = 6)	Standard deviation (±)	Recovery (%)
5	4.7	0.3	94.0	—	—	—
10	11.2	0.8	112.0	9.6	2.0	96.0
50	55	4.2	110.0	59	2.2	118.0
100	98	7.2	98.0	110	5.3	110.0
200	188	9.5	94.0	—	—	—
500	470	26.7	94.0	526	51.7	105.2
Mean			100.3			107.3
C.V. (%)			8.4			8.6

5-ISMN. The results obtained with the procedure described are given in Tables I and II.

Day-to-day precision

The day-to-day precision was checked in plasma and urine by determining two concentrations (10 and 100 ng/ml) of 2-ISMN and 5-ISMN, in duplicate, every day for one week. The results obtained with the procedure described are given in Tables III and IV.

TABLE III

DAY-TO-DAY PRECISION AND RECOVERY OF 2-ISOSORBIDE MONONITRATE IN SPIKED PLASMA AND URINE SAMPLES

Amount added (ng/ml)	In plasma			In urine		
	Mean amount found (ng/ml) (n = 8)	Standard deviation (±)	Recovery (%)	Mean amount found (ng/ml) (n = 8)	Standard deviation (±)	Recovery (%)
10	10.6	0.7	106	10.0	0.5	100
100	105	7.8	105	97	4.6	97

TABLE IV

DAY-TO-DAY PRECISION AND RECOVERY OF 5-ISOSORBIDE MONONITRATE IN SPIKED PLASMA AND URINE SAMPLES

Amount added (ng/ml)	In plasma			In urine		
	Mean amount found (ng/ml) (n = 8)	Standard deviation (±)	Recovery (%)	Mean amount found (ng/ml) (n = 8)	Standard deviation (±)	Recovery (%)
10	9.6	1.1	96	10.5	1.3	105
100	104	9.9	104	99	9.6	99

Analysis of control samples

Four plasma samples spiked with concentrations of 2-ISMN and 5-ISMN unknown to the analyst were prepared in duplicate. The accuracy demonstrated by calculating the recovery for each concentration is 102.1 ± 5.6% and 94.0 ± 5.7%, respectively, for 2-ISMN and 5-ISMN.

Plasma and urine interference

Fig. 2 shows the chromatograms of an extract of human plasma (1 ml) and of the same extract spiked with 10 ng of 2-ISMN and 5-ISMN. No interference from normal plasma components was recorded. 2-ISMN and 5-ISMN are well separated from the normal components of the urine extract.

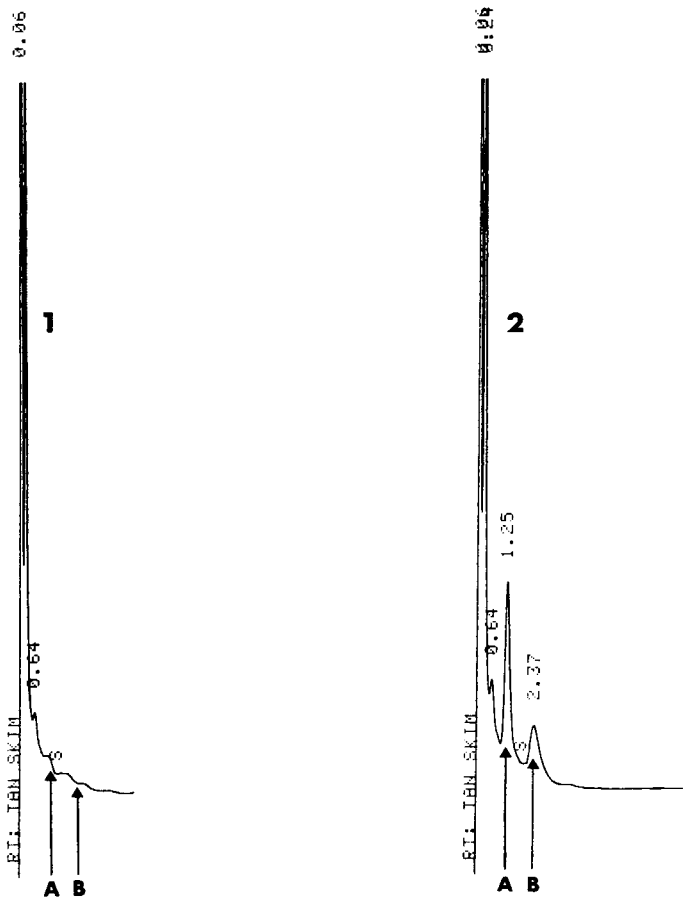

Fig. 2. Examples of chromatograms: (1) human plasma blank (1 ml plasma); and (2) the same plasma spiked with 10 ng of 2-ISMN (A) and 5-ISMN (B).

Storage stability of 2-ISMN and 5-ISMN in human plasma

No decrease in the 2-ISMN and 5-ISMN content (10 ng/ml and 100 ng/ml) was observed in plasma samples when stored frozen for 1, 8 and 15 days, 1, 3, 6 and 9 months, and 1 year at −20°C.

Application

The present method was used to determine the plasma concentrations and the urinary excretion of the mononitrate metabolites (2-ISMN and 5-ISMN) after oral administration of ISDN to a healthy subject. The corresponding plasma concentrations of the parent drug (ISDN) have also been determined [1]. Fig. 3 shows the plasma concentration curves of 2-ISMN and 5-ISMN and Fig. 4 the urinary excretion of the free and conjugated mononitrates after a single dose of 5 mg of ISDN in the form of one Isoket tablet. In the 0–104 h urine, 14% of the administered dose was recovered as mononitrate metabolites. Only 0.3% corresponds to free 2-ISMN and 2.2% to free 5-ISMN.

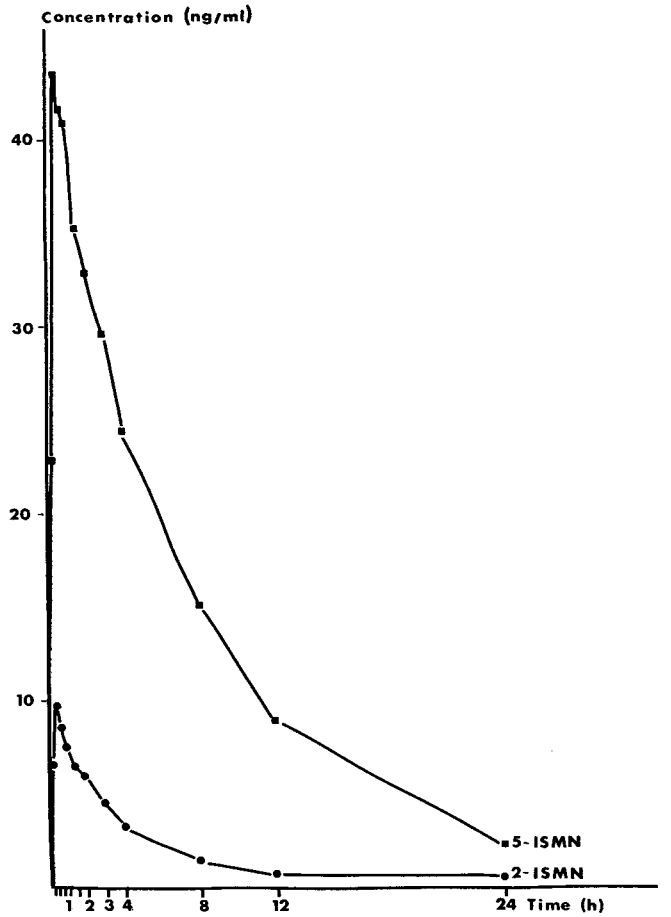

Fig. 3. 2- and 5-isosorbide mononitrate plasma concentrations in a healthy subject after oral administration of 5 mg of ISDN as one Isoket tablet. (•), 2-ISMN; (▪), 5-ISMN.

Enzymatic hydrolysis suggests that about 11.1% is excreted as 5-ISMN glucuronide whereas only 0.4% of the dose is excreted as 2-ISMN glucuronide.

CONCLUSION

The proposed gas chromatographic technique permits the quantitative assay of 2-ISMN and 5-ISMN in human plasma and urine and can be applied for the determination of free and conjugated 2-ISMN and 5-ISMN as metabolites of ISDN.

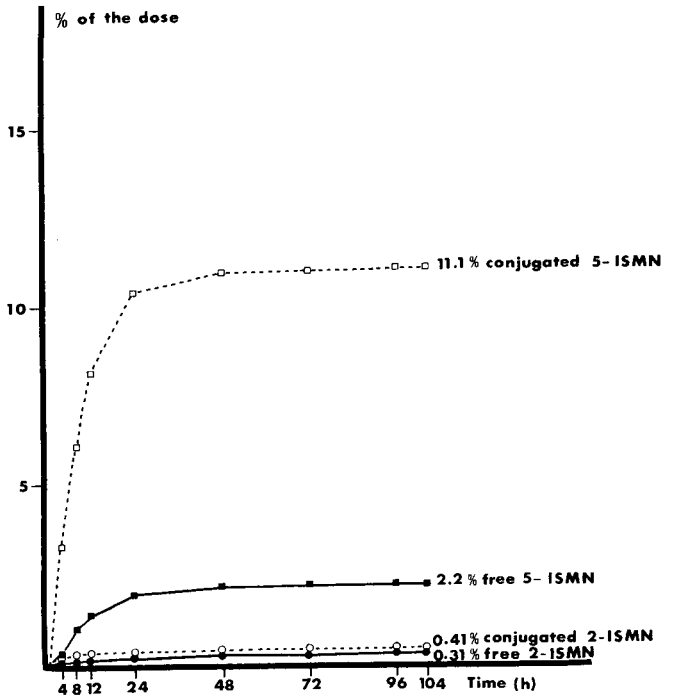

Fig. 4. Cumulative urinary excretion in a healthy subject after oral administration of 5 mg of ISDN as one Isoket tablet. (•), Free 2-ISMN; (○), conjugated 2-ISMN; (▪), free 5-ISMN; (▫), conjugated 5-ISMN.

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CHROMBIO. 1886

GAS AND LIQUID CHROMATOGRAPHIC ANALYSES OF NIMODIPINE CALCIUM ANTAGONIST IN BLOOD PLASMA AND CEREBROSPINAL FLUID*

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SUMMARY

Gas (GC) and liquid chromatographic (LC) assay procedures were developed for analysis of nimodipine (1,4-dihydropyridine calcium antagonist, BAY e 9736) in blood plasma at low nanogram concentration levels. To avoid decomposition during gas chromatography, nimodipine was oxidized to nimodipine pyridine (P) analogue before it was chromatographed on the OV-17 column and quantitated using an electron-capture detector. In contrast, the LC procedure involved chromatographic separation and quantitation of the underivatized nimodipine and of the endogenous P analogue using a 3-μm Spherisorb ODS column and UV detection. The same plasma extract and three alternative internal standards were used for both assays. Taking into consideration the fact that the GC assay result includes endogenous P analogue as well as nimodipine, good correlation between GC and LC assay data was obtained. Comparison of the results observed with the two procedures confirmed the accuracy of each procedure and provided an alternative when one of the assay results was subject to patient plasma constituent interference. The LC assay was also used for analysis of the demethylated metabolites of nimodipine. To detect sub-nanogram concentrations of nimodipine in cerebrospinal fluid a combined LC—GC procedure using an LC clean-up step and a GC quantitation step was also developed. The above GC and LC procedures were used to obtain preliminary pharmacokinetic data.

INTRODUCTION

Nimodipine [BAY e 9736, isopropyl-2-methoxyethyl-1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridine dicarboxylate] is a potent calcium

*Dedicated to the memory of Professor Dr. Otto Bayer.

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antagonist which selectively inhibits serotonin- [1] and thromboxane-induced contractions in animal cerebral arteries [2]. In contrast to a structurally related drug, nifedipine, which is used in the treatment of ischemic heart disease, nimodipine yields a marked alteration in cerebrovascular tone without the degree of peripheral vasodilation associated with nifedipine and other calcium antagonists [3–6]. The effect of nimodipine on cerebrovascular tone is most noticeable in the smaller cerebral arteries [7].

Since peroral administration of nimodipine yields relatively low nimodipine plasma concentrations, analytical procedures which can detect nimodipine in blood plasma in the 1—50 ng/ml concentration range are essential. Two independent analytical procedures were developed to meet this requirement. One of these procedures involves gas chromatography (GC) with electron-capture detection which takes advantage of the relatively sensitive response of the electron-capture detector to the nitro group. Similar GC procedures for analysis of the structurally related nifedipine [8–10] and nicardipine [11, 12] were previously reported. As observed earlier [9—12] dihydropyridine compounds are relatively unstable at GC temperatures. Consequently, the GC procedure described in this paper requires oxidation of nimodipine by nitrogen dioxide to the pyridine (P) analogue which does not decompose during GC separation. Although GC assay procedures for analysis of underivatized nifedipine were reported [8, 13, 14], application of similar GC procedures to analysis of underivatized less volatile nimodipine yielded partial (5—20%) conversion during chromatography to the nimodipine P analogue. Furthermore, the observed GC detection limit of nimodipine was about five times higher (less sensitive) than the detection limit of nimodipine P analogue.

In order to assay nimodipine directly without conversion to the P analogue, a high performance liquid chromatographic (LC) procedure was developed. The LC procedure involves separation of nimodipine from the P analogue and demethylated metabolites on a 3-μm Spherisorb ODS column and UV detection of the column eluate.

It was also necessary to develop a procedure which could detect sub-nanogram concentrations of nimodipine in cerebrospinal fluid. An LC clean-up step and a GC quantitation were used for this purpose.

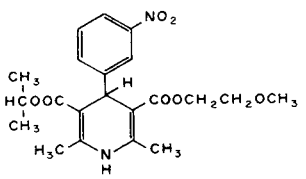
This paper describes the relative accuracy, precision, sensitivity and selectivity of the GC and LC assay procedures. Comparison of the analytical results observed with GC and LC assays of blood plasma obtained during preliminary pharmacokinetic studies is also presented.

EXPERIMENTAL

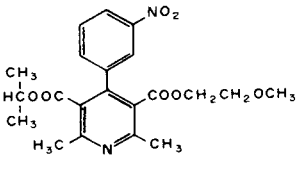
Standards, reagents and solvents

Nimodipine and the appropriate reference and internal standards (see Fig. 1) were obtained from Bayer (Wuppertal, F.R.G.). Stock solutions were prepared by dissolving 10 mg of each substance in 10 ml of methanol or isopropanol and were stored in amber glass volumetrics at 4°C. The mg/ml stock solutions were found to be stable for several weeks but the ng/μl dilutions of the stock solution were freshly prepared every two weeks.

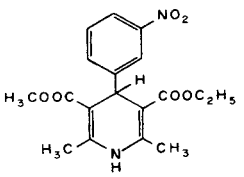
All GC and LC reagents and solvents were analytical or LC grade (Burdick



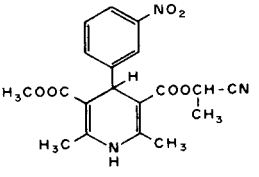
NIMODIPINE



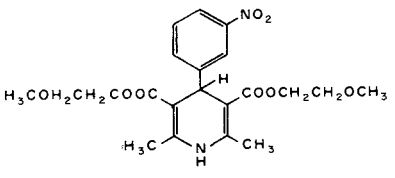
PYRIDINE ANALOG (PA)



NITRENDIPINE



INTERNAL STANDARD B



INTERNAL STANDARD C

Fig. 1. Chemical structure of nimodipine, nimodipine pyridine analogue, and internal standards.

and Jackson Labs., Muskegon, MI, U.S.A.). Diethyl ether was distilled before use. A lecture bottle of nitrogen dioxide was supplied by E. Merck (Darmstadt, F.R.G.) and nanograde toluene by Mallinckrodt (St. Louis, MO, U.S.A.).

Instrumentation and chromatographic conditions

Gas chromatography. GC analysis was carried out on either a Hewlett-Packard Model 5840A gas chromatograph equipped with a ^{63}Ni electron-capture detector and a glass column (1.8 m × 3 mm I.D.) which was packed with 3% SP 2250 DB (Supelco, Bellefonte, PA, U.S.A.) on Gas-Chrom Q, 100—120 mesh (Applied Science Labs., State College, PA, U.S.A.) or a Varian Model 6000 gas chromatograph equipped with a ^{63}Ni electron-capture detector and an Analabs (North Haven, CT, U.S.A.) glass column (1.8 m × 2 mm I.D.) packed with 2% OV-17 on Anakrom Q, 100—120 mesh. Both instruments were operated isothermally at 250—260°C. Carrier gas flow-rate was 25—30 ml/min; argon—methane gas was used with the Hewlett-Packard and nitrogen with the Varian instrument. The Hewlett-Packard chromatograph injection port and detector temperatures were 270°C. The Varian injection port and detector temperatures were 280°C and 320°C, respectively. Sample volumes of 1—2 μl were injected by a Hewlett-Packard 7672A automatic sampling system or a Varian Model 8000 autosampler interfaced with a Spectra Physics SP 4100 (Mountain View, CA, U.S.A.) computing integrator.

Liquid chromatography. A Kratos Model SF 773 (Westwood, NJ, U.S.A.)

UV detector and a Kratos Model 250-1 or Waters Assoc. Model M-45 (Milford, MA, U.S.A.) pump were used for LC analysis. LC samples were injected on an Applied Science Labs. Excalibar (15 cm × 4.6 mm, 3-μm Spherisorb ODS) column using either a Waters Assoc. Wisp 710B or a Perkin-Elmer 1SS-100 (Norwalk, CT, U.S.A.) autoinjector. Two alternative chromatographic solvents were used for column elution. Solvent A consisted of a mixture of acetonitrile—water (57:43, v/v) while solvent B was a mixture of methanol—water (66:34, v/v). Both solvents were pumped at a flow-rate of 0.5 ml/min. The UV detector was set at either 238 nm or 218 nm wavelength, and a 0.004 to 0.002 a.u.f.s. detector sensitivity range. Chromatographic peaks were quantitated using either a Spectra Physics SP 4100 computing integrator or a Waters Assoc. Data Module in the peak area or peak height mode of calculation. Selection of the calculation mode was based on the observed peak shape, resolution and baseline.

Analytical procedures

To prevent UV light decomposition of dihydropyridine compounds to pyridine derivatives during the analysis, all stock solutions of reference and internal standard compounds were stored in amber glassware. Sample extracts were prepared in a room illuminated by either diffuse daylight or shielded fluorescent light fixtures. Since the same initial sample preparation steps were identical, one 3-ml aliquot of plasma was used for preparation of both the GC and the LC sample extracts. The 3-ml aliquot of plasma was mixed with a 3-ml aliquot of an aqueous solution of internal standard prior to extraction with the organic solvent mixture.

Most samples were assayed by reference to one of the three alternative standards. However, if the selected internal standard was not resolved from a plasma sample constituent in a given plasma sample, one of the two alternative internal standards was used for calculation. The internal standard solution was prepared by aqueous dilution of stock ng/μl isopropanol solution of each internal standard in the same volumetric flask to yield a 20 or a 100 ng/ml concentration of each internal standard. To construct the nimodipine and/or nimodipine metabolite calibration curve the concentration of the compound of interest ranged from 10 to 100 ng/ml of the aqueous solution.

Extraction procedure. The plasma sample diluted with the internal standard mixture was treated with 1 ml of 1 *M* sodium hydroxide and extracted once with 17 ml of diethyl ether–hexane (1:1, v/v) solvent during 5 min shaking. The recovery of nimodipine and internal standards (A, B, C) during the extraction was checked and found to be at least 96% for nimodipine, nitrendipine (A) and internal standard B and 92% for internal standard C.

The organic and aqueous phases were separated by centrifugation at 300 *g* for 10 min. One 10-ml aliquot of the organic phase was withdrawn for LC assay while a 5-ml aliquot was used for GC assay. Both aliquots were evaporated under a gentle steam of nitrogen in a 60°C water bath.

Oxidation of GC extract. Test tubes containing residue of the 5-ml GC aliquot were filled with gaseous nitrogen dioxide, sealed, and stored for 1 h in the dark at room temperature. Subsequently, the nitrogen dioxide was flushed out with argon for 5 min at 60°C. Since nitrogen dioxide is toxic, the filling

and flushing of tubes was carried out in a well ventilated hood. Residue in the flushed tube was dissolved in 1 ml of toluene, vortexed and transferred to an autoinjector vial. Normally 1—2 μl of toluene solution were injected onto the GC column.

LC sample treatment. Test tubes containing residue of the 10-ml LC aliquot were washed with about 1 ml of diethyl ether—hexane (1:1 v/v) which was evaporated again under nitrogen. The final residue was reconstituted in 250 μl of modified chromatographic solvent (the chromatographic solvent diluted with water 1:1, v/v). The dilution was necessary because a relatively large sample injection volume (50 μl) was used and the modified sample injection solvent yielded less band spreading (higher column efficiency) than the undiluted chromatographic solvent. A 50-μl aliquot of the reconstituted sample was injected onto the LC column by the Perkin-Elmer or Waters autoinjector. To assay cerebrospinal fluid (CSF) sample by the LC—GC procedure, the LC nimodipine and internal standard fractions were collected and mixed and evaporated prior to nitrogen dioxide treatment of the dry residue and GC analysis.

RESULTS AND DISCUSSION

Accuracy and precision

Preliminary results obtained with the GC and LC assay procedures yielded sporadic patient plasma constituent interference in both assay procedures. In spite of the selectivity of the GC electron-capture detector and efficiency of the spherical 3-μm LC column support, some patient samples yielded inaccurate GC or LC results. Since the two procedures involve completely different principles the probability that both assays are subject to the same interference and would yield the same error, is rather small. In view of the above observations the two assay procedures serve as parallel controls to each other.

The sporadic interference could be attributed to unknown patient plasma constituents which were not resolved by the GC or LC column from either nimodipine or internal standard peaks. To minimize the chances of interference three alternative internal standards were used. Thus for all practical purposes, only nimodipine peak interference would impair the accuracy of the assay (the chance that all three internal standards would be subject to interference was small).

Fig. 2 illustrates GC separation obtained with plasma extracts spiked with internal standards and nimodipine reference compounds. In general, internal standard B was used as the internal standard. However, nitrendipine (A) was also used for this purpose when excipient peaks overlapping with internal standard B were observed. Note also that since the sample injected onto the column was oxidized prior to GC, GC nimodipine peak includes endogenous nimodipine pyridine analogue as well as nimodipine oxidized to pyridine analogue. Thus, the concentration of nimodipine determined by GC is a sum of nimodipine and P analogue concentrations present in blood plasma.

Figs. 3 and 4 illustrate LC separations of plasma extracts containing internal standards and nimodipine reference compounds chromatographed on the 3-μm

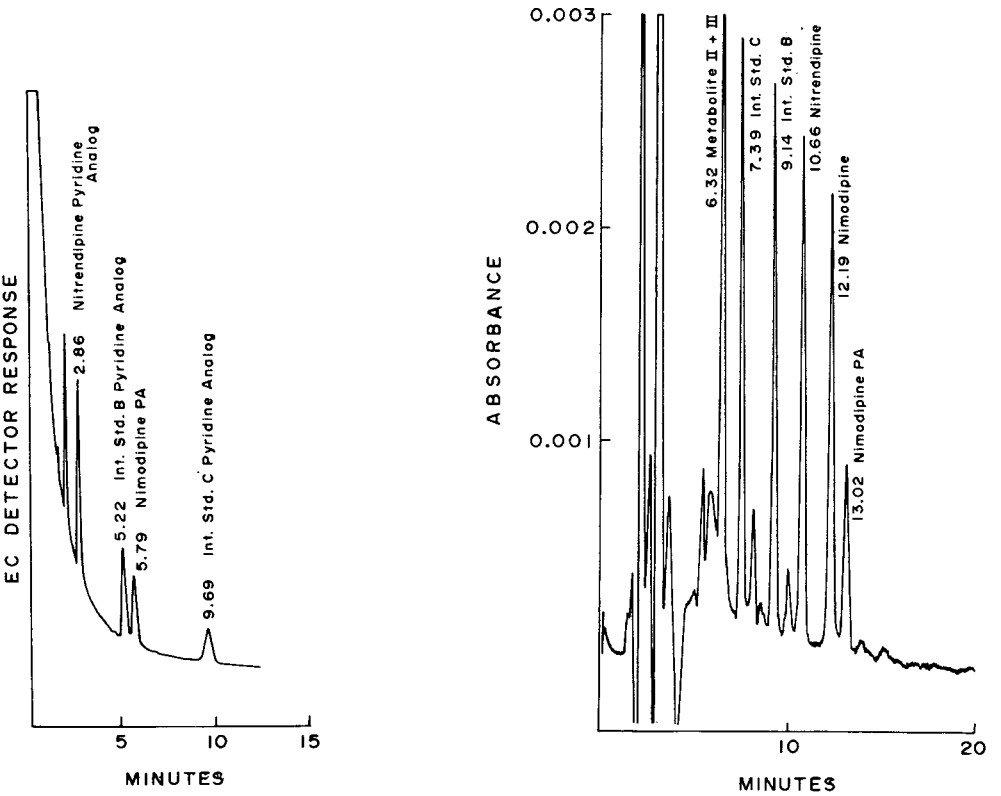


Fig. 2. GC separation of nimodipine and internal standards extracted from plasma and oxidized to pyridine analogues prior to chromatography. Each compound was present in plasma at 20 ng/ml concentration.

Fig. 3. LC separation of nimodipine, nimodipine pyridine analogue, nimodipine metabolites II and III, and internal standards extracted from plasma. Each compound was present in plasma at 20 ng/ml concentration. Spherisorb ODS (3-μm) column was eluted with chromatographic solvent A. The detector was set at 238 nm wavelength, 0.003 a.u.f.s.

ODS column. The same column with two different solvent systems was used to obtain the above chromatograms. Solvent system A was used for analysis of nimodipine and P analogue while solvent system B was used for analysis of nimodipine and two demethylated nimodipine metabolites (M_{II} and M_{III}). Although solvent system A does not resolve M_{II} from M_{III} it was used as an alternative system for assay of some patient samples which were subject to plasma constituent nimodipine and/or P analogue interference.

Figs. 5 and 6 depict the GC and the LC calibration curves. Since the GC assay was used only for analysis of nimodipine after conversion to P analogue while the LC assay was used for analysis of the intact nimodipine as well as endogenous P analogue and two demethylated nimodipine metabolites, the GC calibration required only one calibration curve while the LC calibration required four curves. Linear GC and LC calibration curves were observed within the concentration range of the samples.

Tables I and II present the GC and LC calibration regression line data which

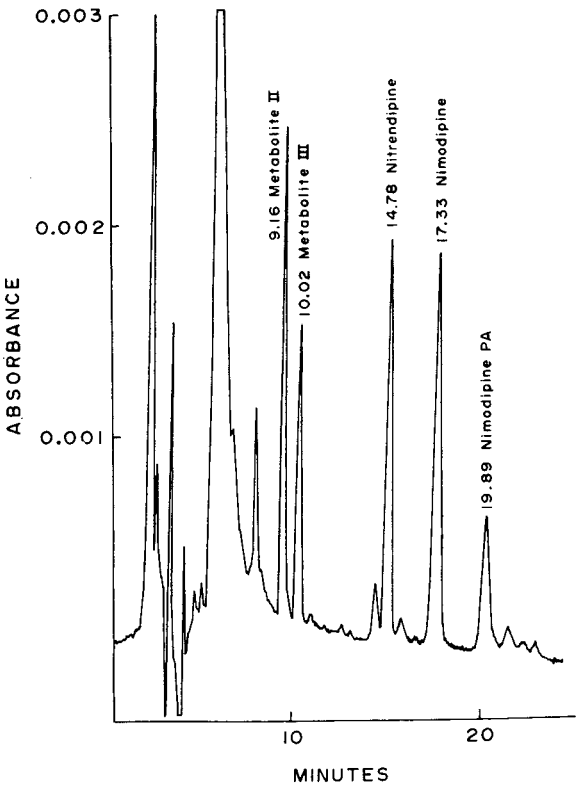


Fig. 4. LC separation of nimodipine, nimodipine pyridine analogue, nimodipine metabolites II and III, and nitrendipine internal standard A extracted from plasma. Each compound was present in plasma at 20 ng/ml concentration. Spherisorb ODS (3-μm) column was eluted with chromatographic solvent B. The detector was set at 238 nm wavelength, 0.003 a.u.f.s.

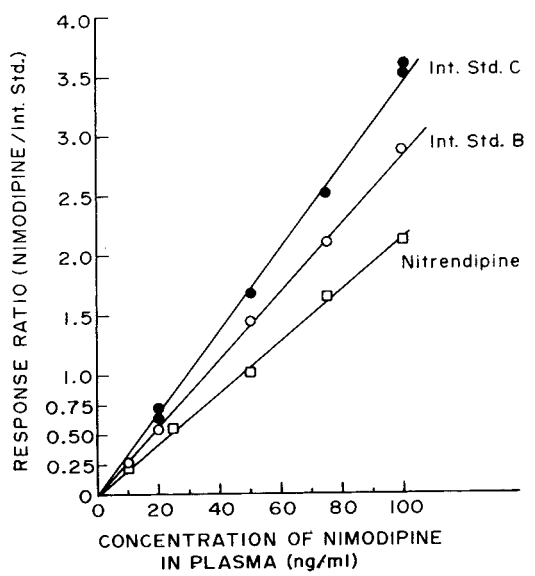


Fig. 5. GC calibration curves of nimodipine obtained by reference to three internal standards.

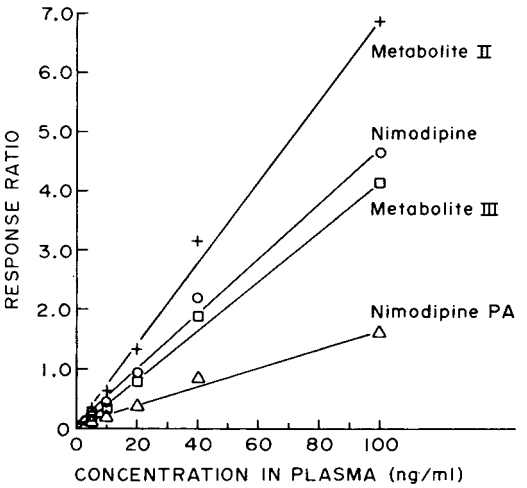


Fig. 6. LC calibration curves of nimodipine, nimodipine pyridine analogue, and nimodipine metabolites II and III, obtained by reference to nitrendipine internal standard A.

TABLE I

GC CALIBRATION CURVE DATA

Peak ratio	Correlation coefficient	Intercept (ng/ml)	Standard error of estimate
Nimodipine/nitrendipine	0.9989	−0.07	1.94
Nimodipine/I.S. B	0.9996	1.11	1.10
Nimodipine/I.S. C	0.9983	2.35	2.41

TABLE II

LC CALIBRATION CURVE DATA

Peak ratio	Correlation coefficient	Intercept (ng/ml)	Standard error of estimate
Nimodipine/nitrendipine	0.9965	0.05	0.18
Nimodipine/I.S. B	0.9926	−0.09	1.32
Nimodipine/I.S. C	0.9996	−1.60	0.28
PA/nitrendipine*	0.9925	0.05	0.09
M_{II}/nitrendipine	0.9970	0.03	0.24
M_{III}/nitrendipine	0.9975	−0.03	0.13

*PA = pyridine analogue.

reflect the accuracy of the GC and LC assay procedures. In general, GC quantitation involved nitrendipine (A) or B internal standards while LC quantitation was based on either internal standard A or C. However, because M_{III} was not resolved completely by LC from internal standards B and C, LC quantitation of M_{II} and M_{III} demethylated nimodipine metabolites was based on internal standard A.

Table III presents the short term (within a day) and long term (between

TABLE III

SHORT- AND LONG-TERM PRECISION OF GC AND LC DATA

Assay	Internal standard	Short-term*				Long-term**			
		n***	Mean	S.D.	R.S.D.(%)	n***	Mean	S.D.	R.S.D.(%)
GC	Nitrendipine	14	0.390	0.016	4.68	14	0.414	0.027	6.63
	C	14	1.697	0.097	5.71	14	1.615	0.093	5.75
LC	Nitrendipine	11	27.2	1.68	6.20	14	28.0	1.400	5.10
	C	11	35.8	1.34	3.85	17	36.2	2.300	6.30

*Within a day.
**Between different days.
***n = number of determinations.

different days) GC and LC precision data. The results presented in Table III are based on peak height determinations. The data presented indicate no significant differences between the GC and LC assay precision. As expected, in general, the single-day assay results are slightly more precise than the assay results obtained on different days.

Selectivity of the GC and LC procedures

The above GC and LC separations illustrate the relative selectivity of both procedures. Both chromatographic procedures resolve compounds of interest from the internal standards and plasma constituents. However, because of chemical instability at GC temperatures only the LC procedure is suitable for quantitative analysis of underivatized nimodipine and the demethylated nimodipine metabolites. Injection of underivatized nimodipine on the GC column using the GC conditions specified in the present procedure yields 20—60% conversion to P analogue. Recent results indicate that decomposition of nimodipine during GC chromatography can be reduced to 5–20% at a higher gas flow-rate and lower injector and column temperature but the detection limit of nimodipine under these conditions is reduced from 1 to about 5 ng/ml.

Apparent decomposition of a structurally related dihydropyridine compound during GC was observed by GC—mass spectrometric (MS) analysis [11]. To circumvent this complication the selectivity of the GC assay was enhanced by use of a thin-layer chromatographic (TLC) separation step prior to GC analysis [12]. The present LC procedure provides an alternative to TLC separation and GC quantitation.

LC separation was also used to enhance the selectivity and sensitivity of the cerebrospinal fluid assay and for detection of the demethylated metabolites of nimodipine which could not be detected by the present GC procedure. Two demethylated metabolites of nimodipine were observed in serum [15, 16]. Both metabolites were detected, resolved, and quantitated by the LC procedure.

Sensitivity of the GC and LC procedures

Based on the signal-to-noise ratio of the GC and LC chromatograms the

detection limit of the procedures described above is about 1 ng/ml. The GC procedure as written specifies injection of 1—2 μl of 1 ml toluene solution. In principle, the GC detection limit could be decreased to 0.2 ng/ml if a smaller volume of toluene and/or a larger injection volume were used. However, because of extraneous peaks, electron-capture detector overload and concurrent enhancement of signal-to-noise ratio, the ultimate practical detection limit of the GC assay is about 0.5 ng/ml for relatively clean sample extracts. Similar limitations apply to potential enhancement of the LC assay sensitivity.

A more effective approach to enhancement of GC and LC assay detection sensitivity involves a combination of an LC clean-up step with a GC quantitation step. This procedure was developed to detect sub-nanogram concentrations of nimodipine in CSF. The effectiveness of this approach is based on the fact that the high capacity and selectivity of the LC column facilitates preparation of a relatively pure nimodipine fraction. This fraction can be concentrated and reacted with nitrogen dioxide in the absence of extraneous compounds to yield pyridine analogue derivative. An external and/or internal standard technique was used to correct for possible partial loss of nimodipine during LC chromatography and nitrogen dioxide reaction. The above approach is also applicable to analysis of samples from the terminal phase of drug elimination. Drug concen-

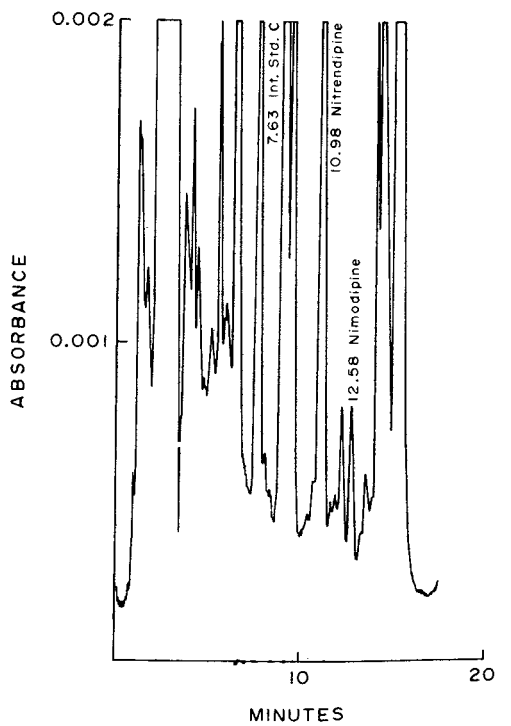


Fig. 7. LC separation of patient CSF extract containing about 0.3 ng of nimodipine per ml of CSF. Nitrendipine and internal standard B were added to CSF prior to extraction to yield 5 ng/ml concentration. The sample injected onto the column was derived from a 2-ml aliquot of CSF. Spherisorb ODS (3-μm) column was eluted with chromatographic solvent A. The detector was set at 238 nm, 0.002 a.u.f.s.

tration in such samples is usually below the detection limit of the direct GC and LC assays.

Some patient CSF samples containing less than ng/ml concentration of nimodipine were estimated directly from the LC chromatogram obtained at high detector sensitivity setting (see Fig. 7). However, subsequent GC analysis of the nimodipine LC fraction enhanced the selectivity, sensitivity, and accuracy of the nimodipine determination. For additional confirmation, some CSF extracts were also analyzed by high resolution, direct inlet MS after LC purification. MS data were quantitated using a multiple ion detection technique based on a deuterated nimodipine internal reference standard.

Table IV presents the CSF results observed by the direct LC, LC—GC and the LC—MS assay procedures. Since each of the above assays is subject to 0.1—0.2 ng/ml error the data presented in Table IV agree within experimental error. It is also of interest to note that the observed concentration of nimodipine in CSF is 10—20 times lower than the concentration of nimodipine in plasma. Since nimodipine is about 95% protein-bound [17] the observed concentration of nimodipine in CSF is approximately equal to the concentration of the free (unbound) nimodipine fraction in plasma. A similar relationship between CSF and plasma concentration of diphenylhydantoin was reported [18, 19].

TABLE IV

COMPARISON OF CEREBROSPINAL FLUID ASSAY BY LC, LC—GC AND LC—MS PROCEDURES

Sample No.	Assay results (ng/ml)		
	LC	LC—GC	LC—MS
621-1	0.3*	0.2	0.3
621-2	0.8	0.8	0.6
644-1	N.P.**	1.0***	0.4
644-2	0.7	0.8	0.4
657-1	N.P.	0.8	0.9
657-2	0.8	0.6	0.8

*2 ml of CSF were used to obtain this estimate.
**N.P. = Not possible to estimate.
***Interference.

Clinical and pharmacokinetic assay results

The GC and LC procedures were applied to analysis of blood plasma collected from patients enrolled in sub-arachnoid hemorrhage and migraine clinical trials and preliminary pharmacokinetic studies in normal volunteers. Statistical analysis of the analytical data collected from patients indicates that there is a correlation between the GC and the combined (nimodipine plus P analogue) LC assay results. Analysis of 80 clinical study plasma samples by the GC and the LC procedures yielded a correlation coefficient of 0.943.

Table V presents preliminary pharmacokinetic study GC and LC assay results. The GC and the combined LC assay results observed with pharmacokinetic data yielded a correlation coefficient of 0.999. It is also of interest to

TABLE V

PRELIMINARY PHARMACOKINETIC STUDY; GC AND LC ASSAY RESULTS BASED ON ONE NORMAL VOLUNTEER

GC vs. LC: Correlation coefficient = 0.999; standard error of estimate = 3.01; intercept = 0.94; slope = 1.00.

Compound(s) assayed	Assay	Concentration (ng/ml) at time (h) after drug administration										
		0.25	0.5	0.75	1.0	1.5	2	3	5	8	24	AUC_{0-8}
Nimodipine + PA*	GC	22.9	126.2	177.7	181.6	86.7	47.5	28.4	13.9	5	2	313.6
Nimodipine	LC	11.9	86.8	116.6	130.6	66.2	34.2	19.6	10.9	4.1	2	224.4
PA	LC	7.0	42.9	56.5	52.1	21.7	11.6	7.5	4.1	2.0	2	90.2
Nimodipine + PA	LC	18.9	129.7	173.1	182.7	87.9	45.8	27.1	15.0	6.1	2	314.6
M_{II}	LC	16.8	143.9	200.6	257.7	126.5	73.4	46.8	25.4	10.9	2	455.3
M_{III}	LC	18.5	189.8	282.8	388.0	172.3	82.8	51.5	24.4	8.5	2	569.8

*PA = pyridine analogue.

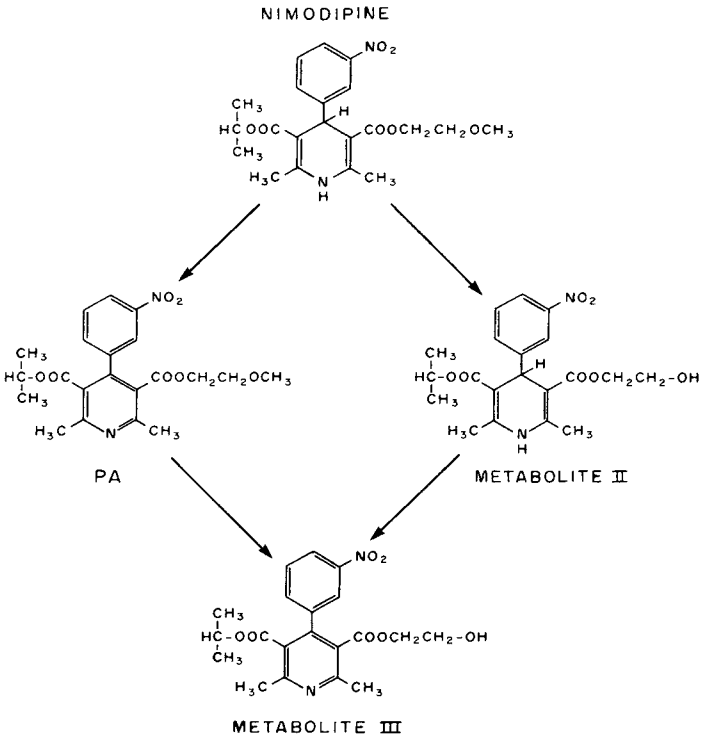


Fig. 8. Initial metabolic reactions of nimodipine.

note that although plasma concentration of nimodipine is considerably greater than the plasma concentration of P analogue, the concentration of the demethylated metabolites is greater than the concentration of nimodipine. The above observation suggests that demethylation occurs more rapidly than oxidation and that the concentration of the demethylated and oxidized metabolite III (M_{III}) is highest because it can form as depicted in Fig. 8 by two sequential reactions. However, the above hypothesis assumes that the elimination and subsequent reaction rates of metabolites observed are similar. It should also be noted that each one of the metabolic products is subject to hydrolysis of each ester side-chain and hydroxylation of methyl groups on the pyridine ring thus the sequence of reactions outlined in Fig. 8 depicts only a fragment of the overall metabolic scheme which was discussed in a previous publication [16]. With the exception of demethylation reaction similar metabolic pathways were proposed to explain nifedipine metabolism [9, 10]. As reported previously the observed dihydropyridine metabolic products are 10--500 times less potent than nimodipine while the oxidized pyridine products did not exhibit vascular activity [15].

However, the analytical procedures described in this publication are not suitable for analysis of the more polar acidic hydrolysis products of metabolism. Thus, additional work is ongoing to elucidate pharmacokinetics and human metabolism of nimodipine.

CONCLUSIONS

Sensitive and precise GC and LC procedures for assay of nimodipine in patient and volunteer plasma were developed. The relative standard deviation and detection limit of both procedures were about 5% and 1 ng/ml, respectively. Comparison of the results observed with both procedures indicates that the GC and LC data can be correlated providing that the GC results are corrected for the presence of endogenous nimodipine pyridine analogue. Since the LC assay determines nimodipine directly it should be used as a reference method.

Greater selectivity and sensitivity were achieved by combining an LC clean-up step with GC quantitation. This procedure allowed measurement of nimodipine in cerebrospinal fluid and plasma below the ng/ml concentration range. The direct GC, LC and the LC—GC procedures are also applicable to analysis of other dihydropyridine calcium antagonists such as nitrendipine.

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APPLICATION OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH SYNCHRONIZED ACCUMULATING RADIOISOTOPE DETECTOR TO ANALYSIS OF GLYCERYL TRINITRATE AND ITS METABOLITES IN RAT PLASMA

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SUMMARY

A new, sensitive, and specific high-performance liquid chromatographic method for the quantitative analysis of [^{14}C]glyceryl trinitrate and its four metabolites in plasma is described. The drugs are extracted from 0.05 ml of plasma with methanol and analyzed by high-performance liquid chromatography using a synchronized accumulating radioisotope detector. The limit of detection is 0.2 ng per injection. The within-day coefficient of variation is 5.9% at a concentration of 27.0 ng per ml of plasma. The method was applied to single-dose pharmacokinetics of glyceryl trinitrate in rat.

INTRODUCTION

Glyceryl trinitrate (GTN) has been used extensively for many years for the treatment of angina pectoris. It is known that GTN is rapidly metabolized by endogenous esterases to glyceryl dinitrates (GDNs) [1], which are about ten times less potent as vasodilators [2], and inactive mononitrates (GMNs) [3]. It is therefore essential to carry out simultaneous analysis of plasma levels of both GTN and its metabolites to perform pharmacokinetic studies of GTN in detail.

The analysis of GTN in plasma has been performed primarily by gas—liquid

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chromatography (GLC) with electron-capture detection [4—6]. Although this method possesses high sensitivity for GTN, it requires extensive clean-up procedures and solvent purification. Furthermore, GTN is thermally unstable and suffers decomposition on the GLC column [5]. It is a problem to achieve an acceptably accurate and reproducible analysis of GTN and its metabolites because the degradation product has a retention time equal to that of 1,2-glyceryl dinitrate (1,2-GDN).

Several high-performance liquid chromatographic (HPLC) methods have been reported for the analysis of GTN and its metabolites [7—10]. Although these methods can separate GTN from its mono- and dinitrate metabolites, the sensitivity and specificity are inadequate for biological samples due to use of spectrophotometric detection. Recently Spanggord and Keck [11] have reported a more sophisticated HPLC method with a thermal-energy analyzer to determine GTN and four degradation products. The disadvantages in this method are the costs of the thermal-energy analyzer detector used, and the lack of adequate accuracy.

Radioactive isotope (RI) tracer techniques have been widely applied to investigate the absorption, distribution, and biotransformation of GTN in animal species [12—14]. In this tracer technique thin-layer chromatography was used for the separation of GTN and its metabolites, but only partial resolution and low sensitivity were obtained. Baba et al. [15] have developed a synchronized accumulating radioisotope detector to detect radioactive substances eluted from an HPLC column. This radio-HPLC system gives satisfactory resolution with high detection efficiency.

The purpose of the present paper is to report a new radio-HPLC method for the analysis of GTN and its metabolites in rat plasma, and its application to the study of single-dose pharmacokinetics of GTN in rat.

MATERIALS AND METHODS

Chemicals and reagents

[U-^{14}C]Glyceryl trinitrate ([^{14}C]GTN) (224.6 μCi/mg) was supplied by Daiichi Kagaku Yakuhin (Tokyo, Japan). 1,2-Glyceryl dinitrate (1,2-GDN), 1,3-glyceryl dinitrate (1,3-GDN), 1-glyceryl mononitrate (1-GMN), and 2-glyceryl mononitrate (2-GMN) were synthesized in this laboratory. The methanol used for the mobile phase was of chromatographic purity and was obtained from Wako (Osaka, Japan). All other chemicals and solvents were analytical grade and were used without further purification.

Radio-HPLC system

The high-performance liquid chromatograph consisted of a Shimadzu (Kyoto, Japan) Model LC-2P solvent metering system, a Shimadzu UVD-2 ultraviolet (UV) detector (detection at 254 nm), an Aloka (Tokyo, Japan) synchronized accumulating radioisotope detector RLC-R17-748, and a Shimadzu two-pen recorder R-12.

A prepacked Chemcopak Zorbax-ODS (250 × 4.6 mm I.D.; particle size 8 μm; Chemco) was used. In order to avoid contamination of the analytical column, a pre-column (50 × 3.2 mm I.D.) tap-filled with Zorbax-ODS (particle

size 8 μm; Chemco) was placed between the injector and the analytical column. The mobile phase consisted of methanol—water (6:4, or 4:6). The mobile phase was degassed by ultrasonic vibration. The assays were performed at ambient temperature with a flow-rate of 1 ml/min. The elute from the HPLC column was mixed with a liquid scintillator* (7.0 ml/min) after UV detection and the resulting solution (8.0 ml/min) was passed through the five counting cells having an effective cell volume of 1.1 ml per cell.

Liquid scintillation counter

Authentic measurements of the radioactivity were conducted with the use of an Aloka LSC-502 liquid scintillation counter and corrections for quenching were achieved by using external standard. The sample was dissolved in 10 ml of scintillation solvent.

Sample preparation for RI-HPLC

To 0.05 ml of rat plasma sample was added 0.01 ml of 1 *M* silver nitrate and extracted three times with 0.2 ml of methanol. The organic layer was collected and condensed to about 0.1 ml. After centrifugation (1000 *g*, 10 min), 10—30 μl of the solution were subjected to RI-HPLC analysis.

Recovery

The absolute extraction recovery of [^{14}C]GTN from rat plasma was measured in the following way. To 0.05-ml portions of pooled blank rat plasma were added 5 nCi of [^{14}C]GTN dissolved in 10 μl of saline. The sample was extracted and concentrated according to the sample preparation procedure. The concentrated sample was subjected to liquid scintillation counting. Absolute extraction recovery was calculated by comparing the radioactivity from the [^{14}C]GTN added with the radioactivity from the plasma extract. The absolute extraction recovery of metabolites was estimated as follows. The accurate disintegration counts in the rat plasma (0.05 ml each) at 8 and 15 min after intravenous administration of [^{14}C]GTN (20 μCi) were made by a liquid scintillation counting method. The same rat plasma (0.05 ml each) was extracted and concentrated according to the sample preparation for RI-HPLC. The concentrated extracts were subjected to liquid scintillation counting. Absolute extraction recovery was calculated by comparing the radioactivity in the plasma with the radioactivity in the plasma extract.

Detection efficiency

The detection efficiency of this RI-HPLC system for [^{14}C]GTN was estimated by comparing the counts under the peak obtained from the injection of known amounts of [^{14}C]GTN with the counts of [^{14}C]GTN measured by a liquid scintillation counter. The detection efficiency for [^{14}C]GTN metabolites was estimated as follows. An accurate disintegration count in the rat plasma sample at 5 min after intravenous administration of [^{14}C]GTN was made by a liquid scintillation counting method. The rat plasma sample was

*Dioxane—toluene—ethyl cellosolve (75:15:10, v/v) containing 100 g of naphthalene, 4 g of 2,5-diphenyloxazole, and 0.4 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene per litre.

then injected into the RI-HPLC system and the total counts under the peaks were calculated. The detection efficiency was estimated by comparing the total counts with the injected accurate counts.

Reproducibility

A male rat was injected intravenously with a single dose of 20 μCi of [^{14}C] GTN. After 5 min the rat was sacrificed and 8 ml of blood were collected. Aliquots (0.5-ml) of the resulting plasma were processed as described by the sample preparation for RI-HPLC. The methanolic preparations of 10 μl of the concentrated sample were injected into the RI-HPLC four times. In addition, four aliquots (0.3 ml each) of the same rat plasma sample were analyzed in replicate according to the sample preparation and RI-HPLC procedures described above.

Pharmacokinetics of a single intravenous dose of [^{14}C] GTN in rat

Three male Wistar-SPF albino rats, weighing approximately 300 g, were injected intravenously into the right jugular vein with a single dose of [^{14}C] GTN (112.3 μCi/kg) dissolved in 0.3 ml of saline. Heparinized blood samples (0.15—0.2 ml) were taken from the left jugular vein at 2, 5, 10, 20, 30, 60, and 120 min after injection. The plasma was obtained by centrifuging the blood at 1000 *g* for 10 min. The plasma samples were divided into two sets. Each plasma sample in one set (0.02 ml) was subjected to liquid scintillation counting and the total radioactivity in the plasma was determined. Each plasma sample in the other set (0.05 ml) was processed as described by the sample preparation for RI-HPLC and then subjected to RI-HPLC analysis. The estimation of the amounts of [^{14}C] GTN and its metabolites in plasma was based on the relative peak intensities on the chromatogram and the total radioactivity measured by a liquid scintillation counter.

RESULTS AND DISCUSSION

It is well known that GTN is rapidly degraded in rat plasma with a half-life of about 20 min at 37°C [16]. Yap et al. [4] reported that this degradation of GTN could be inhibited for at least 1.5 h by the addition of silver nitrate in a 0.05 *M* final concentration. It was decided to add silver nitrate to the rat plasma sample.

The absolute extraction recovery of [^{14}C] GTN from spiked rat plasma was 86.5 ± 0.5% (n = 4). The absolute extraction recovery of total ^{14}C-radioactivity from drug-supplemented rat plasma was 84.7% (n = 2) for the plasma taken 8 min (GTN, GDNs, GMNs), and 81.0% (n = 2) for the plasma taken 15 min (GDNs, GMNs) after administration of [^{14}C] GTN. These data seem to suggest that methanol is an efficient extraction solvent for [^{14}C] GTN and its metabolites ([^{14}C] GDNs, [^{14}C] GMNs) and there is no difference between [^{14}C] GTN and its metabolites in extraction efficiency.

The mobile phases studied included various ratios of methanol—water, which is a simple and commonly used solvent combination for reversed-phase chromatography. The resolution could be regulated by changing the methanol concentration. When the methanol concentration is 60%, the retention times for

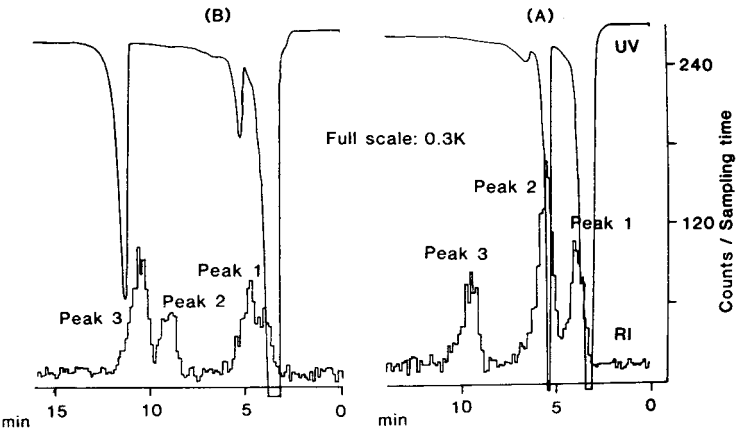

Fig. 1. Radioisotope high-performance liquid chromatograms for an extract of a plasma sample 2 min after intravenous administration of [^{14}C]GTN (112.3 μCi/kg). (A) Mobile phase = 60% methanol; peak 1 = GMNs, Peak 2 = GDNs, peak 3 = GTN. (B) Mobile phase = 40% methanol; peak 1 = GMNs, peak 2 = 1,3-GDN, peak 3 = 1,2-GDN.

GTN, a mixture of GDNs, and a mixture of GMNs are 9.5, 5.4, and 3.7 min, respectively. Using 40% methanol, 1,2 and 1,3-glyceryl dinitrate can be separated with retention times of 10.7 and 9.0 min, respectively. In view of these results 60% and 40% of methanol in water were employed in the analysis. Fig. 1 shows chromatograms of a plasma sample which was collected from a rat at 2 min after intravenous administration of [^{14}C]GTN [methanol 60% (A), 40% (B)]. The retention time of each peak was compared with that of a reference non-labeled compound. The t_R values of peaks 1, 2 and 3 in Fig. 1A were similar to those of GMNs, GDMs and GTN, respectively. The t_R values of peaks 1, 2 and 3 in Fig. 1B were similar to those of reference GMNs, 1,3-GDN and 1,2-GDN, respectively. When a UV detector was used for HPLC effluents, many peaks were found which did not originate from the drug. When the RI detector is used, therefore it is important to clarify in advance which peaks in the HPLC are being referred to.

The detection efficiencies of the RI-HPLC system for authentic [^{14}C]GTN and its metabolites in rat plasma sample were 92.3 ± 2.5 (n = 3) and 89.1 ± 0.9% (n = 4), respectively. The results demonstrate excellent detection efficiencies not only for [^{14}C]GTN but also for its metabolites, and suggest that there is no difference between [^{14}C]GTN and its metabolites in detection efficiency.

The reproducibility in counting of the RI-HPLC was determined by injecting the same test rat plasma sample into the RI-HPLC system four times. The results are shown in Table I. The present method obviously provides very good reproducibility of counting. The within-day precision of the RI-HPLC analysis for [^{14}C]GTN and its metabolites was good, as assessed by conducting replicate (n = 4) analyses of the same rat plasma at 5 min after intravenous administration of [^{14}C]GTN (Table II). It is apparent that the present method has a better reproducibility than the thermal-energy analyzer method (GTN 0.5 ng, C.V. 25%; 13.2 ng, C.V. 12%) [11].

TABLE I

REPRODUCIBILITY IN COUNTING

	Counts		
	GMNs	GDNs	GTN
1	990	1940	329
2	996	1916	372
3	1030	1911	343
4	1046	1952	336
Mean	1016	1930	345
S.D.	26.9	19.5	18.9
C.V. (%)	2.6	1.0	5.5

TABLE II

REPRODUCIBILITY OF ANALYSES OF [^{14}C]GTN AND ITS METABOLITES IN RAT PLASMA

	ng/ml plasma		
	GMNs	GDNs	GTN
1	42.7	118.7	27.3
2	43.7	115.7	27.7
3	42.3	116.7	28.3
4	41.7	116.3	24.7
Mean	42.6	116.9	27.0
S.D.	0.84	1.30	1.59
C.V. (%)	2.0	1.1	5.9

In the RI-HPLC method the detection limit was reported as about 45 pCi for ^{14}C [15]. The lower limit of detection for [^{14}C]GTN was found to be about 0.2 ng injected, since the specific activity of [^{14}C]GTN was 224.6 μCi/mg.

The time course of plasma concentrations of [^{14}C]GTN and its metabolites following intravenous administration of [^{14}C]GTN in three rats is shown in Fig. 2. The half-lives of [^{14}C]GTN and its metabolites are presented in Table III. The data give some insight into interindividual differences. It should be pointed out that the ability to use RI-HPLC which gives satisfactory resolution with high detection efficiency is a great advantage to evaluate interindividual differences of pharmacokinetics in small laboratory animals. The disappearance of [^{14}C]GTN in plasma followed first-order kinetics and was very rapid. By 20 min there was no detectable unchanged [^{14}C]GTN. The elimination half-life of [^{14}C]GTN was 2.2 ± 0.21 min. This value agrees with the values found using a GLC method [17]. The appearance of 1,3-[^{14}C]GDN and 1,2-[^{14}C]GDN was very rapid. In the first blood sample taken 2 min after the administration of [^{14}C]GTN, peak concentration of GDNs was detected.

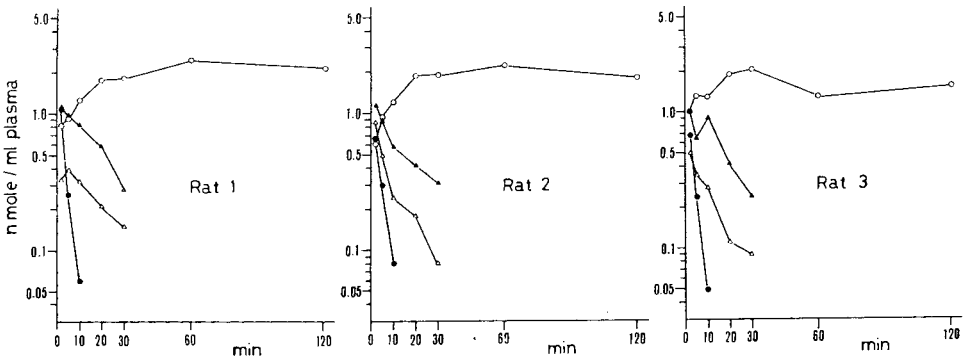

Fig. 2. Plasma concentrations of GTN (•), 1,2-GDN (▲), 1,3-GDN (△), and GMNs (○) as a function of time after intravenous administration of [^{14}C]GTN (112.3 μCi/kg) in male rats.

TABLE III

PLASMA HALF-LIVES OF [^{14}C]GTN AND ITS METABOLITES IN PLASMA AFTER INTRAVENOUS ADMINISTRATION OF [^{14}C]GTN IN MALE RATS

	Min		
	1,2-GDN	1,3-GDN	GTN
1	14.6	21.2	1.9
2	15.3	9.1	2.6
3	14.3	11.1	2.1
Mean	14.7	13.8	2.2
S.E.	0.29	3.75	0.21

Thereafter a monoexponential decline in the plasma levels was observed, and 30 min after [^{14}C]GTN administration the levels were below the limit of detection (0.05 nmol per ml of plasma). Plasma [^{14}C]GMNs levels increased gradually after the administration of [^{14}C]GTN. Peak plasma [^{14}C]GMNs concentrations were generally detected between 30 and 60 min after [^{14}C]GTN administration. The rate of disappearance of [^{14}C]GMNs was slower than of [^{14}C]GTN and [^{14}C]GDNs.

The present synchronized accumulating radioisotope detector HPLC method allows the simple, sensitive, rapid, and selective determination of [^{14}C]GTN and its metabolites simultaneously in rat plasma with good accuracy and precision. The method has made it possible to perform pharmacokinetic studies of GTN and to investigate interindividual variations in small laboratory animals.

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SIMULTANEOUS DETERMINATION OF ELEVEN ANTIEPILEPTIC COMPOUNDS IN SERUM BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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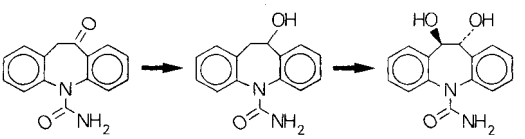
SUMMARY

A high-performance liquid chromatographic method for the simultaneous determination of carbamazepine, CGP 10 000 (a common metabolite of carbamazepine and oxcarbazepine), desmethylmephenytoin, 10,11-epoxycarbamazepine, ethosuximide, GP 47 779 (the active metabolite from oxcarbazepine, a new drug made by Ciba-Geigy), mephenytoin, phenylethylmalonamide, primidone, pheneturide, phenobarbital and phenytoin is described. The serum is extracted with ethyl acetate at pH 3.9 and the dried extract is dissolved in 70% ethanol in water and an aliquot is injected into a Hewlett-Packard 1084 B liquid chromatograph. A reversed-phase (RP-8) column is used with acetonitrile and water as the mobile phase. The eluted drugs are detected at 207 nm. The recovery of the compounds varies from 76% to 95% with the day-to-day precision (C.V.) between 3.8% and 9.8%, the within-day precision between 1.8% and 5.8% and run-to-run precision between 1.0% and 2.6%.

INTRODUCTION

Several high-performance liquid chromatographic (HPLC) methods for the determination of antiepileptic drugs have been published. None includes in a simultaneous program all the drugs routinely analysed in our laboratory. Riedmann et al. [1] described in 1981 the use of HPLC for the measurement of eight antiepileptic drugs and metabolites. Not included were GP 47 779 (Fig. 1), mephenytoin, desmethylmephenytoin and pheneturide. The peak they



Oxcarbazepine GP 47 779 CGP 10 000

Fig. 1. Biotransformation of oxcarbazepine.

named "carbamazepine metabolite" must probably correspond to CGP 10 000 (Fig. 1). Their chromatographic separation time is about 25 min. During the preparation of this paper, Kabra et al. [2] published a fast liquid chromatographic method for five antiepileptic drugs. The chromatography is completed in less than 2.5 min. But in this short time it is not possible to detect simultaneously phenylethylmalonamide, mephenytoin and desmethylmephenytoin. The retention time of 10,11-epoxycarbamazepine is very close to that of the internal standard. GP 47 779, CGP 10 000 and pheneturide are not mentioned. Although CGP 10 000 appears in serum in concentrations between 19% and 220% (mean 52%, $n = 19$) of the carbamazepine concentration, and therefore could cause serious interference, it is not mentioned in recently published simultaneous methods including carbamazepine [2, 3].

The present paper describes our HPLC procedure which has been used routinely in our laboratory for two and a half years. The chromatography of the eleven antiepileptic substances and CGP 10 000 is completed in about 15 min.

MATERIALS AND METHODS

Apparatus

The high-performance liquid chromatograph is a Hewlett-Packard Model 1084 B, equipped with a variable-wavelength detector (190—600 nm). The reversed-phase column, LiChrosorb RP-8, particle size 10 μm, 25 × 0.4 cm (Merck), is gradient-eluted with 18% to 23% acetonitrile in water for 6 min and then isocratically for 9 min until the wash program starts. After 16 min the rate of acetonitrile is 80% and remains so for 2.5 min. After 19.5 min acetonitrile has returned to 18% and after 2 min of equilibration the chromatograph is ready for the next injection. Total time is 21.5 min. The flow-rate is 2.4 ml/min, the column temperature 35°C and the column effluent is monitored at 207 nm.

The diluter used is a Hamilton digital diluter; the Gerhardt shaker with a modified tube holder is suitable for horizontally shaking 35 tubes simultaneously.

Reagents and standards

The buffer is 500 g of ammonium sulfate and 41.4 g of $NaH_2PO_4 \cdot H_2O$ dissolved in 1000 ml of water, pH 3.9 [4].

The internal standard is 100 mg of hexobarbital in 100 ml of ethyl acetate, the working solution 11 ml of internal standard in 2.5 l of ethyl acetate (Merck).

Acetonitrile and ethanol are LiChrosolv reagents from Merck. The water used in the mobile phase is first distilled and then filtered through a Millipore filter type AA with a pore size of 0.8 μm.

The composition of the drug standard mixture is given in Table I. It is stable for at least half a year at room temperature.

Procedure

To 1000 μl of buffer in a 12-ml Pyrex screw-top tube are added 500 μl of

TABLE I

DRUG STANDARD MIXTURE

Drug	Substance concentration in ethanol (mg per 200 ml)	Concentration in serum (40 μl of standard in 500 μl of serum) (μmol/l)
Caffeine	8.01	16.5
Carbamazepine (Ciba-Geigy)	17.04	28.8
CGP 10 000 (Ciba-Geigy)	10.38	15.3
Desmethylmephenytoin (Aldrich)	30.82	60.3
10,11-Epoxycarbamazepine (Ciba-Geigy)	7.14	11.3
Ethosuximide (Park-Davis)	77.65	220.0
GP 47 779 (Ciba-Geigy)	27.74	43.6
Mephenytoin (Sandoz)	16.68	30.5
Phenylethylmalonamide (Imperial Chemical Industries)	19.65	38.1
Primidone (Imperial Chemical Industries)	21.08	38.6
Pheneturide (Sapos)	25.02	48.5
Phenobarbital (Aldrich)	44.84	77.3
Phenytoin (Aldrich)	35.76	56.6

serum with 5 ml of working solution (internal standard) using a Hamilton diluter. For calibration 40 μl of the drug standard are placed in a tube to which drug-free serum is added. The tubes are shaken for 10 min and centrifuged (2500 *g*) for 10 min. The supernatant is decanted into a tube and evaporated to dryness at 37°C by means of a direct air stream. The residue is dissolved in 400 μl of 70% ethanol in water and 10 μl are injected into the liquid chromatograph.

RESULTS

Calibration

The liquid chromatograph is calibrated with a standard containing concentrations within the therapeutic range, with the exception of 10,11-epoxycarbamazepine and mephenytoin. Caffeine is included in the drug standard but is not measured. It is there just to make the standard realistic, as most patients have this compound. Fig. 2 shows a chromatogram of the drug standard which was added to drug-free serum and extracted as a patient serum.

Recovery

The recovery of the drugs is shown in Table II. Due to the high solubility of ethosuximide in water, only 76% is extracted. The recovery of the other compounds ranges from 89% to 95%.

Precision

Table III shows the precision of the method. The only drug with a relatively poor day-to-day precision (C.V. = 9.8%) is ethosuximide. To compensate for its high C.V. we are running serum samples with ethosuximide twice and use

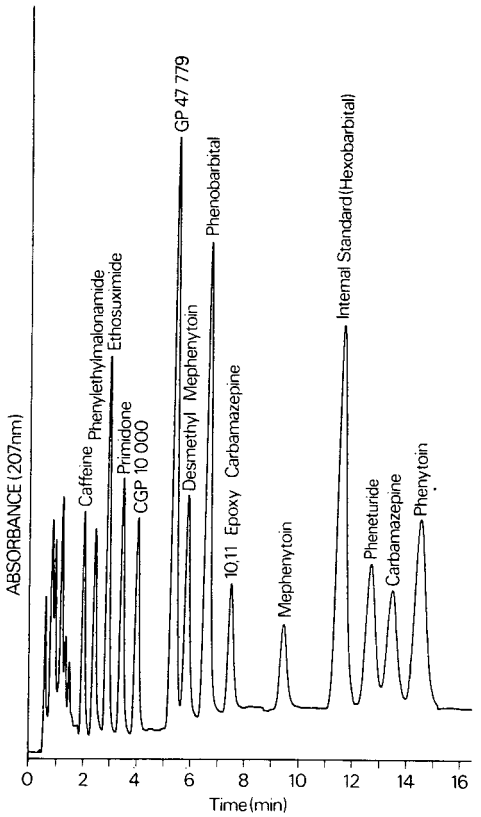


Fig. 2. Chromatographic separation of the drug standard which was added to drug-free serum and extracted as a patient serum. The concentrations are those listed in Table I.

TABLE II

RECOVERY OF DRUGS ADDED TO SERUM

Drug	Concentration (μmol/l)	Mean recovery* (%)
Carbamazepine	38.2	94
CGP 10 000	15.3	89
Desmethylmephenytoin	64.4	94
10,11-Epoxycarbamazepine	16.9	93
Ethosuximide	313.0	76
GP 47 779	43.8	93
Hexobarbital	169.3	90
Mephenytoin	37.8	92
Phenylethylmalonamide	38.0	95
Primidone	40.5	95
Pheneturide	54.5	90
Phenobarbital	76.7	94
Phenytoin	59.1	89

*$n = 7$.

TABLE III

PRECISION OF THE METHOD

n = 30 in every case.

Drug	Range ± S.D. (μmol/l)	C.V. (%)
Day-to-day		
Carbamazepine*	21.7 ± 1.4	6.4
CGP 10 000**	15.4 ± 0.8	5.2
Desmethylmephenytoin**	61.2 ± 2.8	4.6
10,11-Epoxycarbamazepine**	11.6 ± 1.0	8.5
Ethosuximide*	364.4 ± 35.6	9.8
GP 47 779**	44.4 ± 1.7	3.9
Mephenytoin**	30.4 ± 2.2	7.1
Phenylethylmalonamide**	38.7 ± 2.8	7.2
Primidone*	47.7 ± 2.7	5.6
Pheneturide**	73.1 ± 2.5	3.5
Phenobarbital*	66.7 ± 2.5	3.8
Phenytoin*	41.9 ± 1.6	3.8
Within-day		
Carbamazepine*	21.6 ± 1.0	4.6
Ethosuximide*	370.5 ± 21.7	5.8
Primidone*	48.5 ± 1.2	2.4
Phenobarbital*	68.2 ± 1.2	1.8
Phenytoin*	40.5 ± 0.9	2.3
Run-to-run		
Carbamazepine***	28.1 ± 0.7	2.5
Ethosuximide***	391.1 ± 4.6	1.2
Primidone***	38.5 ± 1.0	2.6
Phenobarbital***	91.3 ± 1.5	1.6
Phenytoin***	63.4 ± 0.6	1.0

*Control from Laboratoires Biotrol, Paris, France.
**Serum pool.
***Seronorm Pharmaca, Nyegaard, Norway.

the mean value as a result, when the two results are inside our within-day C.V.

Linearity

The linearity of the method is guaranteed to at least five times the upper therapeutic level.

Interferences

Retention times of the elven antiepileptic substances and other compounds which are also extracted by this method are shown in Table IV. Since methsuximide has a short half-life of 2—4 h [5], and therefore low serum concentrations, we monitor desmethylmethsuximide. The latter has a retention time equal to that of 10,11-epoxycarbamazepine. A selective extraction of 10,11-epoxycarbamazepine from serum in hexane at a pH of 2.0 eliminates this

TABLE IV

RETENTION TIMES FOR SOME COMPOUNDS WHICH ARE EXTRACTED BY THE METHOD

Compound	Retention time (min)
Theobromine	1.6
Theophiline	1.7
Caffeine	2.1
Desethylethadione	2.2
Phenylethylmalonamide	2.5
Ethosuximide	3.0
Primidone	3.5
CGP 10 000	4.1
Sulthiam	4.2
GP 47 779	5.4
Sulfamethoxazole	5.4
Ethadione	5.9
Desmethylmephenytoin	5.9
Phenobarbital	6.6
Desmethylmethsuximide	7.5
10,11-Epoxycarbamazepine	7.5
Butalbital	8.8
Oxcarbazepine	9.2
Mephenytoin	9.5
Hexobarbital	11.6
Methsuximide	12.3
Pheneturide	12.7
Carbamazepine	13.5
Phenytoin	14.5
N_4-Acetylsulfamethoxazole	14.9

interfering peak for the determination of desmethylmethsuximide [6]. Sulfamethoxazole, one of the two components in Bactrim®, has the same retention time as GP 47 779. It is possible to see from the chromatogram whether the patient is taking Bactrim together with oxcarbazepine because a metabolite of one of the two substances in Bactrim gives a peak shortly before the common peak of GP 47 779 and sulfamethoxazole. It is also possible to verify the presence of sulfamethoxazole in a common peak with GP 47 779 by monitoring at 270 nm where sulfamethoxazole has an absorption maximum and GP 47 779 has no absorption. Patients who receive pheneturide have two unidentified peaks appearing in the chromatogram, which we are in the process of identifying.

DISCUSSION

Oxcarbazepine (Fig. 1), a new drug from Ciba-Geigy, has been in clinical trial for the last 8 years in our center. This drug has a very short half-life (~1 h) and therefore the main pharmacological effect is due to GP 47 779 [7] which we are measuring. Our liquid—liquid extraction may be more time-consuming than solid-phase extraction [2]; however, the advantages are that the mate-

rials used are less expensive, the extraction is done at a fixed pH and the concentrations of the drugs are increased, thus less volume has to be injected into the chromatograph. The time required for the preparation of the serum extract is reduced due to the fact that it is possible to decant the ethyl acetate extract directly into the tube where the evaporation takes place, thus avoiding time-consuming pipetting. The buffer used [4], saturated with ammonium sulfate, gives a baseline free from interfering peaks of substances naturally occurring in serum. Our serum extracts are evaporated at 37°C because of the low melting point (64—65°C) of ethosuximide. The other drugs can be heated to 60°C without any loss. With the selected wavelength of 207 nm, the drugs are not measured at their maximum absorption level but on a shoulder, while the maxima are below 200 nm, with the exception of carbamazepine, which has its maximal absorption at 215 and 285 nm. Thus our selected wavelength of 207 nm is a compromise between the carbamazepine maximum at 215 nm and the other substances with maxima below 200 nm. It is important that the chromatograph has gradient facilities. The five substances which elute at the beginning (Fig. 2) are well separated by 18% acetonitrile in water. An isocratic run with this percentage of acetonitrile would give phenytoin a retention time of 27.0 min instead of 14.5 min with the gradient suggested. A chromatographic run takes 21.5 min altogether, the last 6.5 min being a wash program. This is done because the ethyl acetate extract substances, although they do not interfere with the determination, shorten the life-span of the column. In the wash program the column is flushed with 80% acetonitrile in water 2.4 times the total volume of mobile phase within the column. A higher percentage of acetonitrile in the wash program does not seem to prolong the life-span of the column. With this wash program a column can last for half a year with a weekly load of about 200 samples. Only the filter in the inlet has to be replaced when the pressure exceeds about 250 bar. A new column has a pressure of around 100 bar with 2.4 ml/min of 18% acetonitrile in water. The life of the column can be somewhat extended by inverting it. In our experience a shorter column with 5-μm material has a shorter life. The pressure rises faster, resulting in asymmetric peaks and poor separation. But, in the long run, the advantages of shorter analysis time with less acetonitrile might outweigh the disadvantage of more frequent column changes.

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CHROMBIO. 1884

QUANTITATION OF FLUPIRTINE AND ITS ACTIVE ACETYLATED METABOLITE BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING FLUOROMETRIC DETECTION

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SUMMARY

A simple, selective and sensitive procedure is described for the quantitation of flupirtine maleate (FLU) and its active acetylated metabolite (Met. 1) in plasma and urine. Using a 0.5-ml sample, a sensitivity of 10 ng/ml is easily achieved with a reversed-phase octadecylsilane (C_{18}) column, and a high-performance liquid chromatographic system with fluorescence detection. Quantitation from plasma involves addition of an internal standard, protein precipitation with acetonitrile and a sample concentrating step, while for urinalysis the samples are taken through a single extraction with methylene chloride. Analytical recoveries of FLU and Met. 1 from plasma averaged $\geq$ 95%, while from urine only 60 and 50%, respectively, could be recovered. The overall, inter- and intra-day variability for both FLU and Met. 1 averaged 6, 5 and 3%, in plasma, respectively. Standard calibration plots in plasma were linear ($r \geq 0.99$) for FLU (range: 0.01—10.0 μg/ml) and Met. 1 (range: 0.5—25 μg/ml) over the extended range. A slightly modified elution system was employed for quantitation of FLU and Met. 1 in urine.

INTRODUCTION

Flupirtine® [2-amino-3-carbethoxyamino-6-(*p*-fluorobenzylamino)pyridine maleate] (Carter-Wallace Labs., Cranbury, NJ, U.S.A.), is a centrally acting analgesic with a long duration of action and apparently a low addiction and tolerance liability [1]. Its activity appears not to be affected significantly by naloxone and it may therefore lack opiate activity. It is currently being evaluated by the Epilepsy Branch of the National Institute of Neurological and Communicative Disorders and Stroke (NINCDS) at the Clinical Center of the National Institutes of Health as an anti-epileptic agent. A sensitive assay was

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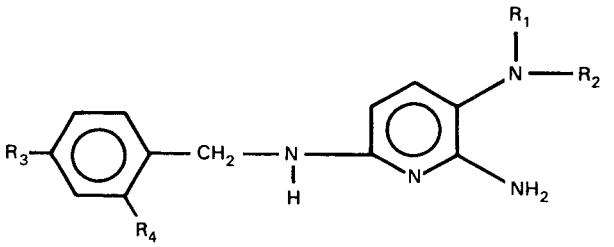

$R_1 = H$	$R_2 = -C(=O)-O-C_2H_5$	$R_3 = F$	$R_4 = H$	(Flupirtine)
$R_1 = H$	$R_2 = -C(=O)-CH_3$	$R_3 = F$	$R_4 = H$	(Metabolite 1)
$R_1 = H$	$R_2 = -C(=O)-O-C_2H_5$	$R_3 = -CH_3$	$R_4 = -CH_3$	(Internal Standard)

Fig. 1. Flupirtine and related substituted pyridine analogues.

desired in order to study its disposition following single oral doses in epileptic patients.

Earlier quantitative procedures for flupirtine maleate (FLU) in biological fluids included measurement of radiolabelled drug [1], thin-layer chromatography [1] and measurement of absorbance via spectrophotometric analysis [1]. These procedures used in preclinical studies lack sensitivity and specificity, and thereby analytical measurements are usually non-specific.

The drug undergoes biotransformation to two primary metabolites which have been identified: (1) an acetylated product and (2) *p*-fluorohippuric acid. The acetylated metabolite [Met. 1: 2-amino-3-acetylamino-6-(*p*-fluorobenzylamino)pyridine] has been shown to possess almost 20—30% analgesic activity of the parent compound. Valid quantitation procedures evaluating the disposition of FLU should be specific enough to resolve the parent drug from any of the metabolites present. Fig. 1 shows the parent compound and other related chemical entities employed in this procedure.

A rapid, reproducible and sensitive assay is described for the quantitation of FLU and its active metabolite (Met. 1) in biological fluids by reversed-phase high-performance liquid chromatography (RP-HPLC) using fluorometric detection, suitable both for routine therapeutic monitoring and/or detailed disposition studies.

MATERIALS AND METHODS

Flupirtine maleate and its dimethyl derivative [2-amino-3-carbethoxyamino-6-(2,4-dimethylbenzylamino)pyridine] used as internal standard (I.S.) were obtained from the Epilepsy Branch of the NINCDS (National Institutes of Health, Bethesda, MD, U.S.A.). Met. 1 was generously supplied by Carter-Wallace Labs.

Both methanol and acetonitrile (Burdick and Jackson Labs., Muskegon, MI,

U.S.A.) used in the assay were glass-distilled and of HPLC grade. Water was double distilled in a glass-distillation apparatus. All HPLC solvents were filtered through a 0.45-μm filter and then degassed prior to use. Stock solutions of FLU, Met. 1 and I.S. were stored at 4°C. Solutions of FLU showed no degradation over a period of three months when kept refrigerated, covered with aluminium foil. The internal standard solution was freshly prepared every two weeks from the stock solution.

Assay method

Quantitation of FLU and Met. 1 from plasma. Plasma, 0.5 ml, was placed in an 4-ml screw cap disposable glass vial (Fisher Scientific, Silver Spring, MD, U.S.A.) which contained 50 μl of I.S. (2.0 μg/ml in methanol). To this, 1 ml of acetonitrile was added using a Repipet® dispenser (Lab Industries, Berkeley, CA, U.S.A.). Each vial was mixed using a vortex mixer for 5 sec and centrifuged for 5 min at 2000 *g*. The supernate was then decanted into another 4-ml disposable screw cap glass vial and evaporated under a gentle stream of air at 37°C. The residue was reconstituted with 150 μl of mobile phase, mixed for 1 min and centrifuged for 2 min at 2000 *g*. A 20-μl aliquot of a sample was injected into the HPLC system.

Quantitation of FLU and Met. 1 from urine. Urine, 0.5 ml, was placed in a 8-ml screw cap disposable glass vial (Fisher Scientific) which contained 50 μl of I.S. and 50 μl of ammonium hydroxide (10% solution of ammonia). The sample was mixed on a vortex mixer for 5 sec and 5 ml of methylene chloride (Burdick and Jackson Labs.) was added using a Repipet dispenser. The vials were then shaken on a reciprocating shaker (Eberbach, Ann Arbor, MI, U.S.A.) for 5 min and centrifuged for another 5 min at 2000 *g*. Approximately 4 ml of the organic layer was drawn and transferred to another disposable vial. The organic phase was slowly evaporated to dryness under a gentle stream of air at 37°C. The residue was treated the same way as with plasma, above.

Chromatographic conditions

The chromatographic analysis of the samples was performed using an isocratic system. An Altex Model 110A single-pump system equipped with a Schoeffel variable-wavelength fluorometric monitor (FS-970, Schoeffel Instrument, Westwood, NJ, U.S.A.) was employed for quantitation of FLU. The excitation (λ_{ex}) and emission (λ_{em}) wavelengths were set at 323 and 370 nm, respectively. The cut-off emission filter used (λ_{em} = 370 nm) has a greater than 89% transmittance at the wavelength. The analysis was performed on a 25 cm × 4.6 mm I.D. Altex Ultrasphere octadecylsilane (ODS) analytical column with a 5-μm particle size (Beckman Instruments, Berkeley, CA, U.S.A.). Preceding the analytical column was a 7 cm × 2.3 mm I.D. guard column packed with Co:Pell ODS (30—38 μm particles) (Whatman, Clifton, NJ, U.S.A.). The mobile phase of methanol—acetontrile—0.005 *M* phosphate buffer (32:32:36, v/v) adjusted to pH 6.7 was used at a flow-rate of 1.4 ml/min as an eluent for plasma samples. The back pressure ranged from 190—210 bar. Standards for FLU and Met. 1 were prepared by spiking plasma and/or urine with FLU and Met. 1 stock solutions to obtain final concentrations ranging from 0.01 to 2.0 μg/ml for FLU and 0.5 to 15.0 μg/ml for Met. 1.

The concentrations of FLU and/or Met. 1 were estimated by comparing the peak area ratios of the drug to the internal standard with area ratios of a simultaneously run standard calibration curve. Analytical recoveries of FLU and Met. 1 were determined by comparison of direct injection of standard aqueous solutions with the injection of the same standards run through the assay procedure.

Inter-day variation was computed by measuring precision in the concentration determined from two plasma quality control standards (0.15 and 1.0 μg/ml) and reproducibility in measured concentration and slope of the standard calibration curves (n = 9) over a three-month period. The intra-day variability was estimated by performing replicate analyses (n = 10) plasma standard containing 0.15 μg/ml of FLU and 4.0 μg/ml of Met. 1 and measuring precision based on peak area ratios of drug and/or Met. 1 to internal standard. Computation of statistical parameters, e.g. means, standard deviation, coefficient of variation (%) and 95% confidence intervals was performed using standard programs available in statistical package software of Hewlett-Packard 85 microcomputer (Hewlett-Packard, Corvallis, OR, U.S.A.).

Selectivity of the assay was also evaluated by checking for possible interference of other anti-epileptic agents that patients might be taking concomittantly.

RESULTS AND DISCUSSION

Typical HPLC chromatograms obtained from control blank (A), spiked (B), and patient plasma (C) are shown in Fig. 2. The eluted peaks are clean, sharp and symmetric, the control or blank plasma did not show any interfering peaks at or near the elution volumes for drug and the internal standard. No attempt

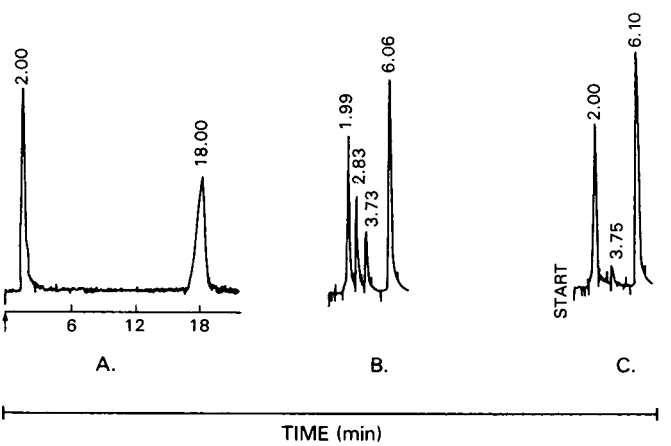

Fig. 2. Representative HPLC chromatograms from plasma samples taken through the assay procedure. (A) Blank plasma; (B) calibration standard containing 0.2 and 5.0 μg/ml of FLU and Met. 1, respectively; (C) patient plasma sample (estimated FLU concentration ca. 0.04 μg/ml). Peaks with retention times relative to the I.S. (retention times in min are in parentheses): solvent front, 0.32 (2.00); Met. 1, 0.46 (2.83); FLU, 0.61 (3.73) and I.S., 1.00 (6.08).

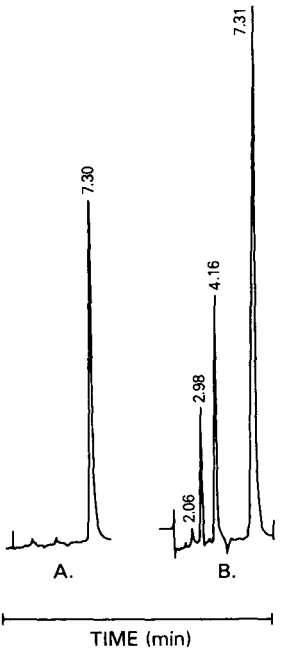

Fig. 3. Representative chromatograms from urinalysis following extraction. (A) Blank urine with I.S.; (B) calibration standard containing 0.8 and 8.0 μg/ml of FLU and Met. 1, respectively. Peaks with relative retention (actual retention times in min are in parentheses): solvent front, 0.29 (2.06); Met. 1, 0.42 (2.98); FLU, 0.57 (4.16) and I.S., 1.00 (7.30).

was made to identify the peak that eluted at approximately 18 min (Fig. 2A from some plasma samples). Retention times for Met. 1, FLU and I.S. were 2.83, 3.73 and 6.08 min, respectively. The relative retention of these compounds is given in Fig. 2.

Fig. 3 shows typical chromatograms obtained from urinalysis following the extraction step of blank urine containing I.S. (A) and a calibration (spiked) standard (B) containing both FLU and Met 1. The elution is clean, peaks are symmetric and sharp. Retention times for Met. 1, FLU and I.S. were 2.98, 4.16 and 7.30 min, respectively. Relative retention times are also shown in Fig. 3.

Fig. 4 shows a mean standard curve (n = 6) in plasma over a two-month period, where peak area ratios are plotted as a function of spiked FLU and Met. 1 concentration. The inset in Fig. 4 shows an excellent linearity when peak height ratio (FLU/I.S.) is regressed as a function of FLU concentration (range 0.01—0.05 μg/ml). This validates linearity at the low range and suggests that either the peak height or peak area ratios may be used to obtain standard calibration plots.

Linearity of the standard curve was established over an extended range of up to 10.0 μg/ml FLU and 25.0 μg/ml Met. 1 in plasma. The mean slope of the standard curves was 1.165 for FLU and 0.053 for Met 1. The variability in the slope of the standard calibration curves (n = 6) for FLU and Met. 1 over a two-month period was 6.2% and 7.8%, respectively. The correlation

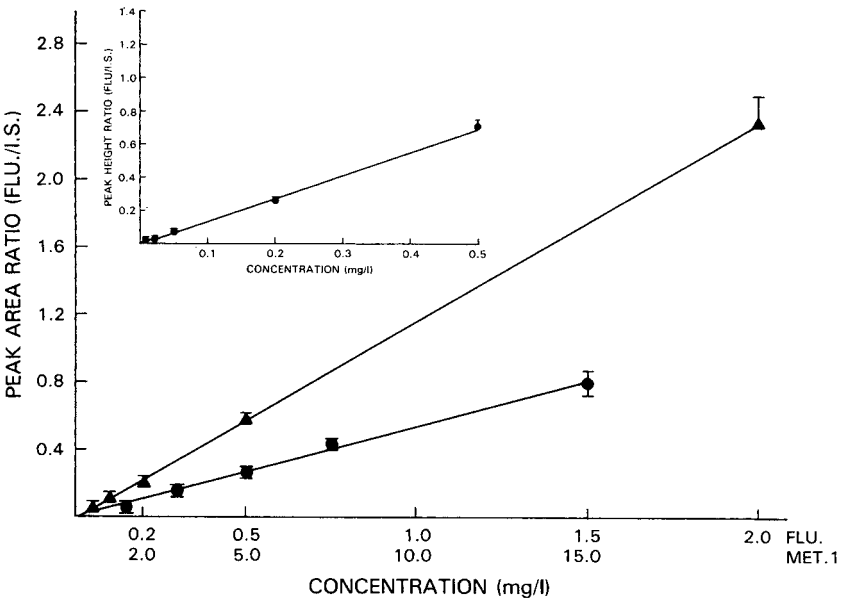

Fig. 4. Mean standard calibration curves for FLU and its active metabolite in plasma over a two-month period. Each datum represents a mean (± S.D.) of six determinations. Key with regression parameters: (▲) FLU (y = 0.011 + 1.165x; r = 0.996) and (●) Met. 1 (y = 0.002 + 0.053x; r = 0.992). Inset: mean (n = 7) standard curve for FLU in plasma over the low concentration range (0.01—0.5 mg/l).

TABLE I

ASSAY REPRODUCIBILITY OF NINE REPLICATES OVER A THREE-MONTH PERIOD FROM PLASMA

	Actual concentration of standards (mg/l)	Measured concentration		
		Mean	S.D.	C.V. (%)
Flupirtine	0.05	0.0496	0.005	10.0
	0.10	0.1031	0.006	6.1
	0.20	0.1840	0.008	4.6
	0.50	0.5082	0.015	2.9
	2.00	1.9656	0.072	3.6
Controls	0.15	0.140	0.006	4.3
	1.00	1.067	0.060	5.6
Met. 1	1.5*	1.335	0.129	9.7
	3.0	2.975	0.099	3.3
	5.0	5.113	0.180	3.5
	7.5	8.067	0.359	4.5
	15.0	14.846	0.299	2.0
Controls	4.0	3.812	0.174	4.6
	10.0	10.546	0.616	5.8

*n = 8 in this case.

coefficients (r) generated by linear regression of peak area ratios of both FLU/I.S. and Met. 1/I.S. on concentration in all cases were 0.99 or better.

The assay sensitivity for a 20-μl injection is at least 10 ng/ml for FLU and 200 ng/ml for Met. 1. This was determined by observing the lowest concentration that maintained an arbitrary signal-to-noise ratio of 3.0. The assay sensitivity for both Met. 1 and FLU can be increased several fold by increasing the injection volume, sample size or decreasing the reconstitution volume.

The assay reproducibility for FLU and Met. 1 determined from nine replicate standard calibration curves in plasma over a three-month period is shown in Table I. As can be seen the overall coefficient of variation (C.V.) for the quantitation procedure was less than 6% for both FLU and Met. 1. Reproducibility data for FLU computed from seven replicate analyses in plasma over the lower concentration range indicate an average variation of 7% (Table II) which is consistent with the data in Table I.

The inter-day variability determined by measuring precision in the measured concentration for two (a low and a high) quality control standards, 0.15 and 1.00 μg/ml for FLU, and 4.0 and 10.0 μg/ml for Met. 1, was approximately 5% for both the parent compound and its metabolite (Table III). The intra-day variation computed similarly by measuring precision in the peak area ratios of FLU/I.S. and Met. 1/I.S. for ten replicates averaged 2.2% and 2.7%, respectively.

TABLE II

ASSAY REPRODUCIBILITY OF SEVEN REPLICATES OVER A TWO-WEEK PERIOD FROM PLASMA

Actual concentration of standard (mg/l)	Peak height ratio		
	Mean	S.D.	C.V. (%)
0.01	0.0146	0.0012	8.2
0.02	0.0260	0.0028	10.7
0.05	0.0700	0.0045	6.4
0.2	0.2530	0.0142	5.6
0.5	0.7130	0.0319	4.5
Mean			7.1
S.D.			2.4

TABLE III

INTER-DAY AND INTRA-DAY VARIABILITY FROM PLASMA

Control	Concentration (mg/l)		Variation (%)	
	FLU	Met. 1	FLU	Met 1
Inter-day variability (n = 9)				
A	0.15	4.00	4.3	4.6
B	1.00	10.00	5.6	5.8
Intra-day variability (n = 10)				
A	0.15	4.0	2.2	2.7

TABLE IV

ANALYTICAL RECOVERY DATA FROM URINE (n = 4)

Compound	Percent recovery		
	Mean	S.D.	C.V. (%)
FLU	60.3	2.3	3.9
Met. 1	49.2	3.9	8.0
I.S.	63.8	1.5	2.3

Analytical recovery from plasma for both FLU and Met. 1 was > 95%. However, the recoveries of Met. 1 and FLU for urinalysis, following extraction with methylene chloride, had a mean of 49.2% and 60.3% (n = 4), respectively. Overall variation in analytical recovery of Met. 1 was approximately two-fold that of FLU (Table IV). The extraction efficiency for the internal standard had a mean of 64%. The standard calibration plots for both FLU and Met. 1 were linear in the range of 0.5--50.0 μg/ml in urine. Sensitivity limit (lower limit of detection) for FLU and Met. 1 in urine was 30 ng/ml and 500 ng/ml, respectively. Extraction efficiencies were not completely optimized since these sensitivities were sufficient for urinalysis.

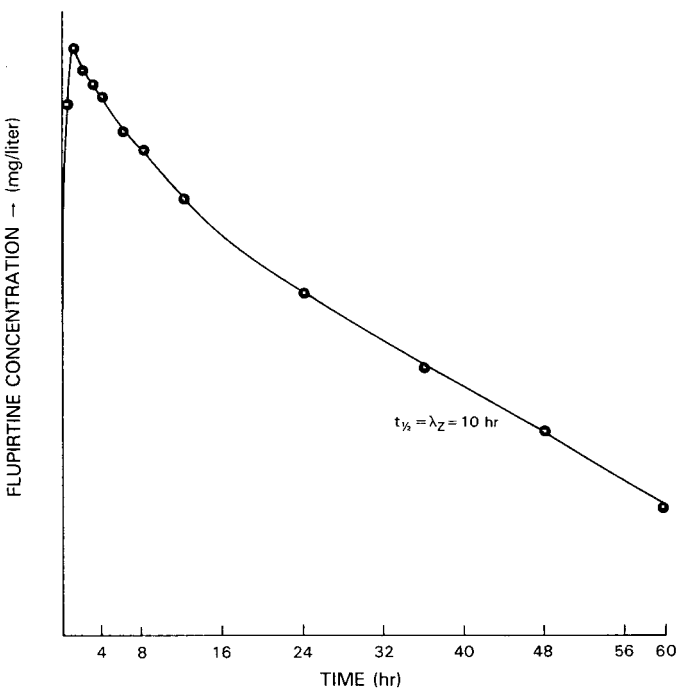

Fig. 5. A representative disposition profile of FLU in epileptic patient over 2.5 days following oral administration of flupirtine maleate.

Selectivity of assay

Carbamazepine and its epoxide, phenytoin, valproic acid, diazepam, clonazepam, aspirin and methylprednisolone were evaluated for possible interference with the assay procedure. None of the compounds chromatographed interfered with FLU and/or Met. 1 peaks. However, at concentrations above 15—20 μg/ml carbamazepine, Met. 1 peak eluted as a shoulder to carbamazepine peak. Therefore, the assay appears to be selective for the quantitation of both FLU and Met. 1 in epileptic patients taking other anti-convulsants concomitantly.

The assay procedure has been successfully used in evaluating the disposition kinetics of FLU in epileptic patients following oral dosing. Fig. 5 shows a representative profile for FLU disposition in one such patient. After relatively quick absorption, the drug decline from plasma appears to be biexponential. The terminal-phase half-life ($t_{1/2}$) in this patient was determined to be approximately 10 h.

The data in the paper clearly demonstrate that the assay is sensitive, rapid, selective, requires only 0.5 ml of the biological sample and should prove useful in further investigations of FLU pharmacokinetics in both man and animals.

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CHROMBIO. 1888

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF ISOXICAM IN HUMAN PLASMA AND URINE

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SUMMARY

A sensitive, selective, and rapid high-performance liquid chromatographic procedure was developed for the determination of isoxicam in human plasma and urine. Acidified plasma or urine were extracted with toluene. Portions of the organic extract were evaporated to dryness, the residue dissolved in tetrahydrofuran (plasma) or acetonitrile (urine) and chromatographed on a μBondapak C_{18} column preceded by a 4—5 cm × 2 mm I.D. column packed with Corasil C_{18}. Quantitation was obtained by UV spectrometry at 320 nm. Linearity in plasma ranged from 0.2 to 10 μg/ml. Recoveries from plasma samples seeded with 1.8, 4 and 8 μg/ml isoxicam were 1.86 ± 0.077, 4.10 ± 0.107 and 8.43 ± 0.154 μg/ml with relative standard deviations of 3.3%, 2.5% and 5.4%, respectively. The linearity in urine ranged from 0.125 to 2 μg/ml. The precision of the method was 3.3—9.0% relative standard deviation over the linear range.

INTRODUCTION

Isoxicam (Fig. 1) is one of the oxicams [1], a class of non-steroidal anti-inflammatory drugs (NSAID) that is structurally distinct from other classes of NSAID. The drug is sparingly soluble in water (2 μg/ml), has a pK_a of 3.65, shows good partitioning in an octanol—pH 4 acetate system (log P = 1.716), and is completely extracted into octanol from 0.1 M hydrochloric acid solution. Isoxicam is a potent long-acting anti-inflammatory agent, highly effective in relieving the signs and symptoms of rheumatoid arthritis and degenerative joint disease. The pharmacology, pharmacokinetics, and clinical efficacy of isoxicam have been recently summarized [2].

In the early stages of development a fluorometric assay procedure was used for the analysis of the drug in biological fluids. Although the method was simple and rapid it lacked specificity. The present report describes an assay for

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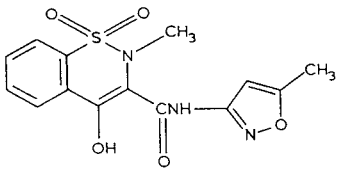
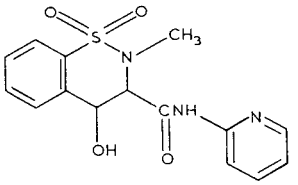
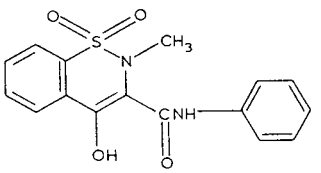


Isoxicam



Piroxican



PD 79,703

Fig. 1. Chemical structures of isoxicam [4-hydroxy-2-methyl-N-(5-methyl-3-isoxazolyl)-2H-1,2-benzothiazine-3-carboxamide 1,1-dioxide], piroxicam [4-hydroxy-2-methyl-N-2-pyridinyl-2H-1,2-benzothiazine-3-carboxamide 1,1-dioxide], and PD 79,703 [4-hydroxy-2-methyl-2H-1,2-benzothiazine-3-carboxanilide 1,1-dioxide].

isoxicam in human plasma and urine based on reversed-phase high-performance liquid chromatography (HPLC). The method has been validated for human biological fluids and it was found to be rapid, precise, and of sufficient sensitivity for its intended use.

EXPERIMENTAL

Chemicals

Tetrahydrofuran (THF) and acetonitrile, distilled in glass grade, were purchased from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Toluene and glacial acetic acid, both reagent grade, were obtained from MCB (Norwood, OH, U.S.A.). Citric acid was dissolved and diluted to 0.2 *M* and hydrochloric acid was diluted to 1 *M* with distilled water, respectively. Both were reagent grade. 1-Heptanesulfonic acid sodium salt was purchased from Eastman Kodak (Rochester, NY, U.S.A.). Isoxicam, piroxicam (internal standard in plasma) and PD 79,703 (internal standard in urine) were used as received from Warner-Lambert/Parke-Davis (Ann Arbor, MI, U.S.A.). The structures of all the compounds are shown in Fig. 1.

Apparatus

An Altex Model 110 pump (Berkeley, CA, U.S.A.) was used for solvent delivery and a Varian Varichrom detector (Palo Alto, CA, U.S.A.) was used for

quantitation. Peak heights were measured with a Shimadzu Chromatopac C-RIA (Kyoto, Japan) and injection was done via a WISP 710B automatic injector, Waters Assoc. (Milford, MA, U.S.A.).

Chromatography

The column was a 300 × 3.9 mm I.D. stainless-steel μBondapak C_{18} (10 μm) obtained from Waters Assoc. A guard column 40 mm × 2 mm I.D. packed with Corasil C_{18} was used. The mobile phase for isoxicam in plasma consisted of 45% THF, 54% water, 1% glacial acetic acid, and 0.005 *M* 1-heptanesulfonic acid. The mobile phase for isoxicam in urine was 50% acetonitrile, 49% water, 1% glacial acetic acid, and 0.005 *M* 1-heptanesulfonic acid. The flow-rate was set at 1.5 ml/min; the wavelength setting was 320 nm.

Sample preparation

Plasma. To 0.5 ml plasma in a 5-ml test tube with a ground-glass stopper were added 0.5 ml water, 0.1 ml isoxicam standard in THF (0.1 ml THF in unknowns), 0.25 ml of 0.2 *M* citric acid and 1 ml internal standard in toluene (2 μg/ml piroxicam). The tube was capped tightly and shaken for 10 min at moderate speed on a variable-speed shaker. After centrifugation for 5 min at 2000 *g*, 0.6 ml of the toluene layer was transferred into a 5-ml conical tube and evaporated to dryness under a stream of clean air, the tube being in a water bath maintained at 67°C. The residue was then dissolved in 0.3 ml of THF and transferred into a limited-volume insert tube. A 30-μl aliquot was injected via the WISP automatic injector.

Urine. To 1 ml of urine 0.1 ml isoxicam standard in THF (0.1 ml THF in unknowns), 0.1 ml of 1 *M* hydrochloric acid and 3 ml of PD 79,703 internal standard in toluene (0.6 μg/ml) were added. The tube was capped tightly and shaken for 10 min on a heavy-duty shaker. Following centrifugation, 2 ml of the toluene layer were transferred into a conical tube and processed as for plasma.

RESULTS

Recovery

Recovery of isoxicam and piroxicam added to plasma was 98% and 90%, respectively. Recovery of isoxicam and PD 79,703 added to urine was 98% and 93%, respectively.

Linearity

A straight line was obtained by plotting μg/ml isoxicam in plasma or urine against the peak height ratio of isoxicam over the internal standard; these data are listed in Table I. The relationship was linear in the range of 0.1—5 μg/ml in plasma and 0.125—2 μg/ml in urine.

Precision

Bulk control standards A, B and C were prepared by adding known amounts of isoxicam to blank plasma at levels of 1.8, 4 and 8 μg/ml, respectively. The precision of the assay was determined by analyzing triplicates of each of these

TABLE I

MEANS, STANDARD DEVIATIONS AND RELATIVE STANDARD DEVIATIONS OF PEAK HEIGHT RATIOS OF ISOXICAM TO INTERNAL STANDARD IN HUMAN PLASMA AND URINE

Concn. (μg/ml)	Mean peak height ratio	S.D.	R.S.D. (%)
Plasma (n = 10)			
0.2	0.035	0.004	10.8
1.0	0.190	0.010	5.26
2.0	0.400	0.013	3.21
3.0	0.610	0.024	4.02
6.0	1.23	0.058	4.76
10.0	2.10	0.093	4.45
Urine (n = 8)			
0.125	0.114	0.010	9.05
0.25	0.239	0.008	3.38
0.5	0.489	0.023	4.79
1.0	0.984	0.033	3.31
2.0	1.966	0.103	5.22

TABLE II

CONCENTRATION OF SEEDED CONTROL SAMPLES OF HUMAN PLASMA ASSAYED DURING A THREE-DAY PERIOD

Day	Concn. (μg/ml)		
	1.8	4.0	8.0
1	1.88	4.20	8.50
	2.04	4.16	8.68
	1.86	4.14	8.44
2	1.86	4.16	8.34
	1.82	3.96	8.38
	1.78	4.24	8.24
3	1.82	3.94	8.58
	1.85	4.02	8.52
	1.79	4.07	8.22
Mean	1.86	4.10	8.43
S.D.	0.077	0.107	0.154
R.S.D. (%)	4.14	2.60	1.82
Difference from theory (%)	3.33	2.50	5.38

TABLE III

BACK-CALCULATED VALUES OF STANDARD CURVES OF ISOXICAM IN HUMAN PLASMA

Day	Concn. (μg/ml)						
	0	0.4	1.0	2.0	5.0	8.0	10.0
1	0	0.40	1.04	2.04	5.24	8.80	10.44
	0	0.44	0.96	1.97	4.82	8.22	9.20
	0	0.40	0.98	2.02	4.92	8.02	9.66
2	0	0.38	0.86	1.94	4.94	8.02	9.90
	0	0.36	0.90	1.90	5.34	8.10	9.82
	0	0.36	0.88	1.70	4.90	8.10	8.68
3	0	0.38	0.92	2.22	5.56	7.98	9.74
	0	0.38	0.92	2.00	4.90	8.16	10.56
	0	0.36	0.92	1.92	5.02	7.98	9.56
Mean		0.38	0.93	1.97	5.07	8.15	9.73
S.D.		0.026	0.055	0.138	0.251	0.256	0.575
R.S.D. (%)		6.77	5.89	7.00	4.95	3.14	5.91
Difference from theory (%)		5.0	7.00	1.50	1.40	1.88	2.70

TABLE IV

BACK-CALCULATED VALUES OF STANDARDS OF ISOXICAM IN HUMAN URINE

	Concn. (μg/ml)					
	0	0.125	0.25	0.5	1.0	2.0
	0	0.11	0.24	0.48	0.99	2.16
	0	0.11	0.24	0.48	1.05	1.88
	0	0.10	0.24	0.52	0.94	1.94
	0	0.11	0.23	0.48	1.02	2.12
	0	0.12	0.26	0.49	0.99	1.93
	0	0.13	0.24	0.50	1.00	1.94
	0	0.11	0.24	0.54	0.97	2.03
	0	0.13	0.24	0.48	1.01	1.93
Mean		0.12	0.24	0.50	1.00	2.00
S.D.		0.011	0.008	0.023	0.033	0.101
R.S.D. (%)		9.3	3.46	4.56	3.30	5.80
Difference from theory (%)		4.0	4.0	0.0	0.0	0.0

controls along with triplicate standard curves on three consecutive days. Relative standard deviations (R.S.D., Table II) of the controls over the three days ranged from 4.1% at the lowest level to 1.8% at the highest level. Percent difference from theory was 4.3, 2.50 and 5.4 for A, B and C, respectively. Concentrations of the standard curves were back-calculated from the slope for the day and the values are listed in Table III. Relative standard deviations ranged from 6.8% at 0.4 μg/ml to 3.1% at 8 μg/ml. Difference from theory ranged from 7.0% to 1.4%.

Blank urine was spiked with 0. 0.125, 0.25, 0.5, 1 and 2 μg/ml isoxicam. Eight replicates at each level were carried through the assay. The results (Table IV) show a back-calculated difference from theory ranging from 0 to 4%. The relative standard deviation ranged from 9.3 to 3.3%.

Selectivity

No interfering peaks were visible in blank plasma at the retention times of isoxicam and piroxicam, 8.1 min and 4.1 min, respectively. Representative chromatograms are shown in Fig. 2. Chromatograms of blank urine and of urine to which isoxicam and internal standard were added as well as chromatograms of pre- and post-dose urine samples are reproduced in Fig. 3.

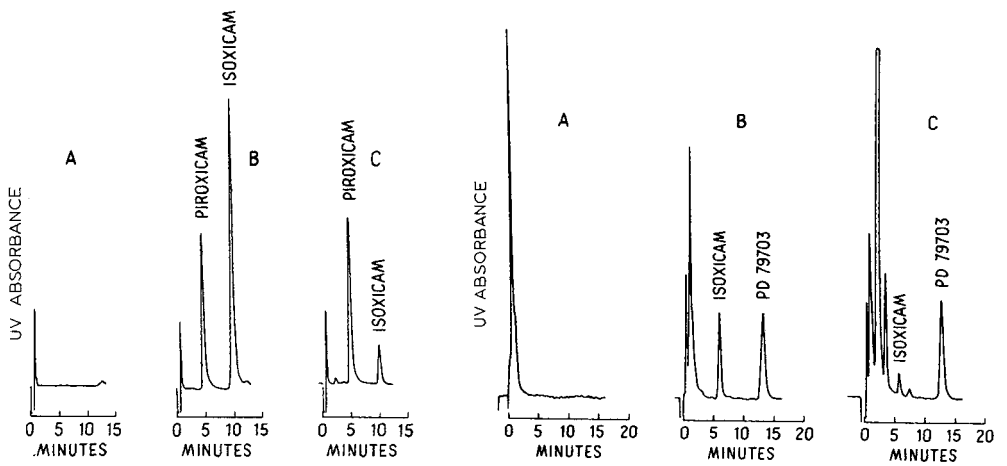


Fig. 2. Chromatograms of isoxicam in human plasma. A, Control plasma; B, plasma spiked with internal standard plus 8 μg/ml isoxicam; C, plasma from a subject 48 h post-dose, isoxicam concentration was found to be 1.2 μg/ml.

Fig. 3. Chromatograms of isoxicam in human urine. (A) Control urine; (B) urine spiked with 1 μg/ml isoxicam plus internal standard; (C) urine from a subject 48—72 h post-dose, isoxicam concentration was found to be 0.28 μg/ml.

Sensitivity

The minimum detectable amounts of isoxicam in plasma, based on three times the standard deviation of the lowest level of precision test was 0.12 μg/ml. That for urine was 0.07 μg/ml, based on twice the baseline noise.

DISCUSSION

Initially all plasma samples were assayed using piroxicam as internal standard. However, occasionally at higher doses, subjects receiving 400 mg, an isoxicam metabolite may interfere with the internal standard. An alternative internal standard, PD 79,703, with a longer retention time than piroxicam was employed where this occurred. PD 79,703 was the internal standard of choice in the case of urine where the metabolite was present in sufficient quantity to preclude the use of piroxicam. However, substitution of acetonitrile for

tetrahydrofuran in the mobile phase, can resolve the isoxicam metabolite from piroxicam. This would make piroxicam a suitable internal standard for the analysis of isoxicam in both plasma and urine. One other consideration, however, is that piroxicam has been on the market for some time and it is possible that patients may have access to both isoxicam and piroxicam.

The method as presented here has been routinely used in our laboratories for the analysis of isoxicam in human plasma and urine in clinical studies. It has proven to be a simple, straightforward procedure with a high degree of reliability, precision and accuracy. The method is sufficiently sensitive to be used in monitoring the levels of isoxicam in biological fluids of patients treated with the drug and in pharmacokinetic studies.

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DETERMINATION OF THE IMIDAZO QUINAZOLINE DERIVATIVE Ro 13-6438 IN BIOLOGICAL FLUIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-performance liquid chromatographic assay has been developed for the imidazo quinazoline derivative Ro 13-6438 [D-(—)-6-chloro-1,5-dihydro-3-methylimidazo(2,1-*b*)-quinazolin-2(3H)-one], which is under clinical investigation as a cardioactive drug.

The drug is extracted from biological fluids into 1-chlorobutane—1-hexanol (90:10) and back-extracted into perchloric acid. This extract is chromatographed directly, using a reversed-phase high-performance liquid chromatographic system with ultraviolet detection at 254 nm. The detection limit in plasma is about 1 ng ml^{-1}, using a 1-ml sample. The assay is rapid, accurate and sufficiently sensitive for the study of the single-dose kinetics of Ro 13-6438 in man following a 7.5-mg intravenous dose.

No instability of the unchanged substance was observed in plasma during storage for one day at room temperature and for five months at —20°C.

INTRODUCTION

Ro 13-6438 (I) is an imidazo quinazoline derivative [D-(—)-6-chloro-1,5-dihydro-3-methylimidazo(2,1-*b*)quinazolin-2(3H)-one, Fig. 1] with positive inotropic properties and is under clinical investigation as a cardiotonic drug.

For pharmacokinetic studies of I a suitable method had to be developed to determine the unchanged drug in biological fluids. High-performance liquid chromatography (HPLC) has proved to be a convenient procedure for this purpose, and, because of its higher selectivity, fluorescence detection is used whenever possible [1—3]. Unfortunately, I has no native fluorescence as extensive experiments with a large number of solvents and different test conditions showed. Therefore, the ultraviolet (UV) absorbance of the substance had to be used for quantitative determination. The UV absorbance of I is pH-

dependent. In aqueous solution at pH 1 two absorption maxima were found, one at 275 nm and a second at 222 nm, the latter having an extinction coefficient about twice that at 275 nm. If the pH was raised, the maximum at 275 nm disappeared; at a pH of 6.5—7.0 and above, a maximum at 250—255 nm could be observed with an extinction coefficient nearly as high as that at 222 nm and a pH of 1.

During the development of the HPLC system, several possibilities were tried. In a preliminary method for pharmacokinetic studies in dogs, normal-phase chromatography was used. Due to endogenous interferences which could not be eliminated and inadequate sensitivity, this approach was not suitable for human plasma. Reversed-phase systems with acidic or neutral to slightly alkaline mobile phases and detection at 222 nm and 254 nm, respectively, were compared. Less base-line noise was encountered at 254 nm; therefore this approach was used to develop an assay for I in biological fluids.

Direct injection of the biological material was not possible [4, 5] due to endogenous interferences. For reversed-phase HPLC, the extraction of a drug from plasma or urine into an organic solvent followed by back-extraction into an aqueous phase, which could then be directly chromatographed, has proved to be an elegant procedure [6, 7]. This approach was applied to I, thus avoiding evaporation and redissolving of the extract.

EXPERIMENTAL

Materials

The following solvents and reagents were used without special purification: *n*-hexane p.a., 1-hexanol for synthesis, methanol p.a., perchloric acid p.a. about 70% (E. Merck, Darmstadt, F.R.G.); 1-chlorobutane HPLC grade (Fisons, Loughborough, U.K.); acetonitrile HPLC grade S (Rathburn, Walkerburn, U.K.).

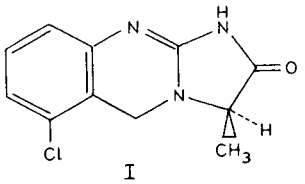
A 0.05 *M* (0.5%) perchloric acid solution was prepared by making up 4.3 ml of perchloric acid (70%) with deionized water to 1000 ml. For extraction, a mixture of one part 1-hexanol and nine parts 1-chlorobutane was used. Buffer solution, pH 6.8 (0.05 *M*), was prepared by dissolving 13.8 g of $NaH_2PO_4 \cdot H_2O$ (p.a. Merck) in about 1950 ml of water (bidistilled), adding about 7 ml of 8 *M* sodium hydroxide, adjusting the pH to 6.8, and making the volume up to 2000 ml with water.

I and the internal standards II and III (for structures see Fig. 1) were first synthesized by Dr. M. Chodnekar and Dr. F. Kienzle of Roche, Basle.

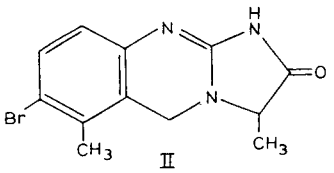
Standard solutions

A stock solution of I in methanol was prepared by dissolving 20 mg of the compound in 100 ml of methanol by ultrasonication. This methanolic solution can be stored at −20°C for more than three months without degradation. Appropriate quantities of the methanolic stock solution were diluted with water to give solutions within the range of 7.5 to 0.05 $\mu g\ ml^{-1}$ of I. These solutions were used as plasma standards by diluting 0.5 ml with blank plasma to 25 ml, covering the concentration range of 150 ng ml^{-1} to 1 ng ml^{-1}.

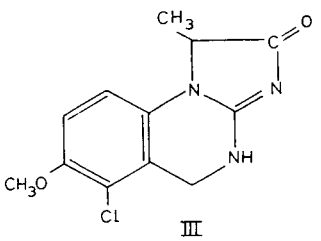
A stock solution of the internal standard was prepared by dissolving 5 mg of



I



II



III

Fig. 1. I = Ro 13-6438 = D-(—)-6-chloro-1,5-dihydro-3-methylimidazo(2,1-*b*)quinazolin-2(3H)-one (Europäische Patentschrift No. 718). II = *rac*-7-bromo-1,5-dihydro-3,6-dimethylimidazo(2,1-*b*)quinazolin-2(3H)-one (Europäische Patentanmeldung No. 21338 A1). III = *rac*-6-chloro-4,5-dihydro-7-methoxy-1-methylimidazo(1,2-*a*)quinazolin-2(1H)-one (Europäische Patentanmeldung No. 46267 A1). The synthesis of compounds I—III is described in ref. 12.

III as the hydrochloride in 10 ml of methanol by ultrasonication. This solution could be stored at −20°C, although crystallization occurred. Prior to use, redissolution was effected by ultrasonication.

Two methanolic solutions of the internal standard were prepared, one containing 100 μg ml^{-1}, the other 25 μg ml^{-1}, 1 ml of each of these solutions was diluted with the extraction mixture to 1000 ml giving the internal standard solution 1 containing 100 ng ml^{-1}, and giving the internal standard solution 2 containing 25 ng ml^{-1}.

The chromatographic system was tested daily by injecting mixtures of 5, 10 and 20 ng of I and 100 ng of III in 1 ml of 0.5% perchloric acid.

Equipment and chromatography

The following system was used for HPLC: an Altex Model 110 A pump (Altex Scientific, Berkeley, CA, U.S.A.); a Rheodyne 7125 valve injector with a 1000-μl loop (Rheodyne, Berkeley, CA, U.S.A.), or a Kontron MSI 660 automatic sample injector with a 1000-μl loop (Kontron, Zürich); a Waters 440 fixed-wavelength detector, wavelength 254 nm (Waters, Milford, MA, U.S.A.); a W + W recorder, Model 1100 or 1200 (Kontron, Zürich); and a Spectra-Physics SP 4100 integrator (Spectra-Physics, Santa Clara, CA, U.S.A.).

The column for chromatography was a 120 mm × 4.6 mm I.D. stainless-steel column (Dr. H. Knauer, Bad Homburg, F.R.G.) packed with Hypersil ODS, 5-μm particle size (Shandon Southern, Astmoore, Cheshire, U.K.). The mobile phase was 155 g of buffer pH 6.8 (0.05 *M*) mixed with 35 g of acetonitrile, degassed by ultrasonication. Using a flow-rate of 2 ml min^{-1}, a pressure of 150—250 bar was obtained. The retention times were approximately 3.9 min for the internal standard III and 4.5 min for I; injection volume was 1000 μl.

Sample preparation

Plasma or urine (1 ml) and the extraction mixtures (5 ml) containing an appropriate amount of the internal standard according to Table I were mixed for 5 min on a tumbler extractor (REAX II, Heidolph Elektra, Keilheim, F.R.G.) at 15 rpm and then centrifuged at 1200 *g* for 5 min. Then 4.5 ml of the organic phase were transferred to a tapered glass tube; 5 ml of *n*-hexane and 1.5 ml of 0.5% perchloric acid were added and mixed for 5 min on a tumbler extractor. After centrifuging for 5 min at 1200 *g*, the organic phase was aspirated and discarded. A portion of the remaining perchloric acid extract was then chromatographed. About 1200 μl were needed to fill the injection valve loop completely (nominal volume 1000 μl).

TABLE I

PLASMA STANDARDS AND AMOUNTS OF INTERNAL STANDARD TO BE USED FOR THE ASSAY

Expected concentration range of I in the unknown samples (ng ml^{-1})	Plasma standards for calibration (ng ml^{-1})	Amount of internal standard III added with the extraction mixture (ng)	Range of the UV detector
150—50	150, 100, 75, 50	500*	0.02
80—5	75, 50, 25, 10, 5	500*	0.01
30 to detection limit	25, 10, 5, 2, 1	125**	0.01

*5 ml of the internal standard solution 1.
**5 ml of the internal standard solution 2.

Calculation

Four to five plasma standards covering the expected concentration range were processed as described above and analysed as calibration samples alongside the unknown samples.

A calibration curve was obtained by least-squares regression of the peak height ratios of I to the internal standard against the concentration of I. This calibration curve was then used to calculate the concentration of I in the unknown samples. The SP 4100 computing integrator was programmed for this calculation.

RESULTS

Characteristics of method

Selectivity. I and the internal standard III were well separated from the main endogenous plasma interferences. Many human blank plasmas were tested and

only in a few were interferences encountered; the levels, however, were insignificant (Fig. 2).

In human urine, the internal standard III was not separated from endogenous urine constituents. Compound II could be used as internal standard (Fig. 1, retention time approximately 9.6 min). However, since only a small fraction of the dose was excreted renally as unchanged drug in man, no attempt was made to modify the assay for urine, and external calibration was applied.

A typical chromatogram of a spiked plasma with 50 ng ml^{-1} I is shown in

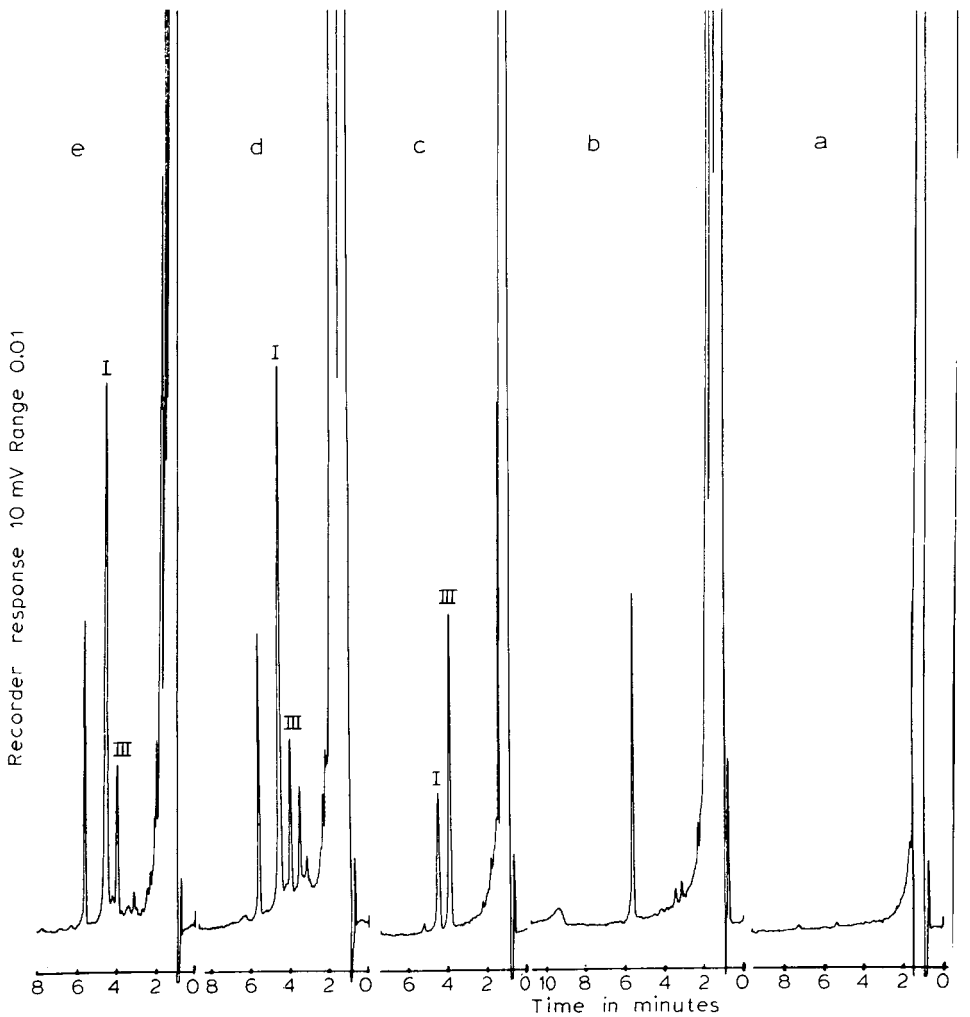


Fig. 2. (a) Chromatogram of 1000 μl of 0.5% perchloric acid. (b) 1000 μl of plasma blank extract. (c) 1000 μl of 0.5% perchloric acid with 5 ng ml^{-1} of I and 100 ng ml^{-1} of III (internal standard). (d) Chromatogram of 1000 μl of perchloric acid extract of plasma standard with 50 ng ml^{-1} of I added. (e) Chromatogram of the extract of a plasma sample of a volunteer taken 30 min following an i.v. dose of 7.5 mg of I. Column: 120 × 4.6 mm, Hypersil ODS, 5 μm. Mobile phase: 155 g of buffer pH 6.8 + 35 g of acetonitrile. Flow-rate: 2.0 ml min^{-1}. UV detection: 254 nm.

Fig. 2, together with a chromatogram of plasma from a volunteer 30 min after administration of a 7.5 mg intravenous (i.v.) dose.

Linearity. A linear response of the UV detector was obtained for up to 2000 ng of I injected in 1000 μl.

Recovery. The recovery of I from plasma was found to be 75% (standard deviation 8%) and was independent of the concentration.

Accuracy. The accuracy of the method, defined as the difference between the amount added to blank plasma and the amount found expressed as a percentage of the amount added, is summarized in Table II.

Precision. The precision of the method is represented by the relative standard deviation of the mean of replicate assays of the same sample. The precision of the method for spiked plasma samples is shown in Table III. For unknown clinical plasma samples, a mean precision of about 2% was found for the concentration range 5—185 ng ml^{-1} [8]; in the range of 5 ng ml^{-1} down to the detection limit the precision was about 8%.

Detection limit. The detection limit for I was dependent on the quality and the age of the column used. Based on a signal-to-noise ratio of 3:1, 0.5 ng of I injected in 1000 μl was detectable. This corresponds to a detection limit in

TABLE II

ACCURACY AND PRECISION OF THE ASSAY OF I IN SPIKED HUMAN PLASMA SAMPLES

Amount added (ng ml^{-1})	Amount found (ng ml^{-1})	n replicates*	Amount found expressed as percentage of amount added (%)	Coefficient of variation of the determinations (%)
90	92.3	11	103	3.2
60	62.6	11	104	2.7
30	32.0	9	107	4.4
15	15.7	5	105	6.2
7.5	8.2	14	109	8.5
3	3.1	13	103	8.3

*Replicates analysed on separate days over a period of eight weeks.

TABLE III

PRECISION DATA FOR I FROM CLINICAL TRIALS

Coefficient of variation was calculated from duplicates with each determination carried out on separate days; number of duplicates = n.

	Concentration range (ng ml^{-1})		
	< 5 (n = 17)	5—185 (n = 56)	10—35 (n = 10)
Plasma	8%	2%	—
Urine	—	—	4%

TABLE IV

STABILITY OF I IN HUMAN PLASMA

Five replicates in all cases. Statistical calculations are based on logarithms of the data [11].

Storage condition	Amount added (ng ml^{-1})	Amount found (ng ml^{-1})	Percentage difference between amount found and amount added (%)	Confidence interval for the difference between the means of the stored and freshly prepared sample (%)
One day at room temperature	40	39.8 (99.4)*	−0.6	−2.5 to +1.2
	10	9.87 (98.7)	−1.3	−3.4 to +1.0
Three months at −20°C	40	40.3 (101)	+1	−0.4 to +2.0
	10	9.8 (98)	−2	−7.2 to +3.5
Five months at −20°C	40	41.6 (104)	+4	+1.8 to +6.4
	10	9.96 (99.6)	−0.4	−2.9 to +2.3

*In parentheses: percentage of amount added.

plasma of about 1 ng ml^{-1}. With new, high-quality columns, this limit could be reduced to about 0.5 ng ml^{-1}.

Due to endogenous interferences, the detection limit in urine was about 10 ng ml^{-1}.

Stability. Plasma samples spiked with 10 and 40 ng ml^{-1} were stored for 24 h at room temperature and for three and five months at −20°C. The results of these stability tests are compiled in Table IV. The data indicate that I is stable in plasma.

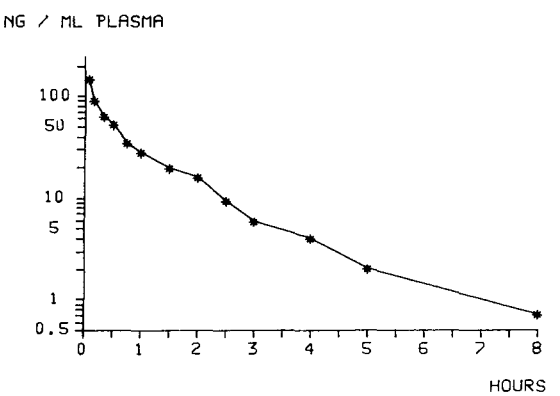


Fig. 3. Plasma concentration—time profile of I of a volunteer (S.H.) following a single i.v. dose of 7.5 mg of the drug as a microsuspension.

A 0.5% perchloric acid solution of I was stored at 4°C for three months, and no measurable degradation was observed. The internal standard III was less stable in 0.5% perchloric acid and fresh solutions should be prepared weekly.

Analysis of plasma and urine samples

The drug I was given to volunteers as a single i.v. dose of 7.5 mg as a microsuspension. The plasma levels of the unchanged drug were determined with the method described. The data for one volunteer are shown in Fig. 3. The corresponding urine data of the volunteer are given in Table V.

TABLE V

CONCENTRATIONS OF I IN URINE OF A VOLUNTEER (S.H.) FOLLOWING A SINGLE i.v. DOSE OF 7.5 mg OF THE COMPOUND AS A MICROSUSPENSION

Collection period (h)	Quantity of urine (ml)	Urine levels of I (ng ml^{-1})
0—1	500	25.6
1—2	108	10.1
2—3	68	<10

In general, following an i.v. dose of 7.5 mg, plasma levels could be determined up to 6—8 h post medication. The assay allowed the calculation of the most important pharmacokinetic parameters.

DISCUSSION

The main problem in developing an assay for I was to find suitable C_{18} reversed-phase material. Several 5-μm particle size materials were tried; for example, Polygosil C_{18}, Nucleosil C_{18}, and Spherisorb ODS 2. These products had good separation properties but there were large batch-to-batch variations which required a modification of the mobile phase for each new batch. Up to the present time, more than five different batches of Hypersil ODS 5-μm particle size have been used and no modification of the mobile phase has been necessary. Compared to normal-phase columns, the lifetime of ODS material is limited. After some days of operation, tailing peaks and a significant decrease in sensitivity were observed. By turning the column (inlet to outlet; replacement of filters) the original conditions were restored. This procedure could be repeated several times. It is interesting to note that no voids at the tops of the columns were observed using Hypersil ODS.

The pH of the buffer used for the mobile phase was reduced from 7.8 to 6.8 but the influence on the lifetime of the column was not significant [9].

The complex extraction procedure developed was necessary due to the polarity of I. Isobutanol was used at first for the extraction mixture, but negative interfering peaks appeared. With 1-hexanol instead of isobutanol, these interferences were eliminated. To improve the extraction yields, *n*-hexane was added to 0.5% perchloric acid for back-extraction. Although the recovery showed some variation from day to day, this did not influence the precision and accuracy of the assay.

The sensitivity of the method depended mainly on the quality of the column. As mentioned previously, detection was possible down to 0.5 ng ml^{-1}, although this limit could not always be attained.

The sensitivity of this assay for I is adequate for pharmacokinetic studies following a single i.v. dose of 7.5 mg of the drug.

To study the elimination phase of I in man a more detailed method which has a 5—10 times lower sensitivity limit than the present assay would be helpful. This limit cannot be reached by HPLC with UV detection.

Other approaches, such as derivatization prior to or post chromatography would have to be tried. It is questionable whether the sensitivity could be significantly improved with gas chromatography—mass spectrometry.

The assay of I was improved using the internal-standard technique [10]. The quantitative consequences of the variation in separation characteristics of a column during a series of determinations could be eliminated by this approach.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF NAFAZATROM IN HUMAN PLASMA USING FLUORESCENCE DETECTION

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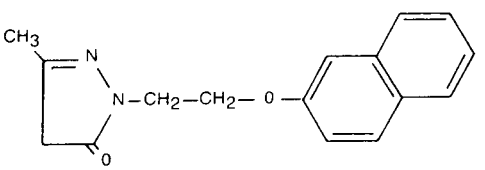
SUMMARY

A rapid, sensitive, and selective high-performance liquid chromatographic assay was developed for determination of the pyrazole derivative nafazatrom (Bay g 6575, NFZ) in human plasma. Separation was obtained using a normal-phase Si-60 column and a mobile phase of methylene chloride—methanol (90:10, v/v) containing 0.25% water. The fluorescence of NFZ was monitored at excitation and emission wavelengths of 232 and 362 nm, respectively. The recovery of NFZ extracted from plasma with methylene chloride was 109 ± 5% (mean ± S.D.) in the concentration range from 5.0 to 500 ng/ml.

The assay was applied to the determination of plasma concentrations of NFZ following administration of the compound to patients in a Phase I clinical trial.

INTRODUCTION

Nafazatrom (NFZ) {3-methyl-1-[2-(2-napthyloxy)-ethyl]-2-pyrazolin-5-one} (Fig. 1) was synthesized by Bayer (Wuppertal-Elberfeld, F.R.G.). NFZ has shown significant antithrombotic and thrombolytic activity in animal models [1]. The compound also has been reported to possess antitumor and anti-



3-Methyl-1-[2-(2-Naphthyloxy)-ethyl]-2-Pyrazolin-5-one

Fig. 1. Chemical name and structure of NFZ.

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metastatic activities [2], and it is currently being investigated in a Phase I clinical trial at Wayne State University (Detroit, MI, U.S.A.). An analytical method was needed to determine NFZ in plasma to characterize its pharmacokinetic behavior. A method using high-performance thin-layer chromatography (HPTLC) reported recently by Ritter [3] has 5.0 ng/ml sensitivity but requires post-chromatographic derivatization, the use of an autospotter optimized for high performance, and densitometric detection.

This report describes a high-performance liquid chromatographic (HPLC) assay with a one-step extraction procedure and fluorescence detection for quantitative determination of NFZ at clinically achievable plasma concentrations.

EXPERIMENTAL

Chemicals and reagents

NFZ was supplied by Miles Pharmaceuticals (West Haven, CT, U.S.A.). Citric acid and disodium phosphate were received from Fischer (Fair Lawn, NJ, U.S.A.). Distilled in glass methanol and methylene chloride were received from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). L-Cysteine hydrochloride (monohydrate) was obtained from Sigma (St. Louis, MO, U.S.A.). Human plasma was obtained from Harper Hospital Blood Bank (Detroit, MI, U.S.A.). Deionized water was used for preparation of all buffers.

Chromatography conditions

The HPLC system consisted of a Model M-45 solvent pump, a Model 710-B WISP automatic injector, and a Model M-730 Data Module (Waters Assoc., Milford, MA, U.S.A.). Separation was obtained using a Hibar-II, LiChrosorb Si 60, 10-μm 250 mm × 4.6 mm column (Anspec Company, Ann Arbor, MI, U.S.A.). The mobile phase consisted of methylene chloride—methanol (90:10) with 0.25% water. A Perkin-Elmer Model 650-10M fluorescence spectrophotometer or Model LC-75 variable-wavelength UV detector (Perkin-Elmer, Norwalk, CT, U.S.A.) was used for quantification. For fluorescence the excitation wavelength was 232 nm, and the emission wavelength was 362 nm. Slit widths of 7 nm, range of 30, and normal gain, response, and mode were used. A wavelength of 280 nm was monitored for UV detection. The chromatographic system was operated at ambient temperature with a flow-rate of 1.0 ml/min. Under these conditions the retention time for NFZ was approximately 4.5 min.

Standard solutions

NFZ standard solutions were prepared by adding an accurately weighed amount of NFZ to methylene chloride and diluting with additional methylene chloride to the working range of the method, 5.0—500 ng/ml. These solutions were injected directly.

NFZ spiking solutions were prepared by adding accurately weighed amounts of NFZ to methanol to yield concentrations from $5.0 \cdot 10^3$ to $5.0 \cdot 10^6$ ng/ml. These solutions were used to prepare plasma standards.

NFZ plasma standards were prepared by adding 10 μl of the appropriate methanol standard solution to 10 ml of plasma to yield plasma concentrations

from 5.0 to 500 ng/ml. After adding 50 μl of a 1 g/ml (w/v) solution of cysteine, the samples were vortexed for 30 sec. The plasma standards were used to establish a calibration curve for the quantification of the concentration of NFZ in plasma from patients.

All standard solutions, spiking solutions, plasma standards, and cysteine solutions were prepared the same day as samples were assayed.

Stability studies

The effect of cysteine on the stability of NFZ in human plasma was evaluated at 21°C as a function of time. Two 10-ml aliquots of plasma were spiked with 10 μl of a $0.9 \cdot 10^6$ ng/ml NFZ standard in methanol. To one of these plasma standards were added 50 μl of a 1 g/ml solution of cysteine in water. Three 1-ml aliquots of each plasma standard were analyzed immediately and at selected times thereafter. Plasma was obtained either from the hospital blood bank (three weeks old) or from a healthy volunteer for immediate use.

One plasma sample (containing added cysteine) from each of four different patients who received NFZ was analyzed as described and then stored at 0°C. The samples were again analyzed ten days later for stability.

The influence of pH on the stability of NFZ was studied between pH 2.0 and 10.0 using 0.1 *M* citric acid buffer at 21°C. To 10 ml of the various buffer solutions were added 10 μl of a $0.9 \cdot 10^6$ ng/ml NFZ standard in methanol. Three 1-ml aliquots of each solution were analyzed immediately and at selected times thereafter.

Plasma samples from a cancer patient given NFZ were analyzed by the above HPLC method and immediately frozen. These samples were packed in dry ice and shipped as soon as possible to Miles Pharmaceuticals. Ten days later the samples were analyzed using an HPTLC method [3].

Sample handling

Because of the potential instability of NFZ in plasma (see results), blood samples were centrifuged immediately after collection to separate plasma from blood cells, and 5 μl of a 1 g/ml cysteine solution were added per ml of plasma. The plasma samples were stored in an ice bath if the analysis was to be performed the same day or frozen at 0°C.

Extraction procedure

Plasma (1 ml), 1 ml of a 0.1 *M* citric acid buffer, pH 7, and 1 ml of methylene chloride were added to a 10-ml centrifuge tube and capped. The test tubes were shaken for 10 min and centrifuged for 10 min at 600 *g* at room temperature. The aqueous phase was discarded and 10—50 μl of the organic phase were chromatographed depending on the concentration expected. All patient samples with expected concentrations higher than 500 ng/ml were diluted (prior to extraction) into the working range 5.0—500 ng/ml with 0.1 *M* citric acid buffer, pH 7.

Preparation of patient samples

Cancer patients were given a twice-daily oral dose of 4000 mg/m^2 of NFZ in tablet form. Blood samples were collected at various time points for three

days via intravenous catheter and transferred to heparinized test tubes.

Duplicate plasma samples were extracted and chromatographed according to the above procedure.

Calculations

The recovery of NFZ in the extraction procedure was calculated by comparison of peak heights of NFZ in the standard solutions with peak heights of the extract of the spiked human plasma. The concentration of NFZ in ng/ml of plasma in an unknown sample was determined by interpolation from calibrations curves of standards processed along with the unknowns.

RESULTS

Sample chromatograms of methylene chloride extracts of control plasma (A), spiked plasma (B), and patient plasma (C) are shown in Fig. 2. No interfering peaks were seen in control plasma obtained from the blood bank or in patient plasma obtained before administration of NFZ.

The linearity, accuracy, and precision of the method were evaluated in plasma over a concentration range from 5.0 to 500 ng/ml NFZ, Table I. Triplicate samples at each of nine concentrations were extracted and chromatographed as described. The least-squares linear regression analysis of the data shown in Table I gives a correlation coefficient of 0.999. The mean coefficient of variation was 2.9%. The percent recovery of NFZ from human plasma was 109 ± 5%, and the limit of detection was 5.0 ng/ml in plasma.

The effect of cysteine on the stability of NFZ in fresh or stored human plasma can be seen in Table II. In fresh plasma NFZ was apparently stable for

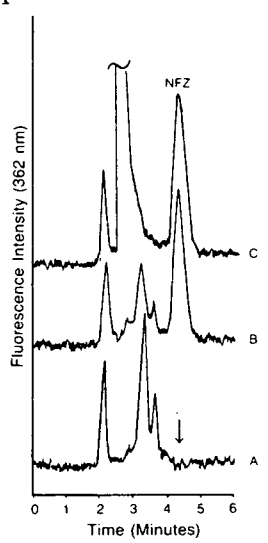

Fig. 2. Chromatograms of plasma extracts of (A) control plasma, (B) control plasma containing 54 ng/ml NFZ and (C) patient plasma 8 h after the second oral dose of 4000 mg NFZ per m² body surface area. Column: Hibar-II, LiChrosorb Si 60, 10-μm, 250 mm × 4.6 4.6 mm; solvent: methylene chloride—methanol (90:10, v/v) with 0.25% water; flow-rate: 1.0 ml/min.

TABLE I

LINEARITY AND PRECISION OF THE HPLC ASSAY OF NFZ IN PLASMA

NFZ concentration (ng/ml)		Coefficient of variation (%)	Recovery*** (%)
Theoretical*	Found** (mean ± S.D.)		
4.9	5.0 ± 0.0	0.1	100
9.7	10.2 ± 0.8	7.8	110
16.2	17.1 ± 0.7	4.1	114
26.8	27.3 ± 0.8	2.9	103
54.0	57.0 ± 0.7	1.2	114
108.0	114.7 ± 3.3	2.9	114
180.0	180.2 ± 5.0	2.8	106
300.0	303.9 ± 4.0	1.3	107
501.0	496.3 ± 16.4	3.3	109
	Mean ± S.D.	2.9	109 ± 5

*Theoretical concentrations were based on the amount of NFZ in methanol added to plasma.
**The concentration found was calculated from the calibration curve for each of three plasma samples per concentration. The mean, standard deviation, and coefficient of variation (n = 3) are tabulated.
***The percent recovery was calculated from peak heights of plasma standards divided by peak heights of standard solutions in methylene chloride containing the corresponding concentrations.

TABLE II

STABILITY OF NFZ SPIKED IN FRESH OR AGED PLASMA WITH OR WITHOUT ADDED CYSTEINE

Time (h)	Fresh plasma*		Aged plasma**	
	With cysteine	Without cysteine	With cysteine	Without cysteine
0	96, 100***	106, 101	106	105
1	106, 105	103, 105	103	94
2	114, 101	89, 104	98	84
3	104, 101	110, 101	99	77
4	106, 99	97, 103	97	74
5	101, 101	103, 100	105	66

*Obtained from a healthy volunteer immediately before use.
**Obtained from blood bank after three weeks of storage.
***Percent of added 90 ng NFZ per ml plasma remaining.

5 h whether cysteine was added or not. However, in stored plasma from the hospital blood bank (three weeks old) the NFZ concentration decreased 34% in 5 h if cysteine were not added.

The stability of NFZ in frozen plasma samples with added cysteine was investigated. The recovery after ten days based on the concentration found in the initial assay was 100.3 ± 2.9% (mean ± S.D., n = 4).

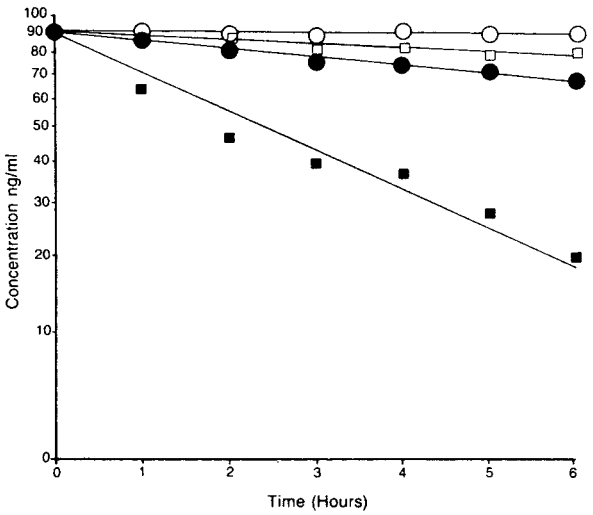

Fig. 3. The effect of pH on the stability of NFZ (90 ng/ml) in 0.1 *M* citric acid buffer. Key: (○) pH 7.0; (□) pH 4.0; (●) pH 2.0; (■) pH 10.0.

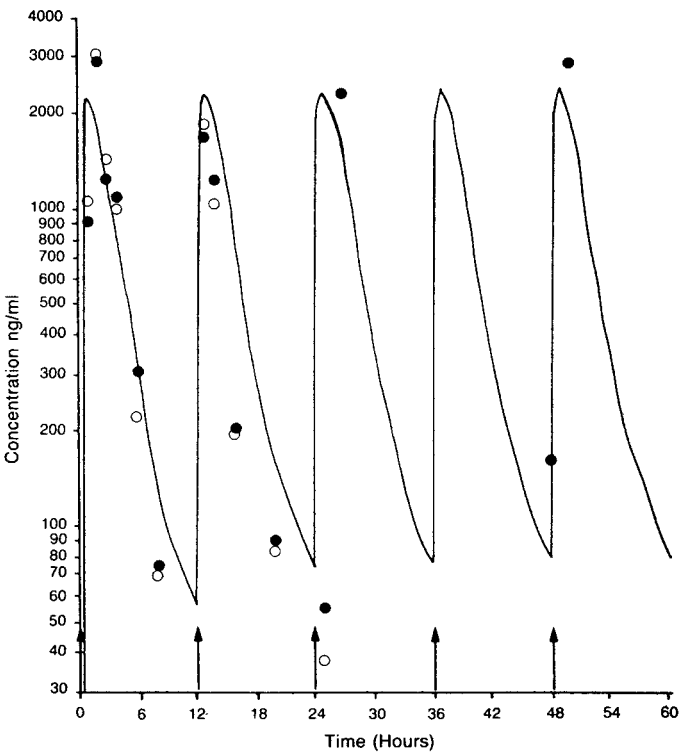

Fig. 4. Plasma concentration—time curve in cancer patient after repeated oral doses of 4000 mg NFZ/m² per 12 h. Key: (●) HPLC results described herein; (○) HPTLC results performed at Miles Pharmaceuticals Laboratory; arrows represent time of dose; curve is a computer simulation.

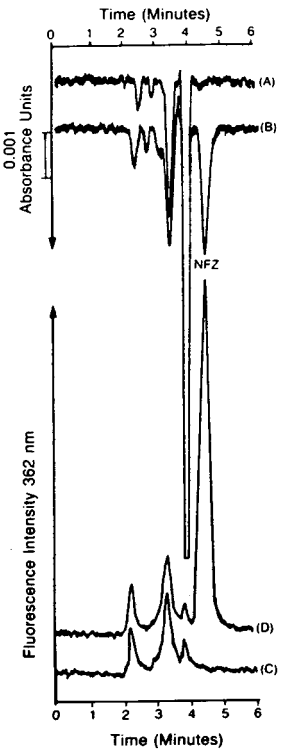

Fig. 5. Chromatograms of (A) blank methylene chloride, and (B) 56 ng/ml NFZ standard in methylene chloride by UV detection compared to (C) blank methylene chloride and (D) 56 ng/ml NFZ standard in methylene chloride by fluorescence detection. UV detector: LC-75 (Perkin-Elmer) 228 nm, 0.01 a.u.f.s.; fluorescence detector: 650-10M (Perkin-Elmer); excitation 232 nm, emission 362 nm; range 30.

The influence of pH on the stability of NFZ is shown in Fig. 3. The apparent linear degradation is much more rapid at extreme acidic or basic conditions. For this reason plasma samples were diluted 1:1 with a 0.1 *M* citric acid buffer, pH 7.0, before extraction.

Fig. 4 shows the concentration versus time curve for samples assayed both by the HPLC procedure described herein, and the HPTLC method performed at Miles Pharmaceuticals Laboratory. The ratio of the concentrations determined by HPLC to those by HPTLC was 1.09 ± 0.21 (mean ± S.D., n = 11).

Fig. 5 shows a direct comparison of UV absorption and fluorescence intensity with both instruments set at maximum sensitivity. A 56 ng/ml NFZ standard in methylene chloride was analyzed, and both UV (optimized at 228 nm) and fluorescence were monitored simultaneously. The fluorescence intensity is approximately three times greater than UV absorbance.

DISCUSSION

Due to the influence of the naphthylene ring on the chemical properties of NFZ, it can be extracted easily from an aqueous phase with methylene chloride. Chloroform also extracted approximately 100% of the NFZ from plasma, but a small co-extracted peak limited the sensitivity of the assay. Hexane or heptane did not extract NFZ from plasma.

The method described herein has a sensitivity limit of 5.0 ng/ml NFZ in plasma. Therefore, a concentration step was unnecessary. This low level of detection can be attributed partially to the excellent fluorescence properties of the drug.

The addition of cysteine to stored plasma samples enhances the stability of NFZ. Since fresh human plasma contains about 4 mg L-cysteine per l [4], the protective effect of adding more cysteine is not apparent. However, in stored plasma it may be speculated that L-cysteine becomes oxidized, leaving the pyrazolinone ring unprotected from oxidation. Thus, adding cysteine to standards prepared in stored plasma has a protective effect (Table II), and as a safeguard a large excess of cysteine is added to all plasma samples.

NFZ was found to be most stable near physiological pH. It is known that NFZ exists in either enol or enol-tautomeric forms due to the acidic hydrogens on the pyrazolinone ring [5]. NFZ has a pK_a of 8.24 determined by the —OH group on the pyrazolinone ring. Therefore, at physiological pH the majority of the drug will be in the keto form. Our studies show that the drug is most stable in solutions buffered at a pH which favors this form.

The correlation coefficient of 0.999 indicates a high degree of linearity in the concentration range from 5.0 to 500 ng/ml. The mean coefficient of variation of 2.9% demonstrates excellent precision. The percent recovery of NFZ from human plasma was 109 ± 5% and the limit of detection was 5.0 ng/ml in plasma.

The validity of the quantitative method has been confirmed by comparing results from samples analyzed by this HPLC method and a HPTLC method with good correlation.

The developed method is both specific and sensitive enough to measure the disappearance of NFZ from human plasma after drug administration [6]. It is a simple one-step extraction and analysis that requires basic HPLC equipment.

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Note

Quantitation of methanol formed in cell culture cytotoxicity assays and as a metabolite in microsome suspensions

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Many important cancer chemotherapeutic agents, mutagens and carcinogens are known to undergo a chemical or metabolic conversion to a methylating intermediate [1, 2]. Included in this category are methylnitrosamine, nitrosourea, hydrazine, azo, diazo, azoxy, diazotate, nitrosocarbamate, nitrosonitroguanidine and triazene containing compounds [3], which are precursors to the methyldiazonium ion. The pharmacologic activity of these agents is thought to result from the small fraction of methyldiazonium ion that reacts with cellular macromolecules. The fraction, over 95%, of methyldiazonium ion that does not covalently bind to large molecules reacts with water to form methanol [4]. Thus the amount of methanol formed in these reactions is a good measure of the amount of pharmacologically active species that has been produced. It is of interest to be able to relate the activity of an agent in a cytotoxicity or mutagenicity cell culture assay to the actual amount of active methyldiazonium ion formed during the assay procedure. Similarly, for agents that require metabolic activation, it is important to know the percent conversion of parent agent to active species that occurs in in vitro metabolic systems. This paper reports analytical methods for the measurement of methanol formed in in vitro rat liver microsome preparations and in cell culture assay incubations at concentrations as low as 10 nmol/ml.

There are a number of problems associated with the analysis of trace amounts of methanol in aqueous mixtures. The high volatility and polarity of methanol precludes extensive sample handling and extraction techniques. Derivatization methods for methanol become unreliable in dilute solutions [5, 6] and methanol itself is not readily detected using spectrophotometric or electrical

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detectors. Head-space analysis is unsatisfactory due to large errors in the results, 20—30% error has been reported with methanol [7, 8]. Gas chromatography with flame ionization detection can quantify methanol concentrations of 10^{-4} *M* [9]. Attempts to increase the sensitivity require large sample sizes, > 5 ml, and have lower precision [5].

This paper reports a gas chromatographic—mass spectrometric (GC—MS) assay that employs methane chemical ionization and selected ion monitoring to quantify 0.5 ng methanol per μl injected. Microsomal incubation preparations were found to contain significant amounts of methanol and ethanol as background impurities. Quantitation of methanol formed as a metabolite under these conditions is possible only if the parent agent contained a deuterium-labeled methyl group and ethanol-d_5 was used as an internal standard.

EXPERIMENTAL

Instrumentation

Mass spectra were obtained on a Finnigan 4023 mass spectrometer equipped with a dual chemical ionization (CI) electron impact source and operated in the CI mode. Methane was used as a carrier and reagent gas at an indicated ion chamber pressure of 0.25 torr. Samples were introduced through a gas chromatograph containing a 180 cm × 6.3 mm O.D. glass column packed with Porapak Q-S at a column oven temperature of 110°C. Eluting ions were detected using an Incos 2000 data system capable of monitoring selected ions.

Reagents and materials

1-Methyl-3-(4-methylphenyl)triazene, 1-methyl-3-phenyltriazene and 1-methyl-3-(4-carboxymethylphenyl)triazene were prepared by the method of White et al. [10]. 1-Hydroxymethyl-3-(4-carboxymethylphenyl)triazene was prepared by the method of Gescher et al. [11]. 1-Methyl-1-nitrosourea was from Sigma (St. Louis, MO, U.S.A.). Synthesis of methyl-d_3-benzylhydrazine hydrobromide will be described elsewhere [12]. Methanol-d_4 was from Aldrich (Milwaukee, WI, U.S.A.), ethyl-d_5 alcohol from MSD Isotopes (St. Louis, MO, U.S.A.). Dimethylsulfoxide (DMSO) (Mallinckrodt, St. Louis, MO, U.S.A.) was purified over 10-Å molecular sieves. Ethyl alcohol absolute was from U.S. Industrial Chemicals (New York, NY, U.S.A.), glucose-6-phosphate, NADP and glucose-6-phosphate dehydrogenase from Sigma. Fischer's medium from Bellco Biotechnology (Vineland, NJ, U.S.A.), 10% (v/v) donor horse serum from Gibco Labs. (Grand Island, NY, U.S.A.). Hypo-vials™ were from Pierce (Rockford, IL, U.S.A.).

Mass spectral analysis

Chemical reactions in aqueous solution or cell culture medium and metabolic reactions in microsome suspensions were conducted in 6-ml and 50-ml hypo-vials, respectively. The vials were sealed with PTFE-coated silicone septa. Larger vials were used in the microsome metabolisms to ensure an adequate supply of oxygen. The chemical or metabolic reactions were carried out at 37°C at a pH of 7.4 on 5 ml of reaction medium. The use of septum-sealed vials

prevented extensive loss of methanol which would have occured if the reaction medium were open to the atmosphere. In the chemical and cell culture reactions, a known amount of methanol-d_3 was added as an internal standard. The methanol-d_3 was added to the medium by injection through the septum prior to incubation. After incubation a known amount of ethanol was added as a second internal standard. In the metabolic reactions a known amount of ethanol-d_5 was added as the internal standard after the incubation. After the incubation reactions were complete, the vials were cooled to near 0°C and 1 μl of the solution was removed for injection into the GC—MS instrument. Chromatographic separation of the solvent water, methanol and ethanol was obtained by operating a Porapak Q-S column at 110°C. Elution times observed using methane as a carrier gas at a flow-rate of 35 ml/min were 1.7 min and 4.5 min for methanol and ethanol, respectively. Methanol, methanol-d_3, ethanol and ethanol-d_5 were detected by monitoring the respective protonated molecular ions. The amount of methanol present was quantified by measurement of the GC—MS peak height ratio with the ethanol standard with reference to a standard curve. The ratio of the methanol-d_3 to ethanol was monitored to determine if methanol was lost through evaporation or metabolism during the course of the incubation period and analysis.

Cell culture incubations

The cell incubation experiments were carried out with P388 lymphocytic leukemia cells suspended in Fisher's growth medium at pH 7.4. The medium was supplemented with 10% (v/v) donor horse serum, 100 μg/ml of streptomycin and 100 units per ml of penicillin G. Prior to each incubation, the cells were counted on a hemocytometer and the cell count was adjusted to a

TABLE I

METHANOL ANALYSIS IN P388 LEUKEMIA CELL SUSPENSION AFTER TREATMENT WITH METHYLNITROSOUREA AND METHYLTRIAZENES

Compound	Reaction time (min)	Initial concentration (μmol/5 ml)	Methanol calculated (pmol/μl)	Methanol observed (pmol/μl)	Percent difference*
Blank (DMSO)	15	0.0	0.0	40.6	
1-Methyl-1-nitrosourea	60	2.48	497	531	1.4
1-Methyl-3-phenyltriazene	12	3.62	650	709	2.8
1-Methyl-3-(4-methylphenyl)triazene	40	1.93	422	422	−9.5
1-Methyl-3-(4-carboxymethylphenyl)triazene	76	1.88	372	406	−1.9
1-Hydroxymethyl-3-(4-carboxymethylphenyl)-triazene	88	1.80	359	381	−5.3

*Percent difference = observed − blank value.

concentration of $1 \cdot 10^6$ cells per ml. The cell suspension was placed in a 6-ml hypo-vial and sealed. A 5-μl aqueous solution containing 10 μmol of methanol-d_3 and 86 μmol of ethanol was added by syringe through the septum followed by addition of a 25-μl dimethylsulfoxide—drug solution (see Table I for drug concentrations). Each drug was then incubated in a Dubnoff Metabolic Shaking Incubator at 37°C for a period of time equal to four half-lives (see Table I). At the conclusion of the incubation the mixture was cooled in an ice-bath and 1 μl of the solution was removed and analyzed by GC—MS.

Microsomal metabolism

The metabolism experiments were performed with rat liver microsomes isolated from phenobarbital-induced (6 mg per day for ten days) Fischer (F-344) male rats. The 100,000 *g* rat liver microsomal pellet suspensions were prepared according to the procedure of Fouts [13]. Metabolism reactions were carried out in 50-ml hypo-vials. The metabolism solutions contained 24 mg of microsomal protein (8 mg/ml), 9.4 m*M* glucose-6-phosphate, 3.7 units of glucose-6-phosphate dehydrogenase, 2.9 m*M* NADP, 6.1 m*M* $MgCl_2 \cdot 6H_2O$, 9.5 m*M* phosphate-buffered saline (PBS) "A" (pH 7.4) buffer and 0.11 m*M* methyl-d_3-benzylhydrazine hydrobromide. The solution was placed in the hypo-vial and the metabolism was initiated by addition of the glucose-6-phosphate dehydrogenase and the vial was then immediately sealed. The metabolism solution was incubated at 37°C for 20 min in a Dubnoff Metabolic Shaking incubator. After 20 min the metabolism vial was cooled to 0°C in an ice-bath and the internal standard, $C^2H_3C^2H_2OH$, was added by syringe through the septum. The samples were then analyzed by GC—MS using 1 μl of the metabolism solution.

RESULTS AND DISCUSSION

Reactions in aqueous solution, P388 cell culture medium or microsomal suspension were conducted in sealed vials to prevent loss of methanol by evaporation. Addition of standards and removal of samples from the reaction medium were made using a syringe inserted through a PTFE-coated silicone septum. No sample workup procedures were used because of the difficulties in handling trace amounts of volatile methanol. Instead, the reaction mixture was injected directly onto the GC column and the methanol and internal standards were detected with adequate sensitivity by methane CI-MS using selected ion monitoring. A standard curve for methanol in water gave a slope of 0.876 with a correlation coefficient of 0.966. The curve was linear over a range of 25 to 1100 pmol methanol injected.

The methanol MH^+ ion appears at *m/z* 33. At this low mass some sensitivity is lost due to the presence of an appreciable level of background ion current. An attempt was made to increase the observed mass of methanol by employing a CI reagent gas that formed higher mass association ions. Our most successful effort used ethylenediamine (E) as a reagent gas which was added to the methane carrier gas at a partial pressure that was great enough to give a reagent gas ion plasma consisting primarily of an ethylenediamine dimer $(E—H—E)^+$. When methanol was introduced into this plasma the only ion produced was $(CH_3OH—H—E)^+$. The stability of this ion was presumably due to the fact that

protonated methanol can form two hydrogen bonds to the ethylenediamine amino groups. Unfortunately, the efficiency of the ionization process was low and the net sensitivity was somewhat less than that of methane reagent gas.

We also employed tetramethylsilane as a reagent which has been reported to selectively form trimethylsilyl adduct ions with alcohols [14]. This report was confirmed by our results, but we experienced repeated serious losses of sensitivity related to contamination of the ion source by this reagent gas.

The cytotoxic activity of methylnitrosourea and 1-methyl-3-aryltriazenes can be measured by incubating these agents with suspended P388 leukemia cells in Fischer's medium containing 10% (v/v) donor horse serum at pH 7.4, 37°C for defined periods, usually 30 to 120 min. When the drug effect of treated vs. non-treated cells is related to the drug concentration decrease occurring during the cell exposure period, these agents are found to have equal activity [15]. If the concentration decrease is related to the amount of methyldiazonium ion formed, this provides further evidence that the toxic effect of presumably randomly generated methyldiazonium ion is independent of parent drug structure [16]. The relationship between parent drug concentration decrease, cytotoxicity and methanol formation may be defined more directly by measuring the amount of methanol formed during the assay incubation period.

The methanol assay procedure was tested in two experimental systems, an in vitro cell culture cytotoxicity assay and a microsomal metabolism preparation. The drugs listed in Table I were incubated with P388 cell suspension at 37°C by the method described above. Each drug was incubated for a period of time equal to four half-lives. After incubation the samples were analyzed by GC—MS. Table I shows the measured methanol concentration expected from disappearance of parent drug assuming 100% conversion to methanol. The small amount of methanol observed in the blank was an impurity in the dimethylsulfoxide. The impurity was reduced to the amount observed in the blank by treatment of the dimethylsulfoxide with 10-Å molecular sieves. Acetone has been found to contain less methanol impurity and may be used as an alternative solvent. The observed value less the blank corresponds closely to the expected value of methanol indicating that a single reaction pathway is operating with these agents. The average difference is −2.5% with a 4.4% standard deviation. Triazenes produce a 50% decrease in P388 cell growth rate at a dose that leads to the formation of 80 to 120 μM methanol. The concentrations reported in Table I are below the limits of detectable toxicity.

Methanol was also measured as a product of the phenobarbital-induced rat liver 100,000 *g* microsomal metabolism of 1-methyl-2-benzylhydrazine hydrochloride. Methylhydrazines are converted to azo, azoxy and azoxycarbinol metabolites by cytochrome P-450 enzymes [17]. The proposed azoxycarbinols react chemically to give methyldiazonium ion intermediates and methanol as a stable product [18]. Measurement of methanol formation would indicate the extent of conversion of parent drug to the ultimate active species, methyldiazonium ion. Analysis of the incubation mixture in the absence of substrate showed high levels of both methanol and ethanol. This material was present in the glucose-6-phosphate (methanol) and NADP (ethanol) and could not be

completely and reproducibly removed. Since the amount of methanol formed in this reaction was small, the presence of even low amounts of background methanol and ethanol prevented its measurement.

An accurate analysis of methanol metabolite formation could be made, however, by using a deuterium-labeled substrate, 1-methyl-d_3-2-benzylhydrazine hydrobromide and ethanol-d_5 as a standard. Fig. 1 shows a representative selected ion chromatogram of methanol-d_3, m/z 36, and standard ethanol-d_5 formed during the 20 min incubation of 1.02 μmol (142 μg)

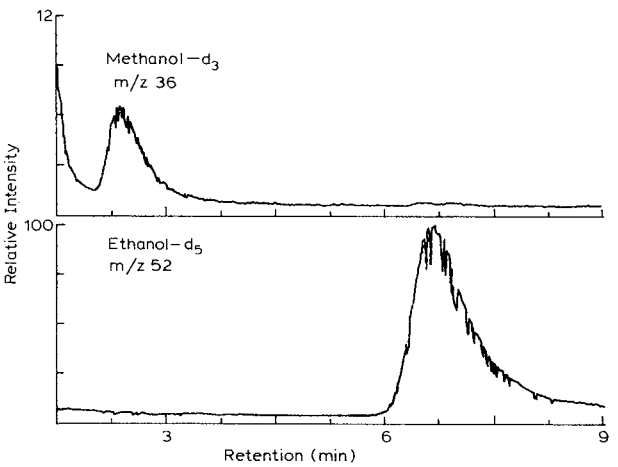

Fig. 1. Selected ion chromatogram of methanol-d_3, m/z 36, and internal standard ethanol-d_5, m/z 52, present in 1 μl of a 100,000 g microsomal metabolism mixture of 1-methyl-d_3-2-benzylhydrazine. The methanol peak at retention time 1.7 min represents 10.9 pmol injected onto a Porapak Q-S column at 110°C.

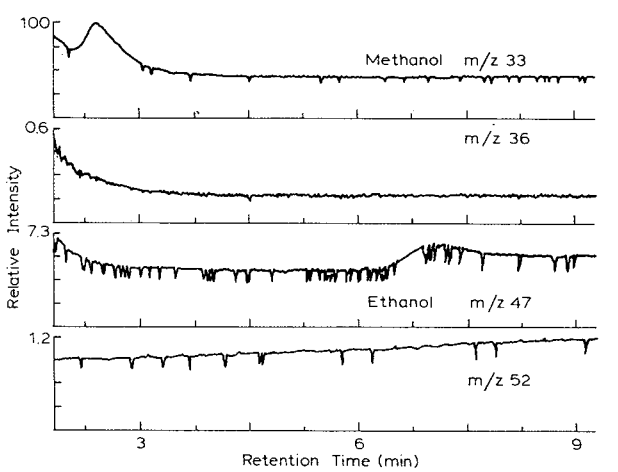

Fig. 2. Selected ion chromatograms from the injection of 1 μl of 100,000 g microsome suspension containing all cofactors and solvents normally present in a drug metabolism experiment. Methanol, m/z 33, and ethanol, m/z 47, are present as significant impurities that could not be completely removed.

deuterated 1-methyl-2-benzylhydrazine in 5 ml of rat liver 100,000 *g* microsome suspension containing 24 mg microsomal protein. The methanol peak of Fig. 1 represents 10.9 pmol injected onto the column or 54.5 nmol of methanol formed in the metabolism reaction, 5.3% of initial substrate. A 5.4% standard deviation was observed on three replicate incubations. Fig. 2 shows a selected ion chromatogram of a 100,000 *g* microsomal suspension that contains all cofactors and solvents but not substrate. Methanol, *m/z* 33, and ethanol, *m/z* 47, are present as impurities at a concentration comparable to the amount of methanol formed in these inefficient metabolism reactions. No interfering peaks are present at the masses of the deuterated variants.

These results demonstrate that methanol can be quantitated in the low pmol range from complex mixtures. This assay can provide a measure of the amount of active methyldiazonium ion formed from cytotoxic and carcinogenic methylating agents and will aid in the understanding of the mechanism of action of these compounds.

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Note

Studies on volatile metabolites of some potentially pathogenic *Bacillus* species, using automated head-space gas chromatography

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Use of head-space gas chromatography (HSGC) has now been extended to diagnostic microbiology. HSGC enables the detection of volatile bacterial metabolites. The technique is simple and, because of its suitability for automation, it has high sample capacity. Automated HSGC, with capillary column, has been utilized in the identification of anaerobic bacteria [1] and for rapid diagnosis of bacteraemia [2].

Several species of *Bacillus* have recently been claimed as causal agents in a variety of pathologic conditions, e.g. abscesses, pneumonia, osteomyelitis, bacteraemia, endocarditis, bovine mastitis and food-poisoning [3]. The recognition of this biochemically heterogeneous group of bacteria is therefore increasingly important. The present investigation was undertaken to evaluate the differential diagnostic capacity of HSGC in regard to some potentially pathogenic species of *Bacillus* by analysis of volatile acidic and neutral metabolites.

EXPERIMENTAL

The study comprised 65 strains, representing altogether seventeen species of *Bacillus* (Table I). All strains were subcultured on blood agar plates and incubated under aerobic conditions in 100-ml flasks containing 20 ml of

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TABLE I

STRAINS OF *BACILLUS* SPECIES INVESTIGATED

Organism	No. of strains from clinical specimens	Reference strains	Total No. of strains
B. alvei	—	NCTC 6352, NCTC 3349	2
B. badius	—	NCTC 10333	1
B. brevis	—	NCTC 2611	1
B. cereus	10	NCTC 2599, NCTC 6474	12
B. circulans	3	NCTC 5846, NCTC 2610	5
B. coagulans	3	NCTC 3991, NCTC 10334	5
B. firmus	1	NCTC 10335, CCM 2212	3
B. laterosporus	—	NCTC 6357, NCTC 2613	2
B. lichteniformis	5	NCTC 10341, NCTC 962	7
B. macerans	1	NCTC 6355, NCTC 1068	3
B. megaterium	—	NCTC 10342, NCTC 5635	2
B. pantothenticus	1	NCTC 8122, NCTC 8162	3
B. polymyxa	3	NCTC 10343, NCTC 4744	5
B. pumilus	—	NCTC 10337, NCTC 2595	2
B. sphaericus	1	NCTC 10338, NCTC 2608	3
B. stearothermophilus	3	NCTC 10339	4
B. subtilis	3	NCTC 3610, NCTC 5398	5
Total	34	31	65

trypticase soy broth (TSB) at 30°C, all cultures in duplicate, for 24 and 48 h respectively. Strains of *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Shigella sonnei*, *Staphylococcus aureus*, *Streptococcus faecalis* and *Yersinia enterocolitica*, all isolated from clinical specimens at the Bacteriology Department, University Hospital, Lund, were incubated at 37°C but otherwise under identical conditions as the *Bacillus* strains. For evaluation of reproducibility, *B. cereus* (NCTC 2599) and *B. macerans* (NCTC 6355) were additionally each incubated in five flasks with TSB for 24 h after which 2-methylpentanoic acid was added as internal standard to a final concentration of 0.005% (w/w). The reference mixture in HSGC analyses was an aqueous solution of acetic (16 m*M*), propionic (6 m*M*), isobutyric (2 m*M*), butyric (4 m*M*), isovaleric (1 m*M*) and valeric (1 m*M*) acids. From the broth cultures, from non-inoculated TSB medium and from the reference solution, 1-ml aliquots were transferred to glass ampoules fitting into the automatic turntable of the HSGC analyzer. To each ampoule were then added five drops of sulphuric acid (25%, w/w, in water) and a saturating amount of solid magnesium sulphate. The ampoules were thereafter sealed with PTFE-lined rubber membranes and aluminium crimp caps.

The gas chromatograph (F 45, Perkin-Elmer) was equipped with a unit for automatic head-space injection, a flame ionization detector at 150°C and a 25-m fused-silica capillary column coated with SP-1000. No attenuation of the detector signal was used. Before the analysis the samples were heated in the automatic turntable of the instrument at 80°C for at least 20 min to ensure temperature equilibration. The flow-rate of the carrier gas (nitrogen) was

0.8 ml/min at a split ratio of 1:12. The column was held at 110°C, the injection needle at 250°C and the injector at 200°C.

RESULTS AND DISCUSSION

With this HSGC system, all the fatty acids in the standard solution could readily be separated within 8 min. Sterile TSB medium incubated under exactly the same conditions as medium inoculated with the *Bacillus* species gave chromatograms which contained few and negligibly small peaks in comparison with those obtained in analyses of broth cultures (Fig. 1).

Chromatograms representing *Bacillus badius*, *B. brevis*, *B. cereus*, *B. circulans*, *B. coagulans*, *B. lichteniformis*, *B. megaterium*, *B. polymyxa*, *B. pumilus*, *B. sphaericus*, *B. stearothermophilus* and *B. subtilis* were all very much alike. After 24 h of incubation the most prominent peaks corresponded to ethanol, and acetic, isobutyric and isovaleric acids. There were also minor yields of propionic acid and butyric acid. *B. firmus* and *B. laterosporus* gave somewhat lower yields of the same fatty acids. Apart from acetic acid, *B. alvei*, *B. macerans* and *B. pantothenticus* produced no detectable amounts of acids even after 48 h of incubation, although heavy growth was observed. The major peak in these chromatograms instead corresponded to ethanol. Typical chromatograms of *B. cereus* and *B. macerans* with the internal standard (2-methylpentanoic acid) added, are shown in Fig. 1. For *B. cereus*, the peak areas related to the internal standard at the five reproducibility studies were in the range 0.20—0.32 (acetic acid), 0.09—0.11 (propionic acid), 0.92—1.20 (isobutyric acid), 0.10—0.14 (butyric acid) and 1.08—1.42 (isovaleric acid). None of the organisms other than *Bacillus* — all of which are common food-poisoning agents — produced more than trace amounts of isobutyric and isovaleric acids.

In addition to *B. anthracis*, several species of *Bacillus* are now recognized as causing a variety of severe infections [4, 5]. *B. cereus*, for example, is considered to be a food-poisoning agent [6] and can cause panophthalmia. Further, *B. subtilis*, *B. lichteniformis* and, though more rarely, also other *Bacillus* species, are occasionally found as clinical or food-borne pathogens [7]. Despite the published reports, the pathogenicity of these organisms has been slow in gaining general acceptance [3].

Bacteria of the genus *Bacillus* are characterized as aerobic spore-formers of gram-type positive, negative or zero [8]. Current taxonomic designations are based on a variety of analytical procedures, including conventional biochemical tests [9, 10], enumerative, sometimes computerized, techniques [11—14], DNA homology studies [15], serology [16] and chromatography [17, 18]. With these procedures, singly or in certain combinations, it is possible to demonstrate differences within and between species. That several *Bacillus* species produce appreciable amounts of isobutyric and isovaleric acids, as demonstrated in our study, does not seem to have been reported previously.

The chromatographic results indicated unity rather than differentiation of the studied species, and thus metabolic homogeneity. Of the seventeen *Bacillus* species studied, fourteen gave virtually identical chromatographic patterns, which could be easily distinguished from those of several other organisms which commonly occur as food-poisoning agents. Gas chromatography appears

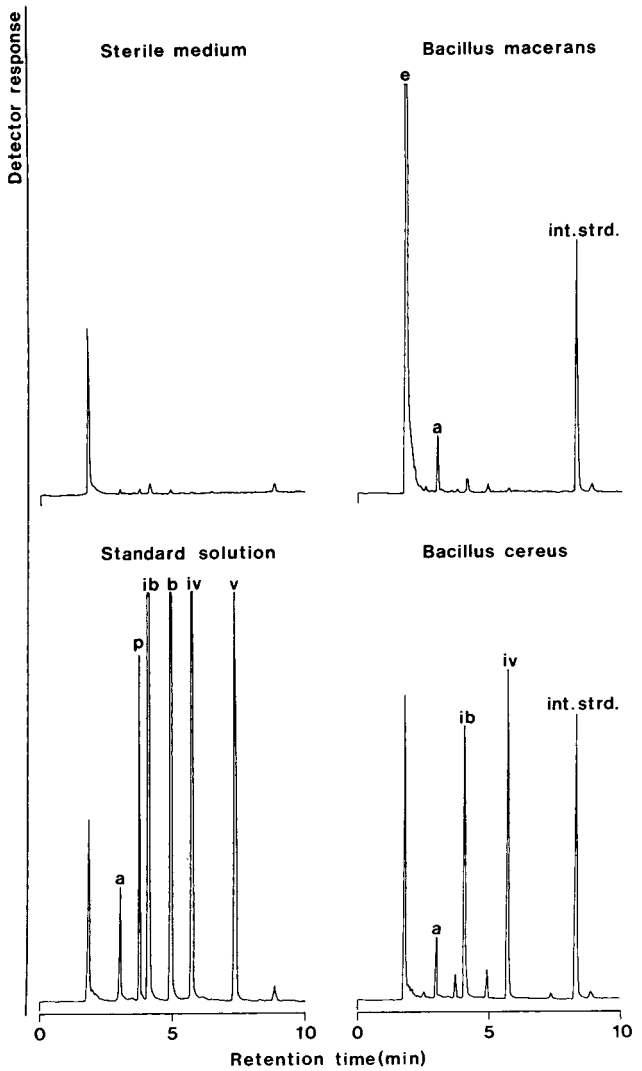

Fig. 1. Chromatograms (head-space gas chromatography) obtained from analyses of a standard solution of fatty acids, of non-inoculated trypticase soy broth medium, and of the same medium incubated for 24 h with *B. cereus* or *B. macerans*. e = Ethanol, a = acetic acid, p = propionic acid, ib = isobutyric acid, b = butyric acid, iv = isovaleric acid, v = valeric acid; int. strd. (Internal standard) = 2-methylpentanoic acid. See text for specification of test conditions.

to be a useful tool in epidemiologic studies when the presence of *Bacillus* is suspected.

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CHROMBIO. 1890

Note

High-performance liquid chromatographic method for the simultaneous determination of myocardial creatine phosphate and adenosine nucleotides

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(First received March 10th, 1983; revised manuscript received August 18th, 1983)

The quantitation of energy stores [creatine phosphate (CP) and adenosine nucleotides, adenosine 5′-triphosphate (ATP), adenosine 5′-diphosphate (ADP) and adenosine 5′-monophosphate (AMP)] is of paramount importance in the study of living-tissues metabolism. This is more so in the heart, which depends to a very great extent on an uninterrupted aerobic metabolism to maintain its normal function. Availability of data on myocardial energy stores is, therefore, indispensable for assessing the responses of the heart to drugs or stresses such as ischemia and hypoxemia.

Until recently this information was not easily obtainable due to a cumbersome and time-consuming methodology. At least two different assays were needed to determine the levels of CP with ATP and ADP with AMP, in relatively large amounts of tissue. Recently, the technique of high-performance liquid chromatography (HPLC) has been successfully applied to this problem [1—7]. Even this approach required the run of two separate assays, one for CP and another for adenosine nucleotides [5]. More recently, simultaneous determination of these compounds has been reported by Harmsen et al. [6, 7]. Their method, however, had to be adapted to our needs for the reasons discussed below. This report is a description of their modified methodology as used at present in our laboratory. It provides an easy, rapid and reliable way of determining energy stores in small quantities of myocardial tissue.

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EXPERIMENTAL

Equipment

The HPLC system was a Beckman 332 model consisting of two Model 110A pumps, a wavelength-selectable detector Model 160 set at 214 nm, a Series 210 universal injection valve and a Model 420 controller/programmer. The latter component allowed the programming of flow-rates and buffer ratios. The results were processed by a Hewlett-Packard integrator Model 3390A.

The column used (25 × 0.46 cm) contained Partisil-10-SAX (10-μm particles) and was provided with a guard column filled with Pellinox-Sax. Buffer A was 0.4 *M* potassium dihydrogen phosphate in 0.4 *M* potassium chloride (pH 4.2 adjusted with potassium hydroxide) and buffer B, 0.05 *M* potassium dihydrogen phosphate (pH 3.3 adjusted with orthophosphoric acid). The characteristics of a typical run are shown in Table I.

TABLE I

CHARACTERISTICS OF THE SAMPLE RUNS

Time (min)	Gradients		
	Flow-rate (ml/min)	Buffer A (%)	Buffer B (%)
0—13	0.5	0	100
13—25	1.5*	30**	70**
25—35	1.5	100*	0*

*Change over 1.5 min.
**Change over 0.5 min.

Reagents, standards and procedure

Either HPLC-grade or the purest available chemicals were used for all solutions. Water was purified in a Barnstead Nanopure II four-cartridge system before making the buffers and other solutions.

CP, ATP, ADP and AMP, obtained from BMC Diagnostics (Dorval, Canada), were used to prepare suitable standards in 0.9 *M* perchloric acid, which were subsequently neutralized with 6 *M* potassium hydroxide.

Tissue samples were obtained from hearts in situ or after perfusion and quickly frozen with a Wollenberger clamp at liquid-nitrogen temperature or immediately homogenized with a Polytron sonicator/homogenizer (Brinkman, Westbury, NY, U.S.A.) in 2.0 ml of 0.9 *M* perchloric acid. The pH was adjusted to 5 with 3.75 *M* potassium carbonate. A 100-μl aliquot of the extract was then applied to the HPLC system. Tissue aliquots and the volume of the homogenate and the extracts could be varied according to the anticipated tissue concentrations and the sensitivity of the detector. In our system the latter was usually set at 0.4 a.u.f.s. (the lowest sensitivity being 2.0 a.u.f.s.).

For enzyme peak shifts, myocardial tissue was homogenized in the presence of 4 mg of glucose per ml of extract and 0.5 *M* magnesium sulfate and the pH adjusted to neutrality with 6 *M* potassium hydroxide. Specific enzymes [1, 7]

were added stepwise followed by incubation at 37°C for 30 min. At the end of each incubation period, aliquots of the extract were then run in the HPLC system.

RESULTS

Fig. 1A shows a chromatogram of a standard solution containing creatine, CP, ATP, ADP and AMP, to illustrate the separation and sharpness of the peaks. The duration of the run was 28 min.

Fig. 1B illustrates a chromatogram of an extract of fresh non-perfused rabbit myocardium.

Peaks were identified by their retention times, by spiking (adding known amounts of the compounds of interest to an aliquot of the extract), and by enzymatic shifting of the peaks. The latter is the most reliable approach, and the results are shown in Fig. 1C—F. In step 1 (Fig. 1C) the ATP peak disappears after the addition of hexokinase, while the ADP peak increases markedly. In step 2 (Fig. 1D) creatine kinase reduces the CP peak while increasing the concentration of creatine. In step 3 (Fig. 1E) ADP is markedly reduced by myokinase with concomitant elevation of AMP. Finally, in step 4 (Fig. 1F) AMP

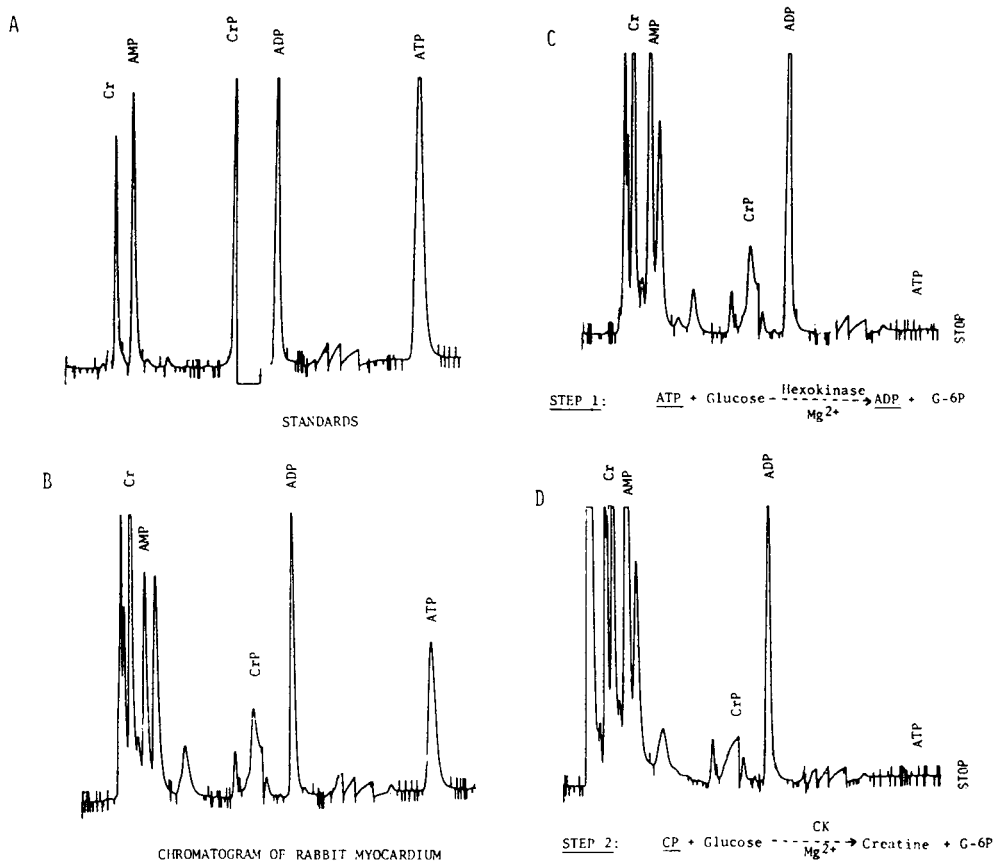

Fig. 1.

(Continued on p. 186)

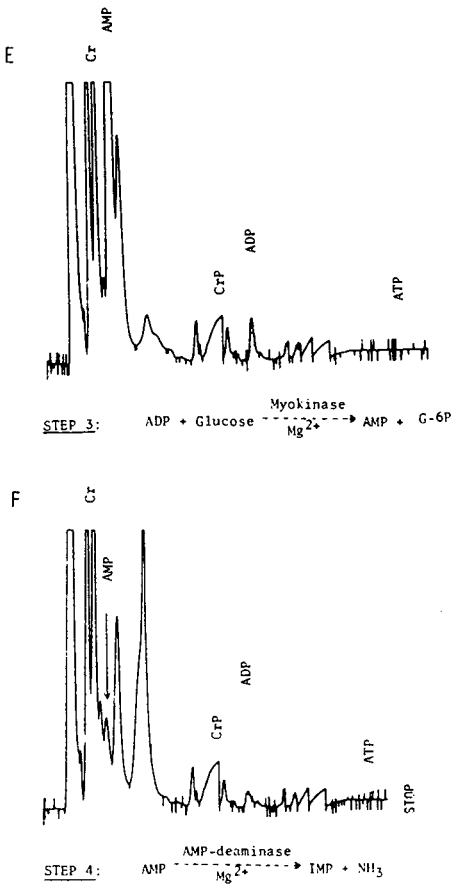

Fig. 1. (A) Run of standards. Cr = creatine; CP = creatine phosphate; ATP, ADP and AMP = adenosine tri-, di-, and monophosphate, respectively. (B) Chromatogram of an extract of non-perfused rabbit myocardium. The duration of the run is around 28 min. (C) The addition of hexokinase in the presence of glucose and Mg^{2+} converts ATP to ADP with changes in the corresponding peaks. (D) After addition of creatine kinase (CK), CP disappears with increase in creatine. (E) The addition of adenylate kinase (myokinase) results in the conversion of ADP to AMP. The ADP peak is markedly decreased; its persistence may be related to the large amounts of ADP present or insufficient incubation time, since it is no longer present in the next step (see Fig. 1F). (F) The final step is the reduction of the AMP peak by AMP deaminase (5-adenylic acid deaminase) via its transformation to inosine monophosphate (IMP) (not identified).

concentration decreases after the addition of AMP deaminase, resulting in the production of inosine monophosphate (IMP) (not identified).

The present method allows determination of the energy stores in as little as 10—15 mg of mycardial tissue. The change in concentration of the energy stores in samples frozen up to four weeks at —30° C was less than 5%. After the addition of known amounts of the different compounds to a tissue extract, the recoveries were 101% for CP, 93% for ATP and 90% for ADP and AMP. The lower limits of detection were (in nmol) 2.27 for CP, 0.343 for ATP, 0.213 for ADP and 0.072 for AMP.

DISCUSSION

Our results show that CP and adenosine nucleotides stores in the myocardium can be determined simultaneously by HPLC in approximately 28 min. Further improvement of this method will shorten the duration of the run and increase its sensitivity. This can be achieved by reducing the volume of the extract, and/or increasing the volume of the injectate and the sensitivity of the detector. The latter, however, has limitations since it can markedly distort the baseline.

Harmsen et al. [7] were very kind to provide us with details of their methodology before publication. However, their method did not work in our system for several reasons. Firstly, the change in buffers (0.01 *M* orthophosphoric acid and 0.75 *M* potassium dihydrogen phosphate) during the run caused exaggerated baseline shifts in our system that could not be compensated; secondly, the use of a strong buffer B (0.75 *M* potassium dihydrogen phosphate) was hard on the pump because of salt precipitation; and thirdly, the high flow-rate (2.0 ml/min) did not allow us to achieve good peak separation.

By changing buffer A from 0.01 *M* orthophosphoric acid to 0.4 *M* potassium dihydrogen phosphate, buffer B from 0.75 *M* potassium dihydrogen phosphate to 0.05 *M* potassium dihydrogen phosphate, and reducing the flow-rate from 2.0 to 1.5 ml/min, we were successful in obtaining similar results.

In our hands, the described method is easy, rapid and accurate. It has the important advantage of permitting the simultaneous determination of CP and adenosine nucleotides in extracts of several tissue samples in the same day. Easier isocratic methods have been described for the separation only of nucleotides, but, as far as we are aware, always excluding creatine phosphate. The advantage of our method is that both CP and nucleotides can be now quantitated in an aliquot of tissue extract processed in a single HPLC run. This method could be easily adapted to other HPLC systems.

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Note

New fluorimetric determination of 17-hydroxycorticosteroids after high-performance liquid chromatography using post-column derivatization with benzamidine

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

Detection of steroids after high-performance liquid chromatography (HPLC) has usually been performed by ultraviolet (UV) absorption at 240 nm of the Δ^4-3-keto group of steroids or by derivatization with fluorescent compounds [1—3]. These methods, however, can not be applied to the analysis of urine in which most corticosteroids are present in tetrahydro form.

Recently, we developed a new fluorimetric method for a determination of urinary corticosteroids using a reaction with glycinamide [4, 5]. This method has the advantage of being highly selective and capable of measuring 17-hydroxycorticosteroids and most 17-deoxycorticosteroids.

Ohkura and Kai [6] have reported a method for the fluorimetric determination of guanidino compounds in which the reaction of guanidino compounds with benzoin, a ketol derivative, under alkaline conditions yields fluorescent diphenylimidazole derivatives. Based on their results we inferred that compounds having a ketolic group might give fluorescent compound(s) when heated with amidine derivative under alkaline conditions. Therefore, we tried a reaction of steroids carrying a ketolic group with benzamidine and found that 17-hydroxycorticosteroids yielded fluorescent compound(s) with excitation and emission maxima of 370 and 480 nm, respectively.

In this paper, application of the new method for the fluorimetric determination of 17-hydroxycorticosteroids separated by HPLC is described.

EXPERIMENTAL

Materials

Benzamidine hydrochloride was the product of Aldrich Chemical Company

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and was purchased from Yashima (Osaka, Japan). Other reagents were of analytical grade and used without further purification.

Standard solution of steroids

Steroids were dissolved in aqueous methanol (methanol—water, 1:1, v/v) at a concentration of 10 μg/ml.

Reagent for fluorimetry

Reagent A is a sodium hydroxide solution (0.4 mol/l) and reagent B is a solution of benzamidine hydrochloride (0.5%, w/v) in a mixture of 2-propanol—water (1:1, v/v).

Mobile phase

Methanol (500 ml) was made up to 1000 ml with reagent-grade water and mixed well. Then air bubbles were removed using a suction pump under reduced pressure.

Apparatus

We used a TRiRotar high-performance liquid chromatograph equipped with a variable-loop injector, spectrofluorimeter equipped with a 30-μl flow cell (Model FP-550F), and a recorder (Model RC-125). The column, Finepak C_{18}, was octadecyl silica, 10 μm particle size, 4.6 mm diameter and 25 cm length. A reciprocal-type pump equipped with two pump heads pumping liquid alternatively (Model SP-2-24) was used to mix reagents A and B. All apparatus was from Japan Spectroscopic Co.

Preparation of sample

Pipette 2 ml of urine sample into a 10-ml glass stoppered test tube and adjust to pH 6.5. Add 0.1 ml of β-glucuronidase (500 Fishman units/ml, from *Escherichia coli*), 0.2 ml of 0.2 *M* phosphate buffer (pH 6.5), and one drop of chloroform to the test tube and mix well. Incubate the mixture for 24 h at 37°C. Add 2 μg of β-methasone (20 μl of 100 μg/ml solution in methanol), as internal standard, and shake solution with 4 ml of methylene chloride for 3 min. Discard the urine layer and wash the organic layer with 0.5 ml of 0.1 *M* sodium hydroxide and 0.5 ml of water successively. After centrifugal separation, transfer 2 ml of the extract to another test tube and evaporate to dryness in a hot water bath at 80°C.

Chromatographic separation and fluorimetric determination of steroids

Add 100 μl of mobile phase to the dried residue of the extract and dissolve the extract, 10 μl of this solution were injected into the chromatograph. The column temperature was kept at 40°C and the flow-rate of the mobile phase was 0.8 ml/min. Effluent from the column is mixed with the mixture of reagents A and B. Reagents A and B were pumped at a flow-rate of 0.5 ml/min; they were mixed using a T-shaped connector. The mixed reagent was added to the effluent from the column via a T-shaped connector and heated at 95°C for 5 min in a PTFE tube (I.D. 0.5 mm, length 30 m) immersed in a water bath, and then cooled to room temperature by passing through a tube immersed in

a water cup. Fluorescence was measured at 480 nm, with excitation at 370 nm; the slit width for both excitation and emission was 20 nm.

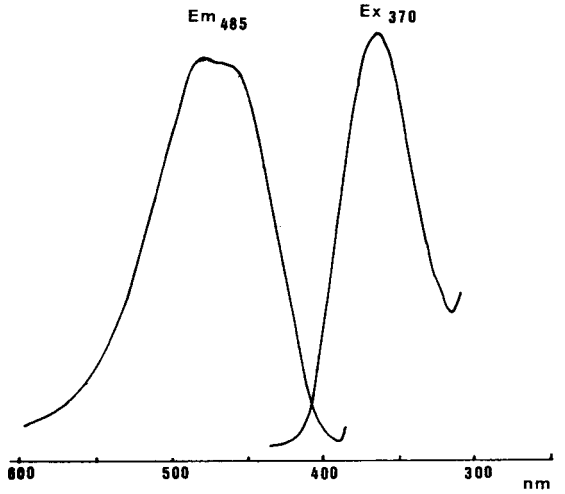

Fig. 1. Excitation and emission spectra of fluorescent compounds. Two millilitres of water, 0.5 ml of 0.4 *M* sodium hydroxide and 0.5 ml of 1% benzamidine were added to a test tube containing cortisol (50 μg per tube) and then the mixture was heated at 95°C for 5 min. The fluorescent compound formed has a maximum at 370 nm for excitation and at 480 nm for emission.

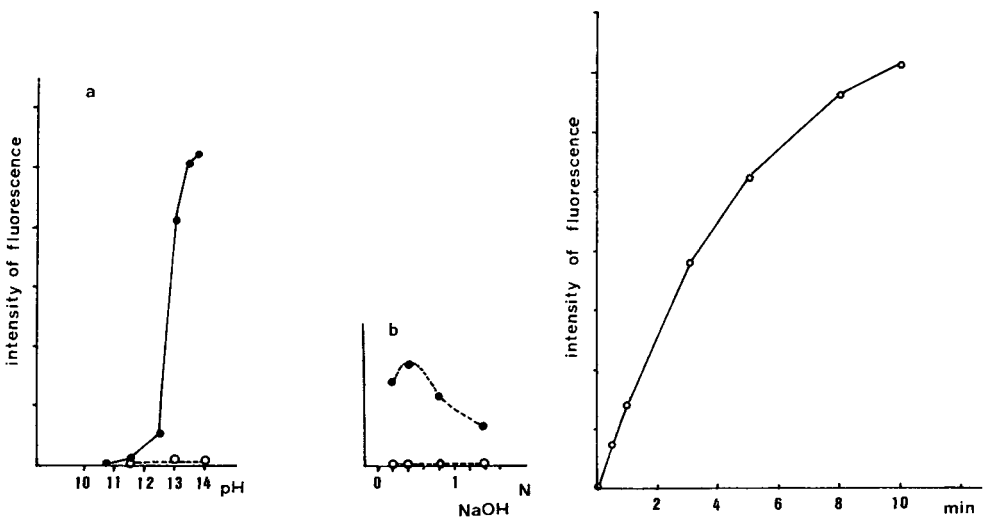

Fig. 2. Effect of pH on the intensity of fluorescence (a) in the test tube, (b) in HPLC analysis. (a) In the test tube, the intensity of fluorescence was highest between pH 13 and 14. (b) In HPLC analysis the concentration of sodium hydroxide in reagent A was examined and the highest fluorescence intensity was found around 0.4 *M*. (•), cortisol; (○), corticosterone.

Fig. 3. Time course of the reaction. Two millilitres of water, 0.5 ml of 0.4 *M* sodium hydroxide and 0.5 ml of 1% benzamidine were added to a test tube containing cortisol (50 μg per tube) and the mixture was heated at 95°C for the specified time, 5 min in the case of HPLC analysis.

RESULTS AND DISCUSSION

Excitation and emission spectra of the fluorescent compounds formed from the reaction of cortisol with benzamidine are shown in Fig. 1. Maximum fluorescence intensity was found at pH between 13 and 14 in the test tube and by the post-label method as shown in Fig. 2.

The time course of the reaction of formation of fluorescent compounds in the test tube is shown in Fig. 3. The selectivity of the reaction utilized in the post-label method is shown in Table I. Compared with the method using glycinamide, which can detect both 17-hydroxy-20-oxo-21-hydroxycorticosteroids and 20-oxo-21-hydroxycorticosteroids, this method is selective for the compounds carrying a dihydroxyacetone side-chain or group.

Chromatograms of a standard mixture and of samples from a normal subject and a subject with Cushing's disease are shown in Fig. 4. This method is based on the reaction of benzamidine with the dihydroxyacetone side-chain of corticosteroids at C-17 so that both Δ^4-3-keto-17-hydroxycorticosteroids and tetrahydro-17-hydroxycorticosteroids could be measured, and at the highest sensitivity of the fluorimeter cortisol in serum could be measured at a level of 5—50 ng per injection as shown in Fig. 5.

The within-assay coefficient of variation (C.V.) calculated for a normal urine sample (five repeated assays) was 2.4% for tetrahydrocortisol and 3.2% for tetrahydrocortisone. The between-assay C.V. was 5.2% for tetrahydrocortisol and 7.8% for tetrahydrocortisone.

TABLE I

SELECTIVITY OF THE REACTION

To determine the selectivity of the reaction using benzamidine, 2 μl of 0.1 m*M* standard solution were injected and the relative peak heights were compared. β-Methasone was used as an index of 100%. For selectivity of the reaction using glycinamide [5], as has been described previously, the reactivity of the reaction is dependent on pH. In this table, reactivity at pH 9.8 is shown and cortisol used as an index of 100%.

Steroids	Reactivity (%) with	
	Benzamidine	Glycinamide
β-Methasone	100	10
Cortisol, cortisone	95	100
11-Deoxycortisol	90	92
Prednisolone	70	65
Tetrahydrocortisone, tetrahydrocortisol	85	80
Tetrahyro-11-deoxycortisol	70	75
Androsterone	0	0
Dehydroepiandrosterone	0	0
Progesterone	0	0.2
Corticosterone	0	65
11-Deoxycorticosterone	0	50
Aldosterone	0	5
16-Hydroxydehydroepiandrosterone	0	2

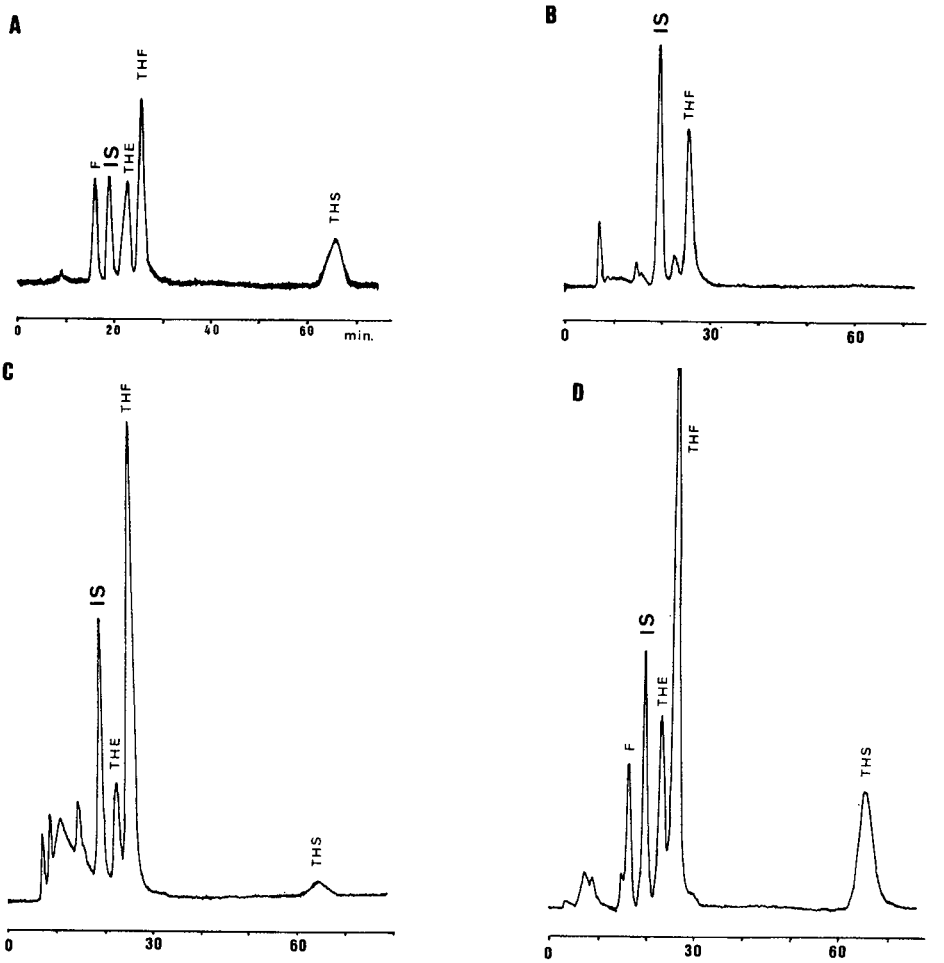

Fig. 4. Chromatograms of standard mixture and patient samples. (A) Chromatogram of 10 μl of standard solution, containing 50 ng each of F, IS, THE and THS, and 100 ng of THF. (B, C) Chromatograms of a urine sample from a normal subject. (D) Chromatogram of a urine sample of a patient with Cushing's disease. F = cortisol, THE = tetrahydrocortisone, THF = tetrahydrocortisol, THS = tetrahydro-11-deoxycortisol, IS = β-methasone (internal standard).

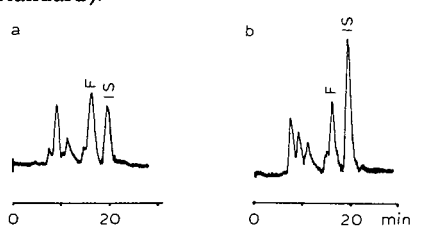

Fig. 5. Chromatograms of serum cortisol (F) assay. To 1 ml of serum sample, internal standard (β-methasone solution of 100 ng or 200 ng, 10 μl or 20 μl of 10 μg/ml) was added and mixed with methylene chloride. After extraction, the organic layer was washed with alkaline solution and water, and then evaporated to dryness. The residue was dissolved in 100 μl of 50% methanol and 30 μl of the solution were injected for HPLC. (a) 100 ng of internal standard were added to 1 ml of serum. (b) 200 ng of internal standard were added to 1 ml of serum.

The present method is more selective than the UV absorption method so an analysis of corticosteroids in biological fluids could be performed by the present fluorimetric method.

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Note

Application of a high-performance gel permeation liquid chromatographic procedure to the determination of binding of prednisolone to high-affinity binding sites in human serum

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It has been established that prednisolone, a synthetic corticoid, binds to corticosteroid-binding globulin (CBG) and albumin (AB) in human serum [1—4]. The binding affinity of the steroid for CBG is high ($K = 3 \cdot 10^7$ ml^{-1}) whereas the capacity is low. In contrast, albumin has a low affinity ($K = 2 \cdot 10^3$ ml^{-1}) for the drug but the binding capacity is high. Various authors [1—3] have attempted to evaluate the binding characteristics of the drug to albumin and CBG in serum by equilibrium dialysis and by employing non-linear regression analysis which is based on an appropriate mathematical model. Nevertheless, there is no report of a direct measurement of the binding of the drug to high-affinity binding sites in serum. Hoffman and Westphal [5] have employed gel permeation chromatography (Sephadex) for the evaluation of binding of hydrocortisone (HC) to CBG in plasma. This is based on the fact that HC bound to AB completely dissociates during the chromatography described. In contrast, HC bound to CBG dissociates slowly on the column. The method, however, is tedious.

This report describes a high-performance liquid chromatographic (HPLC) gel permeation procedure which allows prednisolone bound to AB to completely dissociate during chromatography while the binding of the drug to high-affinity proteins is unaffected.

EXPERIMENTAL

Materials

Prednisolone (U.S.P. reference) and fatty acid free human serum albumin

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(Sigma, St. Louis, MO, U.S.A.) were used for the preparation of solutions. Prednisolone [6,7-^{3}H (nominal)] with a specific acitivity of 1.96 terabecquerels/mmol (New England Nuclear, Montreal, Canada) was used. HPLC solutions were prepared from double-distilled water. Spectrapor 2 membrane tubing (Spectrum Medical Industries, Terminal Annex, Los Angeles, CA, U.S.A.) and 1-ml dialysis chambers (Technilab Instruments, Pequamok, NI, U.S.A.) were used in the equilibrium dialysis studies.

Chromatographic procedure

The constant-volume HPLC system consisted of a pump (Altex Model 110A, Berkeley, CA, U.S.A.), an injector (Rheodyne, Berkeley, CA, U.S.A.) fitted with a 20-μl loop and a UV detector (Waters 440, Milford MA, U.S.A.) set at 280 nm. The column (Bio-Sil TSK-250, 300 × 7.5 mm, particle size 10 ± 2 μm, Bio-Rad Labs., Richmond, CA, U.S.A.) with a molecular mass range of 1000—300,000 was preceded by a guard column (O-SIL, TSK ARD, Bio-Rad Labs.). The chromatographic system was operated at 4°C by placing the mobile phase and column in an ice bath. After use, the column was washed with 0.05% sodium azide and stored at 4°C. The mobile phase consisted of 0.1 *M* sodium sulfate and 0.02 *M* sodium phosphate monobasic adjusted to pH 6.8 with 0.1 *M* sodium hydroxide. Degassing was accomplished by means of a stream of helium bubbled through the mobile phase. A flow-rate of 54 ml/h was used.

General procedure

Aliquots (25 μl) of serum spiked with [^{3}H] prednisolone and cold carrier were placed in glass culture tubes, covered and incubated in a water bath at 37° C for 3 h. Following incubation, the sample was diluted with 1.0 ml mobile phase (at 4°C); 20 μl of the sample were immediately chromatographed. The column eluent was collected stepwise at 1-min intervals and the radioactivity was determined in a beta-counter. Periodic injections of a standard protein mix containing thyroglobulin, chicken ovalbumin, bovine myoglobin, phenylalanine and cyanocobalamin which was supplied by the column manufacturer, were used to monitor column performance.

Dialysis procedure

Incubates of serum containing [^{3}H] prednisolone with carrier prednisolone at a concentration range of 50—800 ng/ml were placed in dialysis chambers separated by dialysis membranes and dialysed against an equal volume of 0.054 *M* phosphate isotonic buffer, pH 7.4. The cells were shaken in a water bath at 37°C for 3 h. Preliminary studies had shown that equilibrium was achieved in this time.

The study of the binding of prednisolone to albumin was carried out under similar conditions. The albumin concentration in buffer was 4% which is the concentration in normal human serum.

RESULTS AND DISCUSSION

Fig. 1a depicts a chromatogram following the injection of an aliquot of prednisolone (200 ng/ml) incubated in 4% albumin. It is evident from the

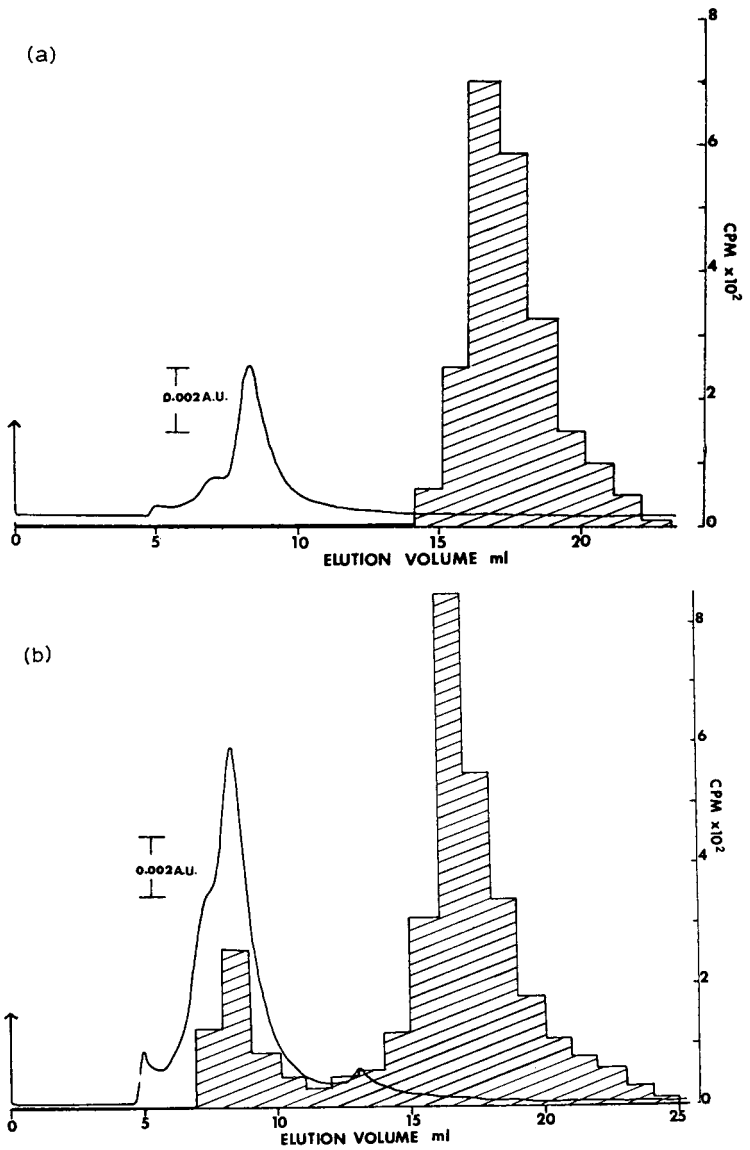

Fig. 1. Chromatograms following injection of an aliquot of prednisolone (200 ng/ml), (a) incubated with albumin (4%) and radioactive tracer, and (b) incubated with serum and radioactive tracer. The histogram represents the radioactivity.

histogram that the drug bound to albumin is completely dissociated during HPLC. Similar results were obtained at lower prednisolone concentrations (25 ng/ml).

Fig. 1b illustrates a chromatogram of prednisolone (200 ng/ml) incubated in human serum. Some of the radioactivity was found in the protein fraction as well as in the prednisolone region. In the former region, the drug is probably

TABLE I

EFFECT OF PREDNISOLONE CONCENTRATION ON THE BINDING IN SERUM (HPLC)

Concentration (ng/ml)	Percent bound	
	Subject 1	Subject 2
25	14.5	26.9
50	13.3	24.9
100	12.5	24.2
200	9.69	20.2
400	8.25	15.1
800	7.5	12.2

TABLE II

COMPARISON OF BINDING OF PREDNISOLONE BY HPLC AND EQUILIBRIUM DIALYSIS

Prednisolone (ng/ml)	HPLC	Equilibrium dialysis	
	Percent bound to high-affinity protein	Percent bound to serum	HPLC value + percent bound to albumin*
50	29.1	87.6	80.4
100	28.1	86.4	79.4
400	19.7	73.3	71.0
800	13.9	65.7	65.2

*The percent bound to albumin as determined by equilibrium dialysis is 51.3% and is independent of concentration. This value is added to the HPLC value.

bound to a high-affinity and low-capacity binding protein such as CBG. The effect of flow-rate on the binding of prednisolone in serum was studied. The amounts bound were found to be constant in the 0.8—1.5 ml/min range, indicating that the drug is not dissociated from this high-affinity binding protein during HPLC.

The relationship between the degree of binding in serum and drug concentration as determined by HPLC, is described in Table I. The concentration-dependent binding noted has also been reported by other authors [1—4].

Replicate analyses of serum containing 25 ng/ml prednisolone demonstrated the reproducibility of the method (C.V. = 4.6%, n = 6). With a concentration of 100 ng/ml, prednisolone binding to serum from six volunteers (HPLC) was found to be 16.97%, C.V. = 32.8. The range was between 8.1 and 23.1%.

Binding values determined by HPLC were compared to results obtained by equilibrium dialysis and are tabulated in Table II. Although it is recognized that amounts bound are not additive, the HPLC values plus albumin are in reasonably good agreement with the total serum binding.

CONCLUSION

In conclusion, by using the conditions described, the drug bound to albumin is completely dissociated during the chromatographic procedure. In contrast, when serum is chromatographed, the percentage bound drug is independent of flow-rate, which indicates that the drug is bound to a high-affinity binding protein such as CBG and does not dissociate appreciably in the experiment. This HPLC gel permeation provides a simple method for the direct measurement of specifically bound drugs. This method should be applicable to the study of binding of other steroids to high-affinity proteins in serum.

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CHROMBIO. 1895

Note

Analysis of retinoids by high-performance liquid chromatography using programmed gradient separation

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(First received April 13th, 1983; revised manuscript received August 8th, 1983)

Vitamin A (retinol), its metabolites and analogues have been heavily focused upon due to their clinical value and utility. Retinol can be used as a reflection of dietary or nutritional status, while the retinol analogues (isotretinoin, tretinoin, etretinate) are being proven as potent dermatologic and anti-tumor agents. High-performance liquid chromatography (HPLC) has been demonstrated to be applicable to the measurement of these compounds in blood. HPLC assays have been reported for retinol [1—4], 13-*cis*-retinoic acid [5, 6], all-*trans*-retinoic acid [7, 8], etretinate [9, 10], plus retinol and retinal isomers [11—15]. Several assay systems have been reported for the isocratic separation of mixtures of retinoids [13—15]. Of these, the methods of Frolick et al. [14] and McClean et al. [15] allow for the measurement of numerous retinoids in biological specimens through the use of single isocratic systems. Using these methods the required time for separation of compounds can run as long as 36 min [14]. The natural and synthetic retinoids, plus their respective major metabolites, have differences in polarity that make chromatographic separation difficult in a short time period. During the long separation times the later eluting peaks become broad and require an integrator. To counteract this

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problem solvent programming has been successfully applied to the analysis of 13-*cis*-retinoic acid and 4-oxo-13-*cis*-retinoic acid in blood samples [5].

In this report we describe a programmed gradient HPLC system for the analysis of multiple retinoids, including retinol, retinal, 13-*cis*-retinoic acid (isotretinoin) and its metabolite, all-*trans*-retinoic acid (tretinoin), etretinate and its metabolites. The use of gradient elution significantly shortens the analysis time, and results in sharp symmetrical peaks which can be measured without an integrator or data module.

EXPERIMENTAL

Reagents

All reagents were of analytical-reagent grade. Acetonitrile was from Burdick and Jackson Labs. (Muskegon MI, U.S.A.). Glacial acetic acid was from Mallinckrodt (Paris, MO, U.S.A.). Retinol, all-*trans*-retinoic acid, retinal, and retinyl acetate were purchased from Sigma (St. Louis MO, U.S.A.). 13-*cis*-Retinoic acid, etretinate, and Ro 10-1670 were gifts from Hoffmann-La Roche (Nutley NJ, U.S.A.).

Chromatography

Measurements were made using a Varian Model 5000 liquid chromatograph equippped with a UV-50 variable-wavelength detector. A Whatman 10-μm Partisil PXS 10/25 ODS-2 column (Whatman, Clifton, NJ, U.S.A.) was used. The programmed mobile phase consisted of a combination of two solutions. Solution A consisted of 0.5% (v/v) acetic acid in acetonitrile. Solution B consisted of 0.5% acetic acid in water. The instrument parameters and program are outlined in Table I. A linear gradient was used between $T_{4\,\mathrm{min}}$ and $T_{6\,\mathrm{min}}$. The total run time was 11 min, resulting in a 5-min hold at the final conditions. A 5-min equilibration time was used between injections.

TABLE I

INSTRUMENTAL PARAMETERS

Flow-rate	3.0 ml/min	
Temperature	ambient	
Absorbance	360 nm (UV), 0.05 a.u.f.s.	
Mobile phase	$T_{0\,\mathrm{min}}$	80% A, 20% B
	$T_{4.0\,\mathrm{min}}$	80% A, 20% B
	$T_{6.0\,\mathrm{min}}$	90% A, 10% B
	$T_{11.0\,\mathrm{min}}$	90% A, 10% B

Specimen preparation

The extraction procedure used was similar to that outlined by Puglisi and de Silva [13], with a single modification. A 1-ml volume of blood or serum was placed in a 15-ml screw-top tube. To this 50 μl of retinyl acetate, 10 mg/l in acetonitrile, were added as internal standard. After vortexing, 2.5 ml of 1 *M* phosphate buffer, pH 6.0, were added. Following a short vortex, 6 ml of diethyl ether were added. The tube was capped and placed on an Eberbach

mechanical shaker (Eberbach, Ann Arbor, MI, U.S.A.) for 15 min at 180 strokes per min. Following centrifugation the ether layer was then removed and evaporated to dryness under nitrogen. The residue was dissolved in 100 μl of solution A. A 50-μl aliquot was injected for analysis.

Calculations

Three blood or serum standards of 250, 500, and 1000 ng/ml (500, 1000, 2000 ng/ml for retinol) were prepared for each compound using 500 μg/ml stock standards in acetonitrile. Calibration curves were made by determining peak height ratios relative to the internal standard.

RESULTS AND DISCUSSION

Typical chromatograms for standard solutions and patient specimens are shown in Fig. 1. As can be observed from Fig. 1, excellent separation of the

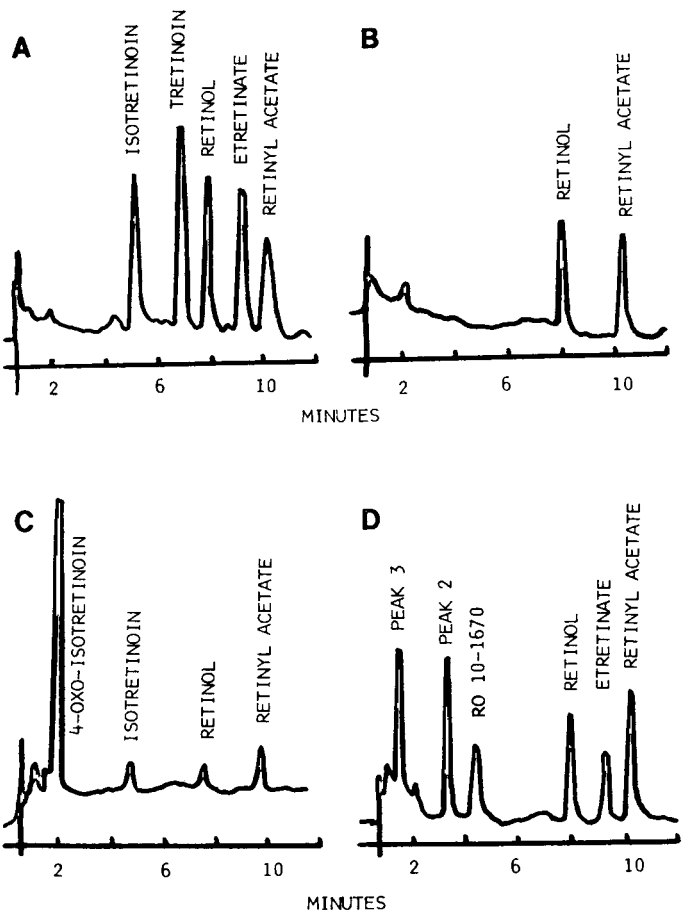

Fig. 1. Reversed-phase chromatograms for whole blood specimens containing retinoids; (A) blood spiked with standards (all-*trans*-retinoic aicd and etretinate, 500 ng/ml; 13-*cis*-retinoic acid and retinol, 2000 ng/ml), (B) normal individual, (C) blood from patient receiving multiple-dose isotretinoin, (D) blood from patient receiving multiple-dose etretinate therapy. Absorbance setting is 0.05 a.u.f.s. for A, B and D, 0.10 for C.

TABLE II

RETENTION TIMES AND RECOVERY FOR RETINOIDS

Compound	Retention time (min)	Recovery* (%)
Ro 10-1670	4.1	78 ± 5
13-*cis*-Retinoic acid	5.1	98 ± 5
All-*trans*-retionic acid	6.9	94 ± 7
Retinol	7.9	86 ± 13
Etretinate	9.1	77 ± 11
Retinyl acetate	9.9	70 ± 5
Peak 2	3.2	—
Peak 3	1.6	—

*Average based on 22 extractions and analyses of a 500 ng/ml blood standard of each compound. This concentration approximates a low-normal value for retinol [15], and a therapeutic level for etretinate [16].

compounds can be achieved using the chromatographic system described here. Although retinal will overlap with etretinate to some extent, this will cause no problem since the normal concentration in blood specimens is too minimal to cause any interference in the determination of etretinate levels. The actual retention times and analytic recoveries are summarized in Table II.

In addition to the presence of etretinate in the blood of patients receiving this drug, at least two metabolites are also found. One of these is Ro 10-1670, the acid metabolite of the parent compound [16, 17] that is also potentially active. However, as has been observed by Hänni et al. [10] and McClean et al. [15], a second metabolite is also evident. This metabolite is Ro 13-7652, the 13-*cis* isomer of Ro 10-1670. The peak height of this second metabolite was consistently larger than the Ro 10-1670 peak in blood samples from patients on chronic etretinate therapy. The exact clinical importance of this metabolite is currently under study. In addition to these two previously observed metabolites, a third metabolite can also be observed. This compound, labelled as peak 3 in Fig. 1D, seems to parallel the level of the other two metabolites in blood from individuals receiving this drug. This peak was not observed in blood from control individuals, nor in specimens taken from individuals receiving placebos during therapeutic trials. When individuals are removed from etretinate therapy, this peak disappears. This metabolite is most probably the 4-hydroxyphenyl retinoic acid analogue, Ro 12-7310, an active major metabolite of etretinate [18].

In patients receiving isotretinoin (13-*cis*-retinoic acid) this assay clearly separates and defines both the parent compound and metabolite, 4-oxo-isotretinoin [19]. The concentration of the metabolite exceeds that of the parent compound 6 h after a single dose [16] or during chronic drug therapy (Fig. 1). McClean et al. [15] have also been able to observe 4-oxo-isotretinoin in patient specimens using HPLC, although the metabolite elutes on the down-side of the solvent front in their system. This could potentially make quantitation difficult. The large solvent front is probably due to the extraction system used.

Although not as rapid as the extraction procedure just discussed above, the method of Puglisi and De Silva [13], as utilized in our assay procedure, results in a small solvent front. This allows for an accurate quantitation of 4-oxo-isotretinoin. In addition the results obtained are unaffected by variations in specimen type. The method presented here works equally well on whole blood, serum, hemolyzed specimens, or plasma collected with any common anticoagulant. The inter- and intra-assay variability (coefficient of variation) for all compounds was less than 10%.

Due to photodecomposition of retinoids, care must be taken to protect samples from direct contact with light. In our laboratory, extraction and analysis are performed in a darkened room with only a minimum of diffuse light allowed. Extraction tubes were wrapped with aluminum foil so as to protect the retinoid compounds.

The assay described here is optimized for the determination of multiple retinoids. The selective determination of retinol in biological specimens can be best performed at 324 nm, at which retinol has maximal absorbance. With this method proposed here retinol levels can be accurately determined using as little as 200 μl of serum, making this method applicable to pediatric specimens.

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CHROMBIO. 1897

Note

Isotachophoretic analysis of iminopeptides in the urine of patients with iminopeptiduria

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Prolidase deficiency is a rare disorder characterized by clinical features such as chronic recurrent infections, mental retardation, splenomegaly, and skin lesions [1]. It has been known that these patients also excrete large amounts of iminopeptides in the urine due to hereditary prolidase deficiency [2—4].

We also reported in a previous paper [4] that a patient with mental retardation and chronic recurrent ulcers on the legs and soles of the feet excreted massive amounts of iminopeptides in her urine. The detection of these iminopeptides has been previously carried out either using an amino acid analyzer, or by paper or thin-layer chromatography [4]. The analysis of some peptides using an isotachophoretic analyzer has been previously reported [5—7], but analysis of iminopeptides has not yet been reported.

It is the purpose of this paper to describe a new, simple and rapid method using isotachophoresis for detecting urinary iminopeptides. The isotachophoretic method presented here has several advantages over previously described techniques. This method can be applied to the screening of patients with prolidase deficiency.

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MATERIALS AND METHODS

Urine samples

The samples of normal human urine were obtained from laboratory personnel. The samples from patients with prolidase deficiency were obtained from two girls (sisters) who were admitted to Okayama University Hospital several years ago. Each of the normal urines and the urines of the patients with prolidase deficiency was directly analyzed by an amino acid analyzer and an isotachophoretic analyzer.

Identification of iminopeptides

A column containing 40 ml of Chelex 100 (Na^+, 100—200 mesh, Bio-Rad) was prepared for the collection of urinary peptides according to the following method. After washing the column with 200 ml of water, 100 ml of a saturated copper sulphate solution were added to the column and allowed to stand overnight. The column was washed well with water and buffered with 0.01 *M* borate buffer (pH 11).

Each 100 ml of the urine samples of two patients and that of a normal human was adjusted to pH 11 with 1 *M* sodium hydroxide, transferred to the buffered Chelex column prepared as above and washed with 100 ml of 0.01 *M* borate buffer (pH 11).

The effluent and washings were combined and evaporated in vacuo below 40°C. The residue containing peptides was dissolved in water, made weakly acidic with 6 *M* hydrochloric acid and filtered. The filtrate was transferred to a column (25 × 2.2 cm) containing 90 ml of Diaion Sk-1 (H^+, 100 mesh, Mitsubishi Kasei Co., Tokyo, Japan), washed with 500 ml of water, and the peptides were then eluted with 2 *M* ammonia. The eluate was evaporated to dryness in vacuo. An aliquot of the residue was analyzed using an amino acid analyzer and an isotachophoretic analyzer.

The rest of the residue was transferred to a column (40 × 1.8 cm) containing 100 ml of Diaion SK-1 (H^+, 100 mesh), washed with water, fractionated with 1100 ml of 0.5 *M* hydrochloric acid and 700 ml of 1 *M* hydrochloric acid, and then washed with water and eluted with 500 ml of 2 *M* ammonia. Each 100 ml of the 0.5 *M* hydrochloric acid and 1 *M* hydrochloric acid eluates, and of the ammonia eluate were evaporated in vacuo below 40°C. The peptides in each fraction were detected by reaction with ninhydrin on paper chromatography. Each of the fractions containing peptides was separated from the others by large-scale paper chromatography (Toyo-Roshi No. 50, 40 × 40 cm) in *n*-butanol—acetic acid—water (4:1:4). All peptides showing the same R_F value were combined, extracted with water, and then evaporated in vacuo.

The peptides contained in the residue were identified according to the following methods. (1) Comparison with authentic samples on isotachophoresis and paper chromatography. (2) Identification of the components of amino acids after hydrolysis of each peptide. (3) Identification the of N-terminal amino acid by dinitrophenylation.

Instrumentation

The capillary apparatus was a Shimadzu IP-1B isotachophoretic analyzer

(Shimadzu Seisakusho, Kyoto, Japan) [8]. The separations were carried out in a capillary tube, 20 × 0.5 cm I.D., which was maintained at a constant temperature of 20°C. The detector cell was 0.5 mm I.D. and 0.05 mm long. The leading electrolyte consisted of 10 m*M* hydrochloric acid and 2-amino-2-methyl-1-propanol (pH 7.50). The terminal electrolyte was 10 m*M* γ-aminobutyric acid and barium hydroxide (pH 10.90). The chart speed was 10 mm/min, migration current was 75 μA.

Reagents

Glycylproline (Gly-Pro) was purchased from Protein Research Foundation, Osaka, Japan. All other chemicals were of analytical grade.

RESULTS AND DISCUSSION

Isotachophoretic analysis of the original urine samples of a normal human and a patient with prolidase deficiency is shown in Fig. 1. The patterns of zones were obviously different between the two samples. The peptide fractions of urine samples of a normal human and of two patients with prolidase deficiency were obtained as described under Materials and methods; isotachophoresis of these peptide fractions is shown in Fig. 2. The isotachophoretic zones detected in the urine of a normal human disappeared after treatment with Chelex-100 and SK-1 (Fig. 2A), but the zones in the urine samples of patients with prolidase deficiency did not disappear (Fig. 2B and C).

The results of analyzing urinary peptides with an amino acid analyzer were also in good agreement with the results obtained using an isotachophoretic analyzer [4]. The results described above indicate that a normal human excretes a very low amount of peptides in the urine; however, patients with

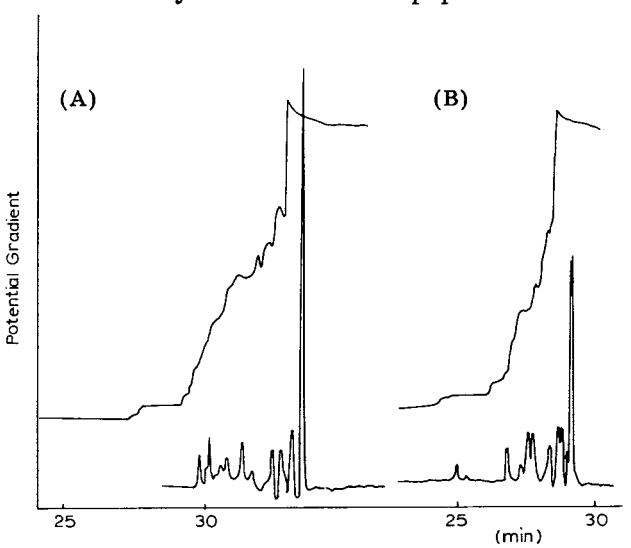

Fig. 1. Isotachophoretic runs of the original urine samples of a patient with prolidase deficiency (A) and of a normal human (B). The leading electrolyte was 0.01 *M* hydrochloric acid and 2-amino-2-methyl-1-propanol (pH 7.50) and the terminal electrolyte was 0.01 *M* γ-aminobutyric acid and barium hydroxide (pH 10.90).

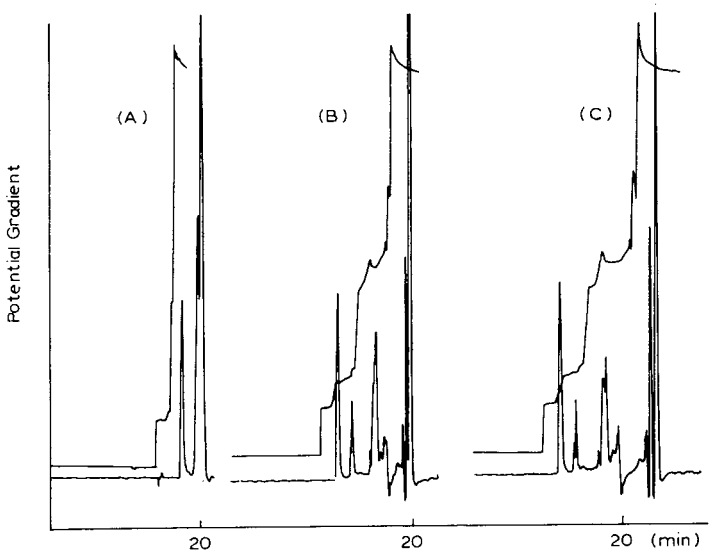

Fig. 2. Isotachophoretic runs of the peptide fractions from the urine of a normal human (A) and patients with prolidase deficiency (B, C). Analytical conditions were as in Fig. 1.

prolidase deficiency excrete massive amounts of peptides in their urine [3, 4].

In order to check the recovery of Gly-Pro during treatment with Chelex-100 and Diaion SK-1, an internal standard of authentic Gly-Pro (10 μmol) was added to the urine samples (2 ml) before chromatography on Chelex-100 and Diaion SK-1. The urine without any addition of Gly-Pro was also processed in parallel. The results indicate a recovery of the added Gly-Pro of about 89.2 ± 6.55% (n = 5).

Each peptide zone from the isotachophoresis was isolated from the urine of the patients, as described under Materials and methods, and identified. The peptides with the same potential gradient as zone c were mainly eluted between 700 and 900 ml of 0.5 M hydrochloric acid and then each peptide in the peptide zone was isolated by paper chromatography. A peptide in zone c was hydrolyzed in 6 M hydrochloric acid, and the hydrolysate dried under reduced pressure. Two amino acids, glycine and proline, were detected in the hydrolysate by paper chromatography and an amino acid analyzer. This peptide also gave the same R_F value as authentic Gly-Pro, and the same potential gradient as Gly-Pro on isotachophoresis, as shown in Fig. 3A and C. This peptide zone on the isotachophoretic chart disappeared upon hydrolysis, and a zone of glycine was newly detected.

Isotachophoretic runs of peptide fractions obtained from the urine of a patient with iminopeptiduria and with Gly-Pro added to the peptide fractions are shown in Fig. 4. Zone c in Fig. 4A and authentic Gly-Pro were made to overlap by adding authentic Gly-Pro to the peptide fractions, resulting in the elongation of zone c as shown in Fig. 4B. These results indicate strongly that zone c of the peptide fractions contains Gly-Pro.

Peptides other than Gly-Pro were also identified by comparison with authentic samples, the determination of the component of amino acids after

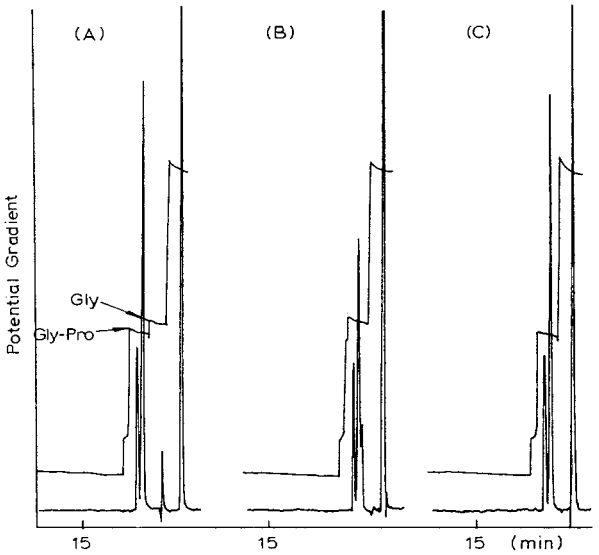

Fig. 3. Isotachophoretic runs of authentic Gly-Pro and Gly (A), the hydrolysate of C (B), and a peptide isolated from peptide fraction (C). Analytical conditions were as in Fig. 1.

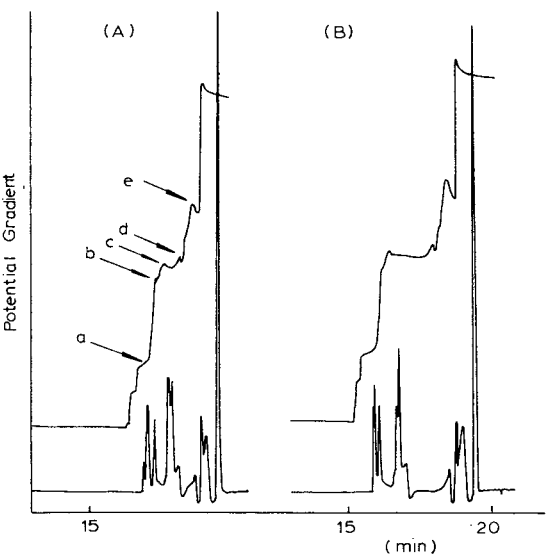

Fig. 4. Isotachophoretic runs of peptide fractions from the urine of a patient with prolidase deficiency (A) and with added authentic Gly-Pro (B). Analytical conditions were as in Fig.1.

hydrolysis of each peptide, and identification of the N-terminal amino acid by dinitrophenylation. It was demonstrated that zone a is a mixture of Glu-Pro and Asp-Pro; zone b, Thr-Pro; zone c, a mixture of Gly-Pro, Leu-Pro, Ileu-Pro, Ala-Pro, Val-Pro, Phe-Pro, Tyr-Pro; zone d, Ser-Pro; and zone e, Pro-Pro. These results indicate that the determination of peptides in each peptide fraction can not be carried out using the isotachophoresis conditions presented

here, but this simple and rapid isotachophoretic method can certainly be applied for the screening of patients with iminopeptiduria.

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Note

Rapid gas chromatographic assay for serum thiopental

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Several methods have been described for thiopental analysis, some of them using gas liquid chromatography (GLC) involving various solvents, extensive extraction procedures and derivatization techniques [1—5]. Also four high-performance liquid chromatographic (HPLC) methods [6—9] have been reported.

This communication describes a simple and rapid GLC method for quantitation of thiopental in serum. With minor modifications to the extraction procedure and chromatographic conditions we used for monitoring anticonvulsant drugs [10, 11], the thiopental samples can be prepared in the same way, therefore only one extraction is needed and the thiopental is analyzed in the underivatized form.

METHODS

Reagents and materials

Thiopental in free form is supplied by Abbott Labs. Methylene chloride and 85% phosphoric acid were pro analysi grade from Merck (Darmstadt, F.R.G.).

Stock solutions of thiopental (1 mg/ml) were prepared in methanol, and in methanol adding 1 ml of 85% phosphoric acid to 100 ml of methanol.

Internal standard solutions were prepared by diluting a stock solution (1 mg/ml) of 5-methylphenylhydantoin from Supelco (Bellefonte, PA, U.S.A.) to 50 μg/ml and 12.5 μg/ml.

Procedure

Internal standard (10 or 2.5 μg) was added to each extraction tube and the methanol was evaporated with a stream of nitrogen. Then 0.5 ml of serum

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sample was poured into the extraction tube. After mixing, the sample was acidified with 100 μl of 85% phosphoric acid and mixed. Then the sample was extracted with 5 ml of methylene chloride for 10 min and centrifuged for 5 min at 2100 *g*. The organic layer was transferred to an 8-ml tube (Kimax 13 × 100 mm 45066) and evaporated to dryness with nitrogen. The extract was reconstituted with 100 μl of methylene chloride (when the amount of internal standard was 10 μg) or with 60 μl (when the amount of internal standard was 2.5 μg), and 1.2 or 2 μl were injected into the GLC system.

Calibration was made by analysing in duplicate serum spiked with 1—5 μg of thiopental and a constant quantity of internal standard (2.5 μg) and 5—30 μg of thiopental and 10 μg of internal standard. Standard curves were constructed plotting the peak height ratio and the peak area ratio of thiopental to the internal standard (2.5 or 10 μg) against 1—5 μg and 5—30 μg of thiopental, respectively. Thiopental concentration was calculated from the standard curves.

The recovery of thiopental from serum was determined by comparing thiopental/internal standard peak area and height ratios in serum with those obtained by direct injection of equal amounts of the thiopental in methanolic solution.

Gas chromatographic conditions

A Perkin Elmer 3920 B chromatograph (Perkin Elmer, Norwalk, CO, U.S.A.) equipped with a flame ionization detector was used. A 1.8-m glass column, 2 mm I.D., was packed with 2% SP 2110—1% SP 2510 DA on 100—120 mesh Supelcoport (Supelco).

The analysis was carried out isothermally with the oven temperature at 205°C, the injector and detector at 250°C, carrier gas (nitrogen flow-rate of 50 ml/min, hydrogen flow-rate of 35 ml/min and air flow-rate of 300 ml/min.

A minigrator M2 (Perkin Elmer) was used to measure the areas of the desired peaks.

RESULTS AND DISCUSSION

A representative chromatogram of an extract of a patient sample is shown in Fig. 1.

Two different amounts (2.5 and 10 μg) of internal standard were used. This method should prove to be widely applicable. A concentration range of 1—30 μg permits quantitation of serum concentrations after administration of a wide range of doses of thiopental.

The linearity of each range was evaluated by a least-squares regression analysis of the ratio of the peak heights and areas of thiopental to internal standard (Fig. 2).

Any conversion to pentobarbital (retention time 1.9 min) was reflected in the chromatograms of the standard curves. No difference was observed after storage at −15°C for six weeks between the concentration of thiopental in methanolic solution and in methanol with 1% of 85% phosphoric acid [8]. Pentobarbital is a minor oxidation metabolite of thiopental in man and pentobarbital serum concentrations of 10% of thiopental concentrations have been

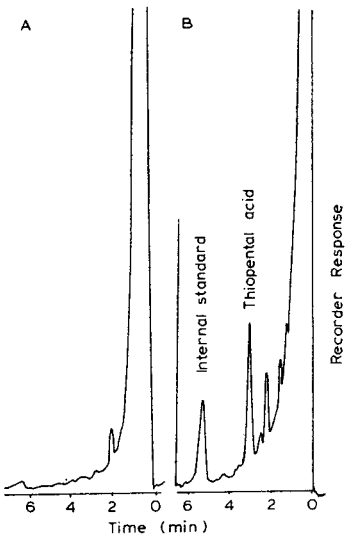

Fig. 1. Gas chromatograms of 0.5-ml serum extracts: (A) serum blank; (B) serum from a patient receiving sodium thiopental, serum level = 2.5 μg.

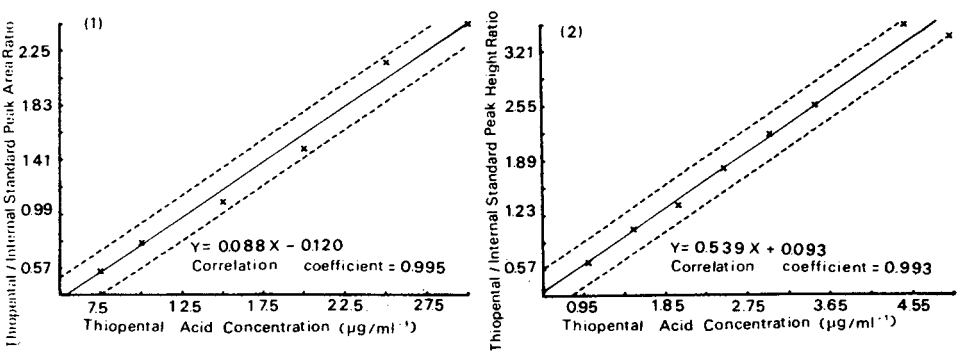

Fig. 2. Standard curves obtained from duplicate analyses of extracts of serum samples spiked with sodium thiopental: (1) high concentration range, derived from 0.5-ml serum samples with 10 μg of internal standard; (2) low concentration range, derived from 0.5-ml serum samples with 2.5 μg of internal standard.

reported during high-dose thiopental therapy [12]. A trace of pentobarbital arising from "in vivo" biotransformation was observed.

The absolute recovery of thiopental was about 95%.

The coefficient of variation obtained by extraction and analysis of six replicates of 15.5 μg was 5.2% while a similar analysis of six replicates of 7.3 μg gave a coefficient of variation of 6.1%.

Several GLC methods with different detection systems have been published for the determination of thiopental in serum. The disadvantage of most of these methods is that some of them analyse the drug in the derivatized form or they need several extraction steps. The method proposed analyses the thiopental in free form and with only one extraction step then, it is simpler than the others.

The sensitivity with the flame ionization detector (0.6 μg/ml) is satisfactory to determine the concentration of thiopental after administration of a wide range of doses in order to optimize the drug dosage selection.

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CHROMBIO. 1885

Note

Improved assay procedure for oxmetidine and its metabolites in plasma, urine and bile samples

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In recently published methods for the assay of oxmetidine (2-[2-(5-methyl-4-imidazolylmethylthio)ethylamino]-5-(3,4-methylenedioxybenzyl)-4-pyrimidone dihydrochloride; SK&F 92994) in various biological fluids [1], the plasma extracts were analysed by a simple modification of the normal phase high-performance liquid chromatographic (HPLC) assay used for cimetidine [2]. The presence of the more polar oxmetidine sulphoxide in extracts of bile and urine prompted the development of a reversed-phase HPLC system [1], for the determination of unchanged drug and metabolites in these fluids.

Recently, however, the manufacturers of the preferred reversed-phase column (Altex Ultrasphere ODS) have changed the method of end capping the octadecylsilane (ODS) packing material. Using the new columns with the original solvent system resulted in broad peaks with little resolution between metabolite, oxmetidine and internal standard. This necessitated the search for a modified solvent system that could be used with the new columns for the analysis of urine and bile samples.

This paper describes the new solvent system, the different internal standard and the modified extraction procedure, which are now considered appropriate for the assay of oxmetidine and its sulphoxide in extracts of urine and bile. The new method may also be used in place of the normal-phase chromatography for extracts of plasma [1].

MATERIALS AND METHODS

Chemicals and reagents

All chemicals used in this study were analytical grade with the following

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exceptions: 1-octanol was puriss (Koch-Light, Colnbrook, U.K.); methanol, water and acetonitrile were HPLC grade (Rathburn, Walkerburn, U.K.); 1-pentanesulphonic acid sodium salt was reagent grade (Kodak, Rochester, NY, (U.S.A.). The solutions of 1 mol l^{-1} carbonate buffer (pH 9.0) and 0.1 mol l^{-1} acetate buffer (pH 5.0) were prepared as described previously [1]. It was necessary to filter the carbonate buffer through 0.45-μm filters in order to reduce the solvent front absorption and baseline shift on the chromatogram. Control human plasma and biological samples for analysis were prepared and stored as previously described [1].

All solvents and solutions for HPLC were filtered through either 0.45-μm membrane filters (Millipore, Bedford, MA, U.S.A., type HA) for aqueous solvents or 0.5-μm membrane filters (Millipore, Type FH) for organic solvents.

The stock solutions of oxmetidine for the plasma and urine assay were prepared by weighing 5.91 and 11.83 mg of the dihydrochloride salt (equivalent to 5 and 10 mg of base), and dissolving same in approximately 0.5 ml water before making the solution to 100 ml volume with methanol. Solutions of oxmetidine sulphoxide and the internal standard (SK&F 93586, 2-[2-(5-methyl-4-imidazolyl-methylthio)-ethylamino]-1-methyl-5-(3,4-methylenedioxybenzyl)-6-pyrimidone dihydrochloride) were similarly prepared by weighing 5.87 and 23.53 mg of the dihydrochloride salt (equivalent to 5 and 20 mg of the respective bases) and dissolving each in appropriate volumes of water and methanol as described. All stock methanolic solutions were stored at −20°C and found to be stable for at least three months under these conditions.

The polypropylene centrifuge tubes (12 ml) and stoppers used for sample extraction were obtained from Henleys Medical Supplies, London, U.K. (Type 300PP and 301PT, respectively).

Extraction procedures for plasma, urine and bile

The plasma extraction was essentially the same as previously reported [1] except that the internal standard SK&F 92909 must be replaced with SK&F 93586. This latter compound (2 μg in 50 μl of methanol) was added to plasma samples before the addition of 1 ml of carbonate buffer to adjust the pH to 9.0. In the final salting out phase 250 μl ethanol replaced the equivalent volume of acetonitrile to make the sample injections compatible with the new solvent system.

The extraction of oxmetidine and its sulphoxide from bile and urine was as described by Lee and McDowall [1] except that an equivalent volume of ethanol was substituted for acetonitrile during the salting out process.

Chromatographic operating conditions

The chromatograph consisted of a Model 6000A pump (Waters Assoc., Milford, MA, U.S.A.). The sample extract was introduced into the system via either a Rheodyne Model 7125 valve injector (Berkeley, CA, U.S.A.) or an automatic injector (Model WISP, Waters Assoc.). Sample extracts that were injected by autosampler were held in spring-loaded microinserts (Type 3-CV, Chromacol, London, U.K.) within 4-ml vials with self-sealing septa (Cat. Nos. 73018 and 73010 respectively, Waters Assoc.). The analytes were separated

by a stainless-steel column 150 mm × 4.6 mm I.D. packed with 5-μm Ultrasphere ODS (Altex Scientific, Berkeley, CA, U.S.A.) and the column eluent was monitored by a Model 441 fixed-wavelength detector fitted with a cadmium lamp and 229-nm filter (Waters Assoc.). A variable-wavelength detector set at 226 nm and 0.04—0.08 absorbance units full scale was also used but proved to be less sensitive. The signal from the detector was fed into a Model 301 integrator (Laboratory Data Control, Stone, U.K.).

The solvent system was a mixture of water—methanol—acetonitrile (45:44:11, v/v) containing 0.095 mol l^{-1} pentanesulphonic acid and prepared as follows: 17.33 g pentanesulphonic acid (sodium salt) was dissolved in 450 ml distilled water, and the pH of the solution was adjusted to 3.0 with 10 mol l^{-1} sulphuric acid; 440 ml methanol and 110 ml acetonitrile were added and dissolved air was removed by the application of reduced pressure. The column was equilibrated by passing solvent through it for approximately 1 h before commencing the analysis. On completion of analysis it is recommended that the column be flushed with filtered methanol for 1—2 h.

At a flow-rate of 1.5 ml min^{-1} the approximate retention times of oxmetidine sulphoxide, oxmetidine and SK&F 93586 (the internal standard) were 4.8, 5.8 and 6.8 min, respectively.

Samples of up to 10 μl of the ethanol extracts were injected onto the chromatograph to obtain separations of the three peaks of interest. The injection of more than 20 μl ethanol often resulted in loss of resolution.

Quantification

The area under each peak was determined by an integrator connected to the UV detector. Peak height measurements can also be used. The ratios of the areas or heights of peaks assigned to oxmetidine and oxmetidine sulphoxide to that of the internal standard in the plasma, bile or urine samples were then used to calculate the concentrations of these compounds, using calibration curves obtained from the corresponding ratios for standards containing known amounts of oxmetidine or oxmetidine sulphoxide.

RESULTS AND DISCUSSION

Recovery of oxmetidine from plasma and urine

The recovery of oxmetidine from plasma and urine samples has been published previously [1]. The substitution of ethanol for acetonitrile did not affect these values and the results of the previous study remain valid.

Selectivity

Typical chromatograms of oxmetidine, its sulphoxide and the internal standard (SK&F 93586) following the injection of a solution of pure standards and extracts of plasma, urine and bile are presented in Figs. 1—4, respectively. These chromatograms were produced by the injection of up to 10 μl of sample extract onto the column; volumes greater than 20 μl ethanol tended to produce asymmetric peaks and poor resolution.

Under normal in vivo conditions oxmetidine sulphoxide is cleared rapidly from the plasma and not usually observed in chromatograms; however, during

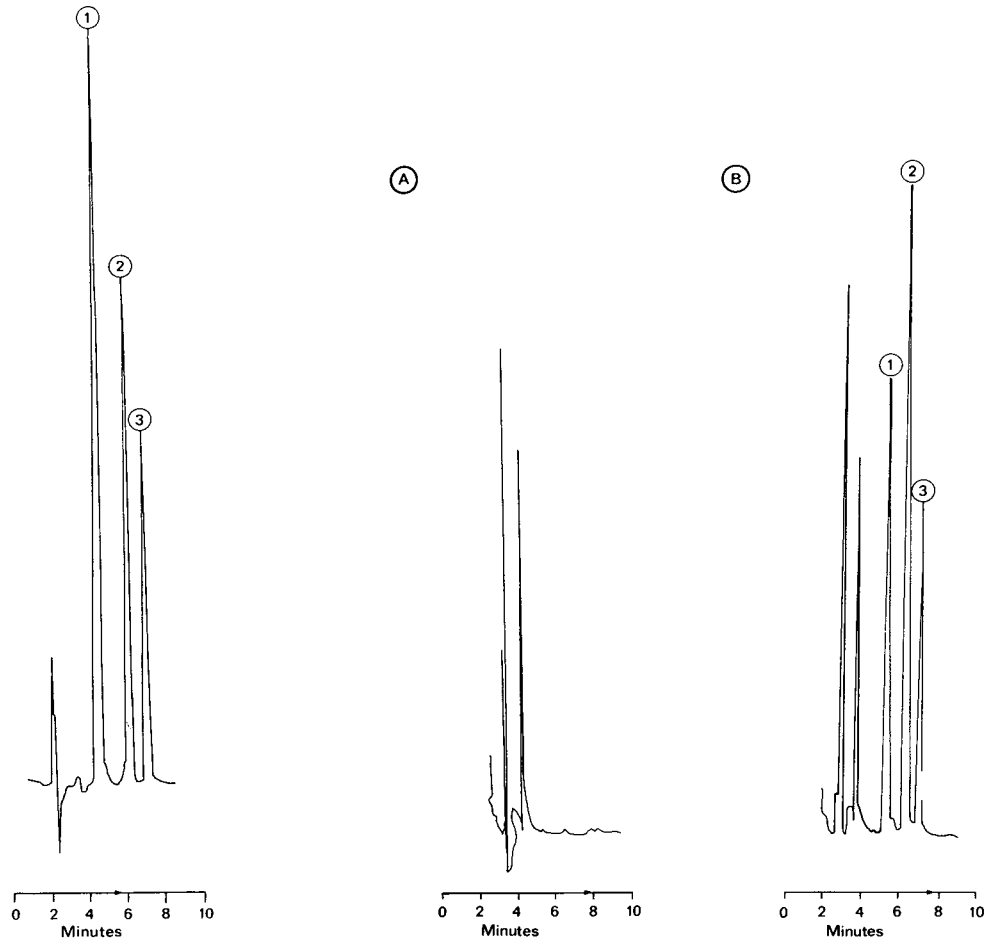

Fig. 1. Reversed-phase chromatogram of pure standards. Peaks: 1 = oxmetidine sulphoxide SK&F 93154; 2 = oxmetidine SK&F 92994; 3 = internal standard SK&F 93586.

Fig. 2. Reversed-phase chromatograms of human plasma extracts. A = Extracted blank plasma; B = extracted sample plasma. Peaks: 1 = oxmetidine sulphoxide SK&F 93154, 2 = oxmetidine SK&F 92994; 3 = internal standard SK&F 93586.

renal insufficiency when clearance is slowed, the metabolite may be present in measureable amounts and this technique can be used to quantify it.

No unwanted peaks with relevant retention times (up to 15 min) were observed for extracts of plasma, urine and bile samples with the exception of a peak with a retention time of approximately 5.1 min found in dog bile extracts. This peak was resolved from oxmetidine sulfoxide and oxmetidine but, at low concentrations of drug and metabolite, affected the quantification of both these compounds (Fig. 4).

Precision and accuracy of the assay

The precision and accuracy of this technique for plasma samples are presented in Table I; the mean concentrations calculated from ten individual

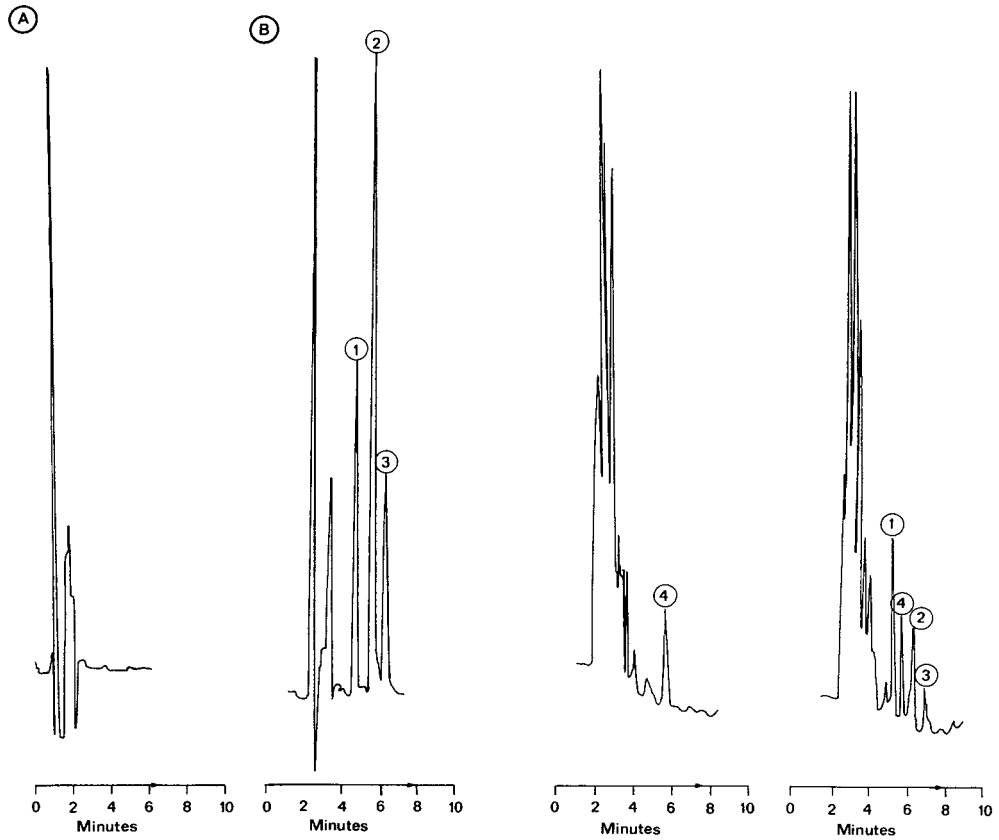

Fig. 3. Reversed-phase chromatograms of human urine extracts. A = Extracted blank urine; B = extracted sample urine. Peaks: 1 = oxmetidine sulphoxide SK&F 93154; 2 = oxmetidine SK&F 92994; 3 = internal standard SK&F 93586.

Fig. 4. Reversed-phase chromatograms of dog bile extracts. Peaks: 1 = oxmetidine sulphoxide SK&F 93154; 2 = oxmetidine SK&F 92994; 3 = internal standard SK&F 93586; 4 = endogenous peak.

TABLE I

PRECISION AND ACCURACY OF THE MODIFIED REVERSED-PHASE HPLC ASSAY OF OXMETIDINE IN HUMAN PLASMA (n = 10)

Oxmetidine concentration (mg l^{-1})	Concentration calculated from peak area ratios (mean ± S.D.) (mg l^{-1})	Coefficient of variation (%)	Bias* (%)
0.25	0.29 ± 0.04	13.8	17.6
0.50	0.55 ± 0.04	7.3	10.0
2.50	2.53 ± 0.07	2.8	−1.2
5.00	5.02 ± 0.06	1.2	0.4
10.00	9.96 ± 0.15	1.5	−0.4

*Bias calculated as $\frac{(\text{mean calculated concentration} - \text{actual concentration}) \times 100}{\text{actual concentration}}$.

TABLE II

PRECISION AND ACCURACY OF THE MODIFIED REVERSED-PHASE HPLC ASSAY OF OXMETIDINE IN HUMAN URINE

Concentration ($mg\ l^{-1}$)	Concentration calculated from peak area ratios (mean ± S.D.) ($mg\ l^{-1}$)	n*	Coefficient of variation (%)	Bias (%)
Oxmetidine				
0.5	0.55 ± 0.08	9	14.5	10.0
5.0	4.92 ± 0.18	10	3.7	—1.6
10.0	10.44 ± 0.23	10	2.2	4.4
Oxmetidine sulphoxide				
0.25	0.23 ± 0.06	9	26.1	—8.0
2.5	2.49 ± 0.26	9	10.4	—0.6
5.0	5.03 ± 0.27	9	5.4	0.6

*n = Number of samples assayed.

TABLE III

PRECISION AND ACCURACY OF THE MODIFIED REVERSED-PHASE HPLC ASSAY OF OXMETIDINE IN DOG BILE

Concentration ($mg\ l^{-1}$)	Concentration calculated from peak area ratios (mean ± S.D.) ($mg\ l^{-1}$)	n*	Coefficient of variation (%)	Bias (%)
Oxmetidine				
0.5	0.62 ± 0.32	5	51.6	24.0
5.0	5.60 ± 0.42	10	7.5	12.6
10.0	11.40 ± 0.65	10	5.7	14.0
Oxmetidine sulphoxide				
0.25	0.38 ± 0.07	10	18.4	51.6
2.5	2.61 ± 0.08	10	3.1	4.4
5.0	5.66 ± 0.08	10	1.4	13.2

*n = Number of samples assayed.

assays of each of five spiked concentrations, are given together with estimates of the precision and accuracy. The precision, as measured by the coefficient of variation (C.V.), was between 1.2 and 7.3% over the concentration range 10.0—0.5 $mg\ l^{-1}$; this was similar to that found with the original normal-phase method. At a concentration of 0.25 $mg\ l^{-1}$ the C.V. was 13.8% which was slightly higher than that of the normal-phase plasma assay (10% C.V. at 0.1 $mg\ l^{-1}$).

The accuracy of the assay as measured by percent bias was very good between 10.0 and 2.5 $mg\ l^{-1}$ (—1.2 to 0.4%), however, at the lower concentrations (0.25 and 0.5 $mg\ l^{-1}$) the bias was 17.6% and 10%, respectively.

The validity of the assay for both oxmetidine and oxmetidine sulphoxide in urine is presented in Table II. The results were essentially similar to those published previously [1].

Table III presents the corresponding precision and accuracy data for oxmetidine and its sulphoxide metabolite calculated from assays of spiked control dog bile. Quantification of the two compounds was complicated by the presence of an endogenous peak, which eluted from the column between oxmetidine sulphoxide and oxmetidine. The coefficient of variation for the assay of bile, for both the drug and metabolite, was greater than for the corresponding concentrations in the urinary assay but still acceptable. At concentrations below 1 mg l^{-1} the assay for both compounds in bile was less reliable and subject to larger variation than in urine.

Comparison of normal phase and reversed-phase HPLC assays for the determination of oxmetidine in plasma

In order that continuity of information is maintained it is essential to show that the results obtained before and after any modification to an analytical

TABLE IV

INDIVIDUAL OXMETIDINE CONCENTRATIONS IN SAME PLASMA SAMPLE DETERMINED BY NORMAL-PHASE AND REVERSED-PHASE ASSAYS

Sample No.	Concentration of oxmetidine (mg l^{-1})		
	Actual concentration	Calculated by normal-phase assay	Calculated by reversed-phase assay
1	0.50	0.55	0.53
2		0.54	0.51
3		0.54	0.53
4		0.55	0.53
5		0.38	0.51
6	1.00	1.02	1.03
7		1.01	1.01
8		0.98	0.99
9		1.02	0.99
10		1.01	0.97
11	4.00	4.04	4.09
12		4.04	4.03
13		4.03	4.01
14		N.R.*	4.02
15		4.00	4.02
16	8.00	8.24	7.98
17		6.88	8.09
18		7.96	7.80
19		8.07	7.93
20		7.93	7.81

*N.R. = no result.

TABLE V

MEAN CONCENTRATION, BIAS, COEFFICIENT OF VARIATION OF OXMETIDINE CONCENTRATIONS DETERMINED IN PLASMA BY NORMAL-PHASE AND REVERSED-PHASE ASSAYS

Actual concentration		Oxmetidine concentration (mg l^{-1})	
		Normal-phase assay	Reversed-phase assay
0.5	Mean	0.51	0.52
	± S.D. (n)*	0.07 (5)	0.01 (5)
	C.V. (%)**	13.7	1.9
	Bias (%)***	2	4
1.0	Mean	1.01	1.00
	± S.D. (n)	0.02 (5)	0.02 (5)
	C.V. (%)	2.0	2
	Bias (%)	1	nil
4.0	Mean	4.03	4.03
	± S.D. (n)	0.02 (4)	0.03 (5)
	C.V. (%)	0.5	0.7
	Bias (%)	0.7	0.7
8.0	Mean	7.82	7.92
	± S.D. (n)	0.54 (5)	0.12 (5)
	C.V. (%)	6.9	1.5
	Bias (%)	−2.3	−1

*n = Number of assays.

**$\text{C.V. (\%)} = \frac{\text{S.D.} \times 100}{\text{mean}}$.

***$\text{Bias (\%)} = \frac{\text{mean calculated concentration} - \text{actual concentration}}{\text{actual concentration}}$.

procedure are comparable. Thus, replicate assays were performed on the same plasma samples by the original normal-phase assay and the new reversed-phase assay (Tables IV and V).

The results show good agreement between the two procedures at all four concentrations; however, at 0.5 and 8.0 mg l^{-1} the reversed-phase assay was more precise (as measured by the coefficient of variation) than the normal-phase assay. The accuracy of the two assays at all concentrations was similar. Thus the reversed-phase assay may be used in confidence in place of the normal-phase assay.

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Note

High-performance liquid chromatographic method for the determination of labetalol in plasma using ultraviolet detection

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Labetalol, 2-hydroxy-5-[1-hydroxy-2-(methyl-3-phenyl-propylamine)ethyl]-benzamine hydrochloride, is a competitive alpha- and beta-adrenergic receptor antagonist [1—4], which has been shown to be effective in the treatment of hypertension [5—8]. The blockade of the alpha-adrenergic receptors in the periphery produces a fall in both systolic and diastolic blood pressure, but a reflex increase in heart rate and cardiac output (baroreceptor-mediated) is prevented by the stronger blocking of the beta receptors. A linear correlation between the plasma concentration and the pharmacological effects of labetalol has been reported [9]. In common with many beta blockers, labetalol is extensively metabolized in the liver, with less than 5% of the dose being excreted unchanged in the urine [10]. Reduced bioavailability of orally administered labetalol due to first-pass metabolism has been indicated [11]. Inter-subject variability in the extent of the first-pass elimination is pronounced and accounts for the large differences in the area under the curve observed between individuals upon administration of the same oral dose of labetalol [11].

Several assays for the measurement of labetalol in biological fluids have been published, the usefulness of which varies with the intended application. The present method involves reversed-phase high-performance liquid chromatography (HPLC) and UV detection of labetalol and the internal standard, propranolol. It possesses the sensitivity and specificity necessary for undertaking the studies that would allow further elucidation of the pharmacokinetic characteristics of this drug. The method is also suitable for monitoring labetalol levels in a routine clinical situation.

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MATERIALS AND METHODS

Reagents

Labetalol hydrochloride was supplied by Schering (Bloomfield, NJ, U.S.A.) and propranolol hydrochloride was supplied by Aldrich (Milwaukee, WI, U.S.A.). Methanol and diethyl ether (anhydrous), both HPLC grade, were obtained from MCB Manufacturing Chemist (Cincinnati, OH, U.S.A.) and J.T. Baker (Phillipsburg, NJ, U.S.A.), respectively. The ammonium acetate and ammonium phosphate were reagent grade.

Instrumentation

An Altex Model 110A solvent metering pump (Altex, Berkeley, CA, U.S.A.), was used as the high-pressure solvent delivery system. The samples were injected with an Altex Model 210 sample injection valve, and their absorbance was measured with an Altex Model 155/10 variable-wavelength UV detector. Separation was achieved on an Ultrasphere octyl 5-μm (150 × 4.6 mm) analytical column (Altex). The absorbances of labetalol and the internal standard were measured at 216 nm (maximum for labetalol).

Chromatographic conditions

The mobile phase consisted of 0.01 *M* ammonium phosphate—methanol (1:1) and the pH of the mixture was adjusted to 3.0 with 15 *M* phosphoric acid. A flow-rate of 1 ml/min was used, generating a back-pressure of approximately 1800 p.s.i. (125 bar). The column and mobile phase were maintained at room temperature.

Procedure

To a 15-ml disposable glass tube containing 0.75 ml of plasma, serum or whole blood, 100 μl of internal standard solution (propranolol, 850 ng/ml) and 0.25 ml of 1 *M* ammonium acetate buffer (pH 9.0) were added. The samples were extracted with 6 ml of diethyl ether by vortexing for 1 min, and separation of the organic layer was achieved by centrifugation at 1500 *g* for 5 min. The organic layer was transferred to a 15-ml screw-capped conical centrifuge tube containing 100 μl of 0.1 *M* hydrochloric acid solution. Labetalol and the internal standard were back-extracted into the aqueous phase by vortexing for 1 min, and the layers were again separated by centrifugation at 1500 *g* for 5 min. A 50-μl aliquot of the aqueous phase was injected onto the column.

RESULTS

Under the specified conditions the retention times of labetalol and the internal standard were 5 and 9 min, respectively. The chromatogram of 0.75 ml of plasma containing 200 ng/ml labetalol and 85 ng/ml propranolol is shown in Fig. 1A. Fig. 1B shows the chromatogram from the plasma of a patient receiving long-term labetalol therapy for hypertension. After analyzing 0.75 ml of plasma of a person receiving no medication, no interference was seen at all (Fig. 2). The limit of detection using 0.75 ml of plasma is 10 ng/ml. The precision and accuracy of the assay are shown in Table I. As it can be seen,

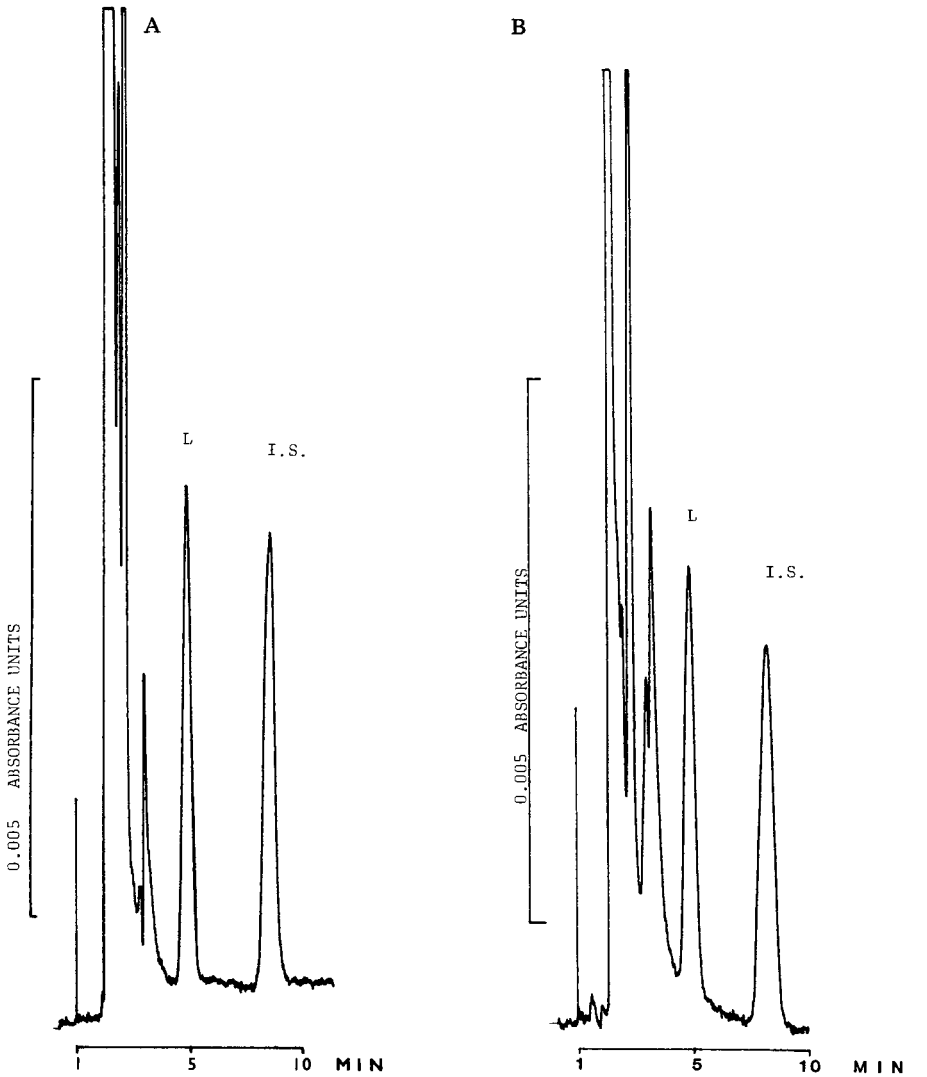

Fig. 1. Chromatograms of (A) 0.75 ml of plasma containing 200 ng/ml labetalol (L) and 85 ng/ml propranolol, internal standard (I.S.) processed as described in the text; (B) plasma sample from a hypertensive patient who was receiving labetalol.

intraday and interday assay coefficients of variation (C.V.) were 4.4% and 7.8%, respectively, at 50 ng/ml and 4.5% and 5.5% at 200 ng/ml. No deviations from linearity were seen over the concentration range 10—1000 ng/ml. The intraday recovery of a 100 ng/ml labetalol serum solution was 80.5% (C.V. = 4.5%, n = 10) and the interday recovery of the same solution was 81.4% (C.V. = 4.8%, n = 7). The selectivity of the assay was assessed by assaying several drugs which may present considerable likelihood of being co-administered with labetalol. As can be seen in Table II most drugs did not show any peak. In the few cases in which a peak was present, the differences in retention time avoided

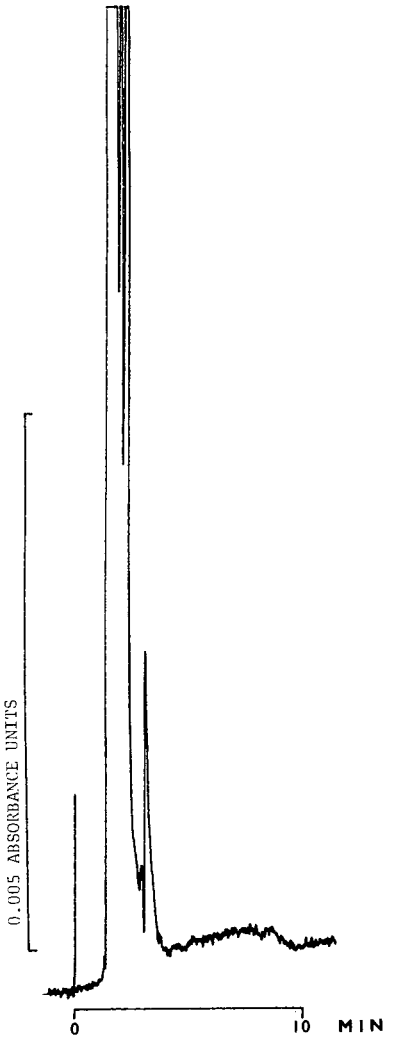

Fig. 2. Chromatogram of 0.75 ml plasma blank processed as described in the text.

possible interferences. Under these experimental conditions, the analytical column has lasted for over 5000 injections.

DISCUSSION

Several analytical methods have been proposed for the determination of labetalol and/or its metabolites in serum [12—16]. Most of these methods, however, contain certain inherent limitations which might limit their general application. Since the plasma concentration of labetalol appears to be related to the pharmacological activity of this drug, the accurate quantitation of the blood levels is useful in optimizing clinical response. Many previous studies on the pharmackinetics of labetalol [9, 12, 17—19] used a spectrophotofluorimetric assay [12] which may have limitations since it does not involve prior

TABLE I

INTRA- AND INTERDAY REPRODUCIBILITY OF REPLICATE STANDARDS AT TWO CONCENTRATIONS

	Within-day		Between-day	
	50 ng/ml	200 ng/ml	50 ng/ml	200 ng/ml
	49.1	208.8	53.5	224.0
	53.1	212.7	55.2	193.9
	53.1	215.0	44.8	207.5
	55.1	198.8	47.5	223.6
	53.1	223.5	49.2	208.4
	49.1	199.5	48.5	219.0
	51.1	209.7		
X =	52.0	209.7	49.8	212.6
S.D. =	2.3	9.5	3.9	11.6
C.V. =	4.4	4.5	7.8	5.5

TABLE II

THE RETENTION TIME OF SOME DRUGS LIKELY TO BE CO-ADMINISTERED WITH LABETALOL THAT WERE ANALYZED BY USING THE ASSAY DESCRIBED

Drug	Retention time (min)	Drug	Retention time (min)
Flurazepam	6	Chlorpromazine	No peak
Doxepin	No peak	Digoxin	No peak
Dilantin	No peak	Nortriptyline	No peak
Librium	6.3	Desipramine	No peak
Phenobarbital	No peak	Chlorthalidone	No peak
Imipramine	No peak	Amitriptyline	No peak
Quinidine	4	Triflupromazine	No peak
Furosemide	No peak	Procainamide	No peak
Diazepam	No peak	Lidocaine	No peak

chromatographic separation. Three HPLC assays have been published [13—15] in which use is made of spectrophotofluorimetric detectors. Their sensitivity is high and good selectivity is also obtained. Nevertheless, all of these methods utilize more tedious methodology; e.g., multiple extractions, dry-ice freezing, chromic acid washing, nitrogen-drying of large volumes (15 ml) of diethyl ether [14] and instrumentation; e.g., post-column alkalinization [15], which are not required by the procedure described herein. In addition fluorescence detectors are somewhat less available than variable-wavelength UV detectors. This might be a limitation if reproduction or utilization of these fluorescence assays is intended. Only one analytical procedure has been reported [16] which combines HPLC separation with UV detection. The sensitivity limit of this assay limits it to the routine clinical monitoring of relatively high serum concentrations and the lack of a back-extraction makes it more susceptible to interferences. It was not reported if the assay had been used to analyze real patient samples.

The assay presented herein, involves extraction of alkalinized samples into an organic solvent (diethyl ether) and then back-extraction into an acidic solution. The extraction of potentially interfering acidic compounds is therefore minimized in the first step and the back-extraction of neutral compounds will be minimized in the second step. The preparation of the samples is fast and their rapid chromatographic analysis allows for the analysis of over 25 samples in a regular work-day. The assay allows for the determination of labetalol in plasma as well as in whole blood. This is particularly important since we have determined from in vitro studies using human blood with 25% hematocrit that labetalol accumulates approximately three-fold in human erythrocytes relative to the plasma concentration. This accumulation will undoubtedly be more significant in vivo when the hematocrit approaches 50%.

The rapidity of the described assay together with its sensitivity and selectivity should make it a strong candidate for routine clinical drug analysis when a rapid determination of labetalol plasma levels is desired. This assay was used to measure labetalol plasma concentrations of ten hypertensive patients who had been receiving chronic labetalol therapy. There were no interferences observed and the plasma levels ranged from 30 to 800 ng/ml. At the same time, the accuracy and sensitivity allows the precise quantitation of blood levels required for detailed pharmacokinetic studies to be undertaken. This method is currently being utilized to investigate first-pass interactions of labetalol with other high-extraction ratio drugs.

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Note

Determination of sodium cromoglycate in human urine by high-performance liquid chromatography on an anion-exchange column

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Sodium cromoglycate {disodium 5,5'-[(2-hydroxytrimethylene)dioxy]bis(4-oxo)-4H-1-benzopyran-2-carboxylic acid} which is also known as disodium cromoglycate and cromolyn sodium, is effective in the treatment of allergic diseases such as asthma [1]. The drug is a highly polar compound with two carboxylic acid groups (pK_a = 2). It is poorly absorbed orally [2] but is completely absorbed from the lung [3]. Sodium cromoglycate is not metabolised; it is cleared rapidly from the plasma and is excreted by man in both the bile and urine in approximately equal proportions [4]. Absorption of the drug by patients in the past has been assessed primarily by determination of the urinary excretion of the drug because of the difficulty of determining the low plasma concentrations following administration of therapeutic doses. Recently a sensitive radioimmunoassay method for the compound in plasma has been developed [5] which has sufficient sensitivity to allow the determination of these low plasma concentrations in patients.

Methods for the determination of sodium cromoglycate in urine include a colorimetric method [2], which was subsequently modified by the introduction of tritiated sodium cromoglycate as internal standard [6], a differential-pulse polarographic method [7] and a reversed-phase ion-pair high-performance liquid chromatographic (HPLC) method [8]. The former methods [2, 6, 7] involve sample concentration procedures and have limits of detection of 0.5 $\mu g\ cm^{-3}$. The latter method [8], which employs direct on-column injection of urine, has a limit of detection of about 0.35 $\mu g\ cm^{-3}$. This note describes an HPLC method which has a limit of detection of 0.05 $\mu g\ cm^{-3}$ and employs an anion-exchange chromatographic column.

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EXPERIMENTAL

Reagents

Diethyl ether was purchased from May & Baker (Dagenham, U.K.). Concentrated hydrochloric acid, sodium chloride, glycine, and orthophosphoric acid (density 1.75 g cm^{-3}) were purchased from Fisons Scientific Apparatus (Loughborough, U.K.), and were analytical quality reagents. The mobile phase was prepared by dilution of 10 cm^3 of orthophosphoric acid to 1 dm^3 with pH adjustment using 5 *M* sodium hydroxide. This solution was 0.9 *M* with respect to phosphate.

Instrumentation

A Spectra-Physics (St. Albans, U.K.) Model 3500B high-performance liquid chromatograph was used. The stainless-steel column (250 × 4.6 mm) was obtained prepacked with Partisil SAX (particle size 10 μm) from Whatman Lab. Sales (Maidstone, U.K.). A Valco injection valve (Spectra-Physics) with a sample loop of 120 mm^3 was used. The mobile phase was phosphate buffer (pH 2.30 ± 0.01) delivered at a constant flow-rate of 3.6 cm^3 min^{-1}. Detection was made at 325 nm with a Schoeffel Model 770 variable-wavelength UV detector (Spectra-Physics), the output from which was recorded at 2 and 10 mV using a Vitatron 2001 twin-pen recorder (Fisons Scientific Equipment, Crawley, U.K.). The column was used at ambient temperature (approximately 22°C) and the column inlet pressure was about 12.5 MPa. Peak heights of sodium cromoglycate were routinely measured manually, but peak areas were also measured using a Columbia Scientific Instruments computing integrator (Kemtronix, Compton, U.K.) during the initial studies. The best straight lines were found by least-squares linear regression.

Sample preparation

Test urine (10 cm^3) was pipetted into glass 30-cm^3 disposable extraction tubes (Payne, London, U.K.) containing sodium chloride (5 g). Water (1.0 cm^3), concentrated hydrochloric acid (1.0 cm^3) and diethyl ether (10 cm^3) were added. Standards were prepared similarly using blank pooled 24-h urine except that aqueous sodium cromoglycate solutions (1.0 cm^3) were added in place of the water added to the test urine. Routinely, standards in the range 0.05—20 μg cm^{-3} were used. Each tube was capped with a plastic aluminium-lined screw-cap, was shaken for 10 min at 200 oscillations per min along the long axis, and was centrifuged at 1540 *g* for 10 min. The diethyl ether layer (9.0 cm^3) was removed and the extraction was repeated with diethyl ether (10 cm^3). The extracts were combined in a tube containing 1.0 cm^3 of 1 *M* glycine—hydrochloric acid buffer (pH 3.5). The tube was capped, shaken and centrifuged. Samples of the lower aqueous phase were injected onto the chromatographic column. Prepared samples could be stored as long as three days at ambient temperature without removal of the diethyl ether before being subjected to the chromatographic separation.

RESULTS

Chromatography

Chromatograms resulting from the analysis of a blank urine and blank urine containing sodium cromoglycate at concentrations of 0.2 and 1.0 $\mu g\ cm^{-3}$ are shown in Fig. 1. Chromatograms resulting from the analysis of urine from patients receiving sodium cromoglycate are shown in Fig. 2. The drug was eluted from the column at about 4.5 min as an asymmetric peak. The retention time was inversely dependent on the concentration of the phosphate in the mobile phase. The pH of the mobile phase also affected the retention of the drug; increasing pH from 1.9 to 5.4 resulted in shorter retention times.

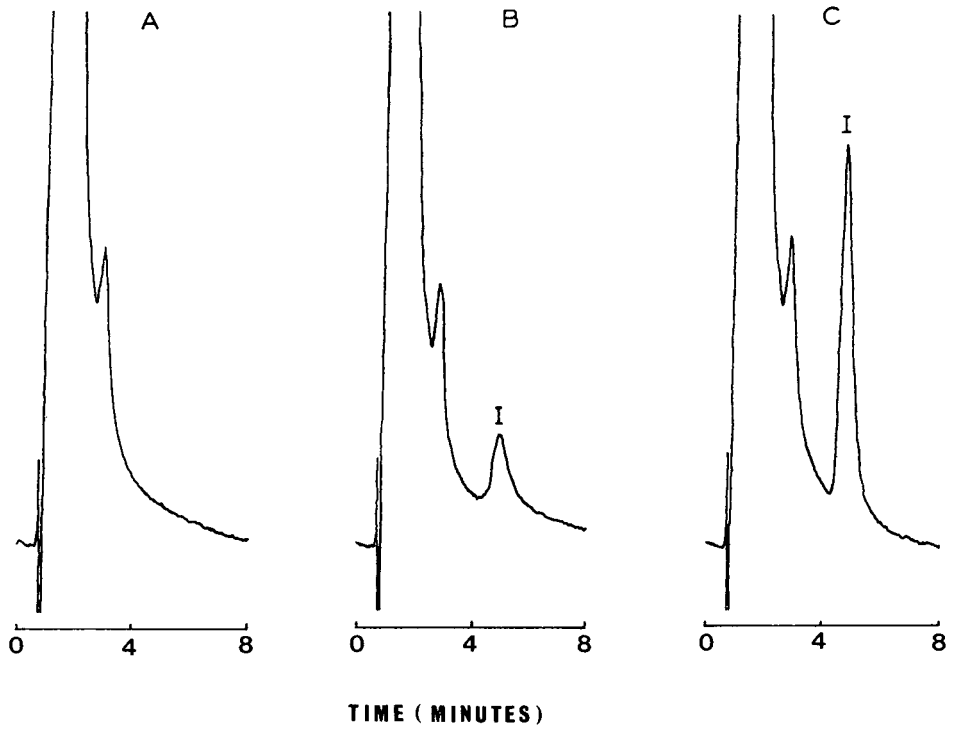

Fig. 1. Chromatograms of blank urine (A) and blank urine containing added sodium cromoglycate (I) at a concentration of (B) 0.2 and (C) 1.0 $\mu g\ cm^{-3}$.

Linearity

Peak area calibration curves were linear, for example, $y = 0.0794x - 0.003$, $r = 0.9977$, $n = 17$, over the concentration range 0.05—20 $\mu g\ cm^{-3}$. Peak height calibration curves were also linear over this concentration range ($y = 14.22x - 4.26$, $r = 0.9986$, $n = 17$) but the peak height data could be more exactly represented by two straight lines over the concentration ranges 0.05—1 and 1—20 $\mu g\ cm^{-3}$ which typically had the properties $y = 10.94x - 0.26$, $r = 0.9970$, $n = 7$ and $y = 14.71x - 10.44$, $r = 0.9988$, $n = 10$, respectively. Hence, whenever peak height measurments were used routinely two standard curves were constructed extending over the appropriate concentration range.

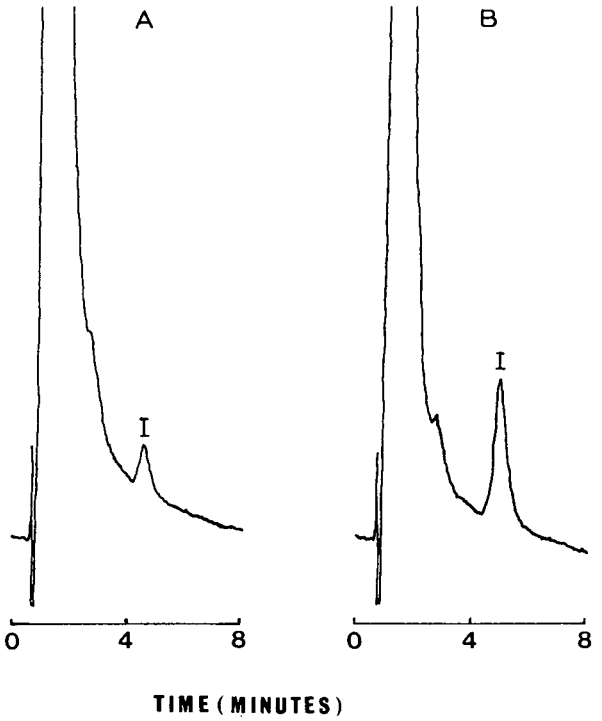

Fig. 2. Chromatograms of urine from patients collected over a 24-h period after administration of 20 mg of sodium cromoglycate (I) by inhalation. Concentration of sodium cromoglycate in samples: (A) 0.10 and (B) 0.34 $\mu g\ cm^{-3}$.

Precision, accuracy and recovery

The intra-day precision and accuracy of the method at sodium cromoglycate concentrationsof 0.22, 1.1 and 5.5 $\mu g\ cm^{-3}$ on two separate days are shown in Table I. The maximum coefficient of variation (C.V.) was 5.8% with a maximum inaccuracy of 11.8%. The efficiency of the extraction procedure is shown in Table II. The extraction efficiency was about 70% and was independent of concentration over the range 0.2—20 $\mu g\ cm^{-3}$.

TABLE I

INTRA-DAY PRECISION AND ACCURACY OF THE SODIUM CROMOGLYCATE METHOD ON TWO SEPARATE DAYS

Results obtained by analysis of six or more spiked urine replicates at each concentration on each day. Peak height determined.

Sodium cromoglycate concentration ($\mu g\ cm^{-3}$)	Coefficient of variation (%)		Accuracy (%)	
	Day 1	Day 2	Day 1	Day 2
0.22	2.3	1.7	99.6	98.2
1.1	4.6	5.8	103.9	111.8
5.5	2.8	2.4	96.2	100.5

TABLE II

EXTRACTION EFFICIENCY OF SODIUM CROMOGLYCATE FROM URINE ON TWO SEPARATE DAYS

Sodium cromoglycate concentration ($\mu g\ cm^{-3}$)	Extraction efficiency (%) (mean ± S.D.)	
	Day 1	Day 2
0.2	71.9 ± 4.2	66.7 ± 5.4
2.0	68.8 ± 7.4	79.7 ± 8.7
6.0	74.6 ± 2.8	Not done
20.0	70.5 ± 4.2	71.4 ± 2.0

Interference

No interfering peaks were observed at the retention time of sodium cromoglycate when urine from normal healthy volunteers or patients was examined. Similarly no interference occurred when drugs likely to be administered to patients concomitantly with sodium cromoglycate were chromatographed. The drugs studied included acetylsalicylic acid, sodium salicylate, phenylbutazone, prednisolone phosphate, hydrocortisone, paracetamol, terbutaline sulphate and theophylline. This latter investigation of the specificity of the method ignored any possible interference from metabolites of these drugs: the drugs were assessed by injection directly onto the chromatographic column.

CONCLUSION

The method described permits the detection of sodium cromoglycate in human urine at concentrations as low as 0.05 $\mu g\ cm^{-3}$ and may be used to determine sodium cromoglycate in urine samples from volunteers collected up to 24 h after administration by inhalation of 20 mg of the compound. A technician may analyse forty samples by the method in two days: automation of sample injection can improve this analysis rate substantially since this time estimate includes manual sample injection. The method has satisfactory accuracy and precision, is simple and has proved to be reliable in routine use during a period of more than three years.

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CHROMBIO. 1898

Note

High-performance liquid chromatographic determination of a new calcium antagonist, fostedil, in plasma and urine using fluorescence detection

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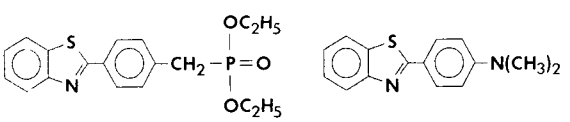
A new calcium entry blocking compound [1—4], fostedil [diethyl-4-(2-benzothiazolyl)-benzyl phosphonate], is currently under investigation at Abbott Labs. as a potential new drug candidate. This compound is being studied under a joint license agreement with Kanebo, Ltd. of Japan and has been reported in the literature as KB-944. We were interested in measuring plasma and urine levels in dogs following administration of the drug. A high-performance liquid chromatographic (HPLC) procedure employing fluorescence detection was developed to measure the concentration of the drug in plasma and urine. The drug was extracted from the biological fluids on Baker 10 Octadecyl (C_{18}) extraction columns (1-ml capacity). This newer extraction system provided a rapid and efficient sample clean-up.

EXPERIMENTAL

HPLC grade acetonitrile and methanol (Burdick and Jackson Labs., Muskegon, MI, U.S.A.) were used. The internal standard (see Fig. 1) was obtained from Abbott Labs. All chemicals and reagents were used as received.

A Waters Assoc. pump and automatic injector were used with a Schoeffel



FOSTEDIL INTERNAL STANDARD

Fig. 1. Structures of fostedil and internal standard.

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FS-970 detector. The column (25 mm × 4.6 mm) was Alltech Octyl (C8), 10-μm particle size. With slight changes in the composition of the mobile phase and/or flow-rate other reversed-phase columns could be used. A Hewlett-Packard recorder was used with peak heights and retention times being determined with a ruler.

The mobile phase was acetonitrile—water (3:2). Minor manipulations of the acetonitrile content may be required to accommodate column efficiency loss or interferences from atypical plasma or urine samples. The mixture was filtered through a 0.4-μm Nucleopore (Pleasanton, CA, U.S.A.) polycarbonate membrane and degassed under house vacuum, while stirring, for about 5 min.

Operating conditions were: flow-rate 1.5 ml/min, chart speed 0.1 in./min. and injection volume was 50—200 μl depending on expected drug concentration. The detector was operated at a range setting of 0.2, a time constant of 6, an excitation wavelength of 290 nm, and a 370-nm emission filter positioned between the sample cell and photomultiplier tube.

The internal standard was 4-(2-benzothiazolyl)-N,N-dimethylbenzenamine. A stock internal standard solution was prepared by dissolving ca. 25 mg in acetonitrile and diluting to 25 ml with acetonitrile. The solution was serially diluted with the mobile phase to a final working concentration of 150 ng/ml.

A methanolic solution of fostedil (1 mg/ml) was diluted (1:10) with methanol and then further diluted with pooled dog plasma or pooled dog urine to concentrations from 10—1000 ng/ml.

Extractions were performed using a Baker 10 Extraction System® with Baker 10 C_{18}, 1-ml columns (Scientific Products, McGraw Park, IL, U.S.A.). This system retains compounds of interest on selected sorbent packings. It has been successfully used by several authors [4, 5]. Solvents and samples are passed through the columns by the application of a vacuum. In this method the disposable extraction columns were first washed with two 1-ml washes of methanol. The vacuum was released and a volume of plasma or urine estimated to contain between 10—1000 ng of fostedil was added to the extraction column. Then 0.1 ml of the working internal standard solution was added to the column. The sample was drawn through the column by vacuum and the drug and internal standard absorbed on the column matrix. The endogenous materials were removed from the matrix by washing three times with a methanol—water mixture. For the plasma samples, the mixture was methanol—water (25:75) and for the urine samples it was methanol—water (55:45). The vacuum was released between each washing.

The drug and internal standard were eluted from the column with 1 ml of methanol. The eluent was collected in a small tube positioned under the column, dried in a water bath (40—45°C) under a gentle stream of air. The residue was reconstituted in 0.3 ml of mobile phase and an aliquot injected into the HPLC system. Peak heights were measured with a ruler and the concentration of fostedil in the unknowns determined by either the internal- or external-standard method.

RESULTS AND DISCUSSION

Originally a solvent extraction procedure was developed to remove the drug

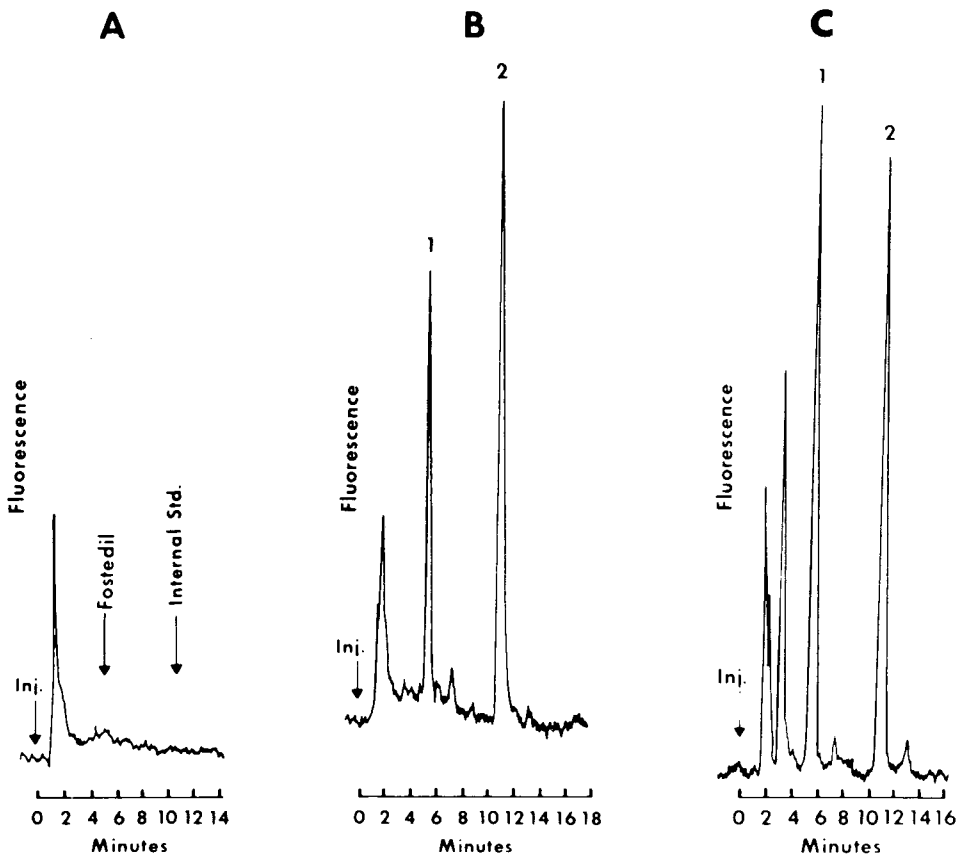

Fig. 2. (A) Chromatogram of extracted blank dog plasma as processed by this method. (B) Chromatogram of extracted dog plasma spiked with fostedil, 50 ng/ml (1) and internal standard (2). (C) Chromatogram of processed dog plasma following p.o. dosing of 12 mg/kg, 6.0 h post dosing.

from the biological fluids. Hexane—isopropyl alcohol (95:5) extracted fostedil quantitatively from the plasma, but resulted in a large background pattern in the chromatogram. Other less polar solvents were tried with hexane and hexane—ethyl acetate (7:3) yielding in the highest recoveries and lowest background pattern in the sample chromatograms.

The Baker 10 Extraction System was also evaluated. This system was found to be simple, fast and reproducible. Cyano, octyl and octyldecyl types of Baker 10 sorbents were screened for extracting efficiency. The following solvents were screened for their eluting properties on these sorbents: methanol, acetonitrile, isopropanol and mobile phase. The best results were obtained with the octyldecyl sorbent with methanol as the eluting solvent.

Typical chromatograms for extracted blank plasma/urine, the 50 ng/ml standard and a dog sample as processed by this method are shown in Figs. 2 and 3 for the plasma and urine assays, respectively. The retention time was 5.5 min for fostedil and 11.0 min for the internal standard. As observed, there may be some variation from sample to sample of the compounds eluting at the void volume.

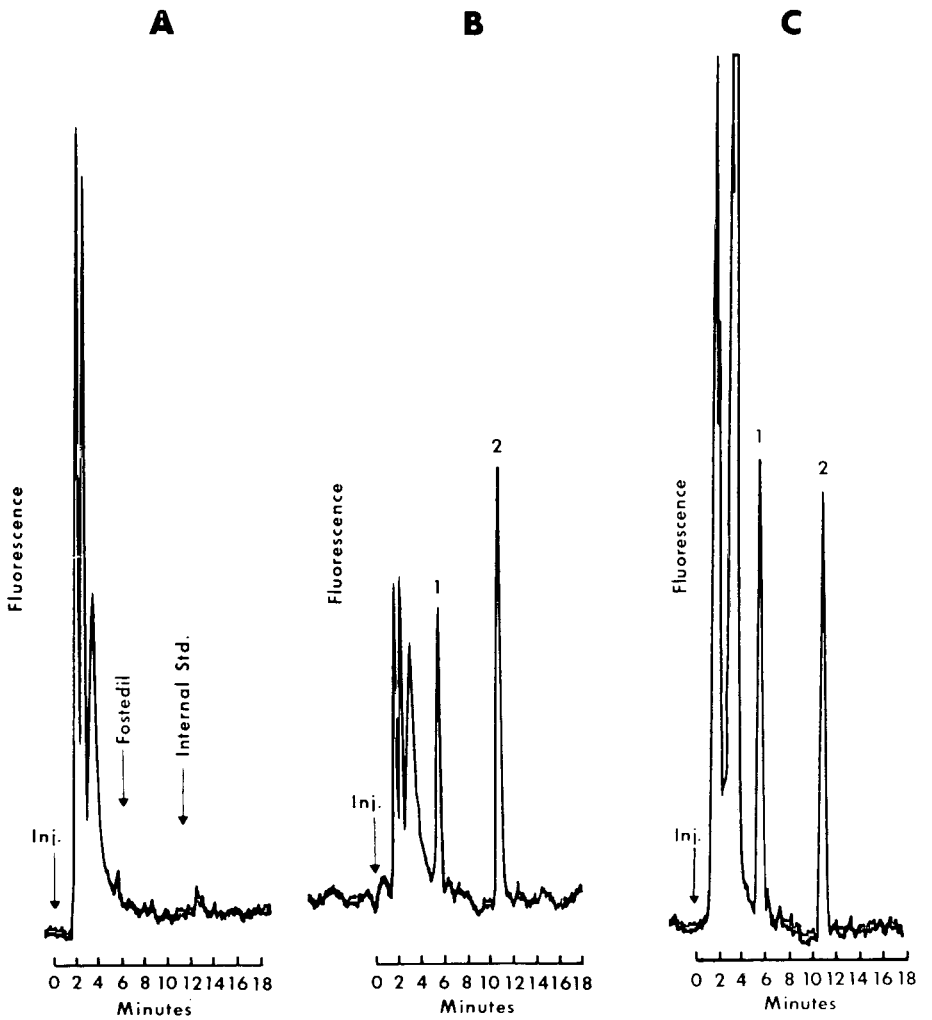

Fig. 3. (A) Chromatogram of extracted blank dog urine as processed by this method. (B) Chromatogram of extracted dog urine spiked with fostedil, 50 ng/ml (1) and internal standard (2). (C) Chromatogram of processed dog urine following p.o. dosing of 12 mg/kg, 0—4 h collection period.

Though an internal standard has been incorporated in this method, we obtained slightly better precision using the external standard technique. Haefelfinger [6] reported that in an HPLC procedure the external calibration is often advantageous. Haefelfinger showed that in HPLC, since the injection volumes are usually large (versus gas chromatography), the precision of the injection is not the main source of variance. Other sources of analytical variance include extraction factors and chromatographic behavior for both the drug and internal standard. If the coefficient of variation (C.V.) of the internal standard is larger than the C.V. of the drug being analyzed then the method will have a higher C.V. value when using internal versus external calibration. In general, samples processed by this procedure have been analyzed by the external

method. However, the use of the internal standard is recommended to guide in peak identification and guard against erroneous results due to sample loss after elution from the column and small within-run changes in HPLC conditions.

The linear response of the fluorescence detector was established by constructing calibration curves from spiked dog plasma and urine. A typical standard curve for the plasma assay yielded a slope of 0.125, a *Y*-intercept of −0.076, and a correlation coefficient of 0.9992. For the urine assay, a typical slope was 0.136, a *Y*-intercept of −0.163, and a correlation coefficient of 0.9990. Analysis of ten standard curves over a six-week period indicated that all correlation coefficients from the linear regression analysis were 0.99 or greater. The detection limit was empirically estimated to be about 5 ng/ml.

The precision of the HPLC assay for fostedil in plasma and urine samples was determined by calculating a mean concentration ± S.D. for each of the six standards from assays of six replicate curves, over a six-week period. The results of this precision study, Table I, show the average C.V. value to be 6.9% for the plasma assay and 7.5% for the urine assay.

TABLE I

PRECISION OF THE ANALYTICAL PROCEDURE FOR DETERMINING FOSTEDIL IN BIOLOGICAL FLUIDS

Theory (ng/ml)	Plasma		Urine	
	Found (ng/ml)	C.V. (%)	Found (ng/ml)	C.V. (%)
10.0	10.6 ± 1.34	12.7	10.1 ± 1.50	14.9
20.0	19.9 ± 1.33	6.7	20.3 ± 0.83	4.1
50.0	46.9 ± 4.27	9.1	47.9 ± 2.71	5.7
200.0	200.1 ± 17.2	8.6	203.6 ± 22.4	11.0
500.0	501.9 ± 12.7	2.5	511.2 ± 22.5	4.4
1000.0	1000.5 ± 14.9	1.5	992.9 ± 47.0	4.7

The recovery of fostedil from the plasma and urine assay procedures was determined by comparing the peak height of the drug from processed samples (plasma/urine) to the peak height of prepared reference samples. The recovery was checked at each standard level (10—1000 ng/ml) for both the plasma and the urine assay. All recoveries were greater than 90%. The recovery of the internal standard was also assessed and found to be greater than 90%.

The stability of room temperature, refrigeration (4°C), and freezer storage (−20°C) of fostedil in dog plasma and urine were assessed. Blank dog plasma and urine were spiked with the drug to 200 ng/ml, and aliquots set at the above stability stations. After 48 h at room temperature and refrigeration storage, no degradation was observed in either the plasma or urine samples.

The frozen samples were tested after one and four months storage. The plasma and urine samples were assayed with freshly prepared reference standards in the corresponding medium. The plasma and urine samples all assayed within the analytical variance of the method. Thus, on storage at −20°C there is no appreciable degradation of fostedil in dog plasma or urine for at least four months.

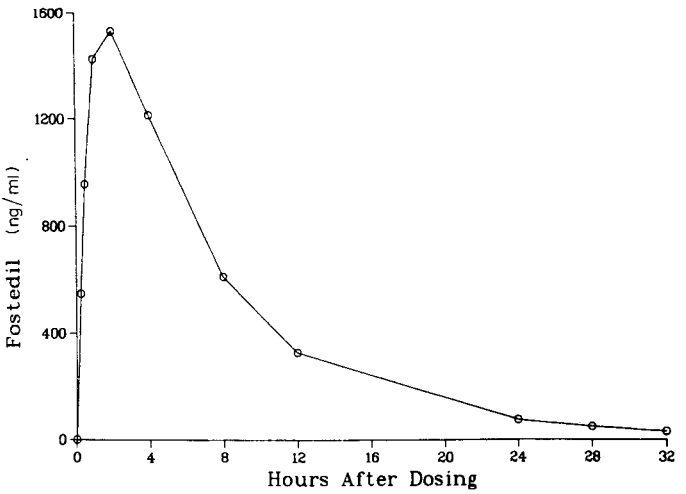

Fig. 4. Average plasma levels of fostedil following oral dose of 12 mg/kg to fifteen dogs.

Several drugs which are often concurrently administered to cardiac patients were also examined for their possible interference with this assay. The following drugs were evaluated on this system: disopyramide, prazosin, methyclothiazide, chlorthiazide, furosemide, hydralazene, procainamide, lidocaine, quinidine, verapamil, flurazepam, prazepam, imiprame and ibuprofen. None were found to interfere with this analysis of fostedil.

This method has been used to analyze over 600 plasma samples and 200 urine samples from dogs. The main metabolites in dog are the 5-, 6- and 7-hydroxy derivatives (i.e. diethyl-4-(5-hydroxybenzothiazol-2-yl)-benzyl phosphonate). Under the chromatographic conditions described here, these metabolites co-elute at a retention of 3.0 min. Following a radiolabeled dose in dogs, over 80% of the radioactivity was recovered in the feces. Thus, renal clearance is not a major route of elimination of the parent compound or the metabolites. A typical plot of plasma concentration versus time post dosing (p.o.) for dogs is shown in Fig. 4. Although all the work for this method was done with dog plasma and urine, generation of acceptable chromatograms and standard curves would serve to validate the procedure for human or other types of samples.

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CHROMBIO. 1899

Note

Determination of primaquine in biological fluids by reversed-phase high-performance liquid chromatography

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Since its introduction in the 1950s primaquine, (8-(4-amino-1-methylbutylamino)-6-methoxyquinoline), has been the drug of choice to treat the tissue schizont stage of *P. vivax* and *P. ovale* malarias. Primaquine levels have been measured in plasma and urine by gas chromatography (GC) [1] and gas chromatography—mass spectrometry (GC—MS) [2]. These methods are complex and require lengthy sample treatment. MS is, in addition, inherently expensive. A high-performance liquid chromatographic (HPLC) method for the determination of primaquine in biological fluids has been developed which is suitable for use in clinical pharmacokinetic studies.

MATERIALS AND METHODS

Chemicals

Primaquine diphosphate was supplied by Aldrich (Gillingham, U.K.) and the internal standard 8-(3-amino-1-methylpropylamino)-6-methoxyquinoline was a gift from the Walter Reed Army Medical Research Centre (Washington, DC, U.S.A.), [^{14}C]primaquine (Specific activity 1.55 mCi/mmol) was synthesised by New England Nuclear (Boston, MA, U.S.A.). Dimethyldichlorosilane, toluene, 880 ammonia and orthophosphoric acid of analytical grade were obtained from B.D.H. (Poole, U.K.). Octanesulphonic acid was supplied by Aldrich. All other reagents were of HPLC grade (Rathburn Chemicals, Walkerburn, U.K.). Glass Culture (Sovirel) tubes and polytetrafluoroethylene (PTFE) lined screw caps were supplied by V.A. Howe (London, U.K.).

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Chromatography

The method was developed on a Spectra Physics liquid chromatograph. The system consisted of an SP 8700 solvent delivery system with an SP 8750 organiser module equipped with a Rheodyne valve injection system and coupled to an SP 8300 fixed-wavelength UV absorbance detector fitted with a 254-nm filter. The separation was carried out on a Partisil ODS III (10 μm particle size) reversed-phase column (20 cm × 0.6 cm O.D., HPLC Technology, Wilmslow, U.K.). The mobile phase consisted of water—acetonitrile—methanol (60:30:10) containing octanesulphonic acid (5×10^{-4} *M*) as an ion-pair reagent, buffered to pH 3.5 with orthophosphoric acid and flowing at 1.5 ml/min.

Extraction procedure

The extraction was carried out in 10-ml capacity glass culture tubes pretreated with dichlorodimethylsilane in toluene (5%, v/v) in order to minimise adsorption. To samples of plasma or urine (0.5—1.0 ml) containing internal standard (0—200 ng) was added ammonia solution (0.88 specific gravity, 2 ml), followed by vortex mixing for 30 sec. This mixture was extracted twice by mechanical tumbling for 10 min with a combination of hexane and ethyl acetate (9:1, total volume 5 ml). After centrifugation (1000 *g* for 10 min) and separation, the organic phases were combined, evaporated to dryness under a steady stream of nitrogen and reconstituted in methanol (50 μl). An aliquot of 5—25 μl was injected onto the column.

Standard curves

Standard curves were prepared by adding known quantities of primaquine (5—200 ng) to a fixed concentration of internal standard (100 ng) in drug-free plasma or urine. Samples were analysed as described above and the peak height ratio of drug to internal standard was plotted against the corresponding weight ratio. Peak height ratios of unknown samples were similarly determined and concentrations calculated from the standard curve. The extraction efficiency of primaquine was calculated from the recovery of ^{14}C-radioactivity following extraction of plasma or urine spiked with [^{14}C]primaquine (10,000 dpm/ml).

Volunteer study

Four healthy male volunteers aged 24—45 years, who were taking no other drugs, each received 45 mg primaquine orally after an overnight fast. Venous blood samples were taken pre-dose and at 0.5, 1, 2, 3, 4, 6, 8, 11 and 24 h. Blood was centrifuged (1000 *g* for 15 min) and the plasma removed and stored at −20° C until the time of analysis. Urine was collected for 1 h pre-dose and from 0—24 h. Urine volume and pH were measured and a sample was frozen and stored (−20° C). All samples were covered with aluminium foil to protect them from the light.

Calculations

The peak plasma concentration of primaquine and the time to attain peak plasma concentrations was measured by inspection and the plasma elimination

half-life was calculated by regression analysis of the post-distributive log linear portion of the plasma concentration versus time curve.

RESULTS AND DISCUSSION

The extraction procedure resulted in simple sample preparation. Chromatograms of an extract of pre-dose plasma sample and a plasma extract obtained after a single 45-mg oral dose of primaquine are shown in Fig. 1 (A and B). The plasma extract from the volunteer receiving primaquine showed a distinct peak with a retention time of 7 min 36 sec corresponding to primaquine (120 ng/ml). This peak was completely resolved from that of the internal standard, retention time 5 min 12 sec. The peak eluting prior to the internal standard was an impurity in the internal standard stock and was not observed in extracts of blank plasma. Chromatograms of blank urine and a urine specimen collected after a single oral dose of primaquine are shown in Fig. 2 (A and B).

The acetylated and carboxylated metabolites of primaquine described by Baker et al. [3] did not interfere with the assay. Although these metabolites appeared in the chromatograms of aqueous stock solutions at retention times of 14 min and 17 min, respectively, they did not appear in any plasma or urine extracts possibly due to poor analytical recoveries under the conditions of the extraction.

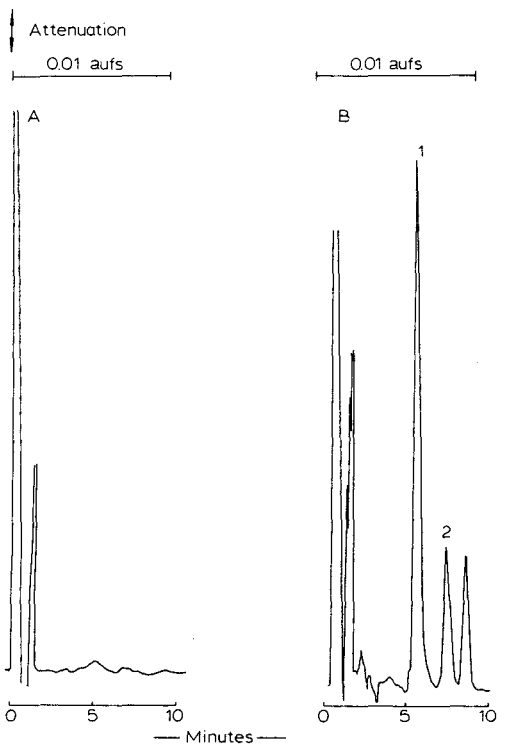

Fig. 1. Chromatograms of a blank plasma extract (A) and an extract of plasma obtained after a single 45-mg oral dose of primaquine (B) (primaquine, 120 ng/ml). Peaks: 1 = internal standard, 2 = primaquine.

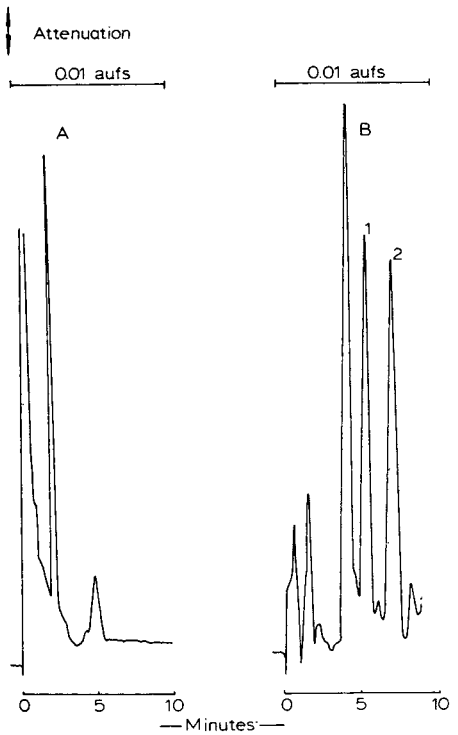

Fig. 2. Chromatograms of a blank urine extract (A) and an extract of urine obtained after a single oral dose of primaquine (B) (primaquine, 154 ng/ml). Peaks: 1 = internal standard, 2 = primaquine.

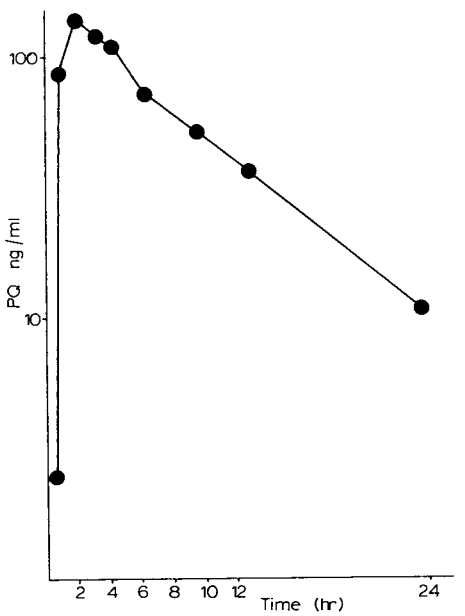

Fig. 3. Plasma levels of primaquine after a 45-mg dose (free base) orally. Volunteer 1.

We found no interference with chromatographic separation from the commonly used antimalarial drugs chloroquine and pyrimethamine or from endogenous compounds in plasma. A clearly resolved component of the extract from plasma obtained from a normal subject receiving primaquine (Fig. 1B) did not appear in blank plasma (Fig. 1A). This may be a metabolite, as yet unidentified. Additionally, in a number of urine extracts a minor endogenous component was seen to elute with a retention time between that of the internal standard and primaquine (Fig. 2). This resulted in a marginally reduced level of sensitivity in these samples.

The extraction solvent, hexane—ethyl acetate (9:1) gave optimal recovery of primaquine with minimal extraction of endogenous compounds. Calibration curves were linear in the range 0—200 ng (r = 0.99) and analytical recovery of primaquine was 93% (± 5, n = 6). The minimal detectable quantity of primaquine in plasma which gave a peak three times baseline noise at the highest detector sensitivity (× 0.0025 a.u.f.s.) corresponded to a level of 1 ng/ml.

The intra- and interassay variation of spiked plasma samples were respectively 8.7% (n = 8) and 5% (n = 5) at 25 ng/ml, and 4.2% (n = 10) and 2.7% (n = 5) at 100 ng/ml.

This assay was applied to a study of the pharmacokinetics of primaquine, following 45 mg orally, in man (all values are means ± standard deviation, n = 4). In all cases plasma primaquine levels could be measured throughout the 24 h of the study. Primaquine was rapidly absorbed reaching peak plasma levels of 150.2 ng/ml (± 28.0) at 2.6 h (± 0.7). The plasma elimination half-life was 6.3 h (± 0.9). Urinary excretion of primaquine over 24 h was 459.5 μg (± 315.0) or 1.1% (± 0.7) of the dose. A typical log plasma concentration versus time curve is shown in Fig. 3.

The advantages of this assay over earlier methods are that it is inexpensive, sample treatment is rapid and simple, sensitivity is increased while selectivity is retained and it is capable of measuring primaquine levels after clinically relevant doses. It may also be possible to quantitate the less stable metabolites of primaquine using this assay or a minimally modified procedure.

ACKNOWLEDGEMENTS

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Note

Determination of timolol in plasma and breast milk using high-performance liquid chromatography with electrochemical detection

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Timolol maleate, (—)-1-(*tert.*-butylamino)-3-[(4-morpholino-1,2,5-thiadiazol-3-yl)oxy]-2-propanol maleate, is a potent non-selective beta-adrenoceptor antagonist [1] effective in the management of systemic hypertension [2], angina pectoris [3] and glaucoma [4]. Because of its high potency timolol is administered in small oral doses, usually 10—45 mg per day [5] and this, in addition to its large apparent volume of distribution, results in the low plasma concentrations generally found in man.

Several methods are available for its measurement in biological fluids, the most widely used being that of Tocco et al. [6] which involves gas chromatography (GC) with electron-capture detection (ECD) and has a limit of sensitivity of 2 ng/ml. Else et al. [7] have used GC with nitrogen-selective flame ionization detection (NFID) and have achieved a similar sensitivity, while Fourtillan et al. [8] have been able to measure plasma concentrations of 0.5 ng/ml using GC—mass spectrometry (MS). Lefebvre et al. [9] have developed a high-performance liquid chromatographic (HPLC) method with UV detection but this displayed a rather poor sensitivity of 40 ng/ml which limited its usefulness in pharmacokinetic studies using normal dosing regimens.

In this paper we describe an accurate and selective HPLC method for the measurement of timolol in plasma and breast milk. It is important to be able to measure timolol in the latter fluid since a number of beta-adrenoceptor antagonists are now used to control high blood pressure in the later stages of pregnancy and it is vital to know the concentration of these drugs in milk since they may be transferred to the new-born during breast feeding. The method uses electrochemical detection and is as sensitive as the GC methods above, with the exception of GC—MS.

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MATERIALS AND METHODS

High-performance liquid chromatography

A Waters Model 6000A solvent delivery system was attached to a reversed-phase column 25 cm × 4.6 mm I.D. The column was packed with Whatman PXS 5/25 Partisil ODS 3, particle size 5 μm (Whatman Chemical Separations, Maidstone, U.K.). Samples were introduced onto the column by means of a Rheodyne loop injector, the volume of the loop being 100 μl. Sample detection was carried out electrochemically using a TL5A thin-layer cell assembly with an LC2A controller (Bioanalytical Systems).

Voltammetry

It was necessary to establish the optimum applied potential to give maximum sensitivity, low signal-to-noise ratio and rapid equilibration. Repeated injections of 50 ng of timolol were made under the conditions described below and the amplitude of the response compared with the applied potential (Fig. 1). The optimum applied potential was found to be +1.2 V and this was maintained throughout the study.

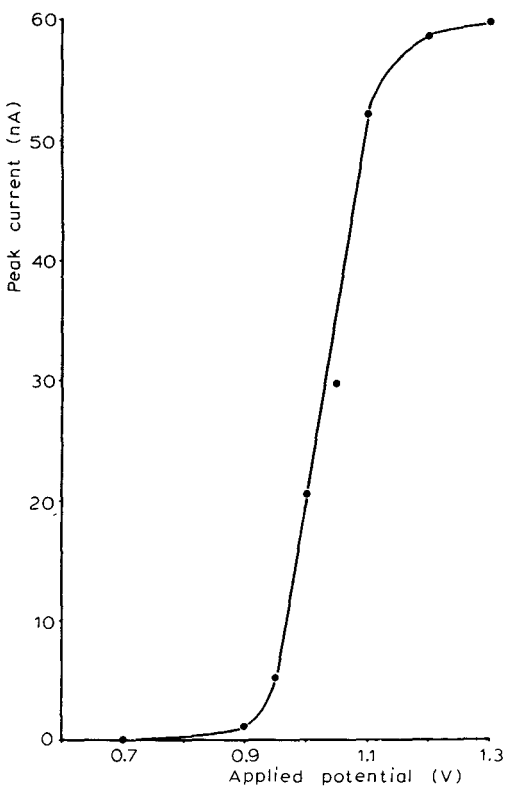

Fig. 1. Voltammogram of repeated injections of 50 ng of timolol.

Reagents and materials

Timolol maleate was supplied by Merck, Sharpe and Dohme, U.K. and

propranolol hydrochloride by ICI (Macclesfield, U.K.). Both drugs were dissolved in glass-distilled water to give stock solutions of 100 μg/ml; from these timolol dilutions of 0.1 and 1.0 μg/ml were made and a propranolol dilution of 1.0 μg/ml. Pindolol was supplied by Sandoz Products (Leeds, U.K.) and was dissolved in methanol to give a stock solution of 100 μg/ml from which a dilution of 1.0 μg/ml in water was made. The stock solutions were replaced after four weeks, 1.0 μg/ml solutions after one week and the 0.1 μg/ml solutions daily. All standards were kept at 4°C when not in use.

Methanol (HPLC grade) was supplied by Fisons Scientific Apparatus (Loughborough, U.K.). All other chemicals were of analytical grade. Glassware was silanized before use.

Extraction of timolol from plasma or serum

To 1.0 ml of plasma or serum was added 0.1 ml of propranolol, internal standard (1.0 μg/ml), followed by 1.0 ml of saturated sodium chloride. The pH was adjusted by the addition of 0.2 ml buffer (1 *M* sodium hydroxide—1 *M* sodium carbonate, 1:2), 3 ml of water-satured diethyl ether were added and the mixture was shaken gently for 15 min. After centrifugation at 300 *g* for 10 min, the organic phase was transferred to a test-tube containing 0.15 ml of 0.1% (v/v) orthophosphoric acid. The tube was vortexed for a few seconds, centrifuged at 300 *g* for 10 min and the organic phase discarded; 1.5 ml of water-saturated hexane were then added to the aqueous phase and the tube was vortexed for 2 min, centrifuged at 300 *g* for 10 min and the organic phase again discarded. Aliquots, usually 0.1 ml, of the orthophosphoric acid phase were then injected onto the chromatographic column. The mobile phase consisted of a mixture of methanol—0.2 *M* sodium dihydrogen phosphate—88% orthophosphoric acid (sp. gr. 1.75)—water (500:200:3:297) pumped at a flow-rate of 1.0 ml/min.

Extraction of timolol from breast milk

To 1.0 ml of breast milk was added 0.05 ml of pindolol, internal standard (1.0 μg/ml), followed by 1.0 ml of saturated sodium chloride and 3.0 ml of water-saturated diethyl ether. The mixture was shaken for 15 min, then centrifuged at 300 *g* for 10 min. The organic phase was transferred to a tube containing 2.0 ml of 0.1% (v/v) orthophosphoric acid, shaken for a further 15 min and centrifuged at 300 *g* for 10 min. The organic phase and the cloudy interface were carefully removed and discarded. To the aqueous phase were added 0.2 ml of the sodium hydroxide—carbonate buffer and 3.0 ml water-saturated diethyl ether. After shaking for 15 min and centrifuging for 10 min as described above, the organic phase was transferred to a test-tube containing 0.15 ml of 0.1% orthophosphoric acid. The tube was vortexed for a few seconds, centrifuged for 10 min as above and the organic phase discarded; 1.5 ml of water-saturated hexane were then added to the acid phase and the mixture vortexed briefly and centrifuged as described. The organic phase was again discarded and an aliquot of the acidic aqueous phase injected onto the column. In this case the mobile phase was composed of the same ingredients in the proportions 300:200:3:497 at a flow-rate of 1.0 ml/min.

RESULTS AND DISCUSSION

Typical chromatograms of blank and spiked plasma are shown in Fig. 2. Under the conditions described in the text the retention times for timolol and propranolol were 4.8 and 8.2 min, respectively. The chromatogram of patient plasma was similar to that of spiked plasma in all respects. The calibration curve was linear from the limit of detection, 2 ng/ml, up to 100 ng/ml and could be described by the equation: $y = 0.02356x + 0.0075$. Typical chromatograms from breast milk are shown in Fig. 3. Under the conditions described in the text the retention times of pindolol and timolol were 7.0 and 13.5 min respectively. Again the chromatogram of patient breast milk was similar to that of spiked breast milk in all respects. The calibration curve for breast milk could be described by the equation: $y = 0.01125x - 0.00563$. The limits of detection and linearity were as for plasma.

The precision and accuracy of the method applied to plasma and breast milk are shown in Table I and the coefficient of variation (C.V.) is less than 10% at the 10 and the 50 ng/ml level. Recoveries of timolol were approximately 80% from plasma and 60% from breast milk.

Plasma concentrations of timolol following a single oral dose of 20 mg were monitored in a young healthy volunteer over a 7-h period (Fig. 4) and the results were consistent with those reported in earlier studies [5, 8]. A peak plasma concentration of 58 ng/ml was achieved after 2 h.

Most pharmacokinetic studies with timolol have used GC as the analytical tool, the method of Tocco et al. being most widely used [6]. That method involves a derivatization step with heptafluorobutyrylimidazole and we feel

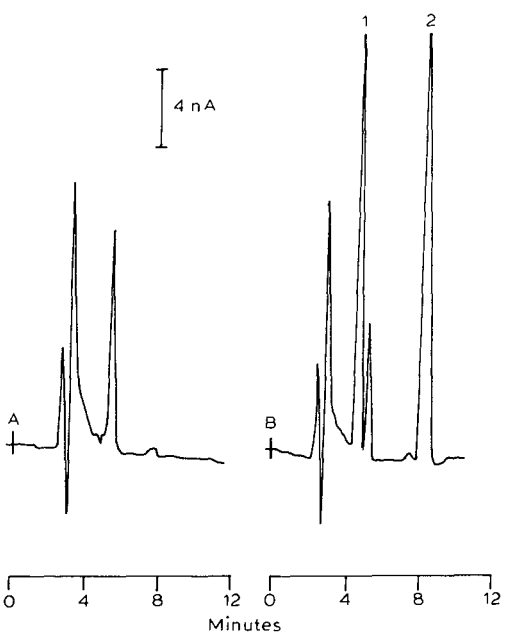

Fig. 2. Timolol in plasma. A, blank plasma; B, plasma containing 50 ng of timolol (1) and 100 ng of propranolol, internal standard (2).

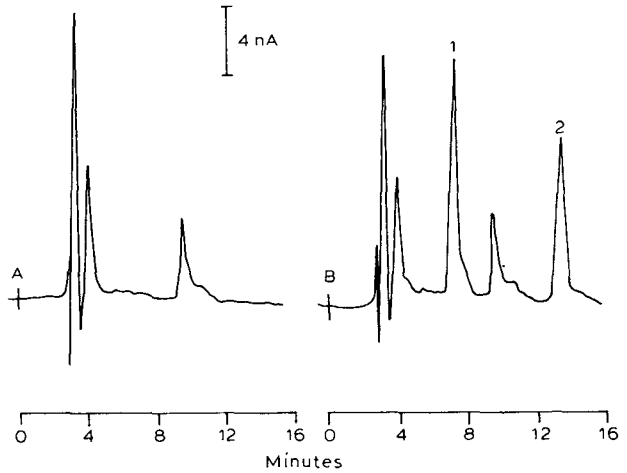

Fig. 3. Timolol in breast milk. A, blank milk; B, milk containing 50 ng pindolol, internal standard (1) and 50 ng timolol (2).

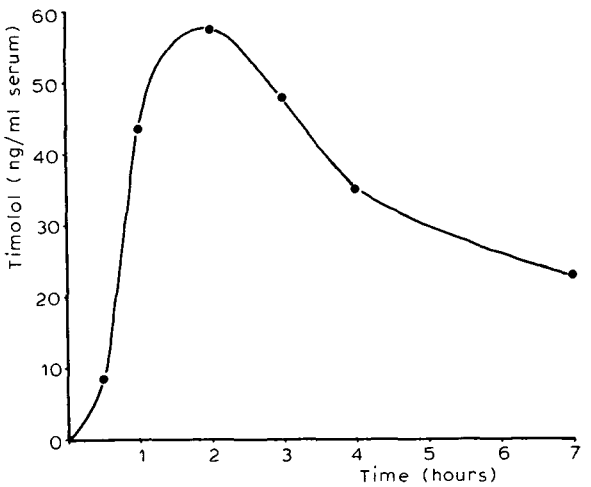

Fig. 4. Plasma concentrations of timolol in a volunteer following a single oral dose of 20 mg timolol maleate.

TABLE I

PRECISION AND ACCURACY OF THE METHOD APPLIED TO PLASMA AND BREAST MILK

	Spiked concentration (ng/ml)	Detected concentration ± S.D. (ng/ml)	C.V. (%)	No. of assays (*n*)
Plasma	10.0	9.8 ± 0.8	8.2	10
	50.0	50.1 ± 3.7	7.4	9
Breast milk	10.0	9.8 ± 0.5	5.1	10
	50.0	50.3 ± 2.3	4.6	9

that the present method is simpler and offers similar sensitivity. The only published method of greater sensitivity is by mass fragmentography and is beyond the reach of most laboratories.

The method described here is both sensitive and accurate and provides a useful alternative to the established GC method for timolol in plasma and breast milk.

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Note

Isolation from urine of 4′-hydroxypropranolol sulfate, a major new propranolol metabolite, by ion-pair extraction

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Sulfate conjugation is a common pathway in the metabolism of many phenolic drugs and drug metabolites [1]. The polar nature of these conjugates makes them difficult to isolate and purify from biological material. This is particularly true, if the metabolite also contains an aliphatic amino group, which renders the molecule zwitter-ion properties. Such a conjugate, 4′-hydroxypropranolol sulfate (HOPS), Fig. 1, was recently identified in urine as a major new propranolol metabolite in both dogs and man [2]. This conjugate was separated from endogenous compounds and other propranolol metabolites by tedious multiple extraction and high-performance liquid chromatographic (HPLC) separation steps.

A previous study of sulfate esters of simple phenols [3], e.g., 2-naphthyl sulfate, has, however, suggested that sulfate conjugates, also of more complex molecules, may be extracted from biological material as ion-pairs, possibly

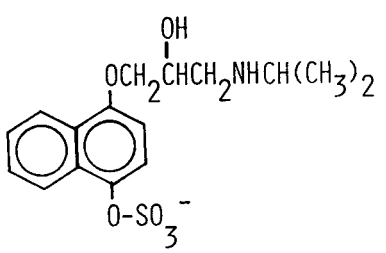

Fig. 1. Chemical structure of 4′-hydroxypropranolol sulfate (HOPS).

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providing a selective and rapid isolation step prior to HPLC separation. Conditions for quantitative extraction of HOPS as an ion-pair with tetrabutylammonium (TBA^+) were therefore investigated and the method applied to determinations of this conjugate in urine. For comparison, the extractability of the corresponding glucuronic acid conjugate, i.e., 4'-hydroxypropranolol glucuronide (HOPG) was also investigated.

EXPERIMENTAL

Chemicals

HOPS and HOPG were isolated and purified from urine of dogs dosed with [4'-^{3}H]propranolol [2, 4]. Their purity was greater than 95% as assessed by HPLC. The specific activity of HOPS was 0.12 μCi/mg and for HOPG 0.07 μCi/mg. Tetrabutylammonium (TBA^+) hydrogen sulfate was obtained from Aldrich (Milwaukee, WI, U.S.A.). The aryl sulfatase (*Aerobacter aerogenes*) was purchased from Sigma (St. Louis, MO, U.S.A.). The chloroform used was of analytical grade and was washed with water prior to its use. The liquid scintillation solution (ACS) was purchased from Amersham (Arlington Heights, IL, U.S.A.).

Instruments

Radioactivity measurements were performed after the addition of 10 ml scintillation cocktail, using a Beckman Model LS-355 liquid scintillation counter.

The HPLC system consisted of a Model 6000 high-pressure pump, a Model U6K injector, a Model 440 UV detector operated at 280 nm (Waters Assoc., Milford, MA, U.S.A.) and a C_{18}-column (5 μm, 25 cm × 4.6 mm I.D., Alltech Assoc., Deerfield, IL, U.S.A.). The mobile phase used was acetonitrile—water (15:85) in 0.01 *M* ammonium acetate.

The gas chromatograph—mass spectrometer was an LKB Model 9000S instrument operated in the electron impact mode at 20 eV, using a glass column (30 cm × 1.5 mm I.D.) packed with 3% OV-1 on Supelcoport 80—100 mesh (Supelco, Bellefonte, PA, U.S.A.).

Extraction constants

A charged organic compound can be transferred from an aqueous phase to an organic phase as an ion-pair. The charged compound is extracted as an association complex with a counter ion, an ion of opposite charge [5, 6]. The extraction of an organic anion, X^-, with a counter ion, TBA^+, can be expressed:

$$TBA^+_{aq} + X^-_{aq} \rightleftharpoons TBA^+X^-_{org} \quad (1)$$

The extraction constant, K_{ex}, can be defined:

$$K_{ex} = \frac{[TBA^+X^-]_{org}}{[TBA^+]_{aq}\,[X^-]_{aq}} \quad (2)$$

$[TBA^+X^-]_{org}$ is the concentration of the ion-pair in the organic phase and

$[TBA^+]_{aq}$ and $[X^-]_{aq}$ are the concentrations of TBA^+ and X^- in the aqueous phase at equilibrium. If no side reactions occur in the system, the distribution ratio, D, for the organic compound, X, can be expressed:

$$D = \frac{[TBA^+X^-]_{org}}{[X^-]_{aq}} = K_{ex} \times [TBA^+]_{aq} \quad (3)$$

or

$$\log D = \log K_{ex} + \log [TBA^+]_{aq} \quad (4)$$

As seen from eqns. 3 and 4, the distribution ratio is determined by the value of K_{ex} as well as the counter ion concentration. K_{ex} is dependent on the nature of the organic phase and the counter ion [5, 6].

HOPS. Chloroform (2 ml) was shaken for 30 min with 2.0 ml aqueous phase containing $1.39 \cdot 10^{-4}$ *M* TBA^+ and $0.8 \cdot 10^{-5}$ to $6.1 \cdot 10^{-5}$ *M* radioactive HOPS and adjusted to pH 12 with a small volume of 5 *M* sodium hydroxide. After centrifugation, the radioactivity content in 1.5 ml of each phase was measured by liquid scintillation spectrometry for the determination of HOPS. Before the addition of 10 ml of scintillation cocktail the organic phase was evaporated to dryness and reconstituted in methanol and the aqueous phase was acidified. The D values were calculated as the ratio of radioactivity in the organic and aqueous phases for each experiment. The K_{ex} values were calculated from eqn. 4 after finding the TBA^+ concentration remaining in the aqueous phase, $[TBA^+]_{aq}$.

HOPG. The determination of K_{ex} for HOPG was performed as for HOPS but with a higher counter ion concentration, 0.529 *M* TBA^+. The HOPG concentrations used were $4.7—38 \cdot 10^{-5}$ *M*. Due to the high salt concentration, smaller aliquots (0.5 ml) of the aqueous phase were counted.

Extractions from urine and HPLC

For the determination of the extent of extraction 2-ml aliquots of human control urine, to which had been added radioactive HOPS (42 μg per sample) or HOPG (173 μg per sample), 2.0 ml of water and 1.0 ml of 0.1 *M* TBA^+, were shaken for 30 min with 5.0 ml of chloroform after the pH of the aqueous phase was corrected to pH 12 with 5 *M* sodium hydroxide. Following centrifugation, 0.5-ml aliquots of the organic phase and 0.2-ml aliquots of the aqueous phase were counted as above.

Urine collections (0—24 h) from five healthy volunteers were collected after a single oral dose of 80 mg propranolol and 40 μCi of [4′-^{3}H]propranolol. Urine samples (2 ml) were extracted as above, 4 ml of the organic phase were evaporated and dissolved in 200 μl of mobile phase. Aliquots (40 μl) were injected into the HPLC column. The peak with the same retention volume as purified HOPS was collected. An aliquot of this peak was used for radioactivity measurement by liquid scintillation spectrometry. Another aliquot was hydrolyzed with 0.5 units of aryl sulfatase at pH 7.1 (0.05 *M* Tris buffer) under nitrogen at 37°C for 15 h [2]. After extraction at pH 9.6 with ethyl acetate and derivatization with trifluoroacetic anhydride [7] the sample was analyzed by gas chromatography—mass spectrometry (GC—MS).

RESULTS AND DISCUSSION

The ion-pair extraction of HOPS and HOPG from an aqueous phase into chloroform was studied using TBA^+ as the counter ion. The extraction constants (K_{ex}) were determined using fixed concentrations of TBA^+ and a range of HOPS and HOPG concentrations, Table I. A pH of 12 in the aqueous phase was chosen in order to avoid protonation of the amino group of the side-chain (pK_a about 9.5). Log K_{ex} for HOPS was found to be 3.95 ± 0.03 (mean ± S.D.) and for HOPG −0.76 ± 0.06. This difference in K_{ex} of more than four orders of magnitude indicates a much greater lipophilicity of the TBA^+ ion-pair with the sulfate than with the glucuronic acid conjugate.

The relationship between the distribution ratios (D) for HOPS and HOPG and the TBA^+ concentration in the aqueous phase at equilibrium (eqn. 4) is expressed in Fig. 2. At TBA^+ concentrations $> 1.1 \cdot 10^{-2}$ M the log D for HOPS is > 2, whereas at TBA^+ concentrations $< 5.7 \cdot 10^{-2}$ M the log D for HOPG is < -2. Thus, at the TBA^+ concentration range of 1.1 to $5.7 \cdot 10^{-2}$ M (shaded area in Fig. 2) there is quantitative extraction of HOPS without extraction of HOPG. The validity of this observation was tested directly by extraction of human urine spiked with radioactive HOPS or HOPG. A TBA^+ concentration of $2.0 \cdot 10^{-2}$ M and a pH of 12 gave 99% extraction of HOPS and negligibe extraction (< 3%) of HOPG. Doubling of the TBA^+ concentration gave identical results. These findings demonstrate the direct applicability of the ion-pair extraction approach to biological samples.

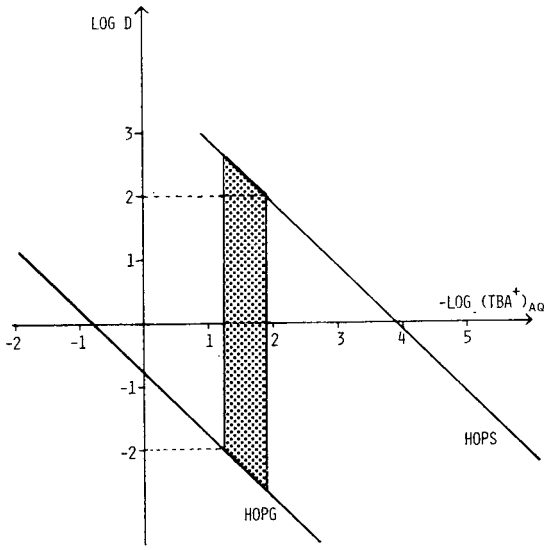
Fig. 3 shows an HPLC tracing of a similar extract of urine from a patient receiving a single 80-mg oral dose of propranolol together with tritium-labeled drug. The shaded peak, which had the same retention volume as purified HOPS, was the only peak containing radioactivity. Although the naphthoxylactic acid metabolite [8, 9] was also extractable as a TBA^+ ion-pair under the conditions

TABLE I

DETERMINATION OF EXTRACTION CONSTANTS, K_{ex}, FOR HOPS AND HOPG

Organic phase: chloroform; aqueous phase: pH 12. C_{X^-} = Initial concentration of X^- in the aqueous phase; C_{TBA^+} = initial concentration of TBA^+ in the aqueous phase; D = distribution ratio for X^-; K_{ex} = extraction constant for X^- as a TBA^+ ion-pair.

Anion X^-	$C_{X^-} \cdot 10^5$	C_{TBA^+}	D	log K_{ex}
HOPS	0.8	$1.39 \cdot 10^{-4}$	1.14	3.93
	1.5	$1.39 \cdot 10^{-4}$	1.11	3.93
	3.0	$1.39 \cdot 10^{-4}$	1.07	3.94
	6.1	$1.39 \cdot 10^{-4}$	1.07	4.00
			Mean ± S.D.	3.95 ± 0.03
HOPG	4.7	0.529	0.080	−0.82
	9.5	0.529	0.116	−0.66
	19	0.529	0.093	−0.76
	38	0.529	0.085	−0.79
			Mean ± S.D.	−0.76 ± 0.06



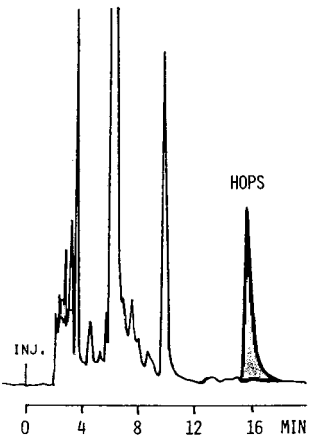


Fig. 2. Distribution ratio as a function of the TBA^+ concentration in the aqueous phase at equilibrium. Log D = log K_{ex} + log $(TBA^+)_{aq}$. Organic phase: chloroform; aqueous phase: pH 12.

Fig. 3. HPLC—UV chromatogram of HOPS after ion-pair extraction into chloroform from human urine following an 80-mg oral dose of propranolol with 40 μCi of [4′-^{3}H]-propranolol. Shaded area contains radioactivity.

used, its elution from the column required pure methanol as the mobile phase. All other peaks in the chromatogram were due to normal urinary constituents, as evidenced by their presence in control urine samples and the absence of radioactivity in these peaks.

The identity of HOPS was further established by hydrolysis of the HPLC peak by bacterial aryl sulfatase and confirmation of the presence of 4′-hydroxypropranolol by GC—MS as previously described [2]. There were no interferences by endogenous compounds or by other propranolol metabolites. It was also shown that HOPS was stable for at least 19 h at the pH of 12 used for the extraction. Analysis of urine samples from five patients demonstrated a high correlation (correlation coefficient 0.993) between the radioactivity content of the HOPS peak and its area measured by UV detection (280 nm), indicating that the method is useful for quantitation of HOPS even without radioactive drug. The relative standard deviations for repeated samples were 3.4% and 4.6% (n = 5), respectively, using radioactivity and UV measurements.

Although HOPG can be extracted as an ion-pair, it would require the use of a larger counter ion, e.g., tetrahexylammonium [3]. HOPG, but not HOPS, can, however, also be determined as the aglycon after direct enzymatic hydrolysis of urine [2, 10].

The ion-pair extraction of a sulfate conjugate with zwitter-ion properties described in this report represents a selective and rapid clean-up step before HPLC separation. It should be useful for the isolation of many other sulfate

conjugates of similar structural complexity prior to chemical characterization and quantitation.

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Note

Simple high-performance liquid chromatographic assay for benzylamine oxidation products in cell suspensions

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Benzylamine and benzyl alcohol are useful industrial reagents and the related oxidation products, benzaldehyde and benzoic acid, are important food additives. Benzylamine is also a convenient model substrate for studies of monoamine oxidase function. Several assays are available for each of these compounds [1—3], as well as for the major end product of mammalian metabolism of these compounds, hippuric acid [4], but none has been developed which allows simultaneous assay of these components in cell or tissue extracts. In a recent study of benzylamine metabolism in isolated hepatocytes [5], it was necessary to obtain a comprehensive description of the metabolites at low concentrations in cell incubations under a variety of metabolic conditions. For this purpose, a high-performance liquid chromatographic (HPLC) system was developed which allows resolution of benzaldehyde, benzoate, hippurate, benzylamine and benzyl alcohol. This report describes this simple and sensitive assay and discusses its application to measurement of benzaldehyde, benzoate and hippurate in metabolic studies with cell suspensions.

EXPERIMENTAL

Benzylamine, benzaldehyde, benzoic acid, hippuric acid, collagenase, and HEPES [4-(2-hydroxyethyl)-1-piperizineethanesulfonic acid] were purchased from Sigma (St. Louis, MO, U.S.A.). Benzyl alcohol was from Eastman Organic Chemicals (Rochester, NY, U.S.A.). HPLC-grade methanol was from Baker (Phillipsburg, NJ, U.S.A.). Doubly distilled water was used for chromatography and deionized water was used for media for cell preparation and incubations.

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Isolated hepatocytes were prepared from male white rats (Kng:(SD)Br, King Animal Labs., fed ad libitum) as described previously [6] and incubated at 37°C in rotating round-bottom flasks in Krebs—Henseleit medium supplemented with 12.5 m*M* HEPES [6]. Viability was 95—99% as estimated by exclusion of 0.2% Trypan blue. Reactions were terminated by addition of 0.5 ml 3 *M* perchloric acid per ml incubation mixture, and protein was removed by centrifugation.

HPLC was performed with a Beckman Model 334 gradient chromatograph with an autosampler and a variable-wavelength detector (flow-cell volume, 20 μl).

Separation of the metabolites was obtained on a 5-μm reversed-phase C_{18} column (25 cm × 4.6 mm, Altex, Ultrasphere-ODS) with a gradient formed from solvent A (50 ml methanol, 550 ml 1% acetic acid, 1 ml ethyl acetate, final pH adjusted to 4.2 with 10 *M* potassium hydroxide) and Solvent B (420 ml methanol, 180 ml 1% acetic acid, 1 ml ethyl acetate, final pH adjusted to 4.2 with 10 *M* potassium hydroxide). The program was as follows: 6 min at 100% solvent A, 10-min linear gradient from 100% solvent A to 100% solvent B, hold 4 min at 100% solvent B, 1-min linear gradient from 100% solvent B to 100% solvent A, hold 10 min at 100% solvent A before injecting another sample. Flow-rate was maintained at 1 ml/min and chromatograms were run at room temperature. Injection volumes were routinely 100 or 200 μl; comparison of recoveries of the same amount of standard injected in different volumes (5—200 μl) showed that recovery was independent of injection volume over this range. Quantitation was performed from the absorbance at 250 nm by both peak height measurements and with a Hewlett-Packard Model 3390A integrator. Integration values are presented, but were proportionate to peak height down to the limits of the assay as expressed below.

RESULTS AND DISCUSSION

Because most cellular metabolites are more polar than benzylamine and its metabolites, an inital isocratic period with 100% solvent A allowed rapid elution of these compounds with retention of the compounds of interest. Separation was highly pH-dependent over the range of 3.3 to 6.0, and optimal separation was obtained at 4.2 with conditions as described in Fig. 1. Separation was very reproducible over a period of several months.

Studies of the recoveries of benzaldehyde, benzoic acid and hippuric acid from cell incubations (Table I) show good recovery can be obtained by precipitation of protein with acid and directly analyzing the protein-free supernatant following centrifugation. For critical analytical studies, correction for incomplete recovery would be necessary, especially if cell content is not constant for different assays. Some retention of the compounds on the columns occurred, but this was not a problem for routine measurements except for initial runs following prolonged washes with methanol or following analysis of relatively high quantities (> 50 nmol). Carry-over from consecutive runs was typically less than 5%, but was sufficient to require a blank run prior to assay of zero time or control incubations. Standard error for integrations from four successive 20-μl injections was less than ± 5%.

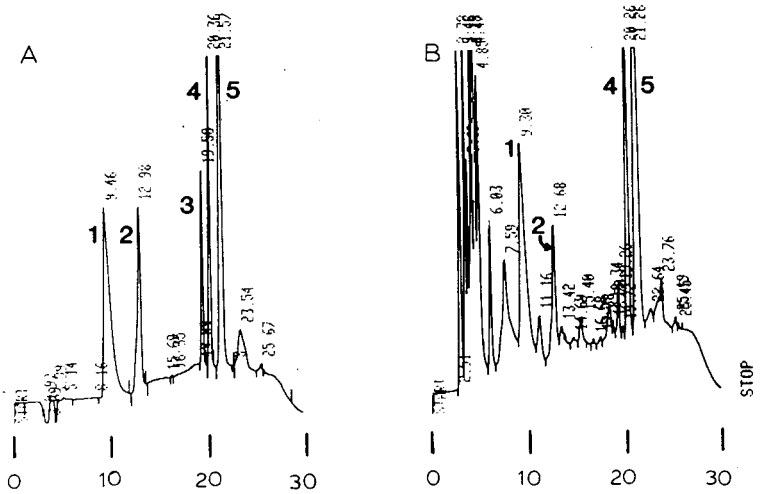

Fig. 1. Separation of benzylamine (1), hippuric acid (2), benzyl alcohol (3), benzoic acid (4) and benzaldehyde (5) by HPLC. (A) 100 μl of a standard mixture of 5 m*M* benzylamine, 0.25 m*M* hippuric acid, 1 m*M* benzyl alcohol, 0.5 m*M* benzoic acid and 0.12 m*M* benzaldehyde were injected on a 5-μm C_{18} column pre-equilibrated with 100% solvent A and run as described in the Experimental section. (B) The metabolic products formed by hepatocytes ($2 \cdot 10^6$ cells per ml) from 5 m*M* benzylamine are separated. Cells were incubated at 37°C for 30 min, and the protein was removed by addition of 3 *M* perchloric acid (0.5 ml per ml incubation) and centrifugation. Injection volume was 200 μl. Retention times for standards were: benzylamine, 9.5 min; hippuric acid, 13.0 min; benzyl alcohol, 19.5 min; benzoic acid, 20.4 min; benzaldehyde, 21.6 min.

TABLE I

RECOVERY OF EXOGENOUS METABOLITES ADDED TO HEPATOCYTE SUSPENSIONS

Metabolites were added at either 2.25 μmol per 10^6 cells or 90 nmol per 10^6 cells; 1-ml aliquots were immediately treated with 0.5 ml of 3 *M* perchloric acid per ml sample and protein was removed by centrifugation. Injection volume for assay was 20 μl. Percent recovery from cells was calculated as the amount recovered from cell suspensions relative to the amount recovered under identical conditions without cells. Values are mean ± standard error for four assays.

Metabolite	Added (nmol)	Recovered —cells (nmol)	Recovered +cells (nmol)	Recovery from cells (%)
Benzaldehyde	30	25.5 ± 1.8	20.9 ± 1.0	82
	1.2	1.19 ± 0.12	1.01 ± 0.03	86
Benzoate	30	29.7 ± 1.5	24.0 ± 1.3	93
	1.2	0.82 ± 0.10	0.86 ± 0.09	105
Hippurate	30	27.2 ± 0.7	24.6 ± 1.3	91
	1.2	1.13 ± 0.04	1.18 ± 0.14	105

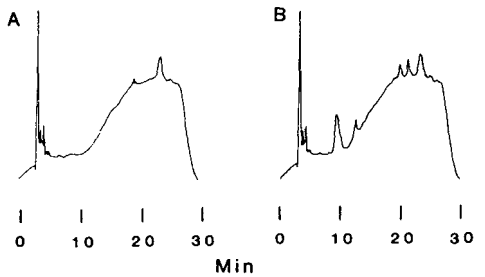

Fig. 2. Illustration of sensitivity of detection of benzylamine oxidation products in isolated hepatocytes. (A) 20 μl of the supernatant from acid-treated hepatocytes (0.5 ml 3 *M* perchloric acid plus 1.0 ml of 10^6 cells per ml) were injected and run as in Fig. 1, except with four-fold higher sensitivity. (B) 20μl of an identical supernatant with added standards, 36 nmol benzylamine, 0.9 nmol hippuric acid, 0.3 nmol benzoic acid and 0.09 nmol benzaldehyde, were run as in (A).

For standards diluted in 1 *M* perchloric acid, quantitation of benzaldehyde was linear over the range of 10 pmol to at least 5 nmol and of benzoate was linear over the range of 50 pmol to at least 25 nmol. Quantitation of hippurate was only linear from 0.25 to 25 nmol because peak broadening occurred with lower amounts. Quantitation of standards added to hepatocytes had a similar sensitivity (see Fig. 2), but in our studies of benzylamine metabolism in hepatocytes [5] only zero-time incubations have concentrations at the lower end of the linear range. In principal, benzylamine and benzyl alcohol could also be quantitated by this approach, but neither assay was found to be practical for cell incubations. The extinction coefficient for benzylamine at 250 nm is relatively low and because of peak broadening and benzylamine retention by the column, acceptable quantitation was not achieved. Benzyl alcohol is not produced from benzylamine at a level detectable in liver cell incubations and consequently, assay of this metabolite has not been pursued.

Benzylamine is metabolized in tissues by monoamine oxidase and is readily assayed in purified preparations of the enzyme by measuring the increased absorbance at 250 nm due to benzaldehyde formation [3]. However, in cells, benzaldehyde is rapidly oxidized to benzoic acid, principally catalyzed by an NAD^+-dependent cytosolic enzyme [7, 8]. Benzoate is further metabolized in mitochondria by conjugation with glycine to form hippurate [9]. Since monoamine oxidase has a rather high K_m value for benzylamine, assay of the mixture of products is necessary for studies with cells and tissues. The current method provides a simple and direct assay for this purpose. The method has been suitable for measuring metabolism of benzylamine, benzaldehyde and benzoate in mitochondria and other subcellular fractions as well as in intact cell suspensions. Acid extracts of tissue samples were stable at −20°C for at least two weeks. The assay is sensitive enough for short incubations (i.e., 3—5 min) and also for systems with low cell concentrations. The entire assay time for the method as described was 31 min between injections. This program can be shortened to 21 min if resolution of benzylamine and benzyl alcohol are not needed.

In conclusion, an HPLC method has been developed which provides a simple

and sensitive assay for oxidation products of benzylamine in cell suspensions and tissues. Suitable quantitation of benzaldehyde, benzoate and hippurate can be obtained in a single run. Direct detection by UV absorption allows accurate quantitation at concentrations of interest in biological tissues.

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