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REVIEW

INVESTIGATIONS OF CATECHOLAMINE METABOLISM USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

ANALYTICAL METHODOLOGY AND CLINICAL APPLICATIONS

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(Received November 20th, 1981)

CONTENTS

1. INTRODUCTION

The three principal catecholamines, norepinephrine (NE), epinephrine (E),

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and dopamine (DA) perform a number of important neuronal and hormonal functions in mammalian systems.

Measurements of the levels of the parent amines, their acidic and neutral metabolites, as well as the activities of related biosynthetic enzymes, have been widely used as diagnostic and prognostic tools for a variety of disease states, among them: hypertension [1], Parkinson's disease [2], affective disorders [3], heart disease [4], neural crest tumors [5—9], hyper- and hypothyroidism [10], muscular dystrophy [11], and familial dysautonomia [12].

The degradation of catecholamines in the body takes place via two reactions: the O-methylation at the 3-position of the catechol group and oxidative deamination of the alkylamine side chain. The two enzymes responsible for the above reactions, catechol-O-methyltransferase (COMT) and monoamine oxidase (MAO) are widely distributed throughout the body; the highest COMT activity is found in the liver and that of MAO in the brain and the heart. The catabolites of greatest clinical utility are: vanillylmandelic acid (VMA), homovanillic acid (HVA), 3-methoxy-4-hydroxyphenylglycol (MHPG), and the metanephrines (normetanephrine, NMN; metanephrine, MN; 3-methoxytyramine, 3-MT).

Catecholamines and their metabolites are found in physiological fluids in two forms: free and as conjugates of sulfuric and glucuronic acids. Conjugation is catalyzed by the transferase enzymes found primarily in the liver and, to a smaller extent, in the brain. The ratio of the free and conjugated forms varies with the type of physiological sample and in some cases the conjugated form predominates by far. For example, MHPG, the principal metabolite of NE in mammals, appears as the free, non-conjugated molecule and as the sulfate conjugate (MHPG·SO_4) and β-conjugate of glucuronic acid (MHPG·Glu). The conjugated forms predominate in the urine and it has been postulated that, while the β-glucuronide form results from the metabolism of systemic NE, the sulfate form reflects the central NE metabolism.

Because of the varying physiological distribution of catecholamines and their metabolites, the clinical interpretation of data must be done carefully. Since urinary catecholamine metabolites reflect both the central and peripheral metabolism, extreme caution must be exercised in relating the fluid composition to a specific area of origin. Because of this, the diagnosis of certain disease states on the basis of altered urinary profiles of catabolites is possible only when gross changes in metabolism exist. Conversely, plasma catecholamines contain useful information concerning the temporary response of the sympathetic nervous system to stress or disease. Except for the direct analysis of brain tissue, only the cerebrospinal fluid (CSF) analysis provides information which is not clouded by peripheral contributions.

The complexity of biological matrices and the extremely low levels of endogenous compounds require the use of an efficient separation technique and sensitive detection devices. High-performance liquid chromatography (HPLC) with fluorometric and/or thin-layer electrochemical (EC) detection is rapidly becoming the analytical method of choice for the assessment of these neurologically important substances in body fluids and tissue samples.

This review will examine the liquid chromatographic methodology and

current examples of the diagnostic use of this technique in clinical investigations of catecholamine metabolism.

2. SAMPLE HANDLING AND STORAGE

Because of the relatively low levels of catecholamines and their metabolites in various physiological fluids, extreme care must be exercised during sample collection, storage and processing in order to avoid contamination of specimens and/or losses due to decomposition. Thus, all glassware used in handling the samples must be scrupulously washed and acid-cleaned. Due to the known instability of catecholamines in basic media, samples must be acidified prior to storage. In addition, antioxidants such as ascorbate, ethylene-bis(nitrilo)-tetraacetic acid (EDTA), ethylene-bis(oxyethylene-nitrilo)tetraacetic acid (EGTA), sodium bisulfite and thioglycolate are added in order to maintain the compounds in their reduced form. This is particularly important for EC detection since catecholamines are generally monitored in the oxidative mode. Internal standard(s) can be added at this stage to correct for losses which may incur during prolonged storage. Removal of proteins can be achieved by centrifugation of acidified samples.

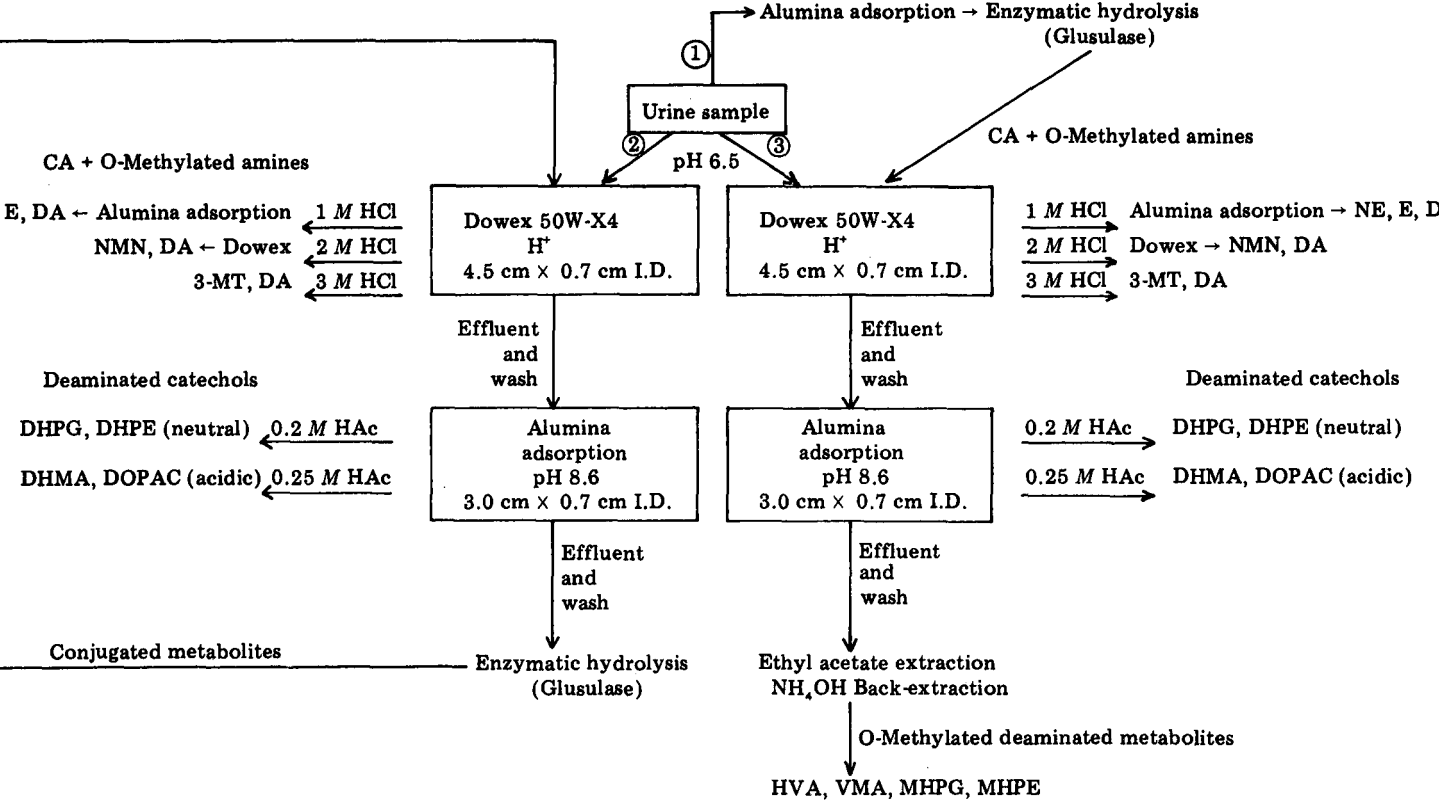
Protein-free specimens or extracts can be safely stored for several months at −70°C to −80°C [13,14]. If, however, samples are to be analyzed within one week of collection, freezing at −20°C will suffice [15].

Since each type of physiological sample requires different pretreatment, a detailed discussion of the preparation of extracts of biological samples is beyond the scope of this review and it can be found elsewhere [9,16—22].

2.1. Post-mortem changes of tissue samples

The determination of catecholamines in tissue samples is often plagued with the problem of post-mortem changes. The extent of these changes depends upon the time elapsed between death and the sample preparation [23, 24], as well as the temperature at which samples were stored prior to processing [25, 26]. While the latter condition can be controlled, the first one will inevitably pose problems in investigations of catecholamines in human brain. Dopamine levels are known to be affected by both factors [25]. However, the rate of decline is reduced if the brain is kept in situ [24, 27] at lower temperature [25]. The reduction in DA levels results in concomitant increase in 3-methoxytyramine (3-MT). This reaction is favored by decreased activity of monoamine oxidase (MAO), by a reduction in tissue pO_2, and by maintained activity of catechol-O-methyl transferase (COMT). However, these problems can be circumvented by destroying the enzymes by means of microwave irradiation [28]. However, less problems are encountered when working with brains of experimental animals since homogenization of tissues in acid or microwave irradiation performed immediately after decapitation minimizes the post-mortem changes [29].

The levels of deaminated metabolites are less affected by the above-mentioned factors. However, prolonged exposure of dissected samples to oxygen is known to increase the levels of 3,4-dihydroxyphenylacetic acid (DOPAC)


Alumina adsorption → Enzymatic hydrolysis
(Glusulase)
①
Urine sample
②
③
pH 6.5
CA + O-Methylated amines
CA + O-Methylated amines
E, DA ← Alumina adsorption
1 M HCl
NMN, DA ← Dowex
2 M HCl
3-MT, DA
3 M HCl
Dowex 50W-X4
H+
4.5 cm × 0.7 cm I.D.
Dowex 50W-X4
H+
4.5 cm × 0.7 cm I.D.
1 M HCl
Alumina adsorption → NE, E, D
2 M HCl
Dowex → NMN, DA
3 M HCl
3-MT, DA
Effluent
and
wash
Effluent
and
wash
Deaminated catechols
Deaminated catechols
DHPG, DHPE (neutral)
0.2 M HAc
DHMA, DOPAC (acidic)
0.25 M HAc
Alumina
adsorption
pH 8.6
3.0 cm × 0.7 cm I.D.
Alumina
adsorption
pH 8.6
3.0 cm × 0.7 cm I.D.
0.2 M HAc
DHPG, DHPE (neutral)
0.25 M HAc
DHMA, DOPAC (acidic)
Effluent
and
wash
Effluent
and
wash
Conjugated metabolites
Enzymatic hydrolysis
(Glusulase)
Ethyl acetate extraction
NH4OH Back-extraction
O-Methylated deaminated metabolites
HVA, VMA, MHPG, MHPE

[30] and HVA [24] due to the higher activity of MAO. In conclusion, assessment of catecholamine levels in samples of human brain requires storage of the body at 4°C and immediate homogenization after dissection. The analysis of tissues from experimental animals is less affected by the post-mortem changes, since dissection and homogenization can be carried out immediately after decapitation.

3. SAMPLE FRACTIONATION SCHEMES FOR CATECHOLAMINES AND RELATED COMPOUNDS

Physiological fluids represent a complex mixture of catecholamines and their major and minor metabolites superimposed upon a background of other potentially interfering compounds present in considerably higher amounts. In addition, compounds involved in the catecholamine metabolic pathways differ significantly among themselves in their chemical, physical and spectroscopic properties. Since in many studies of catecholamine turnover, the measurement of the acidic, neutral or basic metabolites is of greater interest than the monitoring of parent amines or activities of relevant enzymes, specific isolation procedures have been developed for the assessment of isolated compounds or groups of compounds. In spite of the complexity of the problem, it is possible to incorporate all separation schemes for individual compounds or groups of compounds into a general, integrated procedure which will enable sequential analysis of most compounds involved in catecholamine metabolism [31]. This general procedure is outlined in Table 1. The recovery of all the compounds included in this scheme was reported to be in the range of 40—80% [31].

4. METHODS OF ANALYSIS FOR CATECHOLAMINES AND RELATED COMPOUNDS

The amount of attention currently focused on the role of catecholamines in a number of psychiatric and neurological disorders, has prompted the development of a wide number of analytical methods, despite the many difficulties associated with the measurement of the minute amounts of these compounds and some of their metabolites in complex biological matrices. The assessment of quantitative levels requires the use of analytical techniques of high sensitivity and selectivity. Several of them fulfill this requirement with varying success and degree of facility: radioenzymatic procedures [32], the double-isotope derivative analyses [33], gas chromatography alone [34] or coupled with mass spectrometry [35, 36], and more recently, HPLC with fluorometric [37—40] or EC detection [8, 9, 21, 41—48].

Radioenzymatic procedures, which employ the specific radiolabeling of the catechol moiety with 5-adenosylmethionine and catechol-O-methyltransferase (COMT), possess the requisite sensitivity and specificity. However, they require multiple sample handling steps, enzyme preparations and labelled compounds which makes them tedious, time-consuming and expensive for routine analysis. In addition, the enzymatic reaction may be inhibited by the sample components and compounds which are structurally related to catecholamines

can be a potential source of error. The double-isotope derivative methods are also plagued with similar problems.

Earlier gas chromatographic methods employing the electron-capture detector have not achieved wide popularity due to the problems associated with derivatization of complex matrices and the vagaries of the electron-capture detector. In this procedure catecholamines and their metabolites are treated with halogenated anhydrides and the resulting volatile derivatives possess excellent electron-capturing properties [49]. Although the absolute sensitivity of this detector is high, the usable sensitivity is usually decreased due to the sample background arising from the derivatization reagents and contaminants from the sample matrix and solvents. More recently, the tandem operation of gas chromatography and mass spectrometry has emerged as an extremely sensitive method, in spite of the problems associated with the derivatization, high-cost instrumentation and the need for a high level of technical expertise. However, the mass spectrometer, which is considered as a highly specialized detector for gas chromatography, offers three distinct advantages:

(a) the sample does not have to be highly purified when only certain molecular ions are of interest;

(b) deuterated reference compounds, which behave identically as their non-deuterated analogues, can be used and thus high precision can be achieved;

(c) with halogenated anhydrides as derivatizing reagents, excellent fragmentation patterns with prominent molecular ions are obtained.

4.1. Liquid chromatographic methods

HPLC, particularly in its reversed-phase (RP) mode, offers another approach for circumventing the problems commonly associated with other methods of analysis. This technique is ideally suited for the determination of thermally labile biological molecules since it affords high resolution and rapid analyses without prior derivatization. However, the use of this technique could be extended to the investigations of catecholamines only after the introduction of highly sensitive fluorometric and EC detectors.

Several modes of HPLC have been used for the analysis of catecholamines and their metabolites: ion-exchange, reversed-phase and reversed-phase with ion-pairing (RP—IP). More recent examples of the use of these methods in various applications are outlined in Table 2.

Although the development of chemically-bonded microparticulate ion-exchange materials has resulted in improved column stability, increased efficiency and faster analyses, the success of this mode in catecholamine research has been overshadowed by the increasingly popular reversed phases. Therefore, the ion-exchange mode will be discussed briefly.

Generally speaking, weak cation exchangers have been used for the analysis of the amines, and anion exchangers for the acidic metabolites. The ion-exchange packings suffer from several drawbacks: columns are less efficient, less reproducible and stable than the reversed-phase columns and because of the limited number of packings available, the choice of selectivities is rather limited. In addition, neither the cation- nor anion-exchange mode can afford simultaneous separation of a wide range of neurochemical substances.

Sample	Sample preparation	Compounds analyzed	LC mode	Column	Mobile phase	Detection	References
Plasma	Alumina adsorption	NE, E, DHBA (IS), DA	RP—IP	μBondapak C_{18}	6.8 g sodium acetate, 100 mg EDTA, 1 g heptanesulfonic acid, 70 ml acetonitrile per liter; pH 4.8	EC, +0.72 V	33
Plasma	Alumina adsorption	NE, E, DHBA (IS), DA	RP—IP	Ultrasphere ODS	0.1 *M* sodium acetate, 0.02 *M* citric acid, 100 mg/l sodium octyl sulfate, 50 mg/l EDTA, 10% methanol	EC, +0.60 V	34
Rat brain homogenate	Homogenization in $HClO_4$	NE, E, DHBA (IS), DA	CE	Vydac SC	Citrate—acetate buffer	EC, +0.50 V	49
Rabbit brain homogenate	Homogenization in $HClO_4$; alumina adsorption; heptanol—$HClO_4$ extraction	NE, E, DA, 5-HT	RP	Micropak MCH 10 ODS	0.1 *M* perchloric acid—acetonitrile (99:1)	Fluorescence, 200 nm excitation/ 320—400 nm emission	26
Plasma	Alumina adsorption	NE, E, DHBA (IS), DA	RP	Strong cation-exchange	0.008 *M* citric acid, 0.012 *M* sodium acetate, 0.01 *M* EDTA; pH 5.2	EC, +0.50 V	35
Rat brain homogenate	Sonication in $HClO_4$	NE, DHBA(IS) DA, AA, DHPG, MHPG, DBA, NMN, DOPAC	RP—IP	Ultrasphere ion-pair C_{18}	1 vol. methanol + 9 vol. 0.1 *M* KH_2PO_4 (pH 3.0), 0.2 m*M* sodium octyl-sulfonate, 0.1 m*M* EDTA	EC, +0.72 V	41

(Continued on p. 8)

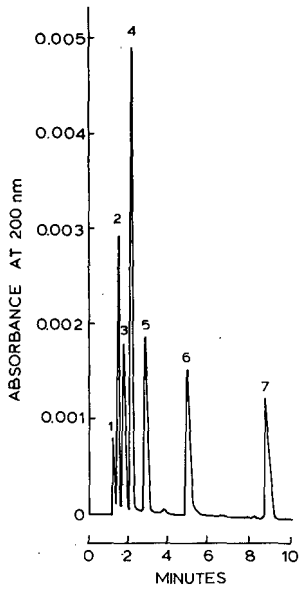
TABLE 2 (continued)

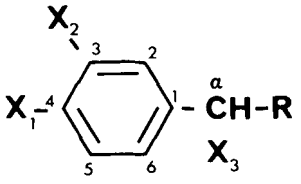
Sample	Sample preparation	Compounds analyzed	LC mode	Column	Mobile phase	Detection	References
Human plasma	Alumina adsorption	NE, E, DHBA (IS), DA	RP—IP	Ultrasphere ODS	0.0347 *M* KH_2PO_4, 0.030 *M* citric acid, 3.0 m*M* sodium octyl sulfate + 14% (v/v) methanol	EC, +0.50 V	8
Tissues, red blood cells	Homogenization in 0.15 *M* KCl, incubation with DA	COMT	RP—IP	Chromegabond, MC-18	Phosphate—citrate buffer (pH 4.4) + 50 mg sodium octyl sulfate + 90 ml methanol per liter	EC, +0.85 V	50
Serum	Incubation with DA; alumina adsorption	DβH	RP—IP	E.M. Labs. RP-18	Phosphate—citrate buffer (0.15 *M*, pH 4.4) + 23 mg sodium octyl sulfate + 35 ml methanol per liter	EC, +0.75 V	51
Rat brain homogenate	Sonication in $HClO_4$	TRP, 5-HT, 5-HIAA, HVA, 5-HTOL, MEL	RP	μBondapak C_{18}	(1) 88% 0.01 *M* sodium acetate (pH 4.25)—12% methanol (2) 85% 0.01 *M* sodium acetate (pH 4.50)—15% methanol (3) 65% 0.01 *M* sodium acetate (pH 4.25)—35% methanol	EC, +0.70 V Fluorescence, 254 nm excitation/ 360 nm emission	52

Mouse brain homogenate, mouse brain perfusate	Homogenization in $HClO_4$; ethyl acetate extraction	MHPG	RP	Rheodyne RP-2 μBondapak C_{18} LiChrosorb	10 m*M* potassium phosphate buffer (pH 7.0)	EC, +0.80 V	55
Human CSF	Ethyl acetate extraction	MHPG (free) MHPG·SO_4	RP	μBondapak C_{18}	Low-strength, 0.1 *M* KH_2PO_4, pH 2.50; high-strength, methanol—water (3:2, v/v); gradient, linear from 0—60% of high-strength eluent in 45 min	EC, +0.70 V	42
Mouse brain homogenate	Homogenization in $HClO_4$; diethyl ether extraction	DOPAC, HVA, VA	RP	μBondapak C_{18}	0.05 *M* sodium acetate buffer (pH 5.0)	EC, + 0.70 V	56
Human urine	Alumina adsorption; heating in alkaline buffer	NE, E, DA, D	SCE	Zipax SCX	50 m*M* NaH_2PO_4 + 50 ml acetonitrile per liter	Fluorescence 400 nm excitation/ 490 nm emission	57
Human urine	Alumina adsorption; THI reaction	NE, E, α-MD, ISO, DA, D, DOPAC, DOMA	RP—IP	Partisil PXS 10/25 ODS	1% acetic acid, 0.0001% sodium dodecyl sulfate, 10% methanol	Fluorescence	58
Human urine	Ion-exchange, alumina adsorption	CA and minor and major metabolites	RP—IP	μBondapak C_{18}	See article	Liquid scintillation	31 59
Animal serum	Incubation with L-DOPA; amberlite CG-	L-amino acid decarboxylase	RP	Yanapak ODS-T	0.01 *M* KH_2PO_4, pH 3.2	EC	59

4.1.1. Reversed-phase liquid chromatography

As evidenced by the ever growing number of publications, the reversed-phase mode of HPLC is experiencing a tremendous increase in popularity due to the high column stability, efficiency and speed of analysis, minimal reequilibration time in gradient elution, and the ability to separate a wide range of compounds through the use of secondary equilibria [60]. The great potential of this technique for catecholamine analysis was first demonstrated by Molnár and Horváth in 1976 [61]. By exploiting the hydrophobic interactions, the authors have separated a mixture of the amines and some metabolites in a single chromatographic run, using isocratic elution with a neat aqueous phosphate buffer (Fig. 1). Since most catecholamine metabolites possess a wide range of polarities, a relatively high carbon content of the stationary phase is necessary for sufficient retention. Molnár and Horváth [62] have also investigated the effects of various ring and side-chain constituents (Fig. 2) on the capacity factors of most catecholamines and related compounds. The results are shown in Table 3. The extent of retention of these compounds on reversed-phase columns is affected significantly by the eluent pH. At low pH values (approximately 2), the amino groups are fully protonated while the dissociation of the carboxyl groups is suppressed [61]. The effects of pH on the extent of retention of the amines and their acidic metabolites are shown in





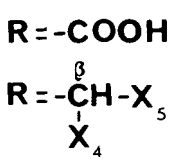


Fig. 1. Separation of some catecholamines and related compounds. Chromatographic conditions: column, 5 μm LiChrosorb RP-18; eluent, 0.1 *M* phosphate buffer, pH 2.1; flow-rate, 2.0 ml/min; temperature, 70°C; inlet pressure, 160 bar. Peaks: 1, norepinephrine; 2, epinephrine; 3, dopamine; 4, tyramine; 5, phenylethanolamine; 6, phenylethylamine; 7, dimethyldopamine. (Reproduced from ref. 62 with permission.)

Fig. 2. Generalized structural formulae for catecholamines and metabolites. (Reproduced from ref. 62 with permission.)

TABLE 3

STRUCTURES AND CAPACITY FACTORS, k', FOR CATECHOLAMINES AND METABOLITES*

Symbol	Name	Structure				Capacity factor k'
		X_1	X_2	X_3	R	
Amines						
NE	Norepinephrine	OH	OH	OH	CH_2NH_2	0.145
OCT	Octopamine	OH	H	OH	CH_2NH_2	0.26
E	Epinephrine	OH	OH	OH	CH_2NHCH_3	0.28
NMN	Normetanephrine	OH	OCH_3	OH	CH_2NH_2	0.48
SYN	Synephrine	OH	H	OH	$CH(CH_3)NH_2$	0.51
DA	Dopamine	OH	OH	H	CH_2NH_2	0.56
MN	Metanephrine	OH	OCH_3	OH	CH_2NHCH_3	0.93
T	Tyramine	OH	H	H	CH_2NH_2	0.96
PEOA	Phenylethanolamine	H	H	OH	CH_2NH_2	1.03
3MDA	3-O-Methyldopamine	OH	OCH_3	H	CH_2NH_2	1.86
PEA	Phenylethylamine	H	H	H	CH_2NH_2	4.06
NIE	Norisoephedrine	H	H	NH_2	$CH(OH)CH_3$	5.17
EPH	Ephedrine	H	H	OH	$CH(CH_3)NHCH_3$	6.76
DMDA	Dimethyldopamine	OCH_3	OCH_3	H	CH_2NH_2	9.09
Acids						
DOMA	Dihydroxymandelic	OH	OH	OH	COOH	0.51
POMA	*p*-Hydroxymandelic	OH	H	OH	COOH	0.87
MOMA	*m*-Hydroxymandelic	H	OH	OH	COOH	1.66
VMA	Vanillylmandelic	OH	OCH_3	OH	COOH	1.69
DOBA**	3,4-Dihydroxybenzoic	OH	OH	—	—	2.66
DOPAC	Dihydroxyphenylacetic	OH	OH	H	COOH	4.42
MA	Mandelic	H	H	OH	COOH	5.57
POPAC	*p*-Hydroxyphenylacetic	OH	H	H	COOH	7.57
VA**	Vanillic	OH	OCH_3	—	—	9.13
HVA	Homovanillic	OH	OCH_3	H	COOH	14.67
PAC	Phenylacetic	H	H	H	COOH	47.80

*Reproduced from ref. 62 with permission.
**The general formula in Fig. 2 is not applicable to the structures of these compounds.

Fig. 3a and b, respectively. It is evident that by suppressing the degree of dissociation, the interaction between the solute molecules and the hydrocarbonaceous stationary phase is enhanced due to increased solute hydrophobicity. Thus, the acidic metabolites are retained longer at lower pH values, while the opposite is true for the amines [61]. The effect of the ionic strength of the medium on the extent of retardation is less pronounced than that of the pH (Fig. 4). This indicates that, under the chromatographic conditions used, the hydrophobic interactions are more important than the coulombic forces.

The effect of temperature on retention of catecholamines and related compounds is illustrated with the Van 't Hoff plots (Fig. 5). The k' values are approximately halved by a 30—40°C increase in temperature. However, the relative retention is not changed significantly.

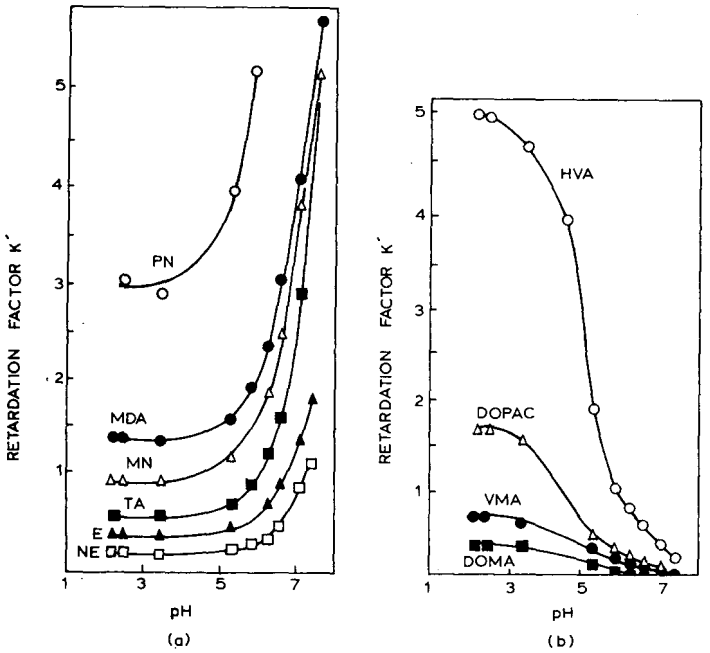


Fig. 3. (a) Effect of pH on the retention of catecholamines. Peaks: E, epinephrine; MN, metanephrine; NE, norepinephrine; MDA, 3-O-methyldopamine; PN, paranephrine; TA, tyramine. (b) Effect of pH on the retention of acidic metabolites. Peaks: DOMA, 3,4-dihydroxymandelic acid; DOPAC, 3,4-dihydroxyphenyl acetic acid; HVA, homovanillic acid; VMA, vanillylmandelic acid. Chromatographic conditions: column, Partisil 10/25 ODS; eluent, 0.1 *M* phosphate buffer; flow-rate, 1.0 ml/min; inlet pressure, 100 atm; temperature, 25°C. (Reproduced from ref. 61 with permission.)

4.1.1.1. Ion-pair reversed-phase chromatography. From the pK_a values for the three major catecholamines it is evident that they are protonated in the pH range commonly employed with the reversed phases [2—7]. The existence of charged moieties results in diminished retention; this is particularly evident in the case of NE which elutes near the void volume, as shown in Fig. 6A. The extent of retention of NE is not improved by changing the eluent pH from 2 to 7.5. In addition, all metabolites with the pK_a values below the pH of the eluent would be present mostly in their dissociated form and would thus elute with minimal retention. Increase in the ionic strength of the eluent upon addition of citrate ions improves the peak shapes by facilitating the protonic equilibria with no effect on retention (Fig. 6B). Therefore, although the reversed-phase mode offers higher efficiency and faster analyses, it still exhibits a discriminatory effect with respect to a wider range of catecholamine metabolites.

The retention of basic solutes such as catecholamines can be augmented by increasing the eluent pH. However, this is undesirable for the following reasons:

(a) catecholamines are notoriously unstable at elevated pH values;

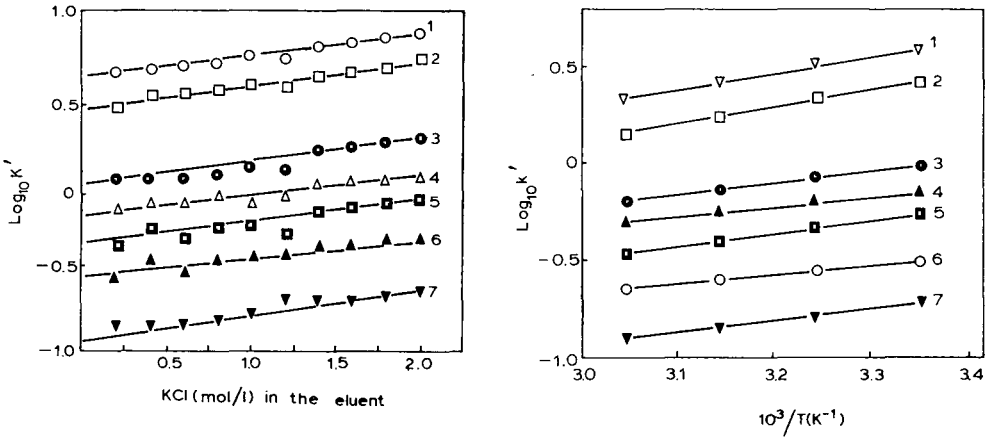


Fig. 4. Effect of ionic strength on the retardation factor; the concentration of KCl in a 50 m*M* KH_2PO_4 solution was varied. Peaks: 1, paramephrine; 2, homovanillic acid; 3, 3-O-methyldopamine; 4, 3,4-dihydroxyphenylacetic acid; 5, tyramine; 6, tyrosine; 7, DOPA. Chromatographic conditions as in Fig. 3. (Reproduced from ref. 61 with permission.)

Fig. 5. Effect of temperature on retardation factor. Peaks: 1, homovanillic alcohol; 2, homovanillic acid; 3, 3-O-methyldopamine; 4, metanephrine; 5, tyramine; 6, dopamine; 7, vanillylmandelic acid. Eluent, 0.05 *M* KH_2PO_4; all other chromatographic conditions as in Fig. 3. (Reproduced from ref. 61 with permission.)

(b) at the same pH (basic), the acidic metabolites can not be efficiently separated (they would elute near the void volume);

(c) with the currently available silica-based reversed phases, dissolution of the support takes place in alkaline media.

The retention of charged solutes can be altered dramatically upon addition of ionic surfactants (ion-pairing reagents, ion-association reagents) to the mobile phase. This technique, which combines the best features of reversed phase (high resolution, high column stability) and ion exchange (improved separation), is finding widespread acceptance under a multitude of names, the most common one being ion-pairing (IP). By exploiting the interplay between the electrostatic and hydrophobic forces, the capacity factors of basic solutes can be greatly modulated by addition of anionic surfactants and those of acidic metabolites can be altered upon addition of cation-pairing reagents. Because of the experimental difficulties associated with obtaining umambiguous physicochemical evidence, the underlying thermodynamic equilibria are a subject of considerable controversy. The retention is believed to occur by dynamic ion exchange [63], ion-pair formation in the mobile phase [64], or the dynamic complex exchange [65].

The main equilibria involved in ion-pair chromatography of catecholamines are the following [8]:

(a) ion-pair formation in the mobile phase with the anion-pairing reagent, L^-

$$CA^+_m + L^-_m \leftrightarrows (CA^+L^-)_m \quad (1)$$

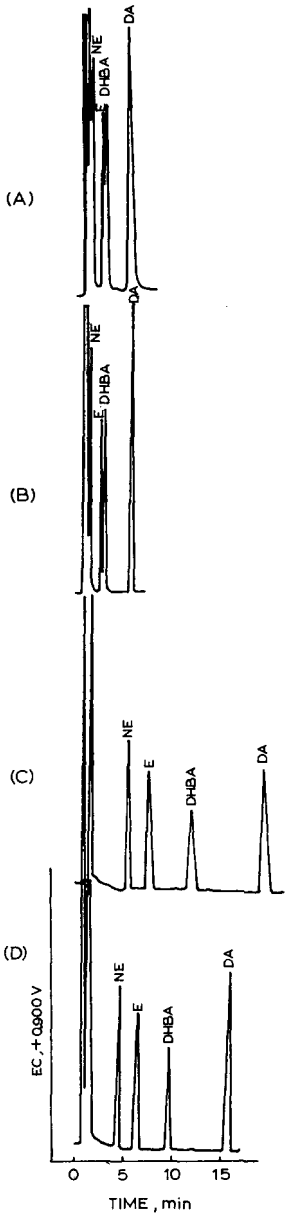


Fig. 6. Separation of a synthetic mixture of norepinephrine (NE), epinephrine (E), 3,4-dihydroxybenzylamine (DHBA) and dopamine (DA). Chromatographic conditions: column, Ultrasphere ODS (15 cm × 4.6 mm I.D.), 5 μm average particle size; flow-rate, 1.2 ml/min; temperature, ambient; detection, amperometric at +0.500 V vs. Ag/AgCl; detector sensitivity, 7.5 nA full scale; eluent, (A) 0.0347 *M* KH_2PO_4, pH 4.85; (B) 0.0347 *M* KH_2PO_4, 0.030 *M* citric acid, pH 4.85; (C) 0.0347 *M* KH_2PO_4, 3.0 m*M* sodium octyl sulfate, 14% methanol (v/v), pH 4.85; (D) 0.0347 *M* KH_2PO_4, 3.0 m*M* sodium octyl sulfate, 0.030 *M* citric acid, 14% methanol (v/v), pH 4.85. (Reproduced from ref. 8 with permission.)

Followed by reversible binding of the ion-pair to the hydrocarbonaceous surface:

$$(CA^+L^-)_m \leftrightarrows (CA^+L^-)_s \quad (2)$$

where the subscripts m and s refer to the mobile phase and stationary phase, respectively;

(b) dynamic ion exchange in which the solute molecule forms a complex with the ligand already adsorbed on the nonpolar surface:

$$CA^+_m + L^-_s \leftrightarrows (CA^+L^-)_s \quad (3)$$

(c) dynamic complex exchange in which a metathetical exchange occurs between the ion-pair formed in the mobile phase and the ion-pairing reagent bound to the column:

$$(CA^+L^-)_m + L^-_s \leftrightarrows (CA^+L^-)_s + L^-_m \quad (4)$$

It is important to remember that each model represents a limiting case and "the retention process is not expected to follow any of them over a wide range of chromatographic conditions" [65].

Regardless of the exact type of thermodynamic equilibrium responsible for retention, it is mandatory that the solute molecules be charged for the interaction with the ion-pairing reagent to occur.

The dramatic effect of the ion-pairing reagent, octyl sulfate, on retention of catecholamines is shown in Fig. 6C; the chemical nature and concentration of the pairing reagent are important in determining the extent of retention. However, high concentrations and excessively long chain lengths of the pairing reagent must be avoided since they give rise to excessively long retention and long equilibration times. Octyl sulfate gives excellent selectivity with minimal equilibration time (20 min). However, under the conditions shown in Fig. 6C, DA exhibits excessively long retention which gives rise to considerable peak broadening. This is prohibitive, for example, for the analysis of the free levels of trace amines in plasma samples from normal subjects. The k' values, however, can be adjusted by addition of an organic modifier, such as methanol, to the mobile phase (Fig. 6C). Furthermore, the presence of citrate ions in the mobile phase exerts a pronounced effect on retention (Fig. 6D). This effect is not due to the change in ionic strength, since the same was not observed when citrate was replaced with an equivalent amount of KCl. From the behavior of catecholamines under the chromatographic conditions shown in Fig. 6A, B, C and D, it can be assumed that the ion-pairing takes place on the column surface covered with octyl sulfate. The citrate ions in the mobile phase lower the retention of catecholamines by exerting an electrostatic attraction on the "pairs".

5. DETECTION AND CLINICAL APPLICATIONS

With the increasing ability to achieve efficient and rapid separations of catecholamines and their metabolites, the central problem in biomedical and biochemical investigations becomes the ability to monitor the minute amounts of these compounds with detection systems of sufficient sensitivity and com-

patibility with the chromatographic system. Only two detectors, the fluorometric and the electrochemical, fulfil these requirements successfully. The relative advantages and disadvantages of both detectors will be discussed in greater detail and illustrated with examples of clinical applications.

5.1. Fluorometric detection

Prior to the advent of the EC detector, fluorometric detection was the only method capable of monitoring picomole amounts of catecholamines and their metabolites. Since only relatively few compounds fluoresce naturally, the use of this detection affords yet another level of selectivity to the analytical system [38]. In addition, each group of fluorescent molecules exhibits different excitation and emission spectra: catecholamines have excitation maxima between 200—220 nm and 280—300 nm and an emission maximum at 310—330 nm, while the indolic compounds exhibit the excitation maxima at 210—220 nm and 270—280 nm and an emission maximum at approximately 360 nm. With the currently available sources of UV radiation, efficient columns, and in view of the molar absorptivities and quantum efficiencies of underivatized catecholamines and indoles, the detection limits are 100—500 pg and 5—25 pg, respectively. Even without the future improvements in HPLC design, considerably lower detection limits can be achieved with the fluorescent derivatives of catecholamines and indoles. The most commonly employed fluorogenic reagents are: fluorescamine, dansyl chloride, *o*-phthalaldehyde and ethylenediamine. Another frequently employed derivatization method, the trihydroxyindole (THI), involves catechol oxidation and alkaline rearrangement.

Fluorogenic reactions can be carried out in the following ways:

(a) pre-column: off-line: the reaction is carried out separately before the chromatography; on-line: the reaction is carried out between the injector and the column inlet;

(b) post-column: off-line: derivatization is carried out on collected fractions, after the chromatography; on-line: derivatization is carried out in a specifically designed reactor cell placed between the column outlet and the detector.

Generally speaking, pre-column, off-line derivatization is often used since it does not pose any restrictions on the mobile phase composition, duration and temperature of reaction and product stability, which is not the case with the on-line derivatization. Post-column, on-line derivatization is carried out on separated chromatographic bands. Therefore, the mobile phase composition must be compatible with the reaction medium. In addition, the reaction detector must be carefully designed in order to minimize dispersion.

5.1.1. Selected clinical applications

Measurements of native fluorescence usually do not afford sufficient sensitivity for the analysis of picomole amounts of catecholamines. Enhanced sensitivity can be achieved with THI or *o*-phthalaldehyde (OPA) derivatizations. The RPLC separations of the OPA and THI derivatives of catecholamines detected fluorometrically are illustrated with the extract of a sample of control urine and a purified sample of human plasma shown in Figs. 7 and 8, respectively.

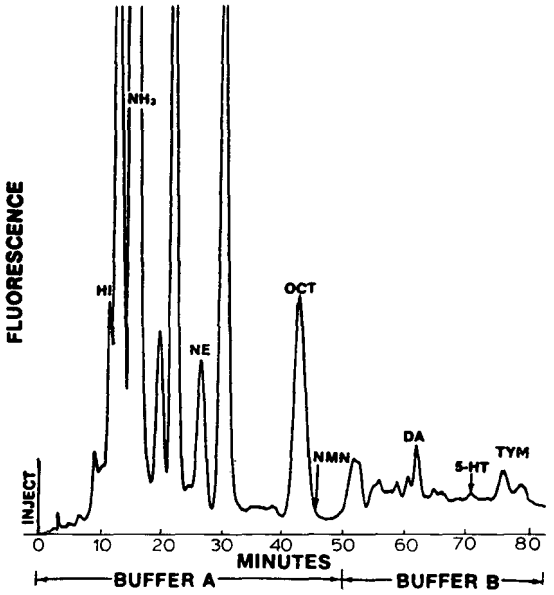


Fig. 7. RPLC separation of OPA derivatives of biogenic amines in control urine. Chromatographic conditions: sample, 15 μl, equivalent to 15 μl urine; column, μBondapak phenyl (300 mm × 4 mm I.D.); buffer, 0.05 *M* NaH_2PO_4, pH 5.10 with 320 ml of methanol added per l for first elution step (A) and 450 ml of methanol added per l for second elution step (B); flow-rate, 1.5 ml/min; detector, Schoeffel FS 970, 0.10 μA full scale, excitation 340 nm, emission 480 nm; temperature, 30°C. The internal standard (IS) is octopamine (OCT). Peaks: HI, histamine; NE, norepinephrine; NMN, normetanephrine; DA, dopamine; 5-HT, serotonin; TYM, tyramine. (Reproduced from ref. 13 with permission.)

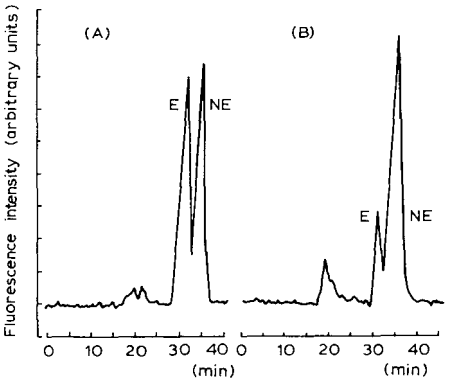


Fig. 8. Elution pattern of fluorometrically detected THI derivatives of epinephrine (E) and norepinephrine (NE). (A) Standard sample of E (0.5 ng) and NE (1.0 ng); (B) plasma sample. Chromatographic conditions: column, Amberlite IRC-50; eluent, succinic acid—boric acid—EDTA (pH 5.4); flow-rate, 0.7 ml/min; temperature, 42°C; detection, excitation 405 nm, emission 500 nm. (Reproduced from ref. 66 with permission.)

Under the chromatographic conditions used, the detection limits for the OPA derivatives were approximately 50—100 pg. It is evident, therefore, that this type of derivatization is not suitable for the analysis of catecholamines in samples of human plasma. However, the post-column THI derivatization shown in Fig. 8 allowed the detection of 20 pg of NE and DA, and 40 pg of NE. Thus, with the latter method it was possible to determine the catecholamine levels in 1—2 ml of human plasma purified by cation-exchange chromatography. It should be pointed out that both methods illustrated with Figs. 7 and 8 could be further improved by reducing the retention volumes and optimizing the method of detection. Since the levels of metabolites often excede those of the parent amines, direct monitoring of native fluorescence can be used for the assessment of metabolite levels in various physiological fluids [38,67]. The comparative features of fluorometric and amperometric detection of the catechol and indolic compounds are illustrated with the analysis of human lumbar cerebrospinal fluid (Fig. 9A and B). Under the conditions used, the phenolic indole (5-HIAA) gives approximately the same

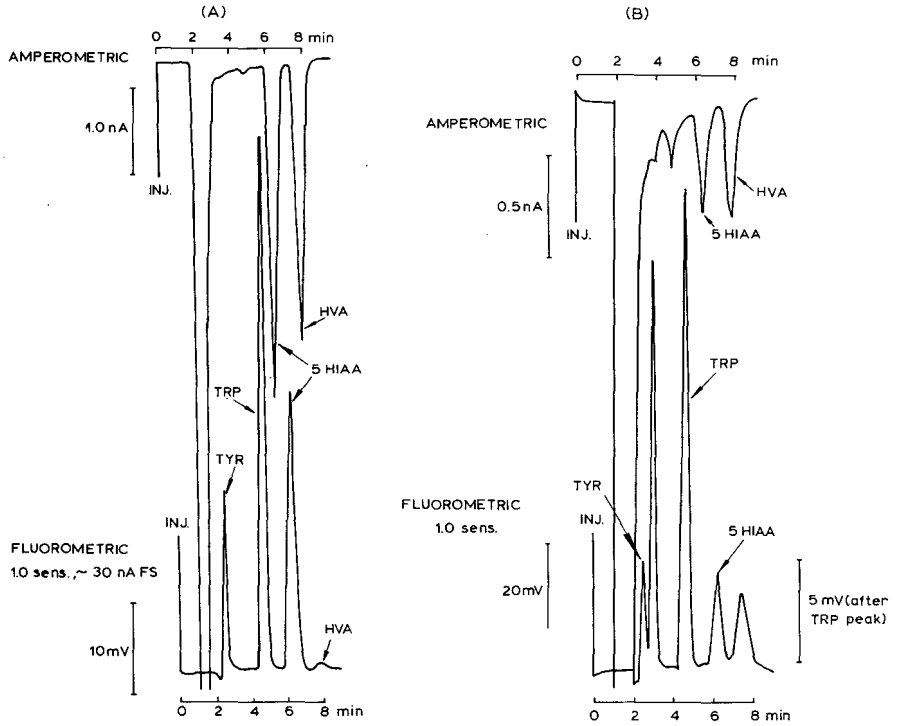


Fig. 9. (A) Chromatogram of reference compounds: 25 ng tyrosine (TYR), 5 ng tryptophan (TRP), 5 ng 5-hydroxyindole-3-acetic acid (5-HIAA), 10 ng homovanillic acid (HVA). TYR was not usually separated from the amperometric solvent front. (B) Chromatogram of a 20-μl unprocessed lumbar CSF sample. Concentrations: TYR, 1.04 mg/ml; TRP, 396 ng/ml; 5-HIAA, 32.6 ng/ml; HVA, 89.7 ng/ml. The fluorometric peaks immediately following the TYR and 5-HIAA peaks are unidentified. Chromatographic conditions: column, μBondapak C_{18}; eluent, 300 ml methanol + 1700 ml 0.01 *M* sodium acetate, pH 4.0; flow-rate, 1.5 ml/min; temperature, ambient; detection, EC, +0.8 V vs. Ag/AgCl; fluorescence, 254 nm excitation, 360 nm emission. (Reproduced from ref. 67 with permission.)

response with both detection methods while amperometric detection is more sensitive for the catechol HVA. The use of fluorometry is generally advantageous for the determination of indolic compounds. An additional advantage of the fluorometric detection in this application is the virtual absence of solvent front, which is pronounced in the amperometric tracing, and arises from ascorbic acid present in the CSF sample and the synthetic mixture of reference compounds [67]. Since biological matrices contain many compounds, the elucidation of identity of chromatographic bands is a step of paramount importance. Quite often, peak assignments are based only on retention times and co-chromatography with the reference compounds. This is by no means sufficient, even with the most selective sample purification and detection. Every detection device offers different possibilities for on-line characterization. With fluorescence monitoring, which is highly selective for the limited number of naturally fluorescing molecules in physiological samples, excitation and/or emission spectra of the solutes can be obtained and compared with those of the reference compounds [37]. Since each compound or a class of closely related compounds exhibits distinctly different absorption and/or emission characteristics, further proof of peak identity can be obtained. The usefulness of the excitation spectra is illustrated with the analysis of catecholamines in a sample of rat brain homogenate (Fig. 10).

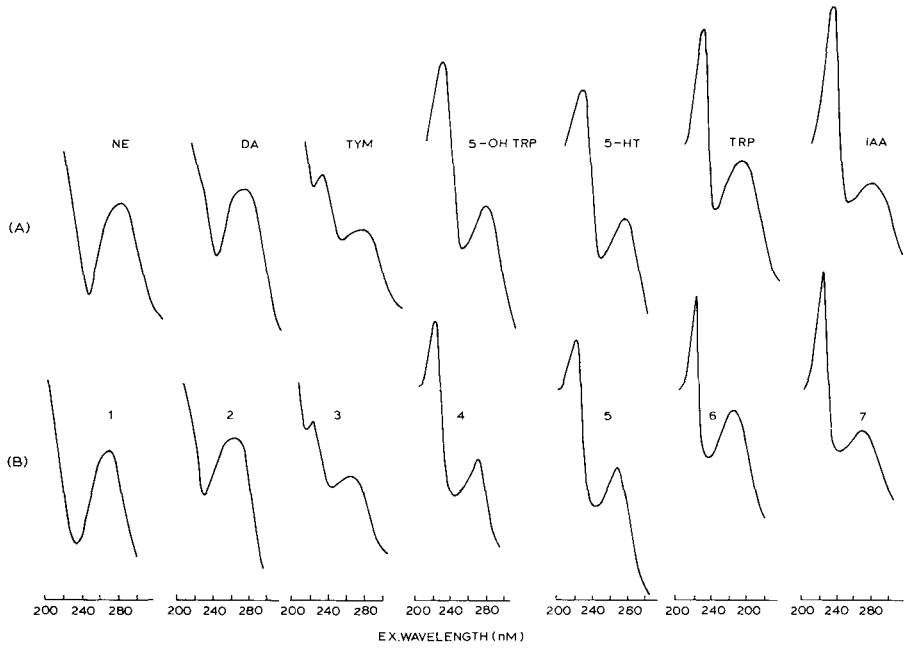


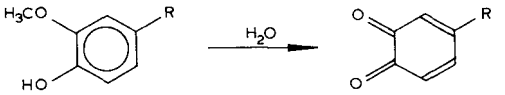
Fig. 10. Comparison of corrected stopped-flow excitation spectra for (A) some reference compounds and (B) chromatographic peaks in a rat brain extract. Scanning rate, 100 nm/min; range, 0.1 μA full scale; chromatographic conditions: column, μBondapak C_{18}; eluents, (low-strength) 0.02 *M* KH_2PO_4, pH 3.7; (high-strength) methanol—water (3:2, v/v); gradient, linear from 0% to 100% of the high-strength eluent in 35 min; flow-rate, 1.5 ml/min; temperature, ambient. (Reproduced from ref. 37 with permission.)

These spectra were obtained by the stopped-flow method, whereby the desired solute is arrested in the detector cell under the stopped-flow conditions and the corrected spectrum is obtained by automatic subtraction of the spectral background arising from the mobile phase, flow cells and the photomultiplier. The marked similarity between the spectra of the sample peaks and those of the reference compounds confirms the identity of the endogenous compounds.

5.2. Electrochemical detection

The use of thin-layer electrochemical reactions is becoming increasingly popular for the determinations of substances of neurochemical interest such as phenols and indoles [43] and related enzymes. The electrochemical reaction(s) involve(s) a direct conversion of chemical information into an electrical signal without the need for intermediate magnetic or optical carriers. Thus catecholamines (a) and vanil compounds (b) can be determined analytically by oxidation to the corresponding orthoquinones at the surface of a graphite electrode, according to the following raction:

(a)  $+ 2H^+ + 2e^-$ (5)

(b)  $+ 2H^+ + ROH + 2e^-$ (6)

The instantaneous anodic current is directly proportional to the number of solute molecules in contact with the interface per unit time. The physicochemical process occurring at the electrode surface poses certain requirements on the nature and properties of the solute and mobile phase: the compounds must be electroactive under the conditions used and the mobile phase must have sufficient electrical conductivity (optimal ionic strength > 0.05 *M*). The latter parameter helps to minimize the internal *iR* drop (where i = current and R = resistance), and to control the electrode potential.

The electrochemical behavior of catecholamines and related compounds is affected by the pH of the medium, as expected from eqns. 5 and 6. According to the Nernst equation, a 60-mV shift in half-wave potential is expected for fast reactions in which the number of electrons transferred across the electrode—solution interface equals the number of protons (lost or gained). This is illustrated with the change in limiting current for NE, E and DA upon change in pH from 3.75 to 7.28 (Fig. 11). At low pH the oxidation of the catechol takes place according to eqn. 5. In the basic range, however, the free base of the alkylamine will attack the aromatic nucleus and the product of the reaction, indoline, will subsequently be oxidized (Fig. 12). If the reaction is fast compared to the residence time of the molecule in the detector cell, a double response will be obtained due to the overall transfer of $4e^-$. Since the extent of this effect depends upon the rate of nucleophilic addition,

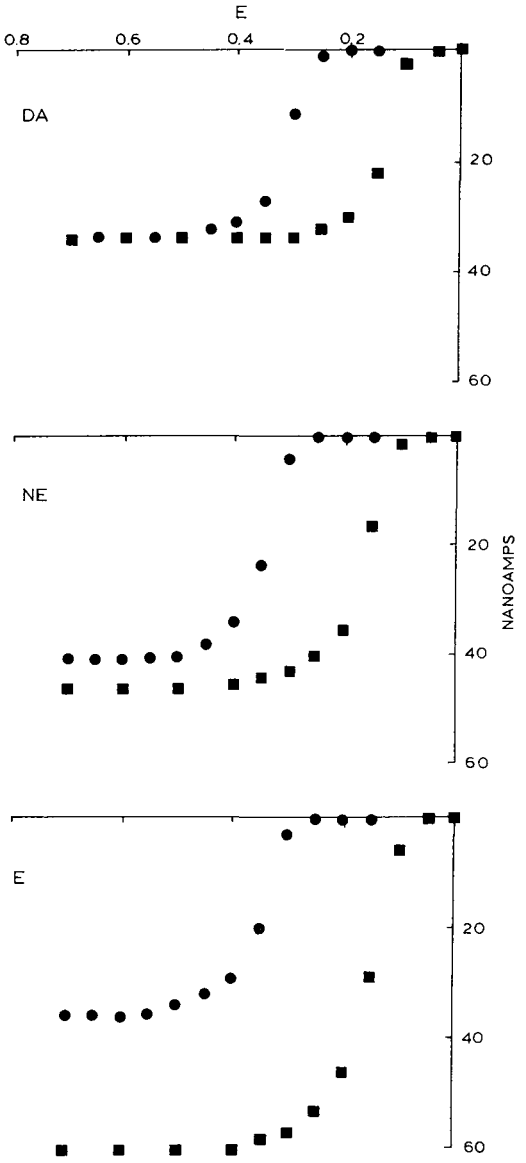


Fig. 11. Hydrodynamic voltammograms obtained from chromatograms of dopamine (DA), norepinephrine (NE), and epinephrine (E) at pH 3.75 (•) and pH 7.28 (▪). Chromatographic conditions: stationary phase; 15 cm × 4.6 mm I.D., Merck C_2 reversed-phase packing; mobile phase, acetate buffer (0.04 *M*, pH 3.75), 0.2 m*M* octyl sulfate; mobile phase flow-rate, 0.40 ml/min; temperature, ambient; amount injected, 20 ng of each catecholamine. Mixing conditions: mixing phase, •, same as mobile phase, ▪, 0.5 *M* Na_2PO_4; mixing phase flow-rate, 0.13 ml/min; reaction coil residence time, 8.1 sec; final pH •, pH 3.75, ▪, pH 7.28. (Reproduced from ref. 68 with permission.)

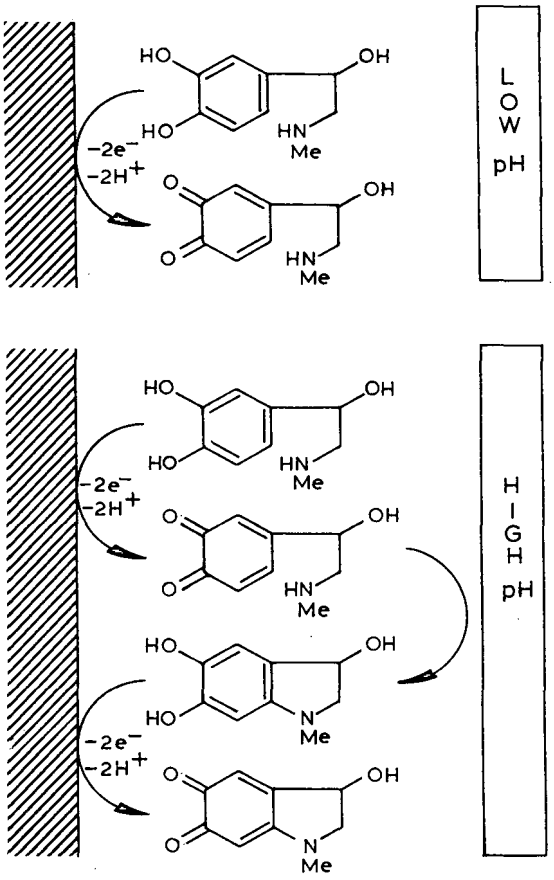


Fig. 12. Mechanisms for the electrochemical oxidation of epinephrine. (Reproduced from ref. 68 with permission.)

the response of NE and DA will not be increased significantly at pH 7.28. It is important to realize that like any other LC detector, the EC detector also suffers from some shortcomings. Although it is possible to vary the applied potential within the useful range of the working electrode material (Fig. 13A and B), the resulting molecular specificity is not adequate when working with complex physiological matrices. Therefore, in order to increase the detection specificity, an isolation step must be incorporated into the analytical procedure.

5.2.1. Selected clinical applications

Since a complete survey of the LC—EC applications in biomedical and biochemical research is beyond the scope of this review, only some examples will be used to demonstrate the tremendous utility of this technique. The determination of plasma catecholamines under standardized conditions is of considerable importance for clinical investigations of disorders such as hypertension, neural crest tumors, neurological disorders such as depression, schizophrenia, Parkinson's disease and many others. If the method is to be used as

a diagnostic tool in clinical applications, prior to the determination of cut-off values, it is necessary to establish the baseline levels in control subjects. As stated previously, many variables must be clearly specified in catecholamine investigations [8].

After the normal levels have been established, it is possible to use this technique to monitor certain disease states, such as pheochromocytoma [9,42].

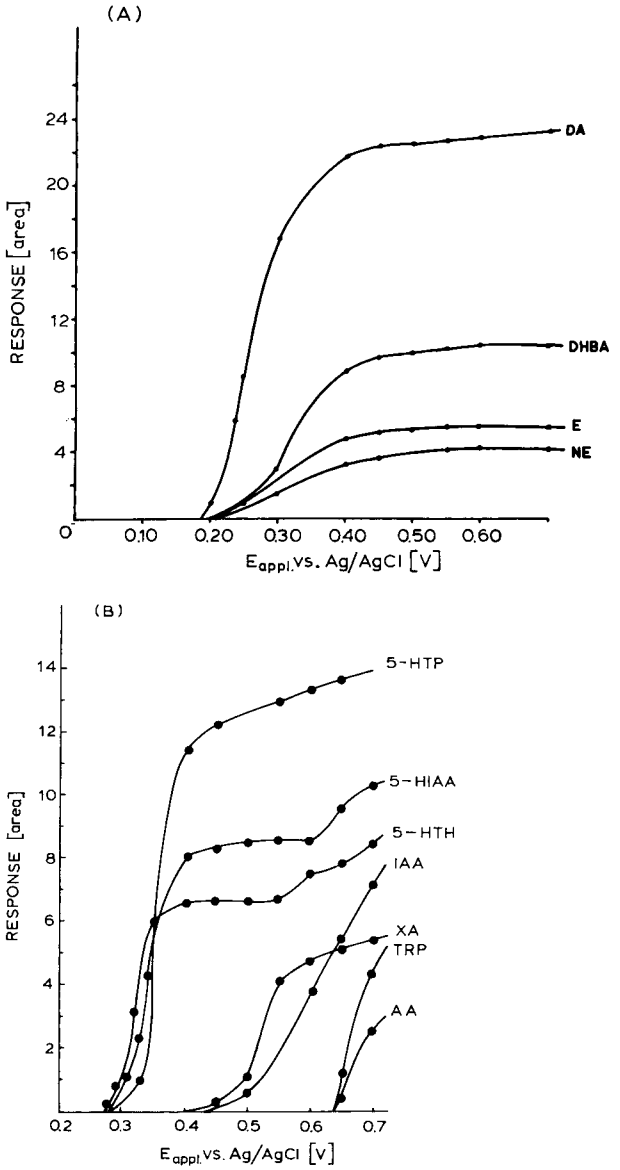


Fig. 13. (A) Voltammograms of the three principal catecholamines and internal standard (DHBA). (Reproduced from ref. 8 with permission.) (B) Voltammograms of some tryptophan metabolites. (Reproduced from ref. 69 with permission.)

Pheochromocytoma is a tumor of the sympathetic nervous system, derived from primitive neural crest tissue. This catecholamine-secreting lesion is a rare cause of hypertension and is fatal if undetected. Since these tumors exhibit tremendous variability in their clinical presentation and have all the symptoms of essential hypertension, a reliable diagnosis is possible only on the basis of altered patterns of catecholamine metabolism. Fig. 14 illustrates the catecholamine levels in the alumina extracts of plasma samples from the same patient before (A, B) and after (C) surgical removal of the tumor. It is evident that this tumor was predominantly NE-secreting. Prior to the final assignment of peak identities and subsequent quantitation, it is mandatory to characterize chromatographic bands by methods which rely on properties other than the retention behavior. It can not be overemphasized that the agreement between the retention times of the peak on the chromatogram and the reference compound is not a proof of identity but merely an indication

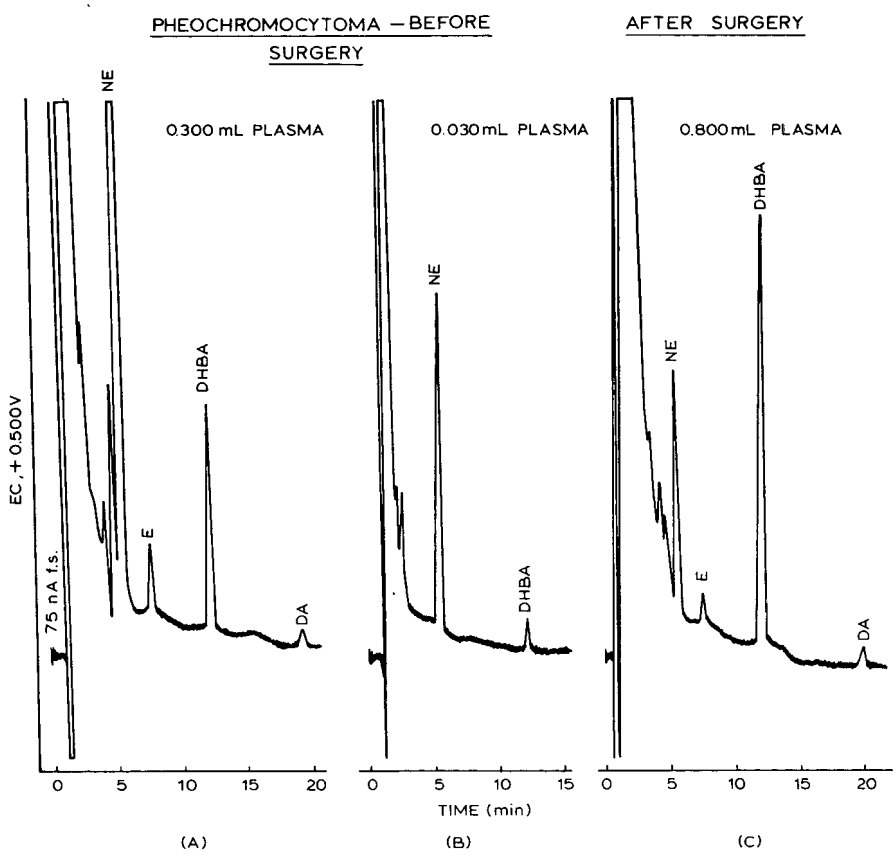


Fig. 14. Chromatograms of plasma samples from a patient with pheochromocytoma, before (A, B) and after (C) surgical removal of the tumor. Chromatographic conditions as in Fig. 6D; volumes of extract injected in (A), (B), and (C), 100 μl; volumes indicated in the figure refer to volumes of plasma extracted by alumina adsorption. Catecholamine levels, (A, B) NE, 14.5 ng/ml; E, 180 pg/ml; DA, 85 pg/ml; (C) NE, 350 pg/ml; E, 25 pg/ml; DA, 40 pg/ml; DHBA is the internal standard. (Reproduced from ref. 8 with permission.)

of it. Amperometric detection offers an attractive possibility for on-line characterization of electroactive solutes by comparison of hydrodynamic voltammograms of the sample component and the reference compound [8, 70]. By making replicate injections of the sample and recording the response (current) at several potentials, the ratios of the current at any given potential to that of the maximal response can be computed. These relative current ratios (ϕ) can be plotted as a function of the applied potential, and the resulting curves for the sample peak and the reference compound compared. Fig. 15 illustrates the use of hydrodynamic voltammograms for characterization of NE in the plasma sample shown in Fig. 14. Careful confirmation of peak identities is essential for ensuring that the right peaks are quantified and that there are no impurities coeluting with the compound of interest. The quantitative data for catecholamines and some metabolites in plasma and urine samples from control normotensive subjects, hypertensive patients and those with pheochromocytoma are given in Table 4. Significant differences in NE levels among the three groups of subjects illustrate the usefulness of these compounds as biochemical determinants of certain disease states.

The aberrations in the metabolic pathways of catecholamines in certain disease states manifest themselves in characteristic urinary excretion patterns of their catabolites (Table 4). Since the levels of VMA, HVA, MHPG and total metanephrines (NMN, MN) are considerably higher than those of the

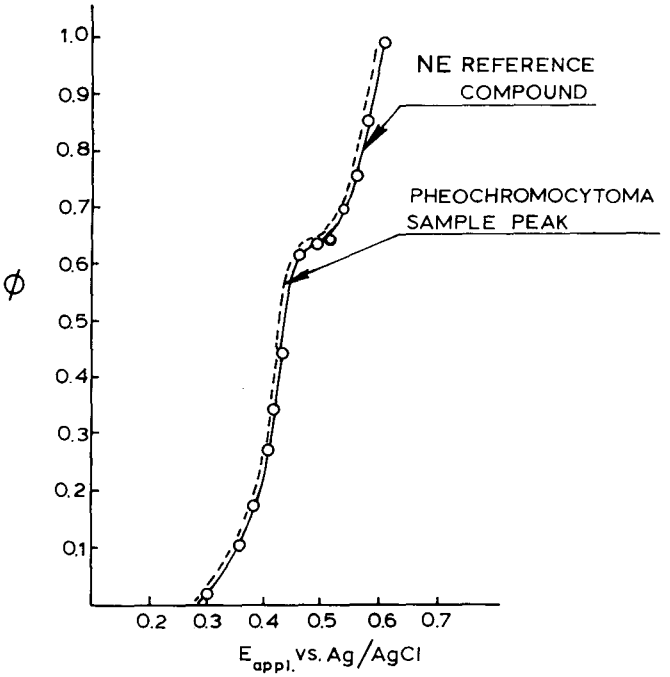


Fig. 15. Hydrodynamic voltammograms for the NE reference compound and the peak in the sample from a patient with pheochromocytoma shown in Fig. 14A; X-axis, oxidation potential vs. Ag/AgCl; Y-axis, ratio of response (current) at a particular potential to the maximal response. (Reproduced from ref. 8 with permission.)

LE 4

ANTITATIVE DATA FOR CATECHOLAMINES AND SOME METABOLITES DETERMINED BY HPLC*

	NE (pg/ml)	E (pg/ml)	DA (pg/ml)	VMA** (μg/mg CRE)	MHPG** free (ng/mg CRE)	MHPG** conjugated (ng/mg CRE)	NMN** (μg/mg CRE)	MN** (μg/mg CRE)
trol subjects motensive)	100—600 $n = 20$	10—80 $n = 20$	10—150 $n = 20$	0.5—3.5 $n = 15$	0.02—0.1 $n = 15$	0.4—0.6 $n = 15$	0.09—0.21 $n = 7$	0.01—0.09 $n = 7$
trol subjects er stress	900—1200 $n = 2$	10—70 $n = 2$	10—50 $n = 2$	—	—	—	—	—
ntial hypertension	1600—1800 $n = 3$	40—80 $n = 3$	200—250 $n = 3$	—	—	—	—	—
ochromocytoma	4000—14500 $n = 3$	80—250 $n = 3$	65—470 $n = 3$	4.5—50.0 $n = 15$	0.07—1.15 $n = 15$	2.4—15.9 $n = 15$	1.5 —27.5 $n = 6$	0.10—1.60 $n = 6$
roblastoma	—	—		5.0—21.0 $n = 5$	0.09—1.50 $n = 5$	2.4—16.0 $n = 5$		
glioneuroma	—	—		—			4.0 $n = 1$	0.79 $n = 1$

esults reproduced from refs. 8, 9, 42, 48 and 71.
urine (referred to creatinine, CRE); all other values pertain to plasma.

parent amines, the determination of these metabolites is an easier analytical problem.

The clinical usefulness of VMA and MHPG as a diagnostic tool for pheochromocytoma is evident from the elevated levels of both metabolites (Fig. 16B) compared to those of the control subject (Fig. 16A). Total metanephrines have also been used as a clinical index of neural crest tumors (Table 4).

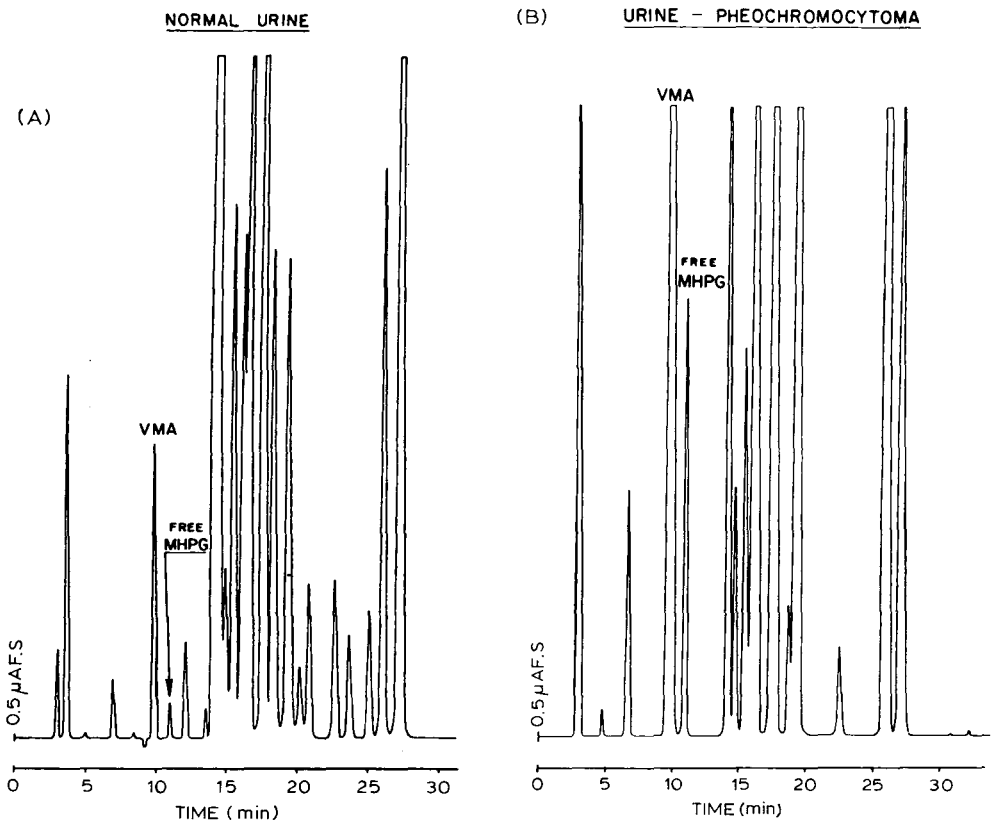


Fig. 16. (A) Chromatogram of the ethyl acetate extract of a urine sample from a healthy subject. Volume injected, 5 μl (15 μl urine); column, μBondapak C_{18}; eluents, (low-strength) 0.1 *M* KH_2PO_4, pH 2.50, (high-strength) acetonitrile—water (3:2, v/v); gradient, linear from 0% to 60% of the high-strength eluent in 45 min; flow-rate, 1.4 ml/min; temperature, ambient; detection, EC, + 1.00 V vs. Ag/AgCl. (B) Chromatogram of the ethyl acetate extract of a urine sample from a patient with clinically diagnosed pheochromocytoma. Chromatographic conditions as same as in (A). (Reproduced from ref. 9 with permission.)

Fig. 17 illustrates the enormously elevated levels of metanephrines in the urine sample from a patient with pheochromocytoma (B) compared to the control subject (A). However, urinary metabolite levels reflect both the CNS and peripheral metabolism. Therefore, the least invasive method for the assessment of monoamine metabolism in the CNS is the analysis of the CSF levels of catecholamines or their metabolites. Fig. 18 illustrates the concurrent

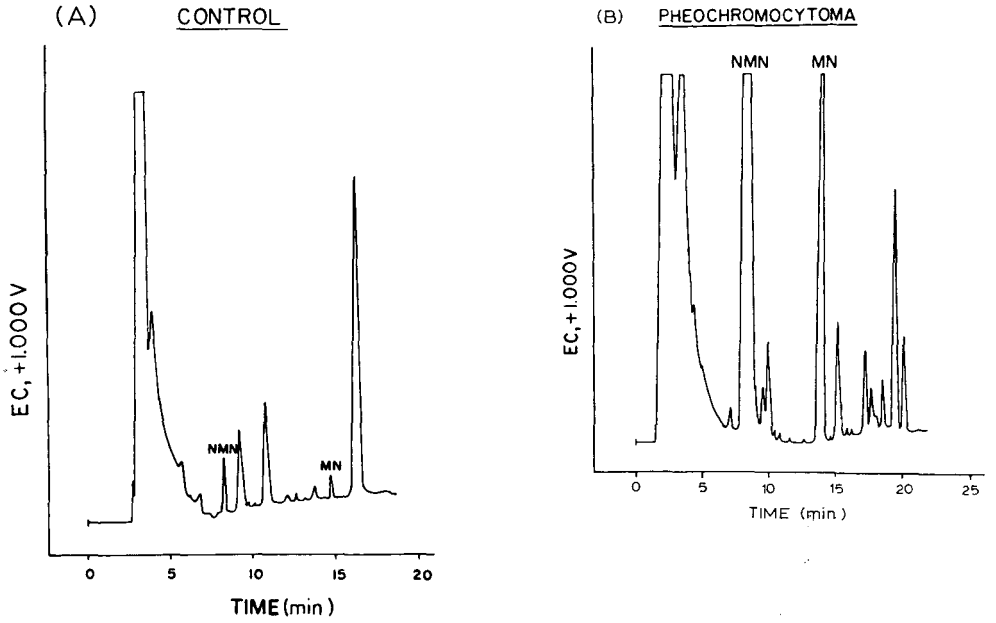
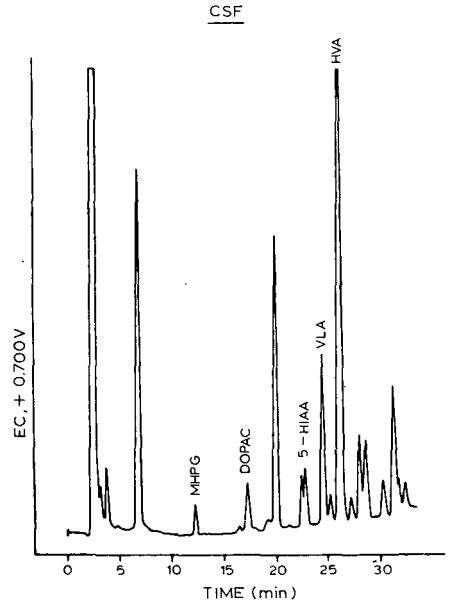


Fig. 17. (A) Chromatogram of the ammonia eluate from urine specimen of a control subject. Volume injected, 20 μl. Chromatographic conditions: column, Ultrasphere ODS; eluents, (low-strength) 0.1 *M* KH_2PO_4, pH 4.56, (high-strength) methanol—water (3:2, v/v); gradient, linear from 0% to 60% in 35 min; flow-rate, 1.0 ml/min; temperature, ambient; detection, EC, + 1.00 V vs. Ag/AgCl; sensitivity, 50 nA; attenuation, × 256. (B) Chromatogram of ammonia eluate from urine specimen of a patient with pheochromocytoma. Volume injected, 15 μl. Chromatographic conditons same as in (A). (Reproduced from ref. 7 with permission.)



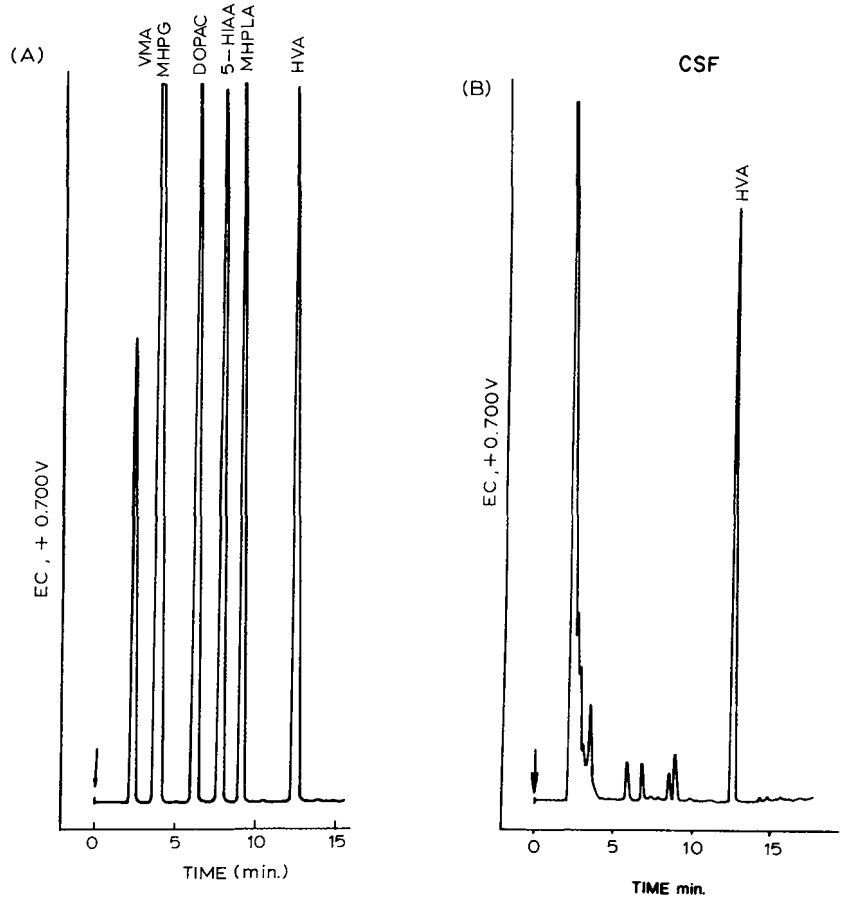


Fig. 19. Chromatogram of synthetic mixture of reference compounds analyzed under isocratic conditions. Chromatographic conditions: column, Ultrasphere ODS (5 μm average particle size); eluent, 25% methanol in 0.08 *M* KH_2PO_4, pH 2.50; flow-rate, 1.2 ml/min; temperature, ambient; detection, amperometric at + 0.700 V vs. Ag/AgCl; detector sensitivity, 75 nA full scale. (B) Chromatogram of the ethyl acetate extract of a CSF sample under isocratic conditions. Chromatographic conditions same as in (A). Volume of the extract injected: 60 μl (180 μl of CSF). (Reproduced from ref. 72 with permission.)

separation of several acidic components of the ethyl acetate extract of an unselected diagnostic specimen of human lumbar CSF. Because of the differences in acidities of various metabolites under study, a gradient elution mode of reversed phase was used. In diseases which involve a derangement in only

Fig. 18. Chromatogram of the ethyl acetate extract of a sample of human lumbar CSF. Volume of the extract injected, 60 μl (180 μl of CSF). Chromatographic conditions: column, Ultrasphere ODS (5 μm average particle size); eluents, (low-strength) 0.10 *M* KH_2PO_4, pH 2.50, (high-strength) methanol—water (3:2, v/v); gradient, linear from 0% to 100% of the high-strength eluent in 35 min; flow-rate, 1.2 ml/min; temperature, ambient; detection, amperometric at + 0.700 V vs. Ag/AgCl; sensitivity, 75 nA full scale. (Reproduced from ref. 72 with permission.)

one neurochemical substance, it is often desirable to monitor one specific compound. For example, in Parkinson's disease, the DA metabolism is of interest and, thus, a rapid, isocratic analysis of HVA is needed. The separation of a synthetic mixture of acidic metabolites, optimized for HVA, is shown in Fig. 19A. The same analysis was applied to the determination of HVA in a CSF sample and a representative chromatogram is shown in Fig. 19B.

The determination of catecholamine levels in the brain using reversed phase with EC detection has been the focus of many investigations [52,74]. The reversed-phase ion-pair separation of a synthetic mixture of reference compounds, and the components of an alumina extract of a rat brain are shown in Fig. 20A and B, respectively. Under the chromatographic conditions used, ascorbic acid and dihydroxyphenylglycol are well resolved from NE and the total analysis time is approximately 10 min. EDTA was reported to be an essential component of the mobile phase, since it decreases dramatically the spread of the solvent front, presumably due to complexation of metal ions leached by the acid from the syringe needle, the injector and the pumping system [41].

The combination of liquid chromatography with electrochemistry is rapidly becoming a method of choice for the determination of catecholamine biosynthetic enzymes, such as COMT [22,50], dopamine-β-hydroxylase [50],

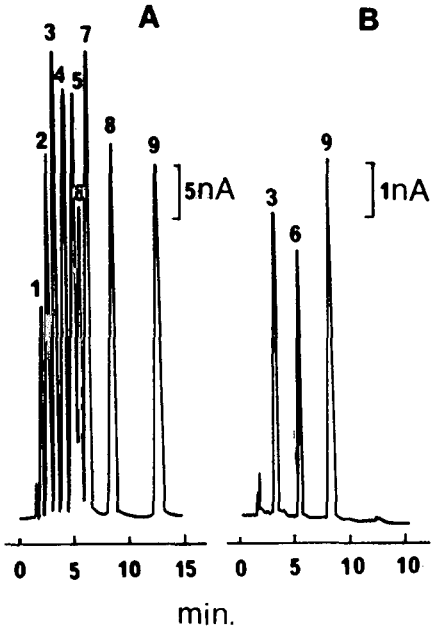
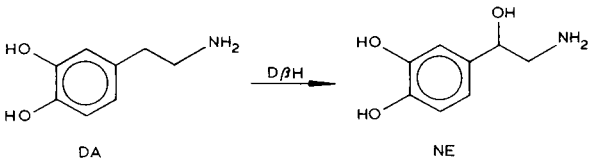


Fig. 20. Chromatogram of reference compounds (A) and of catecholamines from adult rat whole brain with DHBA added as internal standard (B). Chromatographic conditions: column, Ultrasphere ion-pair C_{18}; eluent, 0.1 *M* KH_2PO_4 (pH 3.0)—methanol (9:1), 0.2 m*M* sodium octyl sulfate, 0.1 m*M* EDTA; flow-rate, 1.5 ml/min; temperature, ambient; detection, EC, +0.72 V vs. Ag/AgCl. Peaks: 1, ascorbic acid; 2, dihydroxyphenylglycol; 3, norepinephrine; 4, epinephrine; 5, hydroxymethoxyphenylglycol; 6, dihydroxybenzylamine; 7, normetanephrine; 8, dopamine; 9, dihydroxyphenylacetic acid. (Reproduced from ref. 41 with permission.)

aromatic L-amino acid decarboxylase [59], DOPA decarboxylase [73], etc. The utility of this approach will be illustrated with one example. Dopamine-β-hydroxylase (DβH), which is found in the adrenal medulla and the storage vesicles of noradrenergic neurons, is involved in the terminal step in the biosynthetic pathway of NE:



(7)

The general approach to the assay for both DβH and COMT is shown in Fig. 21; the sample is incubated with the enzyme substrate, DA, the reaction stopped with an acidic protein precipitant, and the product, NE, isolated by alumina adsorption. The substrate, DA, must be added in large excess in order to ensure zero order kinetics and the enzymatically generated NE is measured. In order to avoid saturation of the detector and overloading of the column with a large amount of unreacted DA, Davis and Kissinger [51] have used a novel approach, split column chromatography. Two columns were used with a valve between them, and NE was allowed to pass through both columns. DA was arrested on the first column by switching the valve and shunt to waste. This method is possible only if the two compounds are well separated, which can be achieved by careful selection of the mobile phase and the columns.

An example of the use of this technique for the determination of DβH activity in human serum is shown in Fig. 22.

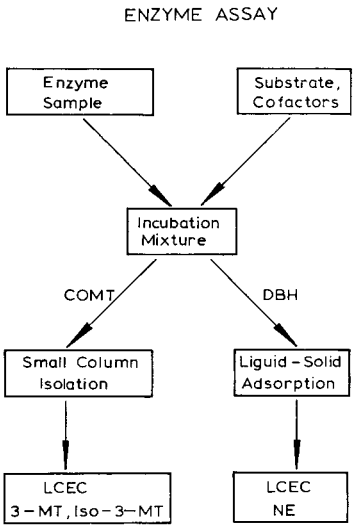


Fig. 21. General approach for the determinations of activities of COMT and DβH. (Reproduced from ref. 12 with permission.)

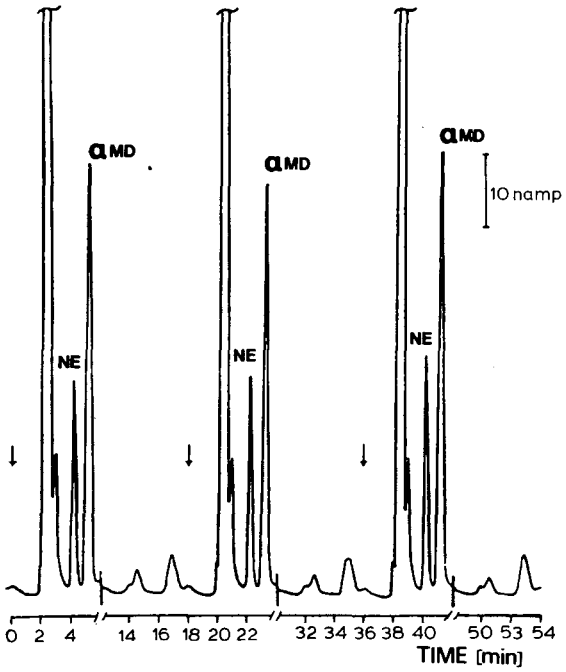


Fig. 22. Chromatograms illustrating the within-run reproducibility for the determination of serum DβH activity. The product, NE, is easily separated from the internal standard, α-methyldopa (αMD). The break in the time axis indicates where the column switching was employed. Chromatographic conditions: column, two RP-18; mobile phase, phosphate—citrate buffer (pH 4.4) with 23 mg of sodium octyl sulphate and 35 ml of methanol added to each liter of buffer; flow-rate, 1.3 ml/min; detection, EC, +0.750 V vs. Ag/AgCl. (Reproduced from ref. 51 with permission.)

6. SUMMARY

High-performance liquid chromatography, particularly in its reversed-phase mode, coupled with electrochemical or fluorometric detection, is becoming increasingly popular as an analytical tool for metabolic profiling of substances of neurochemical interest, such as catecholamines and their metabolites. During the last decade, a continued effort has been made to improve and simplify the analytical methodology for routine use in clinical laboratories where this technique is tremendously needed. New developments in column technology, reliable detectors, simplified sample cleanup procedures, and particularly better understanding of the complex physicochemical phenomena underlying the operation of electrochemical detection, have resulted in a steady and encouraging progress.

The purpose of this review was to describe the current analytical methodology and recent applications of HPLC in the field of catecholamine metabolism. Although this discussion is by no means detailed and complete, it, at least, hints at the impact of this technique on biochemical investigations and its future potential in clinical laboratories.

7. ACKNOWLEDGEMENTS

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SIMULTANEOUS DETERMINATION OF MONOAMINE TRANSMITTERS, PRECURSORS AND METABOLITES IN A SINGLE MOUSE BRAIN

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SUMMARY

A simple and sensitive procedure for simultaneous determination of monoamine transmitters and related substances including precursors and metabolites has been developed for a single mouse brain. The proposed procedure involves (1) primary butanol extraction, (2) separation of the substances into either acid or alkaline aqueous layers according to their physicochemical properties, and (3) determination by means of high-performance liquid chromatography with electrochemical detection. Three transmitters (noradrenaline, dopamine and 5-hydroxytryptamine) and their precursors (tyrosine, 3,4-dihydroxyphenylalanine and tryptophan) and major metabolites (normethanephrine, 3-methoxy-4-hydroxyphenylethylene glycol, 3-methoxytyramine, 3,4-dihydroxyphenylacetic acid, 3-methoxy-4-hydroxyphenylacetic acid and 5-hydroxyindoleacetic acid) were selectively separated and sensitively detected in mouse whole brain sample. Although 3-methoxy-4-hydroxymandelic acid was also separated from other substances by authentic chromatography, the substance was not detected in mouse brain. Changes in levels of these substances were examined for drugs whose effects had been previously confirmed. These changes reflected putative effects of the drugs and confirmed that the procedure is effective for neurochemical research into the transmitter system.

INTRODUCTION

It is generally accepted that monoamines such as noradrenaline (NA), dopamine (DA) and 5-hydroxytryptamine (5-HT) exist in the central nervous system of mammals and function as neurotransmitters. There are many hypotheses concerning these monoamines as causative substances in a variety of neurological [1] and mental disorders [2, 3]. This has led to extensive research con-

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cerning the function and metabolism of these neurotransmitters. In this research, monoamines and related substances have been quantitatively determined in the brains of model animals and the physiological fluid of humans. Drugs used in the treatment of neurological and mental disorders affect the metabolism of catecholamines and indoleamines [4]. However, few studies have reported the simultaneous determination of wide-ranging substances relating to monoamine metabolism, including precursors and metabolites of catecholamines and indoleamines, in the same sample. This kind of assay would, if possible, provide highly important information on the physiological and pathological mechanisms of brain function, because it has been proposed that the central transmission system forms a composite multitransmitter system, which interacts to modulate brain function in a balanced manner [5]. During recent years our experimental focus has been on a simultaneous determination of monoamines and their precursors and metabolites in a single sample of mouse brain.

The most widely used methods of analysis for monoamines and related substances are variations of a fluorometric approach. The procedures involve the physical separation of each substance and conversion to the corresponding fluorescent derivative [6]. The procedures are, unfortunately, insufficiently sensitive for the concurrent determination of monoamine transmitters and metabolites in a single brain sample. Fluorometric detectors have been used in liquid chromatography [7]. However, detection is difficult in simultaneous quantitation because the substances have different fluorescence spectra [6]. Gas chromatography combined with electron-capture detection or mass fragmentography is also used for the determination of monoamines with varying degrees of success [8—10]. In these methods, the substance must first be converted to the volatile derivative. Biogenic substances, including monoamines and related substances, are generally water soluble and difficult to convert to volatile derivatives. It is, therefore, not reasonable to use gas chromatography for monoamine assay if other procedures are available. Recently, radioenzymatic techniques have been introduced for monoamine assay [11, 12]. Although it is extremely sensitive, the radioenzymatic assay possesses a fatal defect for simultaneous determination: it cannot be used to quantify the O-methylated metabolites of catecholamines, which are important in the metabolic pathway of the amines.

Since Takata and Muto [13] introduced the principle and Refshauge et al. [14] demonstrated its application in monoamine assays, high-performance liquid chromatography (HPLC) with electrochemical detection has been extensively used in the quantitation of monoamines and related substances in the brain [15—19]. The comprehensive principle and basic methodology of this new method has recently been reviewed [20]. It has been suggested that electrochemical detection is highly sensitive for substances having phenolic hydroxy groups [7, 13, 20]. All substances involved in monoamine metabolism possess the OH group(s) on their molecules [6] except tryptophan, a precursor amino acid for 5-HT. This amino acid has, fortunately, been reported to be detected electrochemically despite its molecular characteristic of having no OH group [17]. In the present study, therefore, HPLC with electrochemical detection was chosen as the experimental tool for the simultaneous determina-

tion of monoamine transmitters and their metabolites and precursors in a single mouse brain sample.

EXPERIMENTAL

Apparatus

The liquid chromatographic system was set up with commercially available components including a thin-layer voltammetric detector with a glassy carbon electrode (Yanagimoto VMD-101, Kyoto, Japan). The chromatographic column was LiChrosorb RP-18 reversed-phase resin (average particle size, 10 μm) prepacked in a 25 cm × 4.6 mm I.D. stainless-steel column (Altex, Berkely, CA, U.S.A.). The detector potential was set at +700 mV or 600 mV vs. Ag/AgCl reference electrode for amine and metabolite assays, respectively.

Reagents

All chemicals for extraction and chromatography were obtained from a single source (Mallinkrodt, Paris, KT, U.S.A.) and were analytical reagent grade. The reagents were used as obtained commercially without further purification. Each authentic standard was prepared at a concentration of 1 mg/ml in 0.025 *N* hydrochloric acid for amines and precursors, and in 0.05 *M* phosphate buffer (pH 8.5) for metabolites. These standard stock solutions were stable for one month when stored in a refrigerator at 4° C. A working standard was made from the stock solution on the day of assay. The standard solution which contained substances in concentrations similar to those in the samples to be assayed, was taken through the entire procedure for extraction. Isoproterenol (ISO) and 3,4-dihydroxyphenylpropionic acid (DOPPA) were used as internal standards for the amine and metabolite assays, respectively.

Two different types of mobile phases were used according to the purpose of the assay. For the assay of monoamines (including amine metabolites, 3-methoxytyramine (3-MT) and normethanephrine (NMN) and precursor amino acids, a 0.025 *M* citrate buffer (pH 5.0) containing 1% tetrahydrofuran was used. On the other hand, a 0.05 *M* phosphate buffer (pH 6.9) containing 10% methanol was used for the metabolite assay. The mobile phase was pumped at a flow-rate of 1.0 ml/min or 0.7 ml/min for the amine and metabolite assays, respectively.

Animals

The animals were male Swiss—Webster mice each weighing about 35 g at the time of sacrifice. They were maintained on a 12-h light/12-h dark cycle and allowed access to food and water ad libitum. They were sacrificed at a fixed time between 10:00 a.m. and 12:00 noon to exclude daily fluctuation in biogenic amine metabolism. The brain was removed as quickly as possible after decapitation and stored on dry ice until the assay could be performed. In general, the assay was completed within one week after sacrifice.

Drug injections

The following drugs were used; 300 mg/kg of *p*-chlorophenylalanine (PCPA), 1 mg/kg of reserpine (RES), 1 mg/kg of haloperidol (HAL) and α-methyl-*p*-

tyrosine (α-MPT). All drugs were administered intraperitoneally. Time intervals between injection and decapitation were selected to produce the maximum drug effect: 48 h for PCPA; 24 h for RES; 1 h for HAL; and 2 h for α-MPT. Control animals received no injection.

Extraction procedure

Each brain was weighed and transferred to a glass tube containing 200 μl of 0.1 *N* hydrochloric acid, 20 μl of 0.1 *M* EDTA, 500 ng of ISO and 250 ng of DOPPA. Immediately after the addition of 10 ml of butanol, the brain was homogenized by means of a homogenizer (Tissuemizer, Janke and Kunkel, G.F.R.). The homogenate was transferred to a screw-capped glass tube containing 2 g of solid sodium chloride and was shaken in a reciprocal shaker for 60 min. After centrifugation at 4000 rpm for 10 min, 5 ml of the butanol layer was pipetted into another screw-capped tube containing 200 μl of 0.1 *N* hydrochloric acid and 10 ml of *n*-heptane. The tube was shaken again for 10 min and centrifuged at 4000 rpm for 5 min. The organic layer was transferred to another tube and stored overnight in a refrigerator at 4°C. The aqueous layer in this step was used for the assay of monoamines and precursor amino acids. A 10-μl aliquot of the aqueous layer was injected onto the column through a six-port valve equipped with 100-μl sample loop (Rheodyne, Berkeley, CA, U.S.A.). On the next day, 200 μl of 0.1 *M* phosphate buffer (pH 7.5) was added to the organic layer stored in a refrigerator and the tube was shaken for 60 sec on a Vortex mixer. The tube was then centrifuged at 3000 rpm for 60 sec. A 50-μl aliquot of the buffer aqueous layer was taken from the bottom of the tube and injected onto the column for the metabolite assay.

Calculations and statistics

All quantitations were based on peak heights of the resulting chromatogram. Ratios of the peak heights for the substances and the corresponding internal standard were compared for samples and standards taken through the entire extraction procedure, as described previously [14, 15]. Statistical significance was examined by use of the Student's *t*-test.

RESULTS AND DISCUSSION

The present study has demonstrated a simple and sensitive method for the simultaneous determination of wide-ranging substances related to monoamine metabolism in the same sample of mouse brain. The procedure consists of two parts; extraction of the substances by acidic butanol and electrochemical detection following separation by reversed-phase HPLC.

For catecholamine assay, the alumina absorption technique is useful for purification of the sample and has frequently been applied for the brain [14–16] and physiological fluid [21]. However, the procedure is ineffective for indole compounds as well as for 3-methoxylated metabolites of catecholamines. Organic solvent extraction procedures are also used for the pretreatment of monoamine quantitations. Although the solvent extraction is not specific for monoamine transmitters or metabolites, this procedure can be extremely useful if an adequate chromatographic separation is available. Sasa

and Blank [19] have reported successful application of the extraction procedure in liquid chromatography for determination of DA and 5-HT, but not of NA, in mouse brain. In the present study, therefore, the acidic butanol method with minor modifications was used for extraction.

Monoamines and related substances, which had initially been extracted in the butanol layer, were re-extracted in 0.1 *N* hydrochloric acid, and then in alkaline buffer according to their physicochemical properties. It is therefore essential to know the distribution coefficients of the substances for both aqueous layers. Recovery of alkaline compounds (amines) was not influenced by changing the concentration of hydrochloric acid. However, the recovery of 3-methoxy-4-hydroxyphenylethylene glycol (MOPEG), a neutral metabolite of NA, in the hydrochloric acid layer was decreased in proportion to an increase in the concentration. This resulted in a slight increase in recovery in alkaline buffer (Fig. 1). On the other hand, no acid metabolites appeared in the hydrochloric acid layer. While increasing the hydrochloric acid concentration seemed to be convenient for MOPEG determination, the HCl molecule interfered with the proper determination of NA because of its own peak. The concentration of hydrochloric acid for re-extraction was finally set at 0.1 *N* which was the same as previously reported [19].

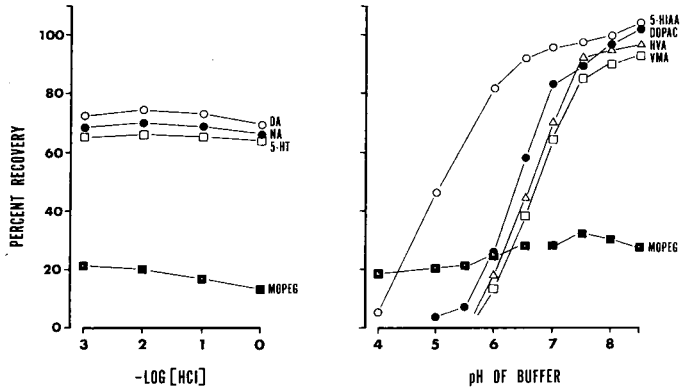

Fig. 1. Changes in recovery rates of monoamine transmitters and metabolites. Left panel shows effects of concentrations of hydrochloric acid on the recoveries of monoamines and a neutral transmitter, MOPEG. Right panel shows effects of pH values of buffer on the recoveries of metabolites.

The pH value of the alkaline buffer affected the recovery of acid metabolites (Fig. 1). While the metabolites recovered well in a high pH buffer, the maximum recovery of MOPEG was at pH 7.5. Since MOPEG is a major metabolite of NA in the brain [22, 23], determination of this substance is essential for understanding the activity of noradrenergic neurons in the central nervous system. The pH value for re-extraction of metabolites was, therefore, decided at 7.5 because of the MOPEG quantitation. Although the recovery rates of other metabolites were lower at this pH value than their maximum, relative deviations for the rates were within 4% and were enough for simultaneous determination of the metabolites.

Under these re-extraction conditions, recovery rates for the substances

TABLE I

RECOVERY RATES OF SUBSTANCES IN THE PRESENCE OF TISSUE SAMPLE

A brain was homogenized in 1 ml of 0.025 *N* hydrochloric acid containing 20 μl of 0.1 *M* EDTA. The homogenate was divided into two equal portions and transferred to two screw-capped glass tubes. Known quantities of authentic standard substances and internal standards were added to one tube, and only internal standards to the other. After additions of sodium chloride and butanol, each tube was subjected to the extraction procedure described in the text. The recovery rates shown in this table were calculated by comparing the peak height of each substances in the sample. The values represent means ± S.D. from six determinations. Abbreviations: DOPA = 3,4-dihydroxyphenylalanine; VMA = 3-methoxy-4-hydroxymandelic acid; DOPAC = 3,4-dihydroxyphenylacetic acid; HVA = 3-methoxy-4-hydroxyphenylacetic acid; 5-HIAA = 5-hydroxyindoleacetic acid.

Precursors		Amines		Metabolites	
Tyrosine	56.7 ± 2.8	NA	60.0 ± 3.7	VMA	68.8 ± 4.3
DOPA	61.9 ± 3.1	DA	64.7 ± 3.4	MOPEG	31.1 ± 2.3
Tryptophan	39.2 ± 4.6	5-HT	54.6 ± 2.9	DOPAC	84.6 ± 2.5
		NMN	61.1 ± 4.2	HVA	76.9 ± 3.2
		3-MT	51.8 ± 3.6	5-HIAA	79.0 ± 4.6

quantified in the present study were calculated in the presence of tissue sample (Table I). The values were higher than those of a previous report [19], while the recovery of catecholamines was less than those obtained by the alumina absorption technique [21]. The recovery rate of each substance was included in the estimation of tissue concentration.

Electrode responses depend upon the applied voltage [8, 13, 20]. Although increases in applied voltages result in more responses, the increases are accompanied by increases in background and noise currents which interfere with suitable determination of the substances. Fig. 2 shows the current—voltage curves for listed substances.

The substances tested in the present study can be classified according to their electrochemical behavior (Fig. 2). The most sensitive substances for electrochemical detection were catechol compounds which have two OH radicals on the benzene nucleus. In this group, the current was initiated at an applied voltage of about +200 mV. 3-Methoxy-4-hydroxy derivatives of catecholamine (3-methoxylated metabolites) required higher voltage for producing the response currents than did catecholamines. The findings suggested that the responses to lower applied voltages were due to the dual oxidation of OH groups on their molecules. Both catecholamines and 3-methoxylated metabolites represented a plateau in the current—voltage curves and further increases of the applied voltages resulted in lowered responses. Such effects might be due to interfering electrochemical reactions among adjacent radicals in the molecule.

5-Hydroxyindole compounds differed from both catecholamines and 3-methoxylated metabolites in their electrochemical responses. In 5-HT and 5-HIAA, the current was initiated at a relatively low applied voltage, and responses gradually increased in proportion to the applied voltage without a plateau (Fig. 2). This response might be due to one molar oxidation of the

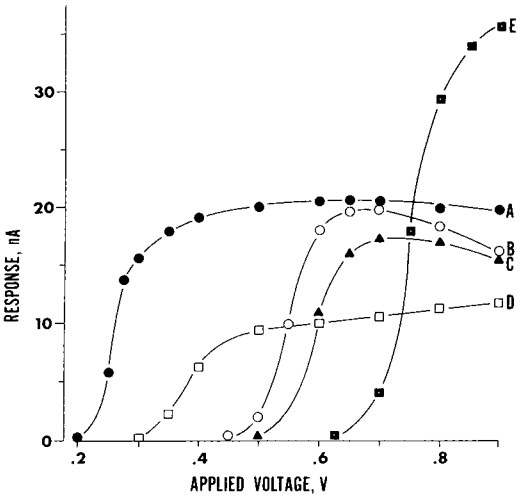

Fig. 2. Current—voltages curves of standard substances. A, for catechol compounds (NA, DA, DOPA and DOPAC) typified with 10 ng of NA; B, for 3-methoxylated amines (3-MT and NMN) typified with 10 ng of 3-MT; C, for 3-methoxylated metabolites (VMA, MOPEG and HVA) typified with 10 ng HVA; D, for indole compounds (5-HT and 5-HIAA) typified with 10 ng of 5-HT; and E, for amino acids (tyrosine and tryptophan) typified with 30 ng of tyrosine.

hydroxy group on the indole nucleus and to the absence of radicals in adjacent positions.

The precursor amino acids, tyrosine and tryptophan, required higher applied voltage to initiate the electrode response than other substances examined. Although tyrosine possesses one phenolic OH, but not tryptophan, in the molecule, both amino acids represented similar responses in the current—voltage curves. This means that the current is generated by the oxidation of not only radicals on nucleus rings but also carboxyl and/or amino groups in these molecules.

The detector response was measured for injections from 0.2 ng to 50 ng and provided a linear calibration for each substance in this range. The overall precision of the procedure was found to be less than ± 4.6% standard deviation for each substance.

Typical chromatograms for monoamine and metabolite assays are shown in Fig. 3. In the chromatographic study mentioned above, eight peaks were detected for the monoamine-related substances on the chromatogram for the monoamine assay in which 20 μl of the hydrochloric acid layer had been injected (Fig. 3A). The identification of each peak was accomplished by the procedure previously reported [17], in which the peak was compared for both chromatographic and electrochemical behavior with the standard substance taken through the entire extraction procedure. In a previous experiment using the butanol extraction method [19], NA could not be detected because of an interfering front peak. This peak can be attributed to the ascorbic acid added to prevent the oxidation of substances during extraction, because, in the present chromatography, this substance had a retention time similar to that of the front peak. With the antioxidant removed, the front peak on the present

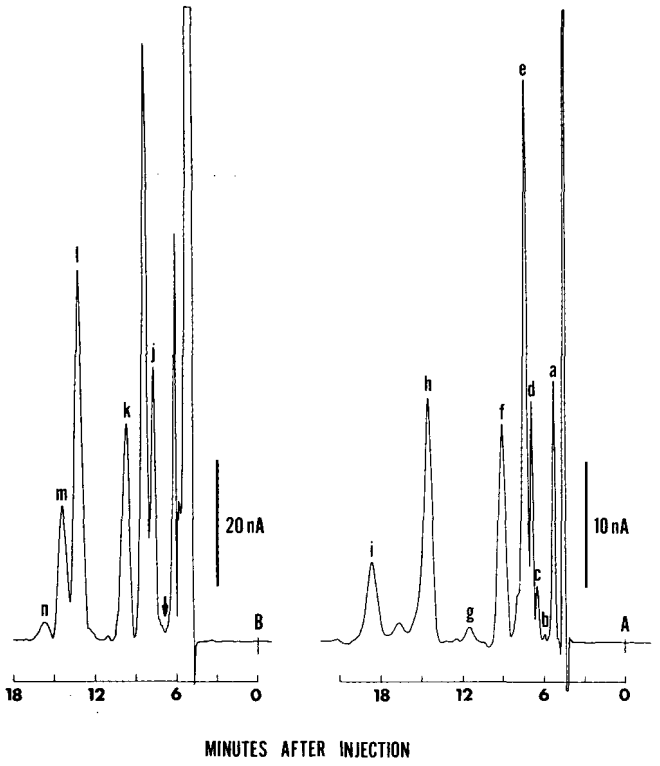

Fig. 3. Typical chromatograms for monoamine (A) and metabolite (B) assay of mouse whole brain. For extraction procedure, see text. Chromatographic conditions: stationary phase, LiChrosorb RP-18 (average particle size, 10 μm) prepacked in a 25 cm × 4.6 mm I.D. stainless-steel column; mobile phase, 0.025 *M* citrate buffer (pH 5.0) containing 1% tetrahydrofuran and 0.05 *M* phosphate buffer (pH 6.9) containing 10% methanol for monoamine and metabolite assays, respectively; detector applied voltage, +700 mV and 600 mV vs. Ag/AgCl reference electrode for monoamine and metabolite assays, respectively. Peaks: a = NA; b = DOPA; c = NMN; d = tyrosine; e = DA; f = isoproterenol (internal standard); g = 3-MT; h = 5-HT; i = tryptophan; j = DOPAC; k = 3,4-dihydroxyphenylpropionic acid (internal standard); l = 5-HIAA; m = HVA; n = MOPEG. An arrow shows a retention time for VMA which was not detected in the present assay. Two recorders with different sensitivities were used for routine determinations.

chromatogram was so small that NA was detected without disturbance.

In previous reports [14—16, 19], 3,4-dihydroxybenzylamine (DHBA) was used as the internal standard substance. Since it has a retention time identical to that of DOPA, the substance is not a suitable standard in the precursor amino acid quantification. Other candidates for the internal standard are α-methyl dopamine [21] and ISO. Because the former has a retention time similar to that of 3-MT, ISO was finally selected as the internal standard in the present study.

In the metabolite assay injected with 50 μl of buffer aqueous sample, five major metabolites were completely separated (Fig. 3B). However, VMA was not detected and MOPEG concentration was low in the present sample of mouse whole brain. These findings suggest that the main metabolic pathway for NA is the formation of MOPEG followed by a rapid conjugation process

with sulfuric acid [6, 22, 23] and that conjugated MOPEG is not extracted in the acidic butanol layer. The minimum detectable amount of each substance by the present procedure was as follows: 200 pg for catechol compounds; 500 pg for 3-methoxylated metabolites and indoles; and 2 ng for precursor amino acids.

In order to ascertain the feasibility of the present procedure, the effects of several drugs were investigated (Table II). These effects have previously been confirmed by a large number of studies.

RES acts primarily by depleting catecholamines and indoleamines from the nervous system as well as the adrenal medulla [4, 24, 25]. In the present study, the drug significantly decreased the concentration of three monoamine transmitters 24 h after a 1 mg/kg intraperitoneal injection. The reducing effect was stronger on NA than on 5-HT. Catecholamine metabolites were also reduced by the RES administration. However, the drug did not affect the intracerebral 5-HIAA level in spite of a significant reduction of the parent transmitter. Although the effect on the 5-HIAA level requires further experiments for confirmation, it is likely that the present effect indicates that the indoleamines are restored more rapidly than catecholamines after RES-induced depletion. These findings also suggest agreement with the possibility that catecholamines play a more important role than 5-HT in the effects of the drug [25].

HAL is a typical neuroleptic drug which is used in the treatment of psychiatric disorders, especially schizophrenia [5]. DA was decreased, while the metabolites were increased, in mouse brain by an injection of the drug. The present results were expected in view of the drug's effect on the dopaminergic system. Vogt [26] has demonstrated similar effects on the dopaminergic system in which the drug decreases the transmitter and increases a metabolite, HVA. These effects have been interpreted as indicating that the drug blocks dopamine receptors [3, 25, 27, 28]. The blockade effect on the receptor results in more DA being released which causes a compensatory increase in the turnover rate of the transmitter. The drug had other effects in the present study, i.e., increasing the levels of MOPEG and 5-HIAA. These findings suggest the possibility that HAL has effects not only on the dopaminergic but also on the noradrenergic and serotonergic systems.

PCPA and α-MPT are biosynthetic inhibitors of indoleamines and catecholamines, respectively. The former blocks tryptophan hydroxylase, the rate limiting enzyme [29]; and the latter, tyrosine hydroxylase [30, 31]. In the present study, PCPA affected the serotonergic, but not the catecholaminergic system, decreasing both levels of 5-HT and 5-HIAA. It has been reported that a 316 mg/kg injection of the drug significantly decreases the 5-HT level without affecting NA [29]. On the other hand, α-MPT affected only the catecholaminergic system, decreasing the concentrations of transmitter substances and metabolites. These results are reasonable for its effects. α-MPT, but not PCPA, influences the levels of precursor amino acids. These results may indicate the difference in substrate availabilities of the enzymes.

The steady-state concentrations of monoamines, precursors and metabolites were simultaneously determined in the same sample of mouse brain by means of HPLC with electrochemical detection. It is impracticable to compare whole values with those of previous reports. The concentrations of three monoamine

TABLE II

EFFECTS OF DRUGS ON MONOAMINE TRANSMITTERS, PRECURSORS AND METABOLITES IN MOUSE WHOLE BRAIN

Values are expressed in ng/g wet tissue and means ± S.D. from six animals.

	Control	RES	HAL	PCPA	α-MPT
Tyrosine	6837 ± 563	6794 ± 289	6918 ± 218	6775 ± 391	7728 ± 431*
DOPA	4.1 ± 0.5	3.3 ± 0.9	6.8 ± 2.4	3.4 ± 0.5	not detectable
Tryptophan	3459 ± 415	3295 ± 247	3623 ± 213	3232 ± 119	3478 ± 372
NA	418 ± 26	194 ± 16*	408 ± 32	458 ± 22	317 ± 17*
NMN	10.9 ± 1.2	11.1 ± 1.4	10.6 ± 1.1	10.5 ± 0.9	6.8 ± 1.0
MOPEG	32.0 ± 5.4	25.8 ± 3.9*	48.3 ± 5.5*	36.2 ± 5.0	13.8 ± 2.5*
DA	887 ± 39	657 ± 38*	714 ± 51*	941 ± 19	526 ± 39*
3-MT	25.2 ± 2.1	14.5 ± 1.6*	47.7 ± 6.6*	21.2 ± 2.9	17.3 ± 1.6*
DOPAC	72.5 ± 5.1	44.8 ± 6.7*	31.2 ± 35*	67.8 ± 5.3	29.5 ± 7.5*
HVA	152 ± 13	103 ± 18*	351 ± 32*	132 ± 17	70 ± 10*
5-HT	429 ± 36	256 ± 16*	425 ± 41	347 ± 20*	433 ± 17
5-HIAA	223 ± 19	239 ± 10	340 ± 27	140 ± 13*	224 ± 16

*Significantly different from control group ($P < 0.01$).

transmitters (NA, DA and 5-HT) were, at least, well within the range of determination reported [19, 32, 33].

The present procedure has merit in the possible determination of a series of substances related to monoamine metabolism from precursor amino acids through end-metabolites in a single brain sample of the mouse, although one problem remains to be resolved. MOPEG, a major metabolite of NA, has the properties of an alcohol and remains, in large part, in the organic layer in the re-extraction process. This resulted in a lower recovery rate in comparison with other substances. Furthermore, a major portion of the substance is converted to a sulfate conjugate [6, 22] which was ineffective for butanol extraction. For these two reasons, the determination of MOPEG was restricted in the present study. However, the effects of drugs were readily seen in the changes in the concentrations of the substances studied, including MOPEG.

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SINGLE-STEP HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE ANALYSIS OF ACETYLATED POLYAMINES

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SUMMARY

A high-performance liquid chromatography method for the determination of urinary acetyl derivatives of the polyamines putrescine, cadaverine and spermidine is described. This procedure utilizes an ion-exchange column for the separation of acetyl derivatives and the compounds are quantitated by fluorescence after reaction with *o*-phthalaldehyde. The steps necessary for sample preparation are minimized, and the assay is both sensitive and reproducible. This chromatographic procedure was used for the determination of the urinary acetylated polyamines of seven normal volunteers and three cancer patients.

INTRODUCTION

The naturally occurring polyamines, putrescine, spermidine, and spermine, are essential for cell growth and viability. All living organisms and tissues studied to date contain polyamines, and polyamine biosynthesis and accumulation is increased in hypertrophy and hyperplasia [1—6]. Russell [7] in 1971 reported that patients with diagnosed metastatic cancer excreted elevated polyamines and that in one patient, the excision of a large solid tumor returned polyamine excretion to near normal values. Elevated urinary polyamine excretion in cancer patients has now been demonstrated by many groups [8—14]. Urinary polyamine analysis can be used for the rapid evaluation of the success or failure of cancer chemotherapy [5]. A two-fold or greater elevation of urinary spermidine within 48 h predicts a partial or complete response with a high degree of accuracy [5, 8, 11].

In a majority of studies reported of urinary polyamine concentrations, samples were hydrolyzed prior to analysis, liberating the parent polyamines from conjugated forms. Over 90% of the human urinary putrescine and spermidine have been identified as the monoacetyl derivatives while 10% or less of

spermine is estimated to be present as conjugated products [15—17]. Although N^1-acetylspermine has been detected in tissues [18] and other conjugates are possible [19], the nature of possible spermine urinary conjugates is unknown.

Enzymes that acetylate polyamines forming the monoacetyl derivatives have been reported in the nucleus and the cytosol of many different rat tissues [20—24]. The enzymes are apparently distinct with differing substrates and products. The substrates for the nuclear N-acetyltransferases are histones and numerous polyamines including putrescine [20—22]. In the presence of spermidine, N^8-acetylspermidine is the only spermidine derivative formed by the nuclear enzyme(s) [25, 26]. The substrates of the cytosolic enzyme are spermine and spermidine forming the N^1-monoacetyl derivatives of these compounds [18, 24]. Putrescine and histones are not substrates for this enzyme. Pretreatment of a rat with carbon tetrachloride increases the cytosolic enzyme activity and the formation of N^1-acetylspermidine and N^1-acetylspermine [18, 23, 24, 27]. The nuclear N-acetyltransferase activity, apparently not induced by carbon tetrachloride, can be stimulated in the kidneys of rats after the administration of growth hormone and ACTH [28].

The acetylated polyamines formed by the N-acetyltransferases do not accumulate to high levels in stimulated tissues, probably due to rapid enzymatic degradation and excretion of the acetyl derivatives from tissues [26, 29—33]. N^1-Acetylspermidine and N^1-acetylspermine are better substrates than the parent compound for the enzyme polyamine oxidase, and the products are putrescine and spermidine, respectively [29]. N^8-Acetylspermidine is not a substrate for polyamine oxidase but is deacetylated by a cytosolic enzyme to form spermidine [30]. Acetylputrescine can also be deacetylated in the cytosol [31] and is a substrate for the enzyme monoamine oxidase forming γ-aminobutyric acid [32].

The concentration of acetylated polyamines excreted in urine will depend on the rate of formation and breakdown of these compounds in the tissues. Seiler et al. [26] studied the influence of cell proliferation and polyamine production on urinary excretion of the acetylated polyamines in rats. Rats pretreated with thioacetamide which markedly increased liver polyamine concentrations resulted in increased N^1-acetylspermidine excretion. Treatment of the rats with agents which cause considerable cell damage such as epidermal UV irradiation or cyclophosphamide injection caused both N^1- and N^8-acetylspermidine excretion to increase. However, rats pretreated with 7,12-dimethylbenzanthracene which caused the formation of mammary tumors did not increase excretion of the acetylpolyamines. Elevated acetylpolyamine excretion, therefore, has not been shown to be a reliable marker for the presence of tumors. In another study, Seiler et al. [33] measured urinary acetylspermidine excretion in hepatoma-bearing rats and found that urinary N^1-acetylspermidine excretion increased exponentially during the time of linear increase in tumor mass. The excretion of N^8-acetylspermidine increased when the tumor mass was 35 g, shortly before the period of observed necrosis.

Acetylpolyamine excretion in human pathology has been less well studied. Abdel-Monem and Ohno [16] found increased urinary acetylpolyamine excretion in all diagnosed cancer patients when compared to normals. In addition, thirteen out of fifteen patients had an elevated N^1- to N^8-acetylspermidine

ratio when compared to normals. Seiler et al. [26] studied urinary acetylpolyamine excretion in two male melanoma patients. In one patient, acetylputrescine excretion was normal, N^8-acetylspermidine excretion was slightly elevated, and N^1-acetylspermidine excretion was several-fold higher than in healthy male controls. In the other patient, monoacetylputrescine, N^1- and N^8-acetylspermidine were excreted in elevated amounts as compared to controls. It seems that extensive studies of intracellular and extracellular acetylpolyamine derivatives are required in order to ascertain the possible significance of nuclear and cytosolic acetylation of polyamines in tumor evolution and growth.

Many reported methods exist for the separation and quantitation of free polyamines [34—40]. However, only a few have been adapted for the separation and quantitation of acetylpolyamines [41—43], and these are rather complex, sometimes requiring two separation steps [41—43]. We report the development of a single-step procedure for the separation and quantitation of urinary acetylpolyamine derivatives which is reproducible and sensitive.

MATERIALS AND METHODS

Chemicals

Putrescine dihydrochloride, spermidine trihydrochloride, spermine tetrahydrochloride, *o*-phthalaldehyde, sulfosalicylic acid and mercaptoethanol were purchased from Sigma (St. Louis, MO, U.S.A.). Sodium citrate was purchased from Mallinckrodt (St. Louis, MO, U.S.A.), sodium chloride from Ashland (Columbus, OH, U.S.A.), sodium hydroxide from Matheson, Coleman and Bell (Norwood, OH, U.S.A.), boric acid from VWR (San Francisco, CA, U.S.A.), and thiodiglycol from Pierce (Rockford, IL, U.S.A.). Acetylpolyamines were prepared by the method of Tabor et al. [44].

Column packing

The Bio-Rad A-9 column was slurry-packed into a 250 × 3 mm column at room temperature with a 1:1 mixture of buffers B and C (see below). Buffer flow-rate was approximately 0.2 ml/min, and the pressure on the column was not allowed to exceed 100 bar during the 8-h packing procedure. Using this method, we have not encountered increased column packing after more than 150 runs, and the resulting peaks in the chromatograms remain sharp.

Elution buffers

The elution system consisted of four buffers. Buffer A was prepared by dissolving 19.6 g of sodium citrate in 1 l of double-distilled water (DDW). The pH was brought to 4.25 with concentrated hydrochloric acid; the final molarity was 0.2 *M* sodium. Buffer B was prepared by adding 2.0 g sodium hydroxide and 8.76 g of sodium chloride to 1 l of DDW. The pH was adjusted to 10.1 with boric acid; the final molarity was 0.2 *M* sodium. Buffer C was prepared by adding 2.0 g sodium hydroxide and 23 g sodium chloride to 1 l DDW. The pH was adjusted to 10.1 with boric acid; the final molarity was 0.44 *M* sodium. Buffer D was prepared by dissolving 12 g sodium hydroxide in 1 l DDW. All solutions were filtered through a 0.45-μm filter and preserved with pentachlorophenol.

o-Phthalaldehyde reagent

o-Phthalaldehyde (OPA) reagent was prepared by adding 25 g potassium hydroxide to 800 ml DDW. Boric acid was used to adjust the pH to 10.4. DDW was then added to bring the solution to 1 l. To the liter of borate buffer solution, 4.5 ml 2-mercaptoethanol, 3.0 ml Brij, 5.8 g potassium thiocyanate and 800 mg OPA dissolved in 20 ml methanol were added, and the solution was mixed. This solution was filtered through a 0.45-μm filter before use.

Urine preparation

Urine samples were collected in polyethylene specimen cups. Ten ml of urine were removed for creatinine analysis and the rest of the urine was acidified to 0.1 *N* hydrochloric acid with concentrated hydrochloric acid. An aliquot (0.8 ml) of acidified urine was added to 0.2 ml of 10% sulfosalicylic acid in a 1.5-ml microcentrifuge tube and vortexed. This solution was then spun on a Beckman B microfuge for 5 min. The supernatant was removed and the pH was brought to 2—3 with 0.25 ml of 0.3 *M* sodium hydroxide. The samples were then frozen until analyzed. Creatinines were determined by the direct (heat clot) procedure (Hycel, Houston, TX, U.S.A.).

Chromatographic separation

High-performance liquid chromatography (HPLC) was accomplished with a component system. The four buffers were contained in 1-l bottles and kept at room temperature. Due to the high pH, buffers B, C, and D were fitted with carbon dioxide traps of sodium hydroxide pellets. All buffers were connected by 0.3-cm tubing to a 4-part distribution timing assembly (Hamilton, Reno, NV, U.S.A.). Buffers were pumped onto the column by a Milton Roy Minipump (Applied Science, State College, PA, U.S.A.), equipped with a pulse dampener. The sample was injected onto the column by a 7010 sample injection valve fitted with a 200-μl sample loop (Rheodyne, Berkeley, CA, U.S.A.). A 250 × 4 mm stainless-steel column (Pierce), packed with approximately 7 g of Bio-Rad A-9 resin (11.5 ± 0.5 μm; Bio-Rad Labs., Richmond, CA, U.S.A.), was used for chromatographic separation. The column was jacketed with a 250-mm microbore column jacket (Altex, Fullerton, CA, U.S.A.) and kept at 60°C by a constant temperature circulating bath (Haake, Saddle Brook, NJ, U.S.A.). All stainless-steel tubing was 0.16 × 0.05 cm. The column outflow was connected to 60 cm of 0.6 × 0.3 mm microbore PTFE-tubing into a 3-way manifold tee (Pierce) where it was mixed with OPA. OPA was pumped by a Model 650 pressure pump (Rainin, Woburn, MA, U.S.A.) equipped with a pulse dampener and carried by 0.16-cm PTFE-tubing (Alltech, Deerfield, IL, U.S.A.) into the 3-way manifold tee. OPA and column effluent were mixed in 185 cm of 0.6 × 0.3 cm PTFE-tubing before entering the detector and were then connected to a waste container by a coiled 9.1-m section of 0.6 × 0.3 cm PTFE-tubing. Fluorescence was measured on a Spectro/Glo Fluorometer (Gilson, Middleton, WI, U.S.A.) equipped with a 5-μl microflow cell and OPA excitation and emission filters. The 100- and 10-mV output of the fluorometer was connected to a 2-channel Omniscribe strip chart recorder (Houston Instruments, Austin, TX, U.S.A.). Pen Y1 of the recorder was connected to the 100-mB output and was run at 0.1 V full-scale,

pen Y2 to the 19-mV output and was run at 1 V full-scale.

The buffer flow-rate was 30 ml/h. Before each run, the column was equilibrated with Buffer A for 15 min. Immediately after sample injection, Buffer B was started and run for 38.5 min. Buffer C was then started and run for 20 min after which the column was washed with Buffer D for 15 min. Total analysis time was 63 min and total run time was 74.5 min. *o*-Phthalaldehyde was also run at 30 ml/h. The presence of the acetylpolyamines was confirmed by collection of the column eluate corresponding to the acetyl derivatives. The acetyl derivatives were then acid hydrolyzed (6 *N* hydrochloric acid, 103°C, 16 h) to form the parent polyamines which were quantitated as previously described [45].

RESULTS

Fig. 1 shows the structures of the naturally occurring acetylpolyamines found in urine. All acetyl derivatives detected which regenerate the parent polyamines are monoacetyl derivatives. Fig. 2 represents a standard separation of the known urinary acetylpolyamines using this method. Arginine, the final basic amino acid to elute from the column, the basic dipeptides anserine, carnosine and homocarnosine, and ammonia elute between 9 and 12 min. There is a large separation of these compounds from acetylputrescine (Ac-Put) which elutes at 23 min and from acetylcadaverine (AcCad) which elutes at 39 min. The two isomers of acetylspermidine are completely separated with N^8 (N^8AcSpd) eluting at 46.5 min and N^1 (N^1AcSpd) at 60 min. The reproducibility of this method in terms of retention time and response is demonstrated in Table I. In all cases, the relative standard deviation was 1.1% or less.

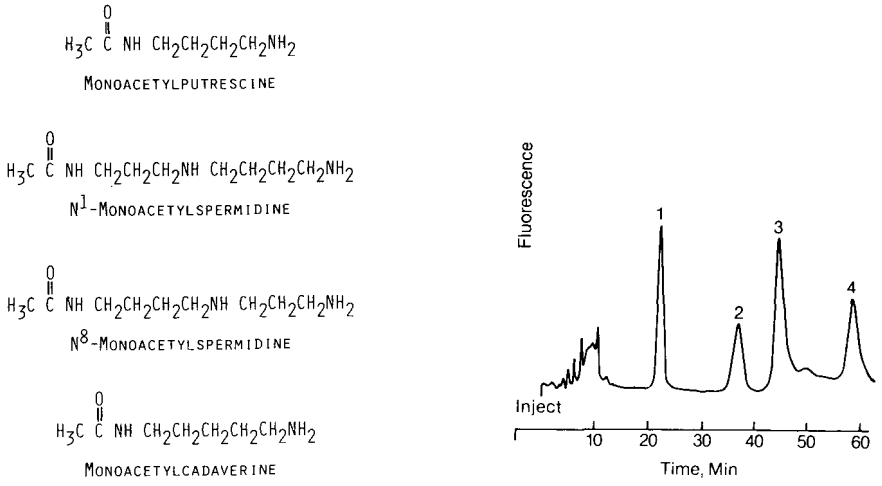

Fig. 1. Structures of naturally occurring acetyl derivatives of diamines and polyamines.

Fig. 2. A standard run of the acetylated polyamines. A 200-μl sample containing 1 nmol of each polyamine was chromatographed as described in Materials and Methods. Peaks: 1 = acetylputrescine; 2 = acetylcadaverine; 3 = N^8-acetylspermidine; 4 = N^1-acetylspermidine.

TABLE I

PRECISION OF ANALYSIS OF RETENTION TIME AND RESPONSE

Retention times and response were determined by measuring standard runs (1 nmol per 200 μl) over a 3-week period of time (n = 6). Amounts as low as 100 pmol can be accurately assayed. During this time, over 100 urine samples were run. All peaks were hand-integrated. R.S.D. (%) = relative standard deviation (%) = $\frac{\text{S.D.}}{\text{mean}} \times 100$.

	Retention time (min)				Response (nmol/200 μ)			
	AcPut	AcCad	N^1 AcSpd	N^8 AcSpd	AcPut	AcCad	N^1 AcSpd	N^8 AcSpd
Average	23.2	38.7	59.8	46.5	1.1	1.0	1.0	0.95
S.D.	0.26	0.43	0.18	0.29	0.05	0.09	0.02	0.03
R.S.D. (%)	1.1	1.1	0.30	0.62	0.05	0.09	0.02	0.03

Typical chromatograms of urine samples from normal volunteers are demonstrated in Fig. 3A and B showing the chromatograms from a dilute urine (30 mg creatinine/dl) and from a concentrated urine (240 mg creatinine/dl), respectively. Fig. 4 shows the separation of the acetylpolyamine derivatives from the urine of a patient with cancer. The separation of the acetylpolyamine derivatives was not affected by urine concentration and no compounds comigrated with the acetylpolyamine derivatives.

The chromatographic procedure was used to determine the acetylpolyamine content of urine of normal volunteers and of cancer patients. The results are shown in Table II. The acetylated polyamines were found in all urines tested with the exception of acetylcadaverine which was sometimes nondetectable. The presence of the acetylpolyamines was confirmed as described in Materials and Methods. Acid hydrolysis of the column eluate corresponding to the acetyl derivatives yielded only the parent polyamine, with the complete disappearance of the acetyl derivative.

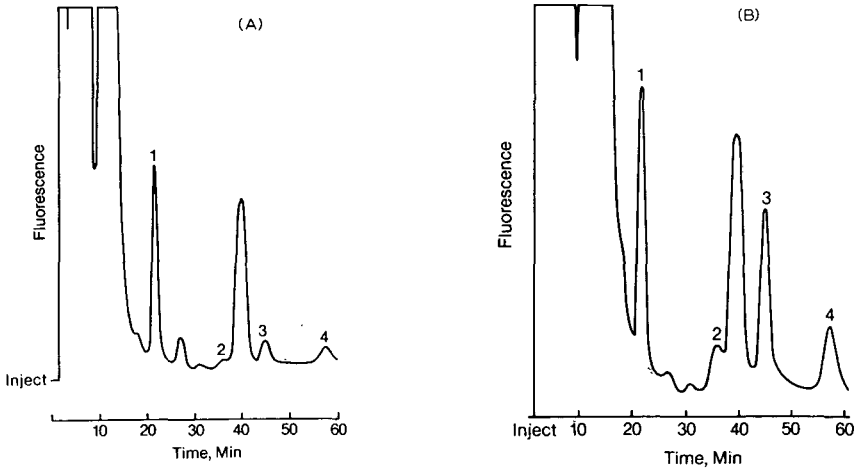

Fig. 3. Separation of acetylpolyamines in normal humans from (A) dilute urine (30 mg creatinine/dl) and (B) concentrated urine (240 mg creatinine/dl). Urine samples (200 μl) were prepared and chromatographed as described in Methods with no sample dilution necessary. Peaks as in Fig. 2.

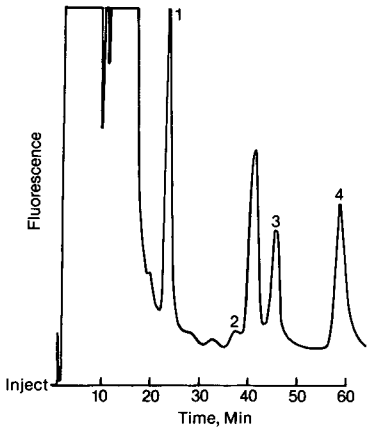

Fig. 4. Separation of a 200-μl urine sample from a male melanoma patient. Acetylpolyamine concentrations are reported in Table II. Peaks as in Fig. 2.

TABLE II

ACETYLPOLYAMINE CONTENT OF NORMAL HUMAN URINE AND URINE OF CANCER PATIENTS

The acetylpolyamine content of urine from six normal volunteers and three cancer patients. All values were standardized by basing acetylpolyamine excretion on urine creatinine as described in Materials and Methods.

Subject	Acetylpolyamine concentration (μg/mg creatinine)			
	AcPut	AcCad	N^1 AcSpd	N^8 AcSpd
Normal volunteers				
1. MM	2.6	0.33	0.66	0.58
2. JE	2.6	0.51	0.77	1.1
3. JR	2.0	TR	0.86	1.6
4. ND	3.0	0.38	0.96	1.0
5. AV	2.6	0.09	0.85	1.3
6. CP	1.3	n.d.*	0.92	0.4
Cancer patients				
1. RR (malignant mesothelioma)	10.7	0.46	2.2	0.45
2. RP (leukemia)	11.1	n.d.*	0.92	0.79
3. DC (malignant melanoma)	7.6	0.54	7.1	2.5

*n.d. = not detectable.

DISCUSSION

This is the first published report of acetylpolyamine excretion determinations as related to creatinine, although creatinine has been shown previously to be a reliable normalizer for urine concentration as related to polyamine

excretion [36, 46]. The ratios of the acetylpolyamine derivatives excreted by normals are in agreement with previously published studies [15, 17, 26, 41]. Cancer patients had greatly elevated urinary acetylputrescine as compared to normals. In two out of three patients, acetylspermidine excretion was elevated with N^1 excretion higher than N^8 excretion. In the patient with leukemia, the two acetylspermidine isomers were not excreted in elevated concentrations (Table II). These results are in agreement with the previous report of Abdel-Monem and Ohno [16], who found that certain cancer patients excrete primarily N^1-acetylspermidine. We are currently performing a large-scale study to examine the urinary excretion of the acetylpolyamines in cancer patients.

The first separation of acetylpolyamine standards was reported in 1960 by Dubin and Rosenthal [47] utilizing Dowex (50 H^+), a polystyrene-based cation-exchange resin. This method totally separated the polyamines from amino acids but the acetylated polyamines were not completely separated from the parent compounds. The eluate from this column was subjected to thin-layer chromatography for confirmation of the presence of acetylated polyamines. Tabor et al. [48] in 1973 described an automated HPLC method for the determination of di- and polyamines. They were able to obtain adequate separation of acetyl derivatives from parent polyamines. However, the run time was 5 h and the method was only sensitive to nanomolar concentrations.

Acetylpolyamine concentrations were initially measured by formation of the dansyl derivatives of the amines, thin-layer chromatography, and measurement of fluorescence [17, 49]. This method is sufficiently sensitive for the detection of acetylated polyamines, although the method has not been automated. Recently, three HPLC methods for the separation and quantitation of acetylpolyamines have been developed [41—43]. The method of Abdel-Monem and Merdink [41] utilizes a three-column system, the first two columns to clean and separate the amines, and the third to quantitate the acetylpolyamines. The HPLC method of Seiler and Knödgen [42] utilizes a single reversed-phase column for separation and detection after ion-pairing the amine-containing sample with octanesulfonate. This method has the advantage of measuring both the parent and acetylpolyamine derivatives. The accurate measurement of urinary acetylputrescine is not possible by this method because it is not separated from unknown interfering compounds. Mach et al. [43] separated the acetylpolyamines by classical ion-exchange techniques on an amino acid analyzer. The measurement of the acetylated and free polyamines using this method requires two separate runs. The first run separates and measures the acetylputrescine concentration and the second, the combined acetylspermidine derivatives and the free polyamines. This method does not separate the two isomers of acetylspermidine with N^1- and N^8-acetylspermidine cochromatographing as a single peak.

The chromatographic system described in this paper uses a single cation-exchange column which allows for the rapid, sensitive and reproducible analysis of all urinary acetylpolyamines, but not the parent compounds. Extensive sample preparation is not necessary and the large capacity of the cation-exchange column permits acetylpolyamine determinations in concentrated urines without sample dilution. We are currently using this method in our

laboratory for the routine measurement of acetylpolyamine excretion in cancer patients and to assess the effects of therapy on acetylpolyamine excretory patterns.

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RAPID HIGH-YIELD PURIFICATION OF NEUROPEPTIDES FROM CANINE INTESTINAL MUSCLE

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SUMMARY

A simplified method for purification of gastrointestinal neuropeptides from relatively small amounts of tissue is described. Sequential adsorption, gel filtration, ion-exchange chromatography, and high-performance liquid chromatography without lyophilization produced good yields of somatostatin, bombesin, and vasoactive intestinal polypeptide immunoreactivity. The method is suitable for simultaneous purification of several small, basic peptides from limited amounts of starting material.

INTRODUCTION

Vasoactive intestinal polypeptide [1], somatostatin [2], and bombesin [3] are polypeptides which were first purified from hog intestine, ovine hypothalamus, and frog skin, respectively. Localization of their immunoreactivities to nerves in brain and gut tissues [4—11] has prompted speculation that they may function as neurotransmitters. Most biological studies to measure effects of these peptides are performed in species other than those from which the peptides were purified, despite evidence for species differences in molecular structure [12—14]. Furthermore, multiple molecular forms of these peptides have been described [14—16]. However, low tissue concentrations and limited tissue availability have discouraged attempts at purification of these peptides from other species. This paper describes a relatively easy and efficient technique for the purification of these peptides, presumably of nervous origin, from the muscle layers of canine small intestines.

EXPERIMENTAL

Equipment

High-performance liquid chromatograph. An Altex Scientific (Berkeley, CA, U.S.A.) Model 312 MP liquid chromatograph equipped with a 210 sample injector and an Altex Hitachi 155-40 detector was employed. All separations were performed on a reversed-phase C-18 Ultrasphere ODS column (5 μm particle size, 250 × 10 mm) also from Altex.

Vacuum centrifuge. A Speed Vac Concentrator (Savant Instruments, Hicksville, NY, U.S.A.) Model SVC100H was used to concentrate purified peptide solutions.

Reagents and chemicals

Acetone, ammonium acetate, and phosphoric acid were from Mallinckrodt (St. Louis, MO, U.S.A.) HPLC-grade water and acetonitrile were from Baker (Phillipsburg, NJ, U.S.A.). Synthetic bombesin, somatostatin-14, somatostatin-28, and vasoactive intestinal polypeptide were from Peninsula Labs. (San Carlos, CA, U.S.A.). Synthetic gastrin releasing peptide was a gift from Dr. J. Rivier (Salk Institute). Amberlite XAD-2 was from Sigma (St. Louis, MO, U.S.A.). CM-Cellulose was from Whatman (Clifton, NJ, U.S.A.). Sephadex G-50 was from Pharmacia (Piscataway, NJ, U.S.A.).

Methods

Radioimmunoassay. Bombesin-like immunoreactivity (BLI), somatostatin-like immunoreactivity (SLI), and vasoactive intestinal polypeptide-like immunoreactivity (VLI) were measured by radioimmunoassay as previously described [17] using antibodies 1078 which is specific for the carboxyl terminus of bombesin, 7812 [18] which is specific for the ring-portion of somatostatin, and 7913 [14] which is specific for the carboxyl terminus of vasoactive intestinal polypeptide.

Tissue extraction. Two dogs weighing 18 and 20 kg were killed by injection with Repose (6 ml i.v.). The entire small intestine was removed and immersed in boiling water (5 ml/g) within 15 min of sacrifice. After boiling for 5 min the small intestine was opened and the mucosa layer was removed from the muscle by scraping. The muscle was blended in 4.5% acetic acid (2 ml/g) for 3 min; then acetone was added (7 ml/g) and the mixture stirred for 6 h at 4° C. The tissue was centrifuged for 30 min at 3000 *g* at 6° C. Acetone was removed from the supernatant by rotary evaporation. The remaining supernatant was centrifuged for 1 h at 3000 *g* at 6° C.

Amberlite XAD-2 chromatography. The concentrated extract was loaded directly onto an Amberlite XAD-2 column (30 × 5 cm) that had been washed with acetone, methanol and equilibrated with water. After loading, the column was rinsed with water until the absorbance at 280 nm (A_{280}) was less than 0.05. The column was then eluted with 100% methanol (1 l) until eluate had an A_{280} of less than 0.01. Glacial acetic acid was added (2 ml) to the pooled methanol eluate which was then concentrated to 65 ml by rotary evaporation and centrifuged for 1 h at 3000 *g* at 6° C.

Sephadex G-50 chromatography. The supernatant was loaded onto a Sepha-

dex G-50 SF column (5 × 140 cm) and eluted with 1% acetic acid at a flow-rate of 80 ml/h. Eluted fractions (10 ml) were assayed for BLI, SLI, and VLI. The elution profile of this column is shown in Fig. 1.

CM-Cellulose chromatography. Fraction I, containing VLI, and fraction II, containing SLI-I and BLI-I (Fig. 1) were separately chromatographed on a CM-cellulose column equilibrated in 0.1 *M* ammonium acetate, pH 5.0. The columns were rinsed with 200 ml 0.1 *M* ammonium acetate, pH 5.0, and eluted with a 300-ml linear gradient of ammonium acetate (0.1—1 *M*, pH 5.0) at a flow-rate of approximately 20 ml/h. Fractions (5 ml) were collected, and assayed for the immunoreactive peptides.

Gradient HPLC. CM-Cellulose-purified BLI-I, SLI-I, and VLI were loaded onto a C-18 reversed-phase HPLC column equilibrated in 0.25 *M* triethylaminophosphate, pH 3 (TEAP). After loading 75—100 ml samples, the column was rinsed with TEAP and then eluted with a linear gradient of acetonitrile from 0% to 30% over 60 min, then at 30% for another 30 min at a flow-rate of 2 ml/min. Fractions of 2 ml were collected and assayed for immunoreactive peptides. Peptides eluted 1—12 min after reaching 30% acetonitrile.

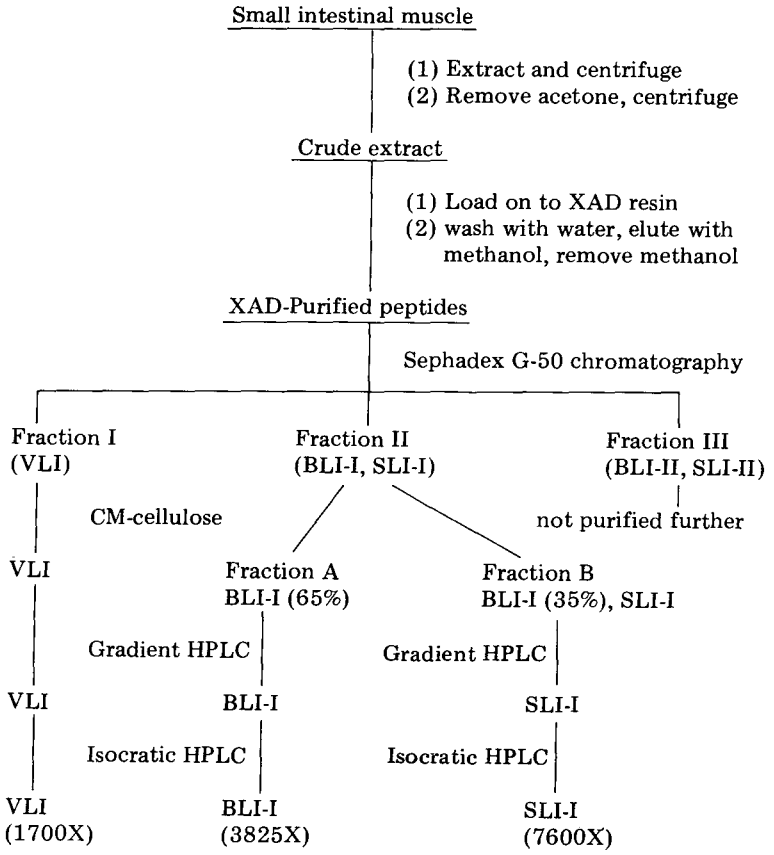

Fig. 1. Flow diagram of the purification procedure.

Isocratic HPLC. Gradient-purified BLI-I, SLI-I, and VLI were each pooled and concentrated to 3 ml in a Speed-Vac Concentrator. These concentrates were chromatographed isocratically on a C-18 reversed-phase HPLC column with 74% TEAP and 26% acetonitrile at a flow-rate of 1 ml/min. Fractions of 1 ml were collected and assayed for immunoreactive peptides.

RESULTS

A flow diagram of the purification procedure is depicted in Fig. 1. As shown in Table I, the acid—acetone extracts of intestinal muscle from two dogs (462 g) yielded 101 nmol of VLI, 16.2 nmol of BLI, and 7.6 nmol of

TABLE I

RECOVERY OF BLI, SLI AND VLI FROM 462 g CANINE SMALL INTESTINAL MUSCLE

Step	Immunoreactivity (nmol)		Step recovery (%)	Cumulative recovery (%)	nmol/A_{280}	Purification ratios (*X*)
	Total BLI	BLI-I				
Extract	16.2		100	100	0.004	1
XAD	14.7		91	91	0.035	8.8
G-50	13.2	9.9*	88	81	0.31	77.5
CM-Cellulose Gradient		9.4	95	77	2.1	525
HPLC Isocratic		5.8	62	36	7.7	1925
HPLC		4.1	80	28	15.3	3825
	Total SLI	SLI-I				
Extract	7.6		100	100	0.002	1
XAD	6.9		90	90	0.016	8.0
G-50	6.3	3.8*	92	83	0.15	75
CM-Cellulose Gradient		3.7	97	80	0.74	370
HPLC Isocratic		3.5	95	76	2.3	1150
HPLC		3.1	89	67	15.2	7600
	VLI					
Extract	101		100	100	0.025	1
XAD	66		65	65	0.16	6.4
G-50	60		91	59	4.0	160
CM-Cellulose Gradient	45		75	44	9.3	372
HPLC Isocratic	40		90	40	33.3	1333
HPLC	31		77	31	30	1700

*Cumulative recovery beyond this step was calculated for the larger components, BLI-I and SLI-I, separated from smaller forms by gel filtration.

SLI. Amberlite XAD-2 chromatography resulted in a 6—8 fold purification of the peptides with minimal losses except for VLI (35% loss). On Sephadex G-50 chromatography (Fig. 2), VLI eluted as a single peak (Fraction I) which was separated from the bulk of A_{280} and from the SLI and BLI peaks. Both

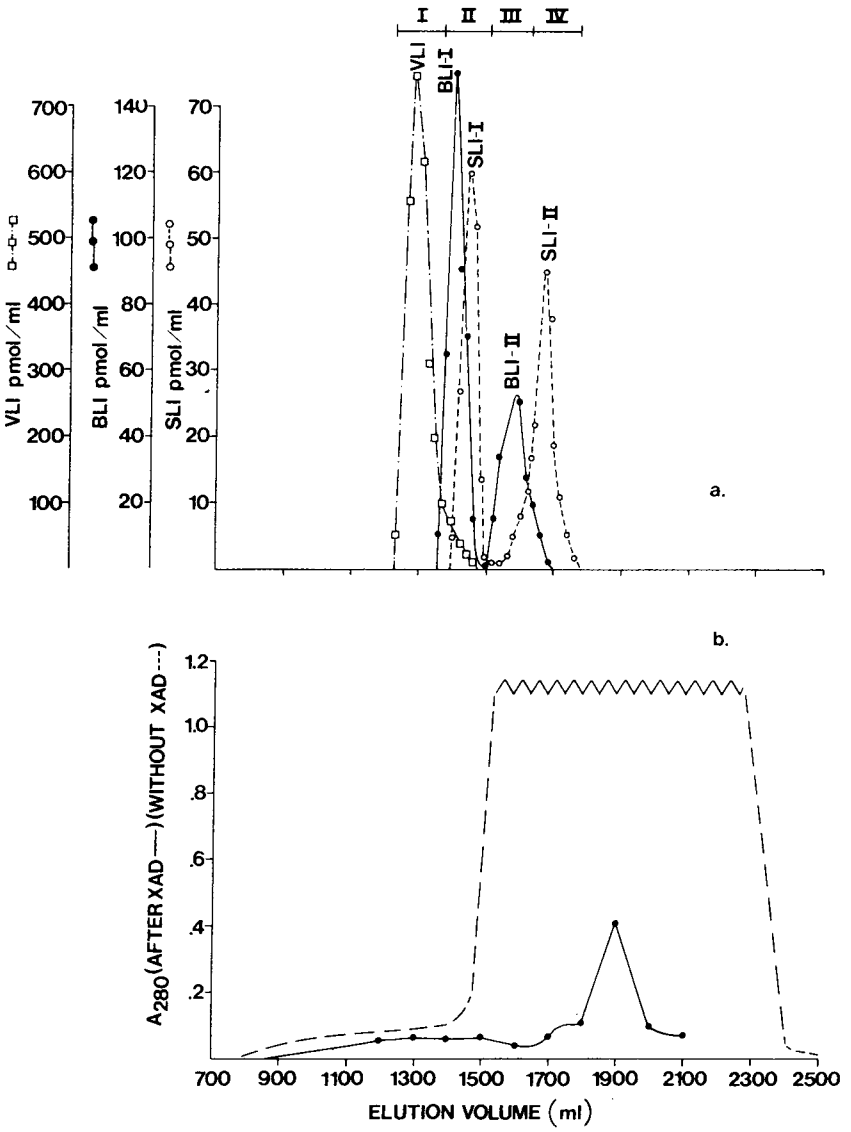

Fig. 2. Chromatography of Amberlite XAD-2 concentrate on a Sephadex G-50 column (140 × 5 cm) eluted with 1% acetic acid. (a) The immunoreactivity profile for VLI (· — · —), BLI (———) and SLI (— — —). (b) The absorption profile at 280 nm for the same XAD-2 concentrate (———), and for an acid—acetone extract from another preparation of intestine that had not been applied to XAD-2 but lyophilized to dryness, dissolved in 1% acetic acid, clarified by centrifugation and then chromatographed on the same column (— — —).

BLI and SLI eluted as two peaks, the larger forms (SLI-I and BLI-I) co-eluted in Fraction II, while the smaller forms (SLI-II and BLI-II) were relatively well separated. The peptides were purified 9—25 fold by Sephadex G-50 chromatography and the recoveries were nearly quantitative.

Fraction I from the Sephadex G-50 chromatography, containing 90% of the VLI, was sequentially purified by CM-cellulose chromatography, gradient HPLC, and isocratic HPLC (see Tables I and II). VLI eluted as a single peak in all these steps with good recovery. However, a contaminant, possibly from the buffer system, co-eluted with the VLI during the isocratic HPLC step, and there was no apparent purification at this step. The final purification of VLI was 1200-fold with a 31% net yield.

BLI and SLI-I, which co-eluted during Sephadex G-50 chromatography, were partially resolved by CM-cellulose chromatography (see Table II). One fraction contained 65% of the BLI-I, and the other contained 35% of the BLI-I and nearly all of the SLI-I. The low recovery of BLI-I following gradient HPLC can be explained because of only 65% of the material recovered from CM-cellulose chromatography was used. Otherwise, the yields for the two HPLC steps were above 80%. Purification of BLI-I was 3825-fold with a net yield of 28% and SLI-I was purified 7600-fold with a net yield of 67%. Unique absorbance peaks were associated with the immunoreactive profile for BLI-I at 13 min (Fig. 3) and for SLI-I at 19 min (Fig. 4) when isocratic elution was done at 26% acetonitrile. The ratios of immunoreactivities to A_{280} suggested that the peptides were nearly pure, assuming the same extinction coefficient for each peptide as their known counterparts.

TABLE II

ELUTION POSITION OF THE IMMUNOREACTIVE PEPTIDES DURING PURIFICATION

	VLI	BLI-I	SLI-I
G-50 peak (%)*	52	59	63
(Range)	(44—66)	(55—65)	(57—67)
CM peak**	0.45	0.33	0.40
(Range)	(0.39—0.52)	(0.15—0.43)	(0.26—0.53)
Gradient HPLC			
(min after 30%)	1	6	12
Isocratic HPLC (min)	9	13	19

*Percentage from protein to salt peak.
**Concentration of ammonium acetate (*M*).

It was found that immunoreactive VIP is homogeneous and similar in gel permeation elution characteristics to porcine VIP while both bombesin and somatostatin immunoreactivities eluted in two major forms (Fig. 2). The larger forms of BLI eluted in a similar position to porcine gastrin releasing peptide while the larger form of SLI eluted in a similar position to porcine somatostatin 28 (Table III). The smaller forms eluted in similar positions testing bombesin (BLI-II) and to somatostatin 14 (SLI-II).

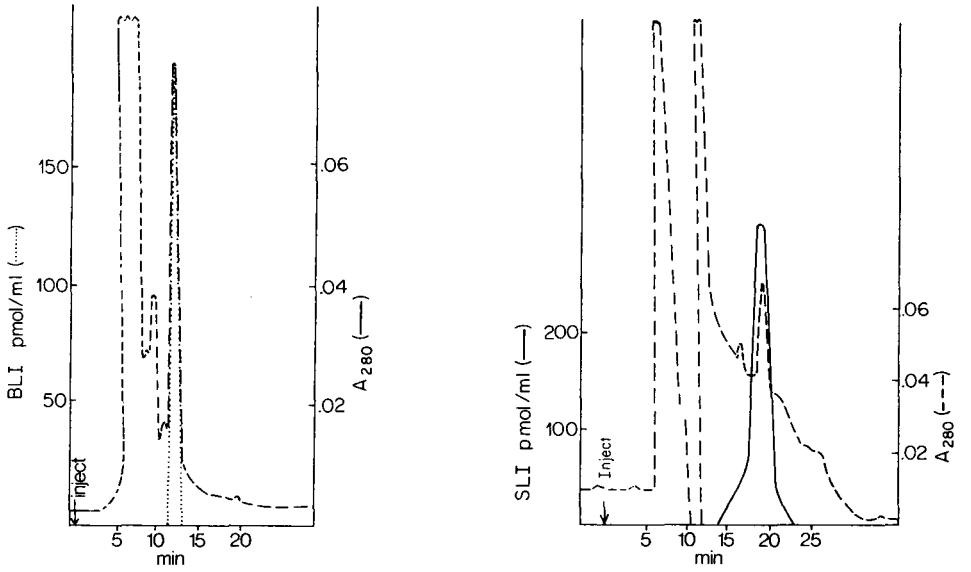

Fig. 3. Isocratic HPLC elution profile of the gradient-purified BLI. The dotted line shows the immunoreactive profile, and the dashed line the absorbance at 280 nm. Details of the chromatography are in the Methods section.

Fig. 4. The isocratic HPLC elution profile of the gradient purified SLI. The dotted line shows the immunoreactive, and the dashed line the absorbance at 280 nm. Details of the chromatography are in the Methods section.

TABLE III

ELUTION POSITIONS OF EXTRACTED PEPTIDES AND STANDARD PEPTIDES ON SEPHADEX G-50 COLUMNS

Peptide	Elution (%)*
Porcine VIP	44
Canine VLI	44
Porcine gastrin releasing peptide	53
Canine BLI-I	56
Porcine somatostatin 28	50
Canine SLI-I	50
Frog bombesin 14	76
Canine BLI-II	83
Porcine somatostatin-14	86
Canine SLI-II	86

*Percentage from protein to salt peak on an analytical G-50 (SF) column (100 × 1 cm) eluted with 3% acetic acid.

DISCUSSION

We have demonstrated that substantial quantities of three neuropeptides can be purified from limited amounts of tissue. Canine intestinal muscle VLI, BLI, and SLI have been purified several thousand times with final recoveries ranging from 28—67%. Indeed, all three peptides would have been isolated with yields of better than 50% if only one peptide had been purified at a time. Such yields suggest that our protocol may be applied to purify peptides from material that is not readily available such as fresh human tissues or unusual hormone-secreting tumors.

Rigorous maintenance of acidic conditions was required to achieve optimal recoveries of these basic peptides. Aside from inevitable losses caused by multiple isolation from single extracts, losses greater than 10% occurred only at steps that required concentration of peptide solutions. Thus, Amberlite XAD-2 chromatography was a particularly useful first step in purification since it resulted in a 6—8 fold purification and concentrated the solution approximately 4-fold without the losses that occur during lyophilization or rotary evaporation.

The HPLC steps resulted in a substantial purification of peptides with minimal losses. Apparent contamination of VLI in the final isocratic HPLC step was of some concern. To avoid further contamination, new HPLC systems for purification of VIP are currently under investigation. This relatively simple, rapid, and efficient protocol may be useful for future purifications of numerous polypeptides from a variety of tissues available only in limited quantities. This should prove to be especially useful in the characterization of human peptides.

The results furthermore indicate that immunoreactive VIP in canine muscle is homogeneous while immunoreactive somatostatin and bombesin are heterogeneous, with about 70% respectively present as large forms. This purification scheme is suitable for purification of both large and small forms of heterogeneous basic neuropeptides.

ACKNOWLEDGEMENTS

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CHROMBIO. 1186

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF PURINE AND PYRIMIDINE BASES, RIBONUCLEOSIDES, DEOXYRIBONUCLEOSIDES AND CYCLIC RIBONUCLEOTIDES IN BIOLOGICAL FLUIDS

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SUMMARY

A method is presented for the separation and quantitative determination of compounds normally related to purine and pyrimidine metabolism in biological material. The retention behaviour of nucleobases, ribonucleosides, deoxyribonucleosides and cyclic ribonucleotides has been systematically investigated by reversed-phase high-performance liquid chromatography using a non-linear gradient. Ultimately a separation of the purine and pyrimidine compounds was achieved in a 35-min run with an average detection limit of 5—10 pmol per injection. Recoveries of standards added to urine, plasma or serum were 96 ± 5%.

INTRODUCTION

A variety of studies has revealed disturbances in purine and pyrimidine metabolism in inborn errors of metabolism [1—4], immune diseases [5—10] and leukemia [11—14]. Analysis of purine and pyrimidine metabolites may allow the pathological mechanisms leading to the clinically observed diseases

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to be elucidated. In addition, measurements of the pools of these metabolites may contribute to more refined diagnosis and further therapeutic approaches.

The present reversed-phase high-performance liquid chromatographic (HPLC) method was designed to determine the pools of nucleobases, nucleosides and cyclic nucleotides in urine, serum and plasma samples. Recently, several HPLC procedures have been presented with sensitive separations of nucleobases and nucleosides [15—19]. The HPLC procedure presented here offers the advantage of measuring various nucleobases, ribonucleosides, deoxyribonucleosides and cyclic nucleotides in a single run.

EXPERIMENTAL

Chemicals

All nucleobases, nucleosides, cyclic AMP and cyclic GMP used for the identification and quantitation of peaks in the UV scans were obtained from Sigma, St. Louis, MO, U.S.A., and from Boehringer, Mannheim, G.F.R. Helium was purchased from Hoekloos, Schiedam, The Netherlands. The other chemicals were from E. Merck, Darmstadt, G.F.R. All chemicals were of pro analysis grade. The water used for the buffer was purified in a Milli-Q System, giving a resistivity of 18 MΩ/cm (Millipore, Bedford, MA, U.S.A.).

Apparatus

The experiments were performed with a Spectra-Physics SP8000 liquid chromatograph (Spectra Physics, Santa Clara, CA, U.S.A.). The apparatus was equipped with a thermostated oven, an auto-injector with a high-pressure sampling Valco valve, an automated data system with integrator, and a two-channel printer-plotter.

Column effluents were monitored with two UV detectors (SP8210, Spectra Physics), one set at wavelength 254 nm and the other at wavelength 280 nm. The flow-cells had volumes of 8 μl.

Pre-packed analytical columns 250 mm × 4.6 mm I.D. were used, packed with Spherisorb 10-ODS (particle size 10 μm; Chrompack, Middelburg, The Netherlands).

Chromatographic conditions

Elutions were performed with a programmed non-linear (NL 2) ternary gradient, starting with 0.05 mol/l potassium phosphate buffer (pH 5.60) and ending with 50% 0.05 mol/l potassium phosphate buffer (pH 5.60), 25% methanol and 25% water (v/v). Before use the phosphate buffer was filtered through a Millipore Type HA membrane filter (pore size 0.45 μm). All mobile phases were degassed by continuous helium purging. The solvent flow-rate was kept constant at 1.5 ml/min, the pressure at ± 38 bars and the temperature at 40°C. The run-time was 35 min.

Sample preparation

Urine samples were filtered through a Millipore Type GS membrane filter (pore size 0.22 μm) and were analysed immediately afterwards.

Serum and plasma samples were deproteinated with perchloric acid (PCA)

at 0°C. Serum or plasma (1 ml) was pipetted into a micro test-tube (Model 3810, Eppendorf, Hamburg, G.F.R.) and kept in an ice-bath. Then 100 μl of 4 mol/l ice-cold PCA was added and the mixture was vortexed for 1 min. The suspension was kept on ice for another 5 min and centrifuged in an Eppendorf centrifuge (Model 3200). After centrifugation, the supernatant was pipetted into another micro test-tube and the pH was adjusted to 6.0—6.8 with an ice-cold mixture of 4 mol/l potassium hydroxide and 1 mol/l dipotassium hydrogen phosphate. After 10 min the precipitated potassium perchlorate was removed by centrifugation and the extract was kept on ice until injection. If the extract was not investigated the same day, it was kept frozen at −23°C.

RESULTS AND DISCUSSION

Chromatography

Fig. 1 illustrates a typical analysis of a standard solution containing several purine and pyrimidine bases, ribonucleosides, deoxyribonucleosides, cyclic

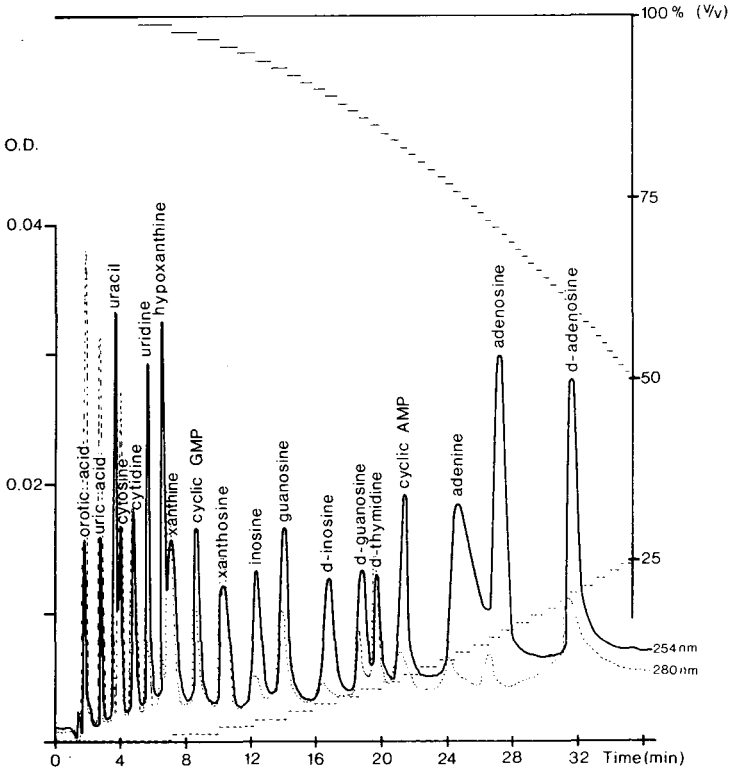


Fig. 1. Elution profile of a test mixture of purine and pyrimidine nucleobases, ribonucleosides, deoxyribonucleosides, cyclic AMP and cyclic GMP. The separation conditions are described in Experimental. Detection was performed at 254 nm and 280 nm. On the scale to the right the percentage of each of the solvents used for the elution is indicated. The 0.05 mol/l potassium phosphate buffer starts at 100% and ends at 50% (indicated by the step-plot with solid lines), whereas the water and methanol each start at 0%, and end at 25% (indicated by the step-plot with broken lines).

AMP and cyclic GMP. The best separation on the Spherisorb 10-ODS columns was achieved using the preprogrammed non-linear gradient described under Experimental. Between two successive runs the system was equilibrated for 15 min under the initial conditions. Some overlap was observed between cytosine and uracil and between hypoxanthine and xanthine. In these cases the absorbance ratios (E_{254nm}/E_{280nm}) of the partially overlapping compounds vary significantly enough to distinguish between the compounds.

Recovery

Standard amounts of purines and pyrimidines were added to human serum (50, 100, 200, 400, 800 and 1000 pmol/ml). At least five samples at each concentration were deproteinated as described under Experimental and analysed. The concentrations and the integrated peak areas were linearly related over the range 50—1000 pmol/ml. For cytosine, uracil, hypoxanthine and xanthine, correlation coefficients were found of 0.987, 0.990, 0.992 and 0.986, respectively. The correlation coefficients found for the other compounds were at least 0.996. The recoveries of all the standards added to serum were 96 ± 5%.

Applications

An application of the present method to biological material is illustrated in Fig. 2A and B. In this figure the UV scans of urine samples are shown from a patient with Lesch-Nyhan syndrome before (Fig. 2A) and after (Fig. 2B) receiving the drug 4-hydroxypyrazolo[3,4-*d*]pyrimidine (allopurinol). The Lesch-Nyhan syndrome is associated with severe deficiency of the enzyme hypoxanthine—guanine phosphoribosyltransferase (HGPRT; EC 2.4.2.8) [2, 20, 21]. The HGPRT deficiency leads to a massive over-production and over-excretion of uric acid [2, 21]. The drug allopurinol is an analogue of hypoxanthine. It is a competitive inhibitor in the formation of the poorly soluble uric acid from hypoxanthine and xanthine, a reaction catalysed by xanthine oxidase. Allopurinol is converted to oxipurinol by xanthine oxidase. As a result, allopurinol is an important agent in the control of hyperuricemia, by giving rise to the formation of the more soluble hypoxanthine and xanthine.

With the HPLC method the effect of allopurinol treatment on uric acid, hypoxanthine and xanthine excretion can easily be studied. In addition, the excreted amounts of free allopurinol and of produced oxipurinol can be detected, so the therapy can be adapted (Fig. 2A and B). Also eventual side-effects on pyrimidine metabolism can be monitored.

In other investigations we are using the HPLC method in the diagnosis of patients with pseudohypoparathyroidism. This is an hereditary disorder, characterised by symptoms and signs of hypoparathyroidism in association with distinctive skeletal and developmental defects [22—26]. The cause of this disease differs, however, from that of hypoparathyroidism. In the latter cases there is deficient parathyroid hormone (PTH) production. In pseudohypoparathyroidism there is excessive secretion of PTH. However, there is a defective response of target-cell receptors or of adenyl cyclase to PTH. Therefore, two principal laboratory tests should be employed, namely the measurement of PTH secretion and the measurement of urinary cyclic AMP excretion following the administration of PTH. In Fig. 3A and B, UV scan patterns are

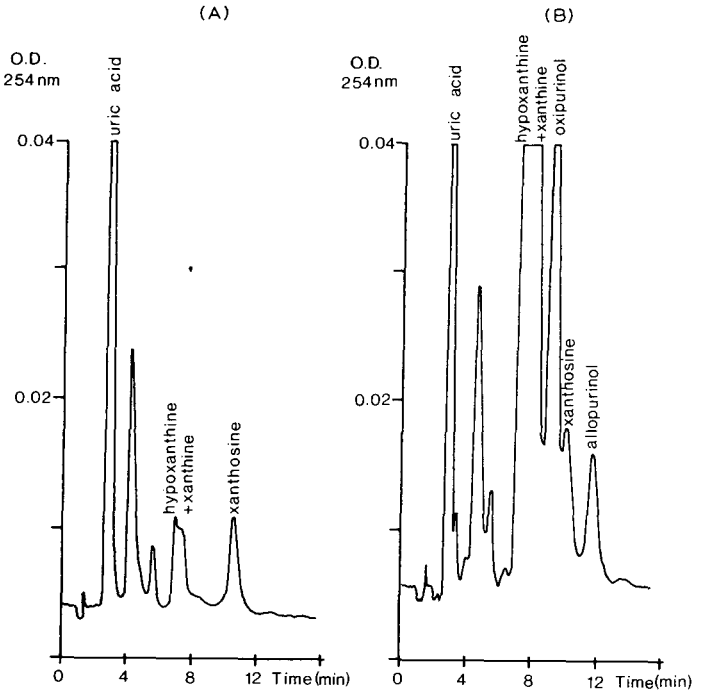


Fig. 2. UV scans of urine samples from a Lesch-Nyhan patient before (A) and after 5 days (B) of treatment with an oral dose of allopurinol, 10 mg/kg body weight per 24 h.

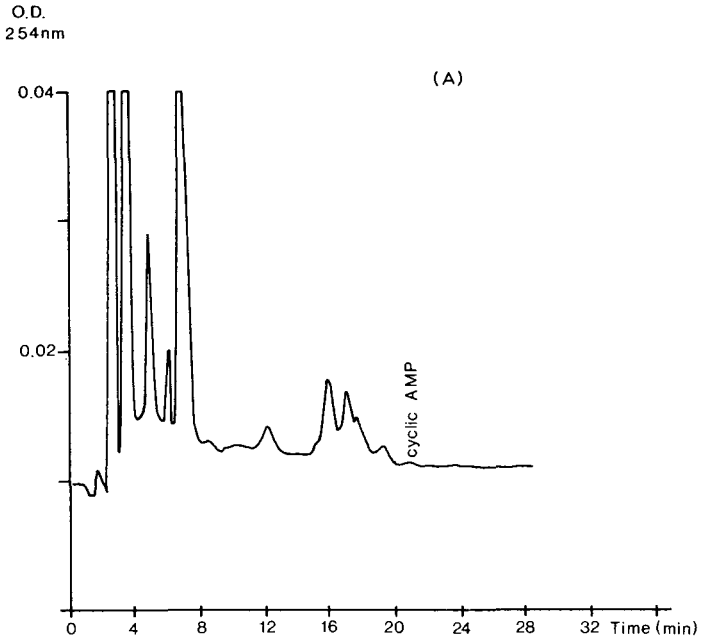
shown of urine from a normal healthy control before and 30 min after administration of PTH. In Fig. 3C and D, UV scan patterns are illustrated from a patient suspected of having pseudohypoparathyroidism before and 30 min after administration of PTH. In contrast to the control, no increase in the cyclic AMP levels was observed in the patient, suggesting that this patient had the disease.

The HPLC system is also used in in-vitro studies of nucleobase and nucleoside metabolism of malignant human T- and B-cell cultures. In this way a more detailed understanding can be obtained of the differences in purine and pyrimidine metabolism known to occur in lymphoblasts of various subsets of acute lymphatic leukemia [11—14]. In Fig. 4A and B an example of such an experiment is given with the Raji cell-line (human Burkitt's lymphoma B-cell). The culture medium was analysed before and after addition of 100 μmol/l adenosine. The major metabolite after transport and metabolism appears to be hypoxanthine (Fig. 4B).

CONCLUSIONS

The HPLC method reported is a useful and accurate analytical tool to determine concentrations of purine and pyrimidine metabolites in a variety of body fluids.

In comparison to previously published methods [15—19], the advantage of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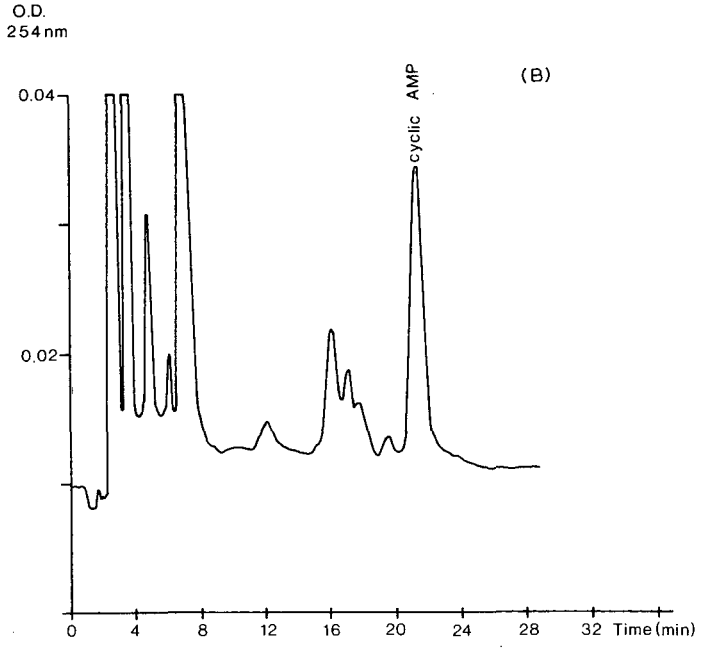
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Fig. 3.

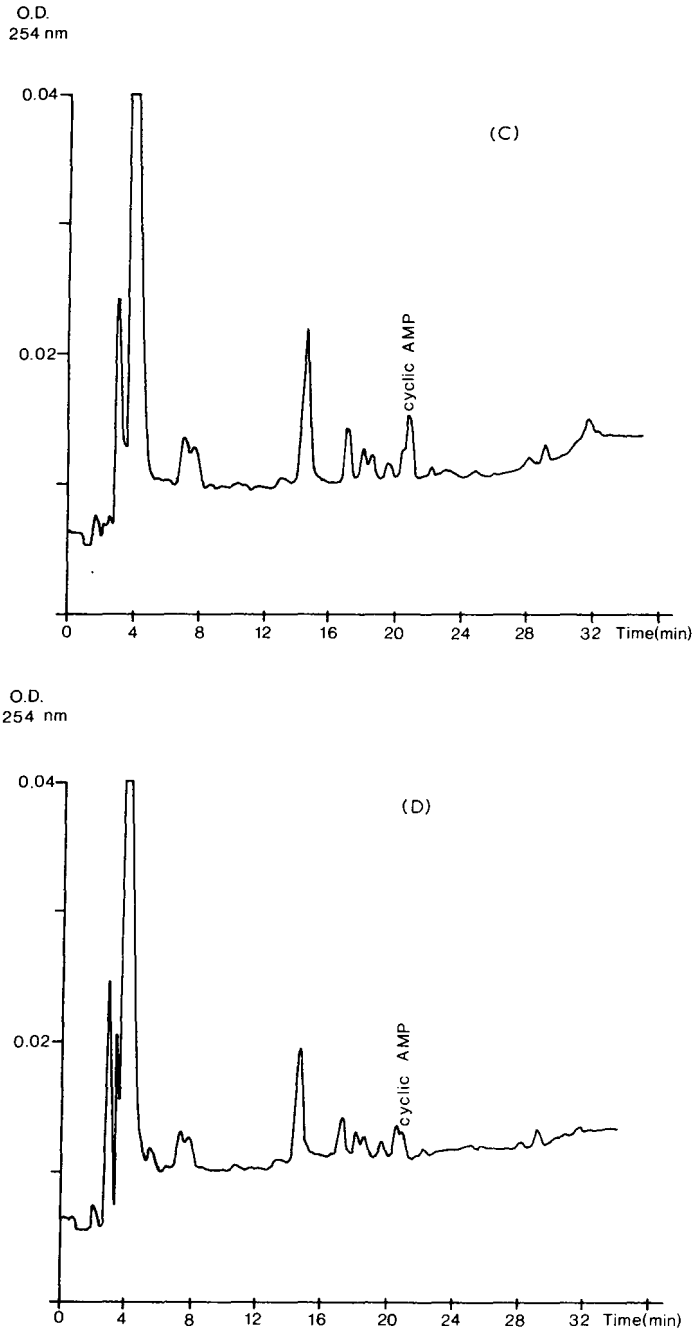

Fig. 3. Excretion of 3',5'-cyclic AMP following an injection of PTH (300 U S.P. units). (A) Urine of a healthy control before administration of PTH. (B) Urine of the control 30 min after the administration. (C) Urine of a patient with pseudohypoparathyroidism before administration of PTH. (D) Urine of the patient 30 min after administration of PTH.

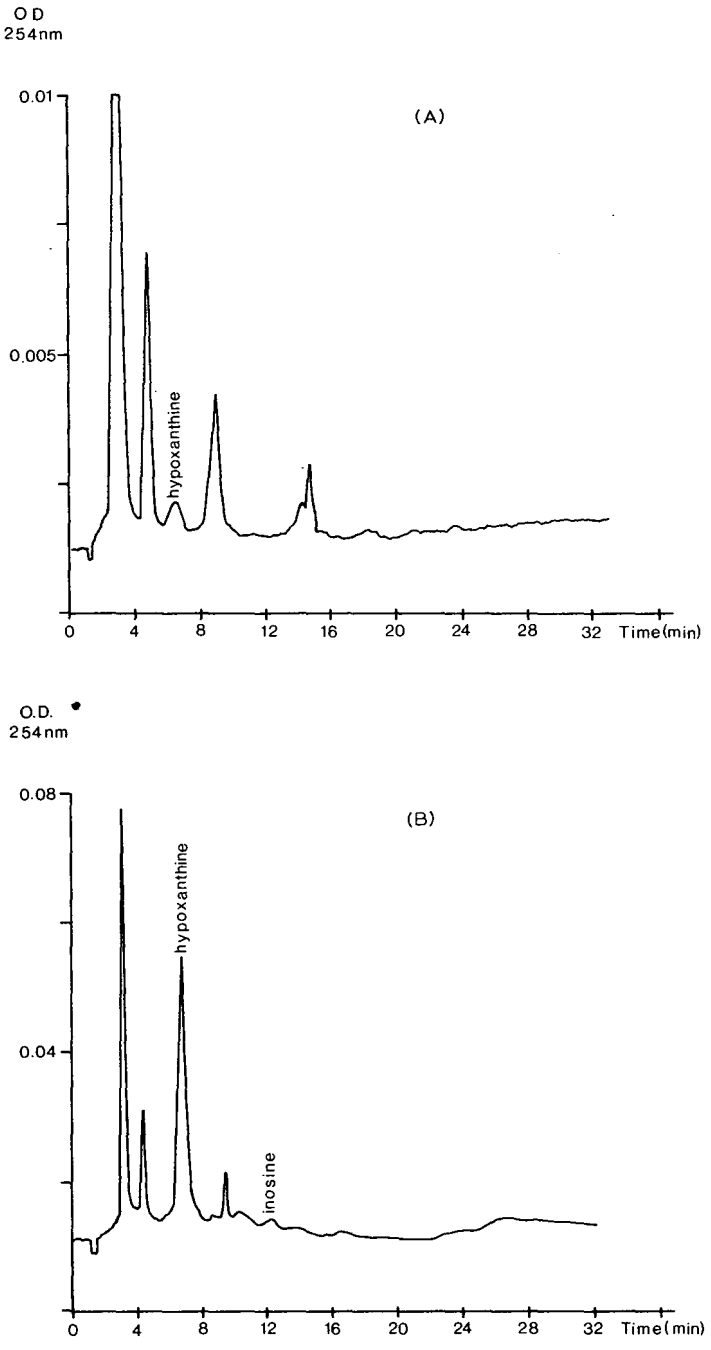

Fig. 4. Elution profile of purine and pyrimidine nucleosides in a culture medium before (A) and 15 h after (B) addition of 100 μmol/l adenosine to a cell culture of a Raji cell-line.

the method presented is that it enables the concentrations of nucleobases, ribonucleosides, deoxyribonucleosides, cyclic AMP and cyclic GMP to be determined in a single run.

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CHROMBIO. 1180

RAPID ASSAY OF CYCLIC AMP PHOSPHODIESTERASE AND 5′-NUCLEOTIDASE BY MEANS OF CHROMATOGRAPHY ON CELLULOSE-NITRATE MEMBRANE STRIPS

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SUMMARY

A simple chromatographic procedure with the use of modified cellulose-nitrate membrane strips, 80 × 40 mm, has been worked out for the rapid isotopic assay of cyclic AMP (cAMP) phosphodiesterase (EC 3.1.4.17) and 5′-AMP nucleotidase (EC 3.1.3.5) in crude extracts of various tissues from animals and plants. The assay is based on enzymatic conversion of the product to adenine, a relatively inert compound which, in contrast to cAMP and 5′-AMP, is strongly adsorbed by the cellulose-nitrate membrane. Due to this property rapid separation of adenine from the unconverted substrate (cAMP or 5′-AMP) is possible. Commercial 5′-nucleotidase and easily obtainable crude extract of adenosine nucleosidase from barley leaves are used as coupling enzymes for the phosphodiesterase assay. The assay of phosphodiesterase in 0.5—2 μl of blood (10^{-5} to $4 \cdot 10^{-5}$ units) has been demonstrated on several examples.

INTRODUCTION

It was recently reported from our laboratory that a number of enzymes can be estimated rapidly by means of chromatography on modified cellulose-nitrate membrane strips [1—3]. This technique has been found to be applicable in the isotopic assay of cyclic AMP (cAMP) phosphodiesterase (EC 3.1.4.17) and 5′-AMP nucleotidase (EC 3.1.3.5) over a broad range of substrate concentrations. The procedure described in this paper is based on our observation that cellulose-nitrate membrane treated with aqueous solutions of aliphatic alcohols strongly adsorb adenine and a few bases but to a much lesser extent the nucleotides derived from them [1, 4]. In this assay the products of these two enzyme actions, 5′-AMP and adenosine, respectively, are converted by coupling enzymes into adenine which in contrast to the substrates is readily adsorbed by the membrane strip. Prepared cellulose-nitrate

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membrane strips, 8 × 40 mm, are used in this method. The application procedure is very simple: 3—5 μl of the incubation mixture are applied onto one end of the strip followed by chromatography with the use of phosphate buffer. The adenine-labeled substrate and the product are separated rapidly and quantitated by counting separately the two halves of the strip. Besides rapidity, the method is advantageous in several aspects in comparison to other phosphodiesterase assays using labeled substrates [5—11]. These advantages are, in particular: (1) fairly good counting efficiency of tritium-labeled compounds on the cellulose-nitrate membrane; (2) high sensitivity; (3) very small amounts of enzyme required (down to $2 \cdot 10^{-5}$ units); (4) insensitivity to a broad range of salt concentrations and pH [2]. This technique seems to be particularly useful for clinical investigations and kinetic studies whenever small amounts of enzyme are available. There is also the potential of adapting it for routine assays by means of automatization.

MATERIALS AND METHODS

Materials

[2,8-^{3}H] Adenosine 3′,5′-cyclic phosphate specific activity 35 Ci/mmol, [adenine U-^{14}C] adenosine 3′,5′-cyclic phosphate specific activity 85 mCi/mmol, [8-^{14}C] adenosine 5′-monophosphate specific activity 53 mCi/mmol, and [8-^{14}C] adenosine specific activity 45 mCi/mmol were from the Radiochemical Centre, Amersham, Great Britain. The [^{3}H] cAMP was purified before use by treatment with 5′-nucleotidase and ascending chromatography on Whatman 3 MM paper with the solvent system *n*-butanol—acetic acid—water (12:5:3). Adenosine 3′,5′-cyclic phosphate was from Calbiochem, San Diego, CA, U.S.A. Cellulose-nitrate membrane sheets (BA 85, 0.45 μm pore size) were from Schleicher and Schüll, Dassel, G.F.R. The sheets were cut into strips 8 × 40 mm in size and modified as described below. 2-Ethoxyethanol was from BDH, Poole, Great Britain. Other chemicals of analytical grade were from Polskie Odczynniki Chemiczne (POCH), Poland.

Source of enzymes

5′-Nucleotidase from *Crotalus adamanteus* venom was from Sigma, St. Louis, MO, U.S.A. The 5′-nucleotidase from barley leaves was a crude preparation obtained in a mixture together with adenosine nucleosidase as described in ref. 2. The partially purified preparations were free of cAMP phosphodiesterase, although, as will be shown in Results, freshly obtained extract of barley leaves showed phosphodiesterase activity. The enzymes dried as described in ref. 12 were kept at room temperature for three years with little loss of activity. Phosphodiesterase from blood was used here as model enzyme for studies of the usefulness of the assay technique. It was obtained from heparinized fresh blood of healthy and ill subjects diluted four times with water to cause osmotic disruption of the cells. It was then diluted several times with 50 m*M* Tris—HCl buffer (pH 7.2) to a concentration of enzyme appropriate to the sensitivity of the method. Phosphodiesterase activity was also examined in freshly prepared crude extracts of barley leaves obtained by rupture of the plant cells as described in ref. 13 and extraction of the enzyme

with the buffer as above followed by centrifugation for 10 min at 10,000 *g*. The supernatant solution was dialyzed against the extraction buffer for 1 h and used with no further purification. As shown in Results, both enzyme preparations appeared to be rather unstable. Commercial 3′,5′-cyclic nucleotide phosphodiesterase from bovine heart and adenosine deaminase from calf intestine were from Sigma.

Phosphodiesterase assay

In this assay the product of diesterase action, 5′-AMP, is converted to adenine in coupled enzymatic reactions with the use of two enzymes — 5′-nucleotidase and adenosine nucleosidase — according to the scheme

$$3',5'\text{-cAMP} \xrightarrow[H_2O]{} 5'\text{-AMP} \xrightarrow[P_i]{} \text{adenosine} \xrightarrow[H_2O]{} \text{adenine} + \text{ribose}$$

The unconverted cAMP and one of the final products, adenine, are rapidly separated by chromatography on cellulose-nitrate membrane strips 8 × 40 mm. Since adenine is adsorbed by the membrane it remains at the origin, while cAMP moves with the front of the chromatographic buffer towards the other end. The labeled product and the unconverted substrate are easily assessed by measuring separately the radioactivity of the two halves of the strip. Owing to the very high specific radioactivity of the applied [^{3}H]cAMP the assay could be elaborated for very small volumes of the incubation mixture and tiny amounts of enzyme. The following standard assay was worked out. The incubation mixture contained in a total volume of 15 μl: 0.5 m*M* [2,8-^{3}H]-cAMP (30,000 cpm); 5 m*M* $MgCl_2$; 0.05 *M* Tris—HCl buffer, pH 7.2; 0.001 unit of 5′-nucleotidase of barley leaves or snake venom; 0.003 unit adenosine nucleosidase; and appropriate amounts of the phosphodiesterase. The tubes, protected from evaporation, were incubated for 30 min at 25° C and the reaction was then stopped by inserting the tubes into boiling water for 30 sec. The tubes were next centrifuged and 3—5 μl of the clear supernatant solution were applied on the membrane strip at a distance of about 7 mm from one of its ends. The strip was then kept for 10 min in a moist atmosphere followed by chromatography with 0.02 *M* K_2HPO_4. The chromatography was done on the bottom of a suitable PTFE or polypropylene box containing a strip of chromatographic paper soaked with the above buffer [2]. The membrane strip was attached by one end to the wet paper in order to allow the buffer to flow along it. The unconverted cAMP moved with the front of the buffer towards the other end of the strip, while the adenine remained adsorbed at the start. When the buffer reached the other end, the strip was dried under an infrared lamp and cut into two pieces of equal length. The pieces A and B, containing the product and the unconverted substrate, respectively, were put into standard scintillation vials and solubilized with 0.5 ml of 2-ethoxyethanol followed by addition of 5 ml of toluene scintillator and measurement of the radioactivity. Blank tubes containing no phosphodiesterase were run simultaneously. The activity of the enzyme was assessed on the basis of percentage of the radioactivity in sector A of the strip and the standard curve, as described in the legend to Fig. 1.

5'-AMP nucleotidase assay

The procedure described for phosphodiesterase assay was adopted for the assay of 5'-nucleotidase. The adenosine produced by the enzyme action was converted by adenosine nucleosidase into adenine. Phosphatase-free adenosine nucleosidase from barley leaves was obtained by further purification of the preparation described above on a Sephadex G-100 column [13]. The incubation mixture contained in a total volume of 15 μl was 0.1 *M* Tris—HCl buffer (pH 7.2), 5 m*M* $MgCl_2$, 0.1 m*M* [8-^{14}C] 5'-AMP (30,000 cpm), adenosine nucleosidase 0.003 unit, and 5'-nucleotidase preparation containing 10^{-5} to $5 \cdot 10^{-5}$ activity units. After an appropriate incubation time at 25° C, during which not more than 30% of the substrate was converted, the reaction was stopped by inserting the tubes for 30 sec into boiling water. Further the procedure was followed as described for phosphodiesterase assay except that the strips were not dissolved prior to counting.

Adenosine nucleosidase assay

The assay of this enzyme is described in ref. 2.

Preparation of cellulose-nitrate membrane strips

The membrane sheets described in Materials were cut into strips, 8 × 40 mm, with a scalpel. In order to make the strips suitable for chromatography, they were inserted for 15 min into a 2% aqueous solution of 2-methyl-1-propanol (isobutanol) heated to 60° C, followed by washing four times with cool distilled water and bathing for 10 min in 5% glycerol at room temperature. The strips were removed with philatelic forceps, pressed gently against a filter paper and dried on PTFE foil at room temperature. The strips were kept at 5° C in closed vials for three years with no change in chromatographic performance. Commercial cellulose-nitrate membrane sheets might differ, however, from batch to batch in performance. To ensure uniform performance of the membranes for a longer period of time it is reasonable to keep a reserve of the same batch in stock. On the other hand, the membranes become hydrophobic during storage. This can be overcome by storing the membranes in the presence of isobutanol vapour; in practice, a strip of filter paper soaked with isobutanol was inserted into each membrane package wrapped into a polyethylene foil. A quantity of 1 ml of isobutanol per litre of package space has been found sufficient. The addition of excessive, uncontrolled amounts of isobutanol poured directly onto the membranes damages them.

RESULTS AND DISCUSSION

The interference of purine-catabolizing enzymes in the phosphodiesterase assay

Accurate assay of the phosphodiesterase in crude biological material is difficult to achieve due to the presence of catabolizing enzymes which degrade the primary product, 5'-AMP. Instant conversion of 5'-AMP to adenine, a compound relatively inert under the assay conditions used, seemed to be a solution to this problem. To achieve this, a large excess of the coupling enzymes is required in the assayed sample. According to refs. 14 and 15, a 100-

fold excess of the first coupling enzyme and several thousand-fold excess of the second enzyme relative to the assayed enzyme are required to obtain a farily accurate measurement in a one-step procedure. The two coupling enzymes — 5′-nucleotidase and adenosine nucleosidase — used in our assay of phosphodiesterase in hemolyzed blood, have been found to be very effective even at low concentrations. This is presumably due to their low Michaelis constants ($1.2 \cdot 10^{-5}\,M$ and $2 \cdot 10^{-6}\,M$, respectively) exceeding by one or two orders of magnitude the K_m values of the potentially interfering enzymes like 5′-AMP deaminase and adenosine deaminase. Hemolyzed blood has been found to contain an appreciable level of adenosine deaminase [2] which, in terms of enzyme activity units, is up five times greater than that of cAMP diesterase. Thus, it seemed to be particularly important to find what level of this interfering enzyme is acceptable in our standard assay. For this purpose the assayed sample was supplemented with commercial adenosine deaminase. The data in Table I allowed us to assess that a supplement of 0.002 unit of commercial adenosine deaminase together with about 0.002 unit of this enzyme introduced with the sample of the hemolyzed blood causes about a 7% underestimation of the phosphodiesterase level measured by the membrane-strip method. This is due to conversion of part of the adenosine into inosine, a compound less effectively adsorbed by the membrane strips. The above deviation is within the range of methodological errors derived from volume and radioactivity measurements and has been considered still acceptable, although near the edge of the given limit. The presence of relatively higher levels of the interfering enzyme would require an increase of the coupling enzyme adenosine nucleosidase in the standard assay in order to fit into the assumed 10% error range. The quantities of the coupling enzymes worked out experimentally and the data in Table I have been found to be in fair agreement with the data calculated on the basis of the known equation combining the Michaelis constant with concentration of the substrate and the velocity of enzymatic conversion:

$$v = \frac{V \cdot s}{K_m + s}$$

Calculation of the results

The mole fraction of cAMP converted by phosphodiesterase was read from a graph (Fig. 1) obtained experimentally from two controls: one with unconverted, and the second with completely converted, substrate in the presence of excess phosphodiesterase. The diagram allows the elimination of errors caused by non-specific binding of cAMP to the membrane and the presence of radioactive contamination in the substrate due to radiolysis of cAMP. The calculation of the enzyme level is described in detail in the legend to Fig. 1. A new graph was routinely plotted every two weeks. Another batch of the modified membrane strip or a fresh portion of the tritium-labeled substrate necessitates a new graph as well.

Examples of diesterase assay

The method has been found to be applicable to the direct assay of cyclic

TABLE I

EFFECT OF ADENOSINE DEAMINASE ON THE DETERMINATION OF cAMP PHOSPHODIESTERASE IN BLOOD, ASSESSED BY PAPER CHROMATOGRAPHY AND THE MEMBRANE STRIP ASSAY

A volume of 300 μl of the standard incubation mixture containing 40 μl of hemolyzed blood from a child with inherited gastrointestinal disorders (patient G in Table II) containing approximately 0.002 unit of cAMP diesterase, 0.012 unit of adenosine deaminase, and 0.5 m*M* [8-^{14}C]cAMP (2,000,000 cpm) instead of the tritium-labeled cAMP used in the standard assay, was divided into six portions. The operation was carried out at 0° C. One portion serving as control for zero time of incubation was immediately denatured by heat treatment. The remaining portions were supplemented with adenosine deaminase or water as specified in the table and incubated at 25° C for 30 min followed by heat denaturation. The sixth portion was exposed to a second incubation for 30 min after addition of 5′-nucleotidase and adenosine nucleosidase as specified. Two 5-μl aliquots of each tube were exposed to chromatography on membrane strips as described in Methods. The quantity of the converted cAMP was calculated as described in the legend to Fig. 1. The residue in the incubation tubes was supplemented with carriers of the compounds indicated in the table and subjected to ascending chromatography on paper Whatman 3 MM using the solvent specified in Methods for purification of commercial [^{3}H]cAMP. The spots visualized by UV light were cut out and the radioactivity was measured. The following R_F values were found: 5′-AMP (0.1), cAMP (0.16), inosine (0.32), adenosine (0.46) and adenine (0.56). The 5′-AMP spot overlapped somewhat that of cAMP, thus the sixth experiment with the additional incubation is the presence of 5′-nucleotidase and adenosine nucleosidase was included. The quantity of substrate converted to the compounds indicated was calculated on the basis of the percentage of radioactivity in the particular spots.

Additives or conditions	Substrate or product in pmoles				
	Paper chromatography				Membrane-strip product
	Substrate cAMP	Products			
		Inosine	Adenosine	Adenine	
Control (no enzyme)	7500	—	—	—	0
None	5115	38	53	2295	2175 ± 98
Adenosine deaminase					
0.0005 unit	5190	60	38	2213	2100 ± 90
0.002 unit	5250	143	30	2078	2025 ± 60
0.005 unit	5265	300	0	1935	1875 ± 38
Second incubation after addition					
0.001 unit 5′-nucleotidase 0.001 unit adenosine nucleosidase	5063	38	0	2390	1875 ± 83

nucleotide diesterase in crude extracts from animals and plants. The diesterase assay in plants is simpler since plants do not have adenosine deaminase, considered the major cause of error. Most plants contain 5′-nucleotidases and 3′-nucleotidases as well as adenosine nucleosidase so that, nolens volens, the incubation of cyclic nucleotide with crude plant extract usually ends with adenine as the final product [2, 4]. In the case of diesterase assay in blood,

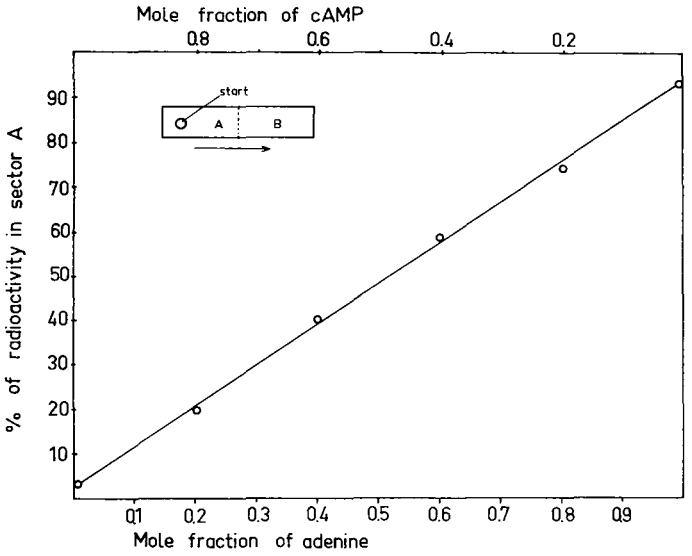

Fig. 1. The standard curve. The points indicated were obtained on the basis of two 30-μl controls, one with zero time of incubation and the second exposed to complete conversion of cAMP to adenine; 30 min of incubation under standard conditions with 0.002 unit of commercial diesterase was satisfactory. The contents of these controls were mixed with each other in ratios to obtain the mole fractions indicated, followed by chromatography on membrane strips and measurement of the radioactivity in both halves (A and B) of the strip (see insert). From the obtained curve the mole fraction of the adenine formed (converted cAMP) was read. The cAMP diesterase (PDE) level (L) in blood expressed in pmoles per min per μl of blood was calculated from the mole fraction of adenine formed (k), time of incubation (t), the volume of undiluted blood (v) and the amount of substrate (m), according to the equation $L = (k \cdot m)/(t \cdot v)$. In routine procedures it was sufficient to draw a graph from two points only, obtained for unconverted and completely converted substrate.

it seemed to be important to know the adenosine deaminase level in order to assess its possible interference. Table II presents examples of routine assay of these two enzymes in blood of children affected with hereditary gastrointestinal disorders. The level of adenosine deaminase is about four to five times greater than that of diesterase. It can be assessed from the data in Table I that the ratio of the levels of these two enzymes as 1:10 is still acceptable within the standard assay.

The stability of diesterase seems to be a critical factor affecting reproducible assay. Since it was observed that the diesterase activity in both blood and barley leaf extract changes noticeably during storage, it seemed important to know the rate of enzyme activity loss in these sources at various temperatures. As shown in Fig. 2 the half-life of this enzyme in the plant extract and in blood at room temperature is about 3.5 and 8 h, respectively, while at 2°C the respective values are 4.5 and 6.5 days. With regard to blood, more often explored as a source of phosphodiesterase in various physiological studies, the curve of enzyme activity decay may be helpful for planning reproducible assay conditions. In contrast to diesterase, the activity of adenosine deaminase changes very little during storage.

TABLE II

LEVELS OF cAMP DIESTERASE AND ADENOSINE DEAMINASE IN BLOOD OF CHILDREN WITH INHERITED GASTROINTESTINAL DISORDERS

The samples of blood were assayed for diesterase and adenosine deaminase as described in Methods and in ref. 2, respectively. Each sample was run in duplicate. The content of each incubation tube was chromatographed on membrane strips in triplicate. The level of the diesterase was calculated from the graph shown in Fig. 1 and the equation given in the legend of Fig. 1.

Subject	pmoles per min per μl of blood (mean ± S.D.)	
	Phosphodiesterase	Adenosine deaminase
10 controls	29—54 (range)	50—120 (range)
A	31 ± 2	96 ± 5
B	48 ± 3	162 ± 6
C	50 ± 3	172 ± 6
D	56 ± 4	162 ± 5
E	60 ± 4	200 ± 6
F	66 ± 4	152 ± 5
G	68 ± 4	248 ± 7
H	75 ± 5	143 ± 5

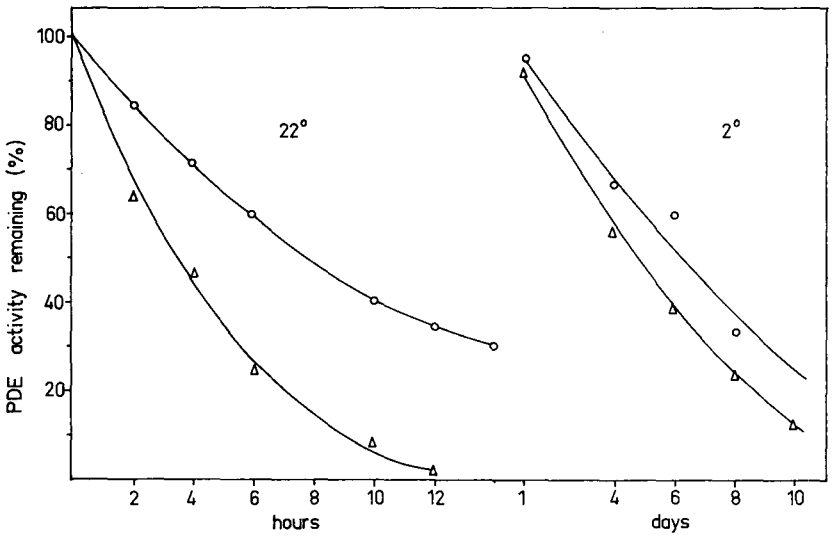

Fig. 2. Time—course of inactivation of cAMP diesterase in blood and barley leaf extract at 2°C and 22°C. Heparinized blood of patient G (see Table II) was kept in small sterile at 2°C and 22°C. The crude extract of barley leaves in 0.1 *M* Tris—HCl buffer (pH 7.2) containing cyclic nucleotide diesterase activity 0.003 unit/ml was handled in a similar manner. At the time indicated, the blood sample was hemolyzed by diluting five times with water. Two 5-μl aliquots of the diluted blood as well as of the barley leaf extract were subjected to standard diesterase assay. The points indicated are mean values of two assays. (○) Blood diesterase; (△) barley leaf diesterase.

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ASSAY OF PRENALTEROL IN PLASMA AND URINE BY GAS CHROMATOGRAPHY—MASS SPECTROMETRY

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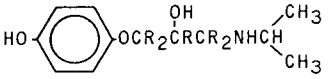
SUMMARY

A sensitive and selective method for the quantitative analysis of prenalterol in plasma and urine is described. Prenalterol and the internal standard, deuterated prenalterol, are extracted with diethyl ether at pH 9.9 under salting-out conditions. Derivatization is performed by means of pentafluoropropionic anhydride in toluene before separation in the gas chromatograph. Detection and quantification of the triacyl derivatives are done by mass spectrometry in the selected ion monitoring mode. The method allows determination of concentrations down to 5 nmol/l (1 ng/ml) in 1 ml of sample, with a relative standard deviation below 10%.

INTRODUCTION

Prenalterol (Fig. 1), the *laevo*-isomer of 4-hydroxyphenoxy-3-isopropylamino-2-propanol, is a selective β_1-adrenoceptor agonist [1]. It has been analysed in plasma by gas chromatography and electron-capture detection of the heptafluorobutyryl derivative after a single extraction step [2]. In low concentra-



Prenalterol R = H

Internal standard (deuterated prenalterol) R = ^{2}H

Fig. 1. Structural formulae of prenalterol and deuterated prenalterol.

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tions interference from endogenous compounds prevents analysis of low concentrations of prenalterol in this way, and back- and re-extraction must be used in the work-up procedure [3].

The molecular structure of prenalterol resembles that of the 4-hydroxy metabolite of propranolol. This metabolite has been assayed in biological materials by gas chromatography—mass spectrometry [4—6]. The purpose of this paper is to describe a method for assaying prenalterol in plasma and urine that involves a single extraction step and determination by gas chromatography—mass spectrometry.

EXPERIMENTAL

Apparatus

A Finnigan MAT 44S mass spectrometer coupled to a Varian 3700 gas chromatograph interfaced by an open split coupling was used for both qualitative and quantitative work. The glass column (2 m × 2 mm I.D.) was filled with 3% OV-17 on Gas Chrom Q (120—140 mesh) (Applied Science Labs., State College, PA, U.S.A.) and operated at 185°C. The temperature of the injection port, the open split coupling and the connection line was kept at 200°C. The ion source temperature was 220°C. The flow-rate of the carrier gas (helium) was 15 ml/min.

Ions were generated by electron impact with an electron energy of 75 eV and an emission current of 0.8 mA, and detected with an electron multiplier voltage of 2000 V. Mass fragmentography was carried out at m/z = 366 for the prenalterol derivative and at m/z = 371 for the internal standard derivative.

Reagents and chemicals

Diethyl ether and toluene, obtained from Fisher Scientific (Pittsburgh, PA, U.S.A.), were purified by distillation. Sodium chloride (analytical grade from Merck, Darmstadt, G.F.R.) was baked at 500°C for 8 h. Pentafluoropropionic anhydride (PFPA) was purchased from Fluka (Buchs, Switzerland) and purified by distillation. Prenalterol was obtained from Ciba-Geigy (Basle, Switzerland) and the internal standard, deuterated prenalterol (H 80/62-$^2H_5$), was obtained from the Department of Organic Chemistry, Hässle, Mölndal, Sweden. Standard solutions of prenalterol and the internal standard were prepared in dilute hydrochloric acid (0.01 mol/l) to produce working standard solutions with concentrations of 1 μmol/l. The carbonate buffer solution, pH 10.8, was prepared by adjusting the pH of a sodium carbonate solution (2 mol/l) by addition of sodium hydrogen carbonate solution (1 mol/l).

Glassware

All glassware was washed in a laboratory dishwasher with detergent at pH 12, rinsed with phosphoric acid solution (pH 2) and deionized water and dried at 60°C.

Determination of the distribution ratio

Distribution ratios (D) for prenalterol between diethyl ether and aqueous buffer solutions (pH 8—12.5, I = 0.10) were determined by shaking in centri-

fuge tubes for 30 min at 25°C. The distribution maximum occurred at pH 9.9, where a distribution ratio of 0.57 was obtained. The concentration of prenalterol was determined by spectrophotometry in the aqueous phase before and after equilibration, and the concentration of prenalterol in the organic phase was calculated from the difference.

The effect of adding sodium chloride to the aqueous phase was also studied. Correction was made for the increase in volume of the aqueous phase after addition of sodium chloride. The pH of the buffer was adjusted to 9.9 as the addition of various amounts of sodium chloride had considerable influence on the pH. The results are shown in Fig. 2.

Analytical procedure

Plasma or urine samples were mixed and centrifuged and 0.1—1.0 ml was transferred to a 15-ml centrifuge tube (fitted with a PTFE-lined screw-cap) containing 100 μl (100 pmol) of the internal standard solution and 0.4—0.5 g of sodium chloride. Sample volumes of less than 1.0 ml were adjusted by adding water. The aqueous phase was buffered to pH 9.9 by adding 100 μl of the carbonate buffer solution, and extracted with 10 ml of diethyl ether. After shaking for 10 min and centrifuging, the organic layer was transferred to a second screw-capped tube and evaporated to dryness at 35°C under a gentle stream of dry nitrogen. The residue was dissolved in 200 μl of toluene and 20 μl of pentafluoropropionic anhydride were added. The reaction mixture was allowed to stand for 60 min at 60°C (or for 90 min at 40°C) and then evaporated to dryness under a gentle stream of dry nitrogen at 35°C. The residue was dissolved in 100 μl of toluene and 4 μl of this solution were injected into the gas chromatograph.

Quantitation

Four reference samples were prepared by adding 100 μl of the prenalterol standard solution (1 μmol/l) to 1.0 ml of blank plasma (or urine). These samples were then analysed according to the analytical procedure. The peak area ratio of the prenalterol derivative over the internal standard derivative was calculated for each chromatogram. The average of the peak area ratios for the reference samples was used for the quantitative evaluation of the authentic samples.

RESULTS AND DISCUSSION

Extraction

Prenalterol is an aminophenol and is preferably extracted at its isoelectric point, calculated to be 10.0 by use of the acid dissociation constants pK_{H_2A} = 9.6 and pK_{HA} = 10.5 (both constants determined by spectrophotometry). This calculated pH optimum is in good agreement with the experimentally found pH optimum. At the isoelectric point the distribution ratio was determined with a weak proton-donating solvent (dichloromethane) and two proton acceptors (ethyl acetate and diethyl ether). The results are summarized in Table I. The highest extraction yield was obtained with ethyl acetate, but, because of excessive interference from coextracted substances with this solvent, diethyl ether was chosen for the extraction procedure. The theoretical recovery of the

TABLE I

DISTRIBUTION OF PRENALTEROL BETWEEN DIFFERENT ORGANIC SOLVENTS AND AQUEOUS BUFFER (I = 0.10) AT pH 9.9.

Solvent	D	log D	Percentage prenalterol at V_{org}/V_{aq} = 8
Diethyl ether	0.5	—0.30	80
Dichloromethane	0.14	—0.84	53
Ethyl acetate	5.6	+0.75	98

extraction using a phase volume ratio (V_{org}/V_{aq}) of 8, as proposed in the method, is 80%. This can be improved by the addition of sodium chloride to the aqueous phase (Fig. 2). Thus, after saturation of the aqueous phase with sodium chloride (0.36 g/ml) and with a phase volume ratio of 8, 98.5% of the prenalterol will be extracted into the organic phase.

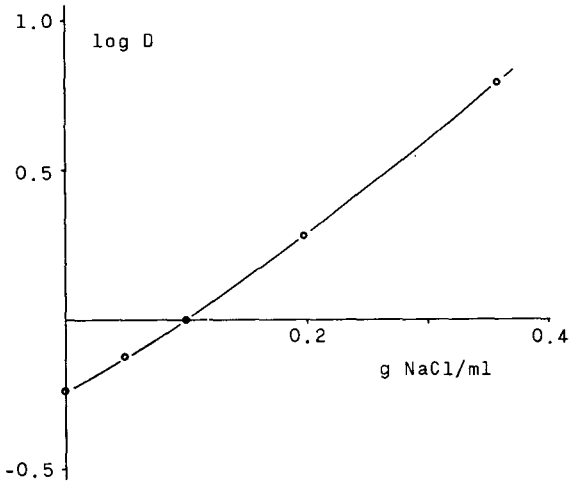

Fig. 2. Distribution ratio (D) of prenalterol between diethyl ether and an aqueous phase (buffer solution pH 9.9) containing sodium chloride.

Derivatization

Trifluoroacylation has been applied to the determination of 4-hydroxypropranolol in a method which involves extraction with buffer to remove the excess of reagent before the gas chromatographic procedure [4]. In order to obtain a derivative with increased stability and avoid the treatment with aqueous buffer, which might be hazardous, we have chosen pentafluoropropionic anhydride as reagent. The pentafluoropropionyl derivative of prenalterol is stable during evaporation of the excess of reagent. The long-term stability of this derivative is good, and a solution in toluene stored in a refrigerator for six days shows no degradation. Gas chromatographic—mass spectrometric determinations during this period gave results with a relative standard deviation of 1.5% (n=6). A corresponding procedure for the trifluoroacyl derivative gave a significantly higher standard deviation.

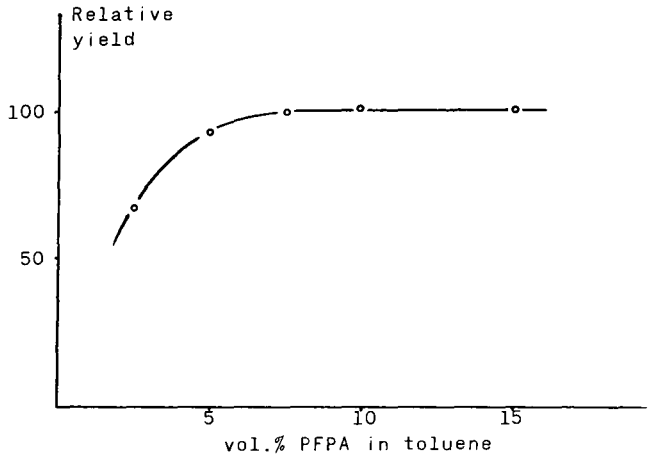

Fig. 3. Influence of the pentafluoropropionic anhydride (PFPA) concentration on the formation of the pentafluoropropionyl derivative of prenalterol. Reaction conditions: 60 min at 60° C.

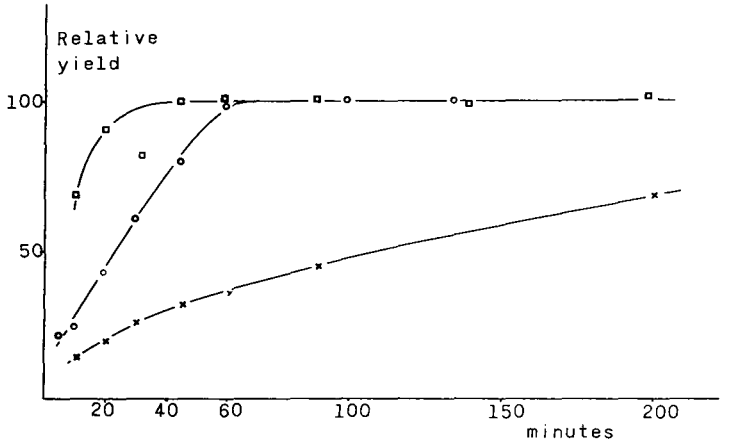

Fig. 4. The formation of the pentafluoropropionyl derivative of prenalterol at different reaction temperatures: X, 22°C; ○, 40°C; □, 60°C.

The influence of reagent concentration and reaction temperature on the pentafluoroacylation of prenalterol in toluene was studied (Figs. 3 and 4) and the structure of the formed derivative was confirmed by mass spectrometry (Fig. 5).

Quantitation

From the electron-impact mass spectrum (Fig. 5) two ions can be selected for quantitative work, m/z = 366 and m/z = 408, both originating from the derivatized aminoalcohol chain, as shown by Garteiz and Walle [7]. The most abundant ion, m/z = 366, was chosen. Consequently, the internal standard, which has an identical fragmentation pattern and incorporates five deuterium atoms in the aminoalcohol chain, will give a corresponding ion with m/z = 371.

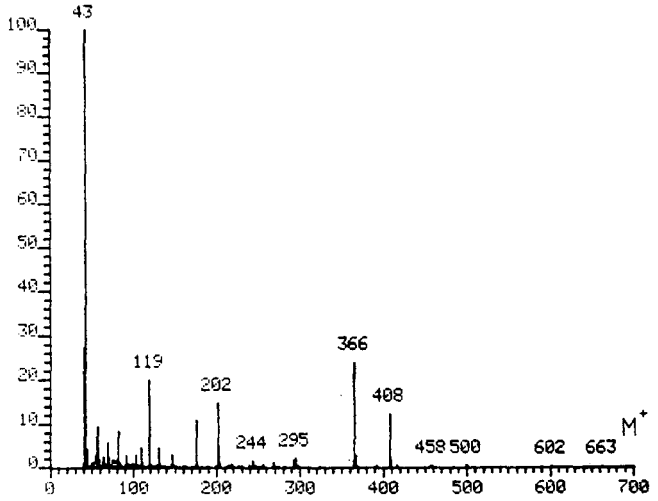

Fig. 5. Mass spectrum of the pentafluoropropionyl derivative of prenalterol. Finnigan MAT 44S, gas chromatography inlet, electron impact, 75 eV.

A chromatogram obtained from analysing an authentic plasma sample is shown in Fig. 6.

Efforts were made to adapt the chemical ionization technique to the quantitation but, although the fragmentation favoured the formation of heavier ions (Fig. 7), these ions were produced in too low yields to give any advantage over the electron-impact ionization technique. Table II shows the relative yield with three of the most commonly used gases in chemical ionization (methane, isobutane and ammonia) compared to electron impact. The mass spectrometer was focused on the most abundant ion in each of the mass spectra (Fig. 7).

Using the electron-impact technique and focusing on m/z = 366, standard

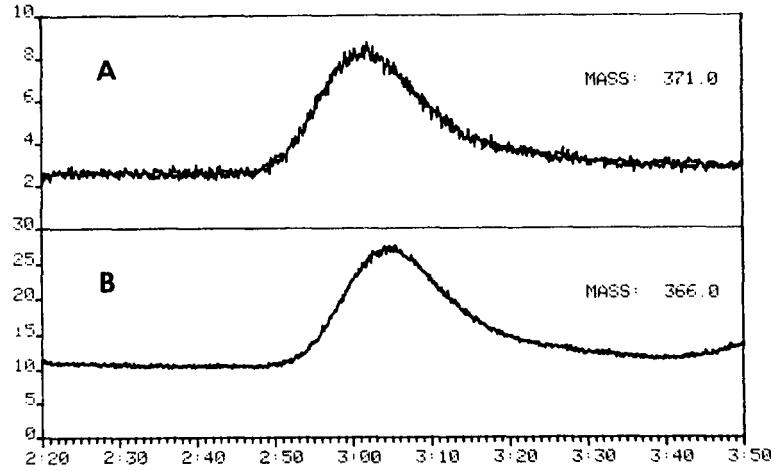

Fig. 6. Selected ion monitoring of the pentafluoropropionyl derivatives of prenalterol (B) and deuterated prenalterol (A). The amount injected on the column corresponds to 4.2 pmol of prenalterol and 2.0 pmol of deuterated prenalterol.

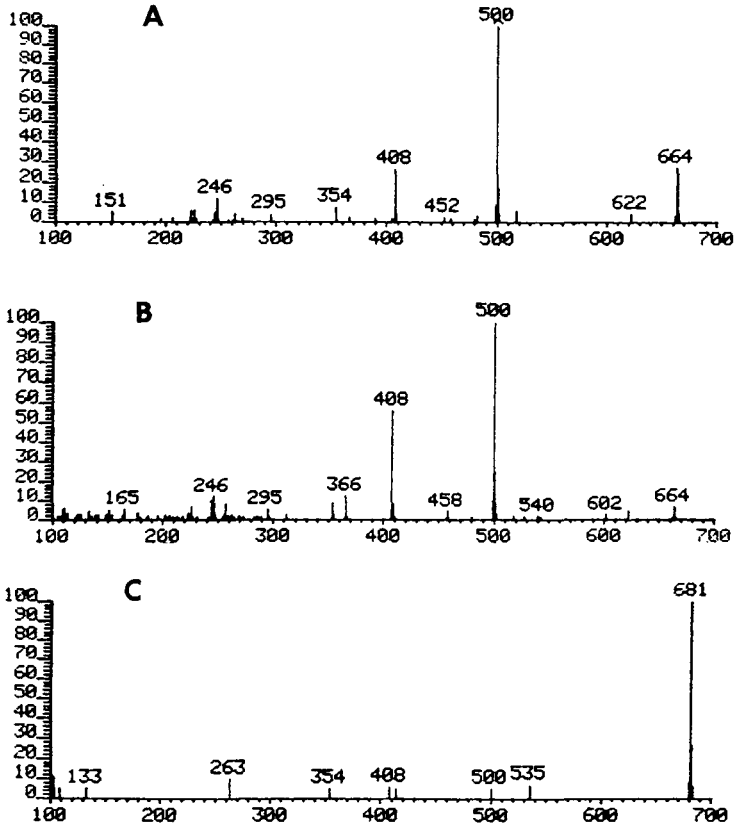

Fig. 7. Chemical-ionization mass spectra of the pentafluoropropionyl derivative of prenalterol with methane (A), isobutane (B) and ammonia (C) as ionization gas.

TABLE II

COMPARISON BETWEEN THE INTENSITY OF THE MOST ABUNDANT ION IN THE MASS SPECTRUM OF THE PFPA DERIVATIVE OF PRENALTEROL USING ELECTRON IMPACT AND CHEMICAL IONIZATION

Ionization mode*	Relative intensity	*m/z*
EI	1.00	366
CI—methane	1.00	500
CI—isobutane	0.25	500
CI—ammonia	0.03	681

*EI = electron impact; CI = chemical ionization.

curves were constructed by analysing plasma and urine samples to which known amounts of prenalterol had been added. The standard curves constructed were straight and passed through the origin, indicating no interferences.

The precision of the method was studied in the concentration range 0—400 nmol/l. The relative standard deviation was 2% in the concentration range

400—100 nmol/l and below 10% down to a concentration of 5 nmol/l of sample. This level (5 nmol/l) was defined as the minimum determinable concentration when 1 ml of sample was used. Long-term inter-assay variation of the method was studied in combination with a stability test for the storage of plasma samples at −18°C. Plasma samples with an added amount of prenalterol to give a concentration of 72 nmol/l were kept at −18°C until the day of analysis. Over a period of six months 60 separate analyses were carried out. The results gave a relative standard deviation of 3.2% at a constant level throughout the period. Regression analysis gave a slope of −0.0028 nmol per l per day.

The method has also been compared with a liquid chromatographic method with electrochemical detection [8]. Thirty-eight authentic plasma samples with concentrations of prenalterol ranging from 5 to 120 nmol/l were assayed by both methods, giving a quotient of 0.99 between the results, and a relative standard deviation of 9%.

ACKNOWLEDGEMENTS

We would like to thank Dr. Bengt-Arne Persson and Prof. Jörgen Vessman for their valuable help and criticism during the preparation of the manuscript.

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CHROMBIO. 1177

SELECTED ION MONITORING OF APOMORPHINE IN WHOLE RAT BRAIN

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SUMMARY

Mass fragmentography was used for quantitation of apomorphine in whole rat brain. Following extraction with 0.45 *N* perchloric acid, the components were derivatized to the O,O-bis(trimethylsilyl) (TMS) derivatives with N,O-bis(trimethylsilyl)trifluoroacetamide at 30°C. The molecular ions of the TMS derivatives of apomorphine (*m/z* 411) and N-*n*-propylnorapomorphine (*m/z* 439), as an internal standard, were assayed simultaneously by selected ion monitoring. Total chromatography time is 5 min. By this method 50 ng/ml can be determined with a coefficient of variation of 12.0%. Application of the method to apomorphine disposition study in whole rat brain was demonstrated.

INTRODUCTION

Apomorphine induces stereotyped behaviour consisting of sniffing, licking or biting in rodents [1]. This behavioral effect is due to a direct action of apomorphine on central dopaminergic receptors [2, 3]. Since antipsychotic drugs such as phenothiazine and butyrophenone analogues block apomorphine-induced stereotypy, this behaviour has been widely used for screening new antipsychotic drugs.

Recent research indicates that apomorphine-induced stereotypy is affected by non-drug factors such as circadian rhythms [4], aging [5, 6], or food deprivation [7, 8]. Effects of non-drug factors on apomorphine-induced stereotypy may be related to the changes in the sensitivity of dopamine receptors or the changes in pharmacokinetics in the body.

In order to determine brain apomorphine levels, it is necessary to develop a sensitive and selective method for determining the compound. Previously reported techniques for measuring apomorphine in biological materials include

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gas—liquid chromatography [9, 10], high-performance liquid chromatography (HPLC) [11, 12], spectrofluorometric assay [13], enzymatic—radioisotopic assay [14], and more recently selected ion monitoring (SIM) [15]. The spectrofluorometric and enzymatic—radioisotopic assays have been predominantly used for determining brain apomorphine concentrations. However, these methods have a limitation in sensitivity and/or selectivity. Therefore, in this study, we applied SIM for measuring apomorphine in whole rat brain.

EXPERIMENTAL

Chemicals and reagents

Apomorphine hydrochloride hemihydrate (APM · HCl) was a gift from Sandoz (Basel, Switzerland). N-*n*-Propylnorapomorphine hydrochloride hemihydrate (PNAPM · HCl) was kindly donated by Dr. R.V. Smith (Drug Dynamics Institute, College of Pharmacy, The University of Texas at Austin, U.S.A.). N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) was obtained from Pierce (Rockford, IL, U.S.A.). Benzene and diethyl ether, analytical grade, and tribasic sodium phosphate and 60% perchloric acid (PCA), reagent grade, were purchased from Katayama (Osaka, Japan).

Extraction of samples

One milliliter of a 0.45 *M* PCA solution of PNAPM (10 μg/ml), as an internal standard, and 4 ml of 0.45 *M* PCA solution were added to whole rat brain samples on an ice bath. The mixture was homogenized using a Kinematica Polytron (setting of 7; PT-10 probe) for 1 min and then the homogenate was shaken for 10 min. After centrifugation (4°C, 8000 *g*, 10 min) the supernatant was transferred to a conical glass tube with PTFE-lined screw-cap. To this tube a mixture of diethyl ether and benzene (5:2, v/v) was added and then shaken for 10 min. After the mixture was centrifuged (4°C, 3000 *g*, 10 min) the organic layer was aspirated and discarded. Three milliliters of the remaining aqueous layer were transferred to a conical glass tube with PTFE-lined screw-cap and adjusted to pH 7.0 ± 0.05 with aqueous saturated tribasic sodium phosphate. Then 5 ml of diethyl ether were added to this tube and the whole was mixed for 15 min. At the end of this period, the mixture was centrifuged (4°C, 1200 *g*, 10 min). A 3-ml volume of the organic layer was taken to dryness under a gentle stream of nitrogen at 30°C.

Preparation of standard curves

With each set of rat brain samples a standard curve was prepared adding 1 ml of APM · HCl solution (0.05, 0.1, 0.3, 0.5, 1.0, 3.0, and 5.0 μg in 1 ml of 0.45 *M* PCA solution), 1 ml of PNAPM · HCl solution (10 μg in 1 ml of 0.45 *M* PCA solution) and 3 ml of 0.45 *M* PCA solution to a drug-free whole rat brain. The standard samples were extracted in the same way as described for whole rat brain samples.

Preparation of derivatives

BSTFA (100 μl) was added to the dried extract, and the mixture was

incubated at 30°C for 60 min. The residual BSTFA was removed in a stream of nitrogen at 30°C, and the dried residue was dissolved in 1 ml of benzene. Of this solution 3 μl were injected onto the gas chromatograph—mass spectrometer.

Gas chromatography—mass spectrometry

Derivatized samples were chromatographed on a silanized glass column (1 m × 2 mm I.D.) packed with 3% OV-17 on silanized Gas-Chrom Q (80—100 mesh, packed by Shimadzu Co., Kyoto, Japan). The column was maintained at 250°C and the injector port at 300 °C. Helium was used as the carrier gas and its flow-rate was set at 25 ml/min.

A Shimadzu AUTO GCMS-6020 fitted with a Shimadzu PAC-500 data system was used. The jet separator was maintained at 300°C. For recording mass spectra the mass spectrometer was operated in the scan mode at an electron energy of 20 eV and 70 eV with an ionization current of 60 μA, scan speed from *m/z* 0 to 800 in 7 sec, and an accelerating voltage setting of 3.5 kV.

Selected ion monitoring

For SIM a JMS-D300 (JEOL, Tokyo, Japan) was used. The instrument was run in the SIM mode at an ionization energy of 20 eV with 300 μA of emission current and an electron multiplier setting of 1.4 kV. The selected ion detector channels were set at *m/z* 411 and 439. These are the molecular ions and the base peaks in the mass spectra of the TMS derivatives of APM (APM derivative) and PNAPM (PNAPM derivative), respectively, at an ionization energy of 20 eV with 300 μA emission current (Fig. 1). Slits were adjusted to a resolution of approximately 500, and filters were set to a peak width of 3 Hz.

Accuracy, precision and sensitivity

Solutions of APM · HCl at 0.05, 0.5 and 4.0 μg/ml and a solution of PNAPM · HCL at 10 μg/ml in 0.45 *M* PCA were prepared. One milliliter of each solution of APM · HCl, 1 ml of the solution of PNAPM · HCl and 3 ml of 0.45 *M* PCA solution were added to a drug-free whole rat brain. The standard samples were analyzed in the same manner as described above. Each sample was analyzed in quintuplicate.

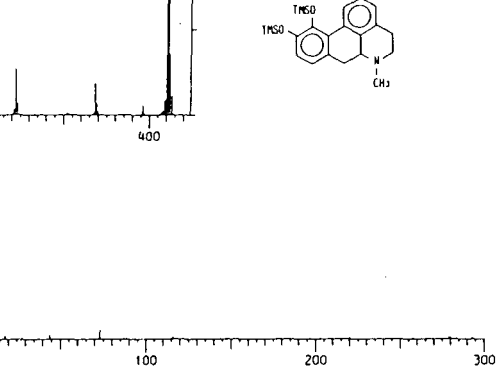
Application of the method to the APM disposition study

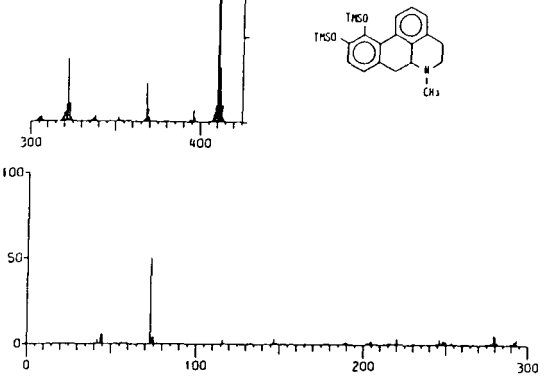
Female Wistar rats, weighing 110—140 g, were sacrificed at 5, 10, 20, 30, 90, and 150 min following subcutaneous administration of 10 mg/kg APM · HCl. Five rats were decapitated at each time interval and brain samples were stored at −20°C until analyzed.

APM concentrations in whole rat brain samples were determined by the method as described.

RESULTS

The TMS derivatives of APM and PNAPM have retention times of 3.12 and 4.15 min, respectively, on the 3% OV-17 column (250°C). The mass spectrum of the APM derivative recorded at 20 eV showed a base peak at *m/z* 411 corre-


TMSO
TMSO
N
CH3
400
100
200
300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TMSO
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CH3
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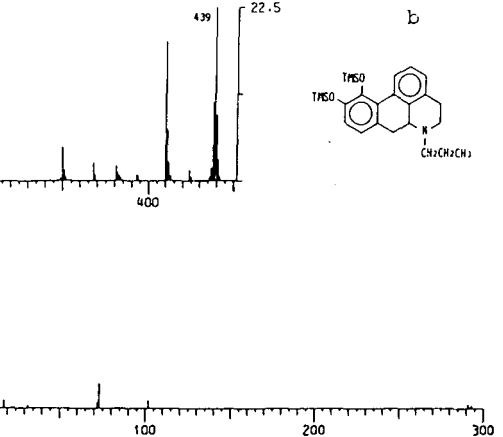
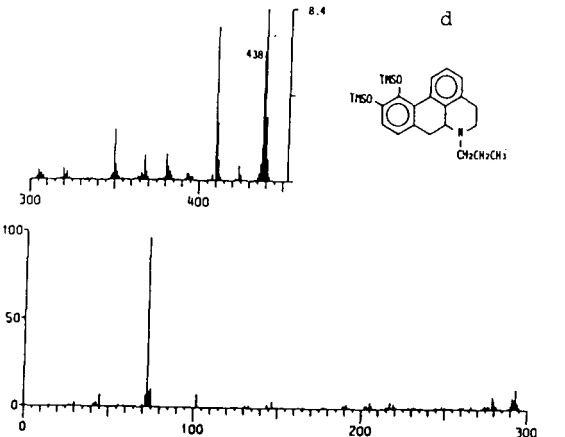

439
22.5
b
TMSO
TMSO
N
CH2CH2CH3
400
100
200
300


438
8.4
d
TMSO
TMSO
N
CH2CH2CH3
300
400
100
50
0
0
100
200
300

sponding to the molecular ion and strong peaks occurring at *m/z* 410 (75.5%), 368 (18.7%) and 322 (27.0%) (Fig. 1a). The fragment ions at *m/z* 410 and *m/z* 368 presumably arise from losses of $H^{\cdot}$ and $CH_2 = N{-}CH_3$ groups, respectively, from the molecular ion. The fragment ion at *m/z* 322 results from the elimination of $Si(CH_3)_4$ from the *m/z* 410 fragment ion. As recorded at 70 eV, the mass spectrum of the APM derivative showed a base peak at *m/z* 410 with a strong molecular ion (*m/z* 411, 91.9%) (Fig. 1c). The fragment ions at *m/z* 368 (22.7%) and *m/z* 322 (36.8%) also appeared (Fig. 1c). The mass spectrum of the PNAPM derivative is characterized by a base peak at *m/z* 439, corresponding to the molecular ion, and strong fragment ions at *m/z* 438 and *m/z* 410 (Fig. 1b and d).

For the purpose of an ion selection employed in the mass fragmentography, a comparison of sensitivity was made by monitoring both the *m/z* 411 ion at 20 eV and the *m/z* 410 ion at 70 eV. These were the base peaks of the APM derivative as recorded at 20 eV and 70 eV, respectively. It was found that the recording of the *m/z* 411 ion at 20 eV was approximately two times more sensitive than the recording of the *m/z* 410 ion at 70 eV. The effect of ionization energy on the peak intensity at *m/z* 411 was also studied. The most intense peak was obtained by monitoring at 20 eV. Therefore, the quantitative assay was performed to record, in the SIM mode, the molecular ions at *m/z*

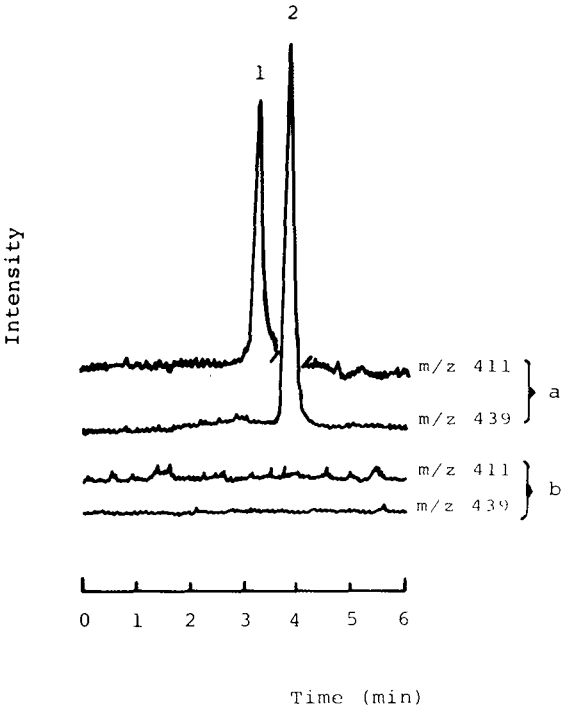


Fig. 2. Selected ion recordings of the ions at *m/z* 411 and *m/z* 439 for (a) the TMS derivatives of extracts of whole rat brain to which were added 0.2 μg/ml APM · HCl and 10 μg/ml PNAPM · HCl, and (b) materials extracted from drug-free whole rat brain. Peaks 1 and 2 correspond to the TMS derivatives of APM and PNAPM, respectively.

411 (APM derivative) and *m/z* 439 (PNAPM derivative, as an internal standard) with an ionization energy of 20 eV. The SIM profile of the TMS derivatives of APM and PNAPM in whole rat brain are shown in Fig. 2 together with the profile of the drug-free whole rat brain extracts. As shown in Fig. 2, no peaks interfering with the monitoring of the ions at *m/z* 411 and *m/z* 439 were present.

TABLE I

ANALYSIS OF REPLICATE SAMPLES, TO WHICH KNOWN AMOUNTS OF APM · HCl WERE ADDED

Added (μg/ml)	Found (μg/ml)	
	Mean ± S.D. (n = 5)	C.V. (%)
0.05	0.050 ± 0.006	12.0
0.50	0.504 ± 0.020	3.97
4.00	4.045 ± 0.102	2.52

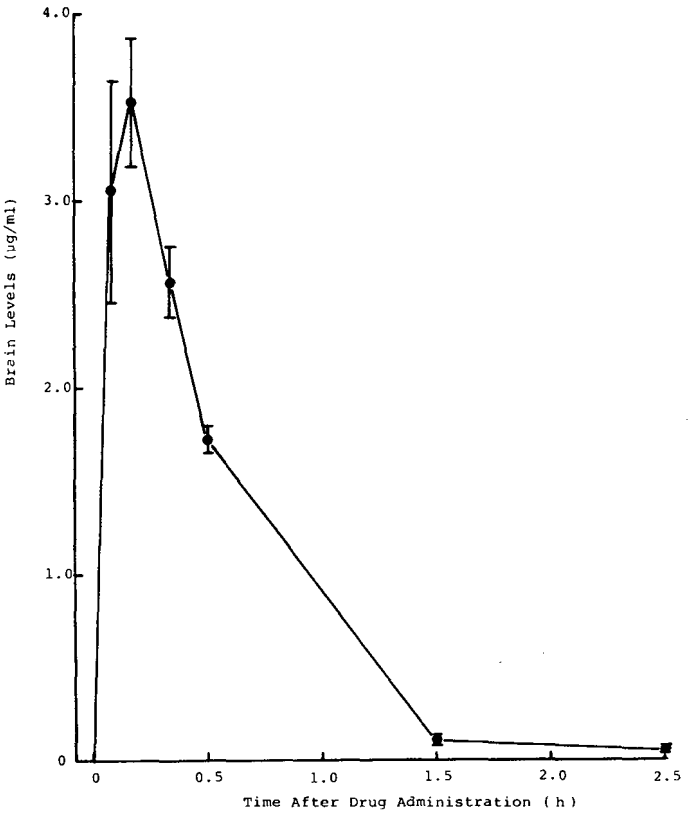


Fig. 3. Concentrations of APM in whole rat brain during 2.5 h after subcutaneous administration of 10 mg/kg APM · HCl. Each point represents the mean ± S.E.M. of five samples.

The standard curve obtained was $y = 2.065x + 0.005$ ($r = 0.999$; $p < 0.01$) and was linear over the concentration range 0.05—5.0 μg/ml APM · HCl.

The accuracy, precision and sensitivity of the method were determined by analysing samples of drug-free whole rat brain to which APM · HCl had been added over the range 0.05—4.0 μg/ml. The results are shown in Table I. The lower limit of detection of the assay was judged to be 0.05 μg/ml, as added APM · HCl concentration to whole rat brain, in which signal intensity was twice as high as the noise value.

As an application of the method to an APM disposition study, a time course of whole rat brain APM levels following subcutaneous administration of 10 mg/kg APM · HCl was examined. The results are shown in Fig. 3. The peak brain APM concentration occurred at 10 min (3.562 ± 0.338 μg/g tissue, mean ± S.E.M.) and then the level of APM declined exponentially. Only a trace amount was detectable at 150 min (0.058 ± 0.016 μg/g).

DISCUSSION

When we re-examined the extraction procedure suggested by Kaul et al. [16] for the estimation of APM in brain tissue, formation of an emulsion and a reduced recovery of APM due to adsorption of the compound on the emulsion were occasionally observed. To remove these defects, we attempted to employ an extraction procedure with 0.45 *M* PCA solution as an extraction medium for APM in the rat brain. No formation of emulsion was observed and satisfactory results were obtained using the extraction procedure indicated above. The acidic extracts obtained were clean enough to permit the estimation of brain APM levels by SIM. No compound interfering with the monitoring of the ions at *m/z* 411 and *m/z* 439 was present in the acidic extracts of drug-free whole rat brain.

PNAPM, a chemical analogue of APM, proved to be satisfactory for use as an internal standard. The similar chemical properties of APM and PNAPM permit their co-extraction and derivatization in adequate yield.

Since the silylation reaction of APM with BSTFA was simple and proceeded at a relatively low temperature (30°C), no decomposition products were found. The silylation reaction time seems to affect the peak intensity of the ion at *m/z* 411. Therefore, the effect of reaction time on the peak intensity of the ion at *m/z* 411 monitored at 20 eV was examined. APM was reacted with BSTFA for 0.33, 0.5, 1, 1.5, 2, and 21 h at 30°C. A constant peak intensity was obtained after 0.5 h.

Since the peak intensity of the ion at *m/z* 411 was also influenced by the ionization energy, the effect of the ionization energy was examined. The most intense peak was obtained when monitoring at 20 eV. Thus, the ionization energy of 20 eV was employed throughout the experiments described in this paper.

Spectrofluorometric methods, which have been used for determining APM concentrations in biological materials [16—18], lack adequate specificity and sensitivity for APM disposition studies in whole rat brain. The selected ion monitoring method is much more sensitive and specific to APM than spectrofluorometric methods.

The range of values obtained for whole rat brain APM concentrations following subcutaneous administration of 10 mg/kg is reasonable when compared with other published data [14] (Fig. 3). As shown in Fig. 3, it is suggested that this selected ion monitoring method is sensitive enough to allow determination of APM concentrations in whole rat brain following an APM · HCl injection at a dosage of less than 10 mg/kg.

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CHROMBIO. 1176

ELECTRON-CAPTURE GAS—LIQUID CHROMATOGRAPHIC DETERMINATION OF TOCAINIDE IN BIOLOGICAL FLUIDS USING FUSED SILICA CAPILLARY COLUMNS

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SUMMARY

Gas—liquid chromatography using capillary columns and electron-capture detection has been employed for the determination of a new antiarrhythmic drug, tocainide, in rat plasma and urine. The drug is extracted from a basified solution along with an internal standard, monoethylglycine xylidide, and subsequently reacted with heptafluorobutyric anhydride. The 50 m × 0.2 mm fused silica capillary column was coated in the laboratory with Carbowax 20M. Linearity of detector response was established in the range of 50—1000 ng of tocainide hydrochloride per 100 μl of plasma or urine. This represented 14—270 pg of the free base at the detector, using a split ratio of 1:25 and an injection volume of 2 μl. The derivatization method and chromatographic assay are well suited for monitoring of plasma or urine samples. The applicability of the method is demonstrated by the analysis of rat plasma collected over a period of 7 h after intravenous administration of 20 mg/kg of tocainide hydrochloride.

INTRODUCTION

Tocainide, 2-amino-2′,6′-propionoxylidide (I), is a primary amine analogue of lidocaine and an effective antiarrhythmic agent with complete oral bioavailability. For many years lidocaine has been the agent of choice for the treatment of ventricular arrhythmias, especially those occurring early after an acute infarction [1]. However, lidocaine cannot be administered orally due to extensive first-pass metabolism in the liver [2]. Tocainide, on the other hand, has been shown in clinical trials to be effective in the treatment of ventricular tachyarrhythmias by both the oral and intravenous routes in man [3—6]. Convenient oral dosage regimens are also possible with tocainide due to its long plasma half-life of approximately 11—14 h [4, 7]. Like many other antiarrhythmic drugs, tocainide exhibits steep plasma concentration—anti-

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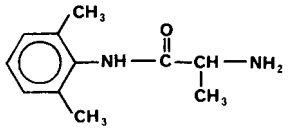
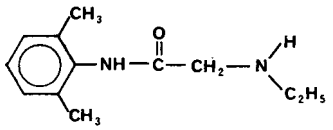
arrhythmic response curves [8] and careful dosage adjustment is necessary for effective treatment.

Analytical methods for the determination of tocainide in biological fluids have included high-performance liquid chromatography (HPLC) [9, 10] and gas–liquid chromatography (GLC) with flame ionization [4] or electron-capture detection [11, 12]. Many of these methods suffer from drawbacks such as tedious sample preparation [9], poor sensitivity [10] or the potential for interference from endogenous substances [11]. Since many of the anti-arrhythmic agents are used in combination with other antiarrhythmics, blood concentration monitoring may become more difficult with conventional packed-column analyses. The limitations of these methods prompted the development of a capillary-column GLC method that combined simple sample preparation, high selectivity, and sufficient sensitivity to measure the levels of tocainide in small volumes of rat plasma.

The analytical methodology described herein employs a fused silica capillary column, wall-coated with Carbowax 20M. Tocainide and the internal standard, monoethylglycine xylidide (II), a metabolite of lidocaine, are extracted and converted to their monoheptafluorobutyrate derivatives for chromatographic assay.



(I)



(II)

EXPERIMENTAL

Materials

Tocainide hydrochloride (Astra Pharmaceuticals, Mississauga, Canada), monoethylglycine xylidide (Astra Pharmaceutical Products, Worcester, MA, U.S.A.), α-bromonaphthalene (ICN Pharmaceuticals, Plainview, NY, U.S.A.), Carbowax 20M (Alltech Assoc., Arlington Heights, IL, U.S.A.) and heptafluorobutyric anhydride (Pierce, Rockford, IL, U.S.A.) were used without further purification. Water and *n*-hexane were of HPLC grade (Fisher Scientific, Vancouver, Canada) and dichloromethane and benzene were distilled in glass (Caledon, Georgetown, Canada).

All analyses were carried out on a Model 5830 reporting gas chromatograph (Hewlett-Packard, Avondale, PA, U.S.A.) equipped with split/splitless

injection modes and a ^{63}Ni electron-capture detector operated in the pulsed mode (150 μsec). Helium carrier gas flow-rate was maintained through the capillary column at 1 ml/min with 25 ml/min passing to the split vent. Argon—methane (95:5) was used as the make-up gas for the detector at a flow-rate of 60 ml/min. The column inlet pressure was 1.84 bar (27 p.s.i.). The injection port and detector temperatures were 240°C and 350°C, respectively and the oven was operated isothermally at 180°C. Samples were introduced into the 50 m × 0.2 mm capillary column (Applied Science Labs., State College, PA, U.S.A.) using the split injection mode.

Preparation of fused silica column

The fused silica capillary column was coated by the mercury plug dynamic method [13]. A 2% solution of Carbowax 20M in dichloromethane was passed through the column under pressure from a coating reservoir (Alltech Assoc.) until the solution completely filled the column. A 10-cm plug of mercury was then forced into the column from the reservoir and allowed to pass through the column along with the stationary phase solution. After the last drop of mercury had left the column, the nitrogen flow-rate was increased and maintained for 3 h to remove the solvent. The coated column was transferred to the oven of the gas chromatograph and with a helium carrier gas flow-rate of 1 ml/min, the oven was heated at a rate of 1°C/min from 40°C to 220°C and maintained at this temperature for 24 h.

Stock solutions

Tocainide hydrochloride (1 μg/ml) and monoethylglycine xylidide hydrochloride (10 μg/ml) were prepared in HPLC-grade water. α-Bromonaphthalene (1 μg/ml) was prepared in *n*-hexane. An aqueous solution of tocainide hydrochloride, equivalent to 25 mg of the base, was rendered alkaline with 1 *N* sodium hydroxide and extracted with five 15-ml portions of benzene. The combined extracts were brought to a final volume of 100 ml and an aliquot was subsequently diluted with benzene to yield a final solution equivalent to 1 μg/ml of tocainide base.

Determination of optimum derivatization conditions

To 1 μg of tocainide base, evaporated from a 1-ml aliquot of the benzene extract, were added 100 μl of *n*-hexane and 30 μl of heptafluorobutyric anhydride. Six such samples were prepared and were heated for 15, 30, 45, 60, 75 or 90 min at 55°C in an aluminum block (Dri-Bath, Thermolyne, Dubuque, IA, U.S.A.). The excess reagent was removed under a gentle stream of clean, dry nitrogen and the residue was dissolved in 200 μl of *n*-hexane containing 0.2 μg of the internal standard, α-bromonaphthalene. A 1-μl aliquot was used for analysis.

Determination of linearity and response in plasma and urine extracts

To five 100-μl aliquots of plasma or urine obtained from untreated male Wistar rats were added 50, 100, 200, 500 or 1000 ng of tocainide hydrochloride from an aqueous solution (1 μg/ml). An aliquot of the internal standard solution, equivalent to 1 μg of monoethylglycine xylidide hydrochloride

(10 μg/ml), was added to each tube. Triplicate samples of each concentration were prepared. An aliquot of 0.5 ml of 1 *N* sodium hydroxide was added to each tube and the total volume of the aqueous phase was brought to 2.0 ml with water. Dichloromethane (5 ml) was added to each tube and the tubes were shaken for 15 min on a rotary shaker (Roto-Rak, Fisher Scientific). After centrifugation at 740 *g* for 10 min, 4 ml of the organic phase were transferred to a 15-ml PTFE-lined screw-capped centrifuge tube (Canlab, Vancouver, Canada) and the contents of the tube were evaporated to dryness in a water bath at 40°C under a gentle stream of clean, dry nitrogen. To the residue were added 100 μl of *n*-hexane and 30 μl of heptafluorobutyric anhydride. The tubes were tightly capped and heated at 55°C for 45 min in an aluminum block. The excess reagent was evaporated under a stream of nitrogen and the residue was reconstituted in 200 μl of *n*-hexane. A 1—2 μl aliquot was used for analysis.

In-vivo study

A solution of tocainide hydrochloride was prepared in normal saline (10 mg/ml) and a dose of 20 mg/kg was administered intravenously to a male Wistar rat (250 g). A jugular vein cannula, inserted one day prior to drug administration was used for dosing and blood collection. The cannula was flushed with an isotonic heparin solution (20 units) after dosing and after each blood collection. Blood samples (200 μl) were collected in Caraway capillary tubes (Sherwood Medical Industries, St. Louis, MO, U.S.A.) and centrifuged at 1000 *g* for 10 min. The plasma was separated and stored at −20°C until required. Tocainide and the internal standard were extracted from a basified solution of plasma (20—100 μl, depending on the volume of plasma available) and were converted to their monoheptafluorobutyrate derivatives as described above.

RESULTS AND DISCUSSION

The utilization of capillary column gas chromatography for the analysis of samples of biological origin has become more prevalent in the recent literature. The technique has been applied for steroid [14, 15] and fatty acid [16] profiling in normal and pathological conditions, and has recently been used for the analysis of a series of ten structurally related equine estrogens in a pharmaceutical product [17]. Many of the problems associated with glass capillary columns, such as fragility and the need for careful surface modification and deactivation, have been overcome with the introduction of fused silica columns in 1979. Such columns are readily coated with the lower polarity stationary phases and, due to the inert surface, facilitate the analysis of many functional groups such as alcohols, phenols, amines and carboxylic acids without derivatization [18]. The method described in the present paper utilizes a fused silica column which was readily coated in the laboratory using the mercury plug dynamic method [13]. Unlike the usual technique of employing a concentrated solution of the liquid phase, a dilute 2% solution of Carbowax 20M was used to deposit a thin film of liquid phase on the column surface.

To determine the optimum conditions required for derivatization of tocainide (I) a series of samples were heated at 55°C for periods varying from 15 to 90 min. The internal standard, α-bromonaphthalene, was chosen as a compound that would not react with heptafluorobutyric anhydride, thereby providing a basis on which to measure the increase in peak area of the tocainide derivative with time. The area ratio of tocainide to the internal standard was maximal and unchanging from 15 to 60 min but was reduced to 65% and less at 75 and 90 min.

For purposes of biological assays, an N-de-ethylated metabolite of lidocaine, monoethylglycine xylidide (II) exhibited a longer retention time than α-bromonaphthalene, and thus would not be subject to potential interference from early eluting peaks in biological samples. A study revealed that the area ratios of the monoheptafluorobutyrate derivatives of tocainide and monoethylglycine xylidide were unchanged over a 7-day period when the derivatives were stored at 4°C.

Detector linearity and assay precision were determined from the calibration curves. Accordingly, triplicate samples of each of five concentrations of tocainide hydrochloride were injected into the gas chromatograph. The data given in Table I show the mean slopes for the calibration curves for plasma and urine. The mean coefficients of variation for plasma and urine were 8.3% and 6.2%, respectively. The differences in the two slope values are due to slight variations in extraction efficiency between plasma and urine. The extraction efficiency for the recovery of tocainide from plasma using similar methodology has been previously reported [11]. Since the present procedure utilizes an internal standard of similar structure to that of tocainide, and quantitation is based on identical handling of standards and samples, it was not considered necessary to determine absolute recovery values again.

The application of the method developed with the fused silica capillary column is illustrated in Figs. 1 and 2. The chromatograms depicted in Fig. 1

TABLE I

CALIBRATION CURVE DATA FOR PLASMA AND URINE

Tocainide* (ng)	Plasma area ratio (± S.D.)**	Urine area ratio (± S.D.)**
50	0.0592 ± 0.0083	0.0612 ± 0.0043
100	0.1100 ± 0.0144	0.1373 ± 0.0074
200	0.2149 ± 0.0020	0.2127 ± 0.0110
500	0.6693 ± 0.0324	0.6376 ± 0.0388
1000	1.2781 ± 0.1157	1.3358 ± 0.1013
Mean slope	1.3058	1.3456
Y-Intercept	−0.0168	−0.0209
Correlation coefficient	0.9989	0.9989

*Each sample contains the same amount of internal standard (1 μg).
**Area ratio determined for drug/internal standard. Standard deviations calculated for three aliquots prepared for each weight of tocainide HCl.

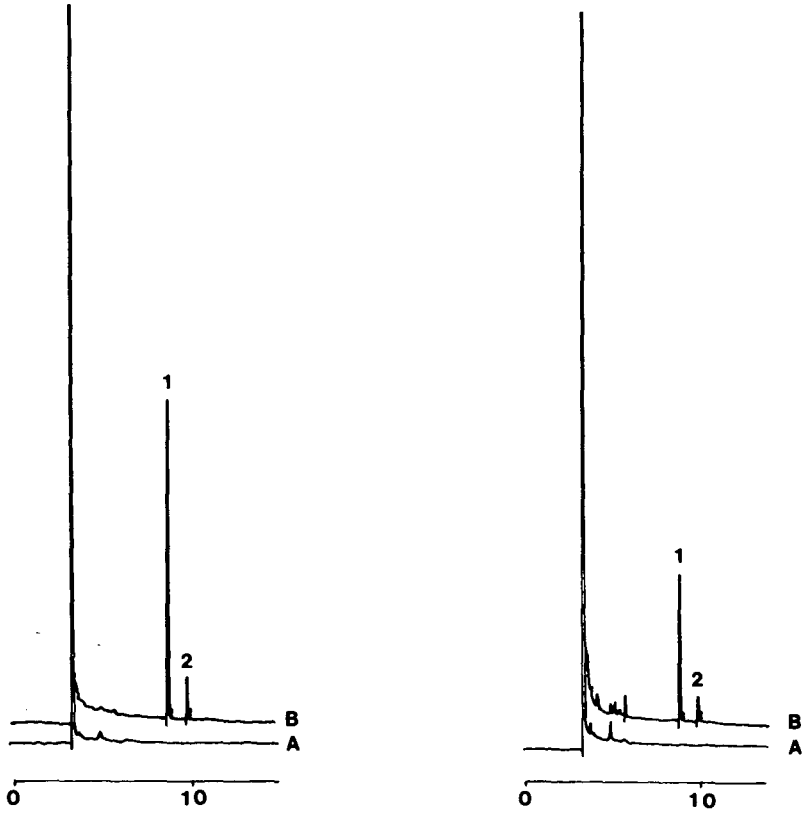


Fig. 1. Chromatograms of (A) rat plasma blank and (B) rat plasma containing 1 μg/ml of tocainide (2) and 10 μg/ml of internal standard, monoethylglycine xylidide (1).

Fig. 2. Chromatograms of (A) rat urine blank and (B) rat urine containing 1 μg/ml of tocainide (2) and 10 μg/ml of internal standard, monoethylglycine xylidide (1).

represent an extraction of blank rat plasma and plasma that contained 1 μg/ml of tocainide hydrochloride. Similarly, Fig. 2 is representative of blank rat urine and urine containing 1 μg/ml of the drug. In both extracts there is no interference from endogenous substances with the peaks due to tocainide or the internal standard. The sensitivity of the method is sufficient to detect as little as 2 pg of tocainide at the detector without appreciable baseline noise. Hence the method allows detection of 1/10 to 1/20 of the amount required for packed-column gas chromatographic methods operated at optimal conditions [11].

The potential for pharmacokinetic investigations in experimental animals is demonstrated by the assay of the drug in rat plasma collected at intervals over a period of 7 h after intravenous administration of a dose of 20 mg/kg to a 250-g male Wistar rat (Fig. 3). The data best fit a two-compartment model, which agrees with previous reports [19], however, further pharmacokinetic parameters were not calculated due to the limited amount of data on a single animal.

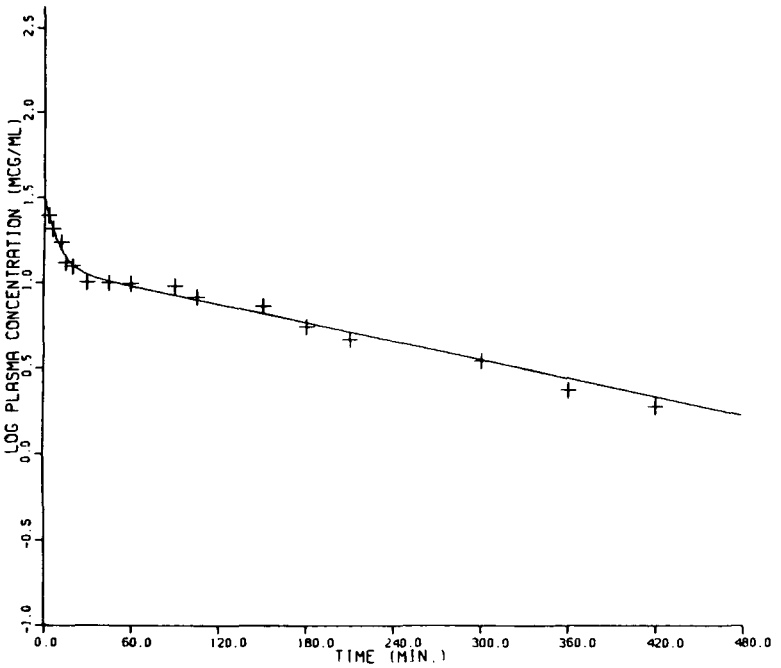


Fig. 3. Plasma tocainide levels in a rat receiving 20 mg/kg of tocainide hydrochloride intravenously.

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SENSITIVE GAS—LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF OXICONAZOLE IN PLASMA

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SUMMARY

A specific and highly sensitive gas—liquid chromatographic method was developed for the determination of oxiconazole in rat, dog, and human plasma. The compound and its internal standard were extracted from plasma at basic pH with *n*-hexane—isoamyl alcohol (98:2, v/v), gas chromatographed on 3% SP-2250/Supelcoport (80—100 mesh) and quantified by means of an electron-capture detector.

Oxiconazole was extracted almost quantitatively from plasma in the concentration range 10—5000 ng/ml. The sensitivity limit was about 1 ng/ml, using a 1-ml specimen.

The method was applied to 13-week tolerance studies in dogs and rats in order to follow oxiconazole concentrations in plasma after oral administration of the compound. The assay was sensitive enough to measure precisely the small amounts of unchanged compound in plasma after intravaginal application of labelled oxiconazole to human volunteers.

INTRODUCTION

Oxiconazole nitrate (Ro 13-8996/001, Sgd 301-76) is being developed by Siegfried, Zofingen, Switzerland and F. Hoffmann-La Roche, Basle, Switzerland. The compound, synthesized by Mixich and Thiele [1], is a new member of a series of 1,4-imidazole derivatives with antimycotic properties [2]. Oxiconazole has, in vitro and in vivo, a broad antifungal spectrum which includes yeasts and dermatophytes [3]. Topical application forms against dermatomycoses and vaginal candidiases are now under clinical evaluation.

Chemically, oxiconazole nitrate is the *Z*-isomer of 2′,4′-dichloro-2-(im-

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idazole-1-yl) acetophenone-O-(2,4-dichlorobenzyl)-oxime nitrate (Fig. 1). Several methods for the determination of imidazole derivatives with antimycotic activity are described in the literature, including photometry [4], microbiology [4—6], thin-layer densitometry [4] and high-performance liquid chromatography [7, 8].

We describe a specific and highly sensitive gas—liquid chromatographic (GLC) method with electron-capture detection for the determination of oxiconazole in dog, rat, and human plasma. The assay was sensitive enough to measure precisely the small amounts of unchanged oxiconazole in plasma after topical application of the compound.

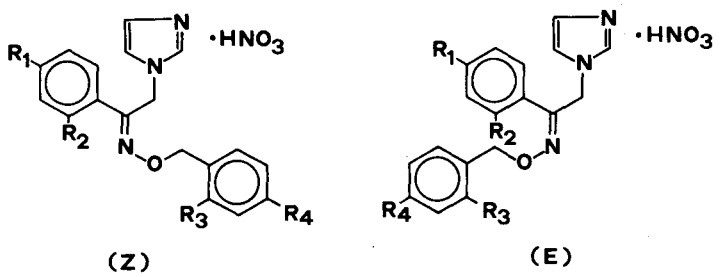

Fig. 1. Chemical structures of oxiconazole nitrate and of some analogues used in the assay.

Compound	Isomer	R_1	R_2	R_3	R_4
Oxiconazole nitrate	*Z*	Cl	Cl	Cl	Cl
Ro 13-8997/001	*E*	Cl	Cl	Cl	Cl
Ro 14-1023/001	*Z*	Cl	Cl	H	Cl
Sgd 149-76	*Z*	I	H	Cl	Cl

EXPERIMENTAL

Materials

n-Hexane (for pesticide residue analysis), isoamyl alcohol (p.a.), ethyl acetate (p.a.), ethanol (p.a.), methanol (p.a.) and potassium hydroxide (p.a.) were purchased from E. Merck, Darmstadt, G.F.R. SP-2250 (3%) on Supelcoport (80—100 mesh) was used as stationary phase (Supelco, Bellefonte, PA, U.S.A.).

Oxiconazole nitrate, Ro 13-8997/001, Ro 14-1023/001 and Sgd 149-76 were supplied by Siegfried, Zofingen, Switzerland.

The priming solution was prepared by extraction of human blank plasma (5 ml) with ethyl acetate (15 ml) on a rotating shaker (15 rpm, 10 min; Heidolph). An aliquot (10 ml) of the organic phase was transferred to a clean glass bottle.

Plasma standards

According to the wide concentration range of oxiconazole present in biological samples, three sets of plasma standards were prepared (see Table I). Using oxiconazole nitrate, a stock solution was prepared corresponding to 1 mg of the free base per ml in ethanol. The working solutions were obtained by dilut-

ing aliquots of the stock solution with methanol. The plasma standards were prepared by diluting 0.1 ml of the corresponding working solution with blank plasma to 10 ml.

The stock solution could be stored in a refrigerator for about four weeks. Working solutions were prepared prior to use. The plasma standards were divided into 2.5-ml aliquots and stored deep-frozen until required for analysis.

TABLE I

CONCENTRATIONS OF OXICONAZOLE IN WORKING SOLUTIONS AND PLASMA STANDARDS

Calibration set 1		Calibration set 2		Calibration set 3	
Working solutions (μg per 0.1 ml)	Plasma standards (ng/ml)	Working solutions (μg per 0.1 ml)	Plasma standards (ng/ml)	Working solutions (μg per 0.1 ml)	Plasma standards (ng/ml)
0.025	2.5	0.4	40	10	1000
0.05	5	0.8	80	20	2000
0.1	10	1.6	160	40	4000
0.2	20	3.2	320	80	8000
0.4	40	6.4	640		
		12.8	1280		

Extraction procedure

Depending on the expected plasma concentration of oxiconazole, an appropriate quantity of internal standard, potassium hydroxide solution (1%) and sample* was added to a ground-glass stoppered centrifuge tube (see Table II). After the addition of 5 ml of *n*-hexane—isoamyl alcohol (98:2, v/v) the sample was extracted by shaking for 15 min on a rotating shaker (15 rpm; Heidolph) and centrifuged for 10 min (10°C, 1700 *g*; Hettich). An aliquot of the organic layer (4 ml) was transferred to a tapered evaporation tube. The aqueous layer remaining was extracted again as described above and a second fraction (5 ml) of the upper phase was retained.

The combined organic phases were concentrated at 40°C by means of a gentle stream of pure nitrogen until only the isoamyl alcohol remained. After the addition of either 1 ml of ethyl acetate or 1 ml of priming solution (see Table II) 2 μl of the resulting solution were injected for GLC analysis.

Gas—liquid chromatography

Depending on the expected plasma concentration of oxiconazole two different gas chromatographic systems were used. Samples containing less than 40 ng oxiconazole per ml were analysed manually on a Packard Model 429 gas chromatograph with an electron capture detector (ECD) (^{63}Ni, 10 mCi) coupled to a Model SP 4100 (Spectra-Physics) computing integrator. All other samples were analysed on a Hewlett-Packard GLC system, consisting of a gas

*Either: Plasma standard (calibration), control plasma (quality control), biological sample (analyses), drug-free plasma (plasma blank) or bidistilled water (reagent blank).

TABLE II

QUANTITIES OF PLASMA SAMPLE AND REAGENTS TO BE USED IN THE ASSAY

	Expected concentration range of oxiconazole (ng/ml)		
	2.5—40	40—1280	1000—8000
Volume of sample (ml)	1	0.5—1	0.1
Amount of internal standard (ng) (dissolved in 20 μl of methanol)	20 (Ro 14-1023) or 75 (Sgd 149-76)	320 (Ro 14-1023) or 1000 (Sgd 149-76)	1000 (Sgd 149-76)
Volume of 1% KOH (ml)	0.5	0.25—0.5	0.05
Type of reconstitution medium	Ethyl acetate	Ethyl acetate	Priming solution

chromatograph with a 15-mCi ^{63}Ni ECD (HP 5700 A), an automatic sampler (HP 7671 A) and a data system for integration and sampler control (HP 3352 B).

The column, a coiled glass tube 1 m × 3 mm I.D., was treated for 10 min with a solution of 2% dimethylchlorosilane in toluene. After having been washed with toluene, methanol and acetone the deactivated column was dried at 100°C and packed with 3% SP-2250 on 80—100 mesh Supelcoport.

The packed column was conditioned for about 10 min at 60°C with a nitrogen flow-rate of 40 ml/min, then "baked" for 3 h at 340°C under "no flow" conditions [9] and then, finally, held at 310°C for 2 days with a nitrogen flow-rate of 40 ml/min.

The instrumental parameters used in this assay were: carrier gases, nitrogen (Packard 429), argon—methane (9:1) (HP 5700 A); carrier gas flow-rates, 40 ml/min; column oven temperature, 270—290°C; injector temperature, 300°C; detector temperature, 350°C.

To maintain the chromatographic system in a deactivated state and, therefore, achieve reproducible chromatography, it was necessary to change the septum, the liner, and the silanized glass-wool at the top of the column every three days.

Calibration and calculation

Only biological samples containing oxiconazole levels within the ranges 2.5—40, 40—1280 or 1000—8000 ng/ml were analysed in the same run. Along with these samples, the corresponding calibration set 1, 2 or 3 consisting of 4—6 plasma standards with appropriate oxiconazole concentrations was carried through the procedure (see Table I). A calibration curve was obtained by a least-squares regression of the peak-height ratios of oxiconazole/internal standard versus oxiconazole concentrations.

This internal standard curve was then used to interpolate unknown concentrations of oxiconazole in the biological samples from the ratio of measured peak heights of the compound and the internal standard.

RESULTS AND DISCUSSION

Specificity and choice of internal standard

The method was developed for the determination of oxiconazole in plasma of three different species; namely, rats, dogs and human volunteers.

Several blank plasma samples from different rats, dogs and human subjects were analysed as described before. In all cases clean plasma extracts were obtained, indicating specificity of the assay with respect to other endogenous components present in plasma (see Fig. 3).

A different metabolic pattern was observed in biological samples depending on the type of species investigated and the quantity of oxiconazole administered. In no case was interference of co-extracted metabolites with the parent compound observed.

Depending on type and amount of co-extracted metabolites, two internal standards with different retention characteristics were used (see Figs. 1 and 2). Whenever possible, Ro 14-1023 was preferred because a short chromatography time (7 min) was obtained even at a column temperature of 270° C (see Figs. 4 and 5). The retention time of Sgd 149-76* was longer than that of Ro 14-1023. The former compound was only used as an internal standard when co-extracted metabolites interfered with Ro 14-1023 (see Fig. 6). An acceptable run time (9 min) was obtained by chromatographing the plasma extracts

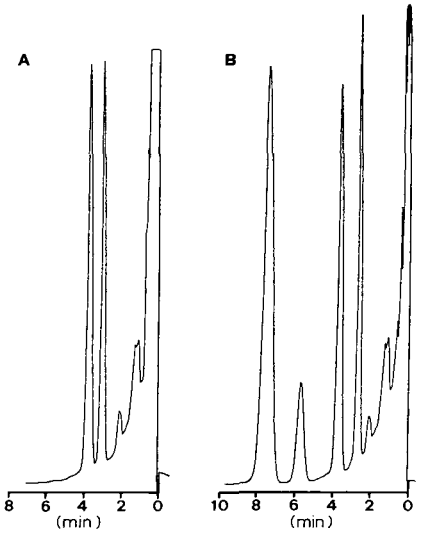

Fig. 2. Chromatograms of spiked blank plasma samples containing: (A) 50 ng of oxiconazole nitrate and 50 ng of Ro 13-8997/001 per ml of plasma; (B) 50 ng of oxiconazole nitrate, 50 ng of Ro 14-1023/001 and 125 ng of Sgd 149-76 per ml of plasma. Oven temperature, 280°C; range, 4; attenuation, 10. Retention times (min): Ro 14-1023, 2.6; Ro 13-8997, 3.0; oxiconazole, 3.7; impurity, 5.7; Sgd 149-76, 7.5.

*Sgd 149-76 contained an impurity which was well separated from the other peaks and did not affect the reproducibility of the assay (see Fig. 2B).

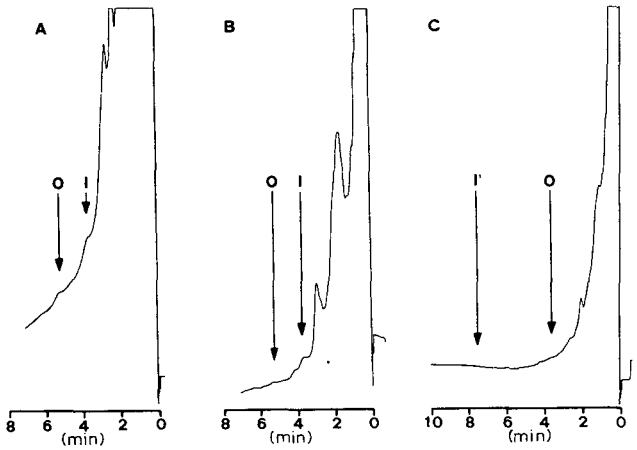

Fig. 3. Chromatograms of blank plasma samples from rat (A), man (B) and dog (C). Oven temperature, 270°C (A and B), 280°C (C); range, 2; attenuation, 10. The arrows indicate the retention times of oxiconazole and the internal standards. O = oxiconazole, I = Ro 14-1023, I' = Sgd 149-76.

at a higher column temperature (280—290°C). Further increase of the temperature (> 290°C) led to decreased deactivation of the chromatographic system in a short time which, in turn, led to deterioration of the peak shape and a decrease of the linear range.

Ro 13-8997, a stereoisomeric form of oxiconazole was tried as an internal standard. Separation from the oxiconazole peak only occurred on well-deactivated columns with high efficiencies (see Fig. 2A). For this reason, further trials with Ro 13-8997 were not continued.

Limit of detection

The limit of detection, defined by a signal-to-noise level of approx. 3:1, was 1 ng of oxiconazole per ml of plasma using a 1-ml specimen. This minimum detectable concentration was equivalent to an absolute amount of 2 pg of oxiconazole per injection, indicating an excellent ECD response of the compound.

The intra-assay precision for the detection limit was determined by analysing four different spiked plasma samples on the same day containing 1 ng/ml oxiconazole. A relative standard deviation (C.V.) of ± 7.3% was obtained.

Linearity

As already mentioned, plasma levels of oxiconazole varied over a wide range (2—2500 ng/ml) when the compound was administered orally to rats and dogs in 13-week tolerance studies. Three linear subranges with different extraction and calibration procedures (see Tables I and II) were established for the following reasons:

(1) Limitation of the absolute amount of oxiconazole injected on to the column in order to prevent "memory effects" (reversible and irreversible adsorption of oxiconazole to the glass wool) and saturation of the ECD.

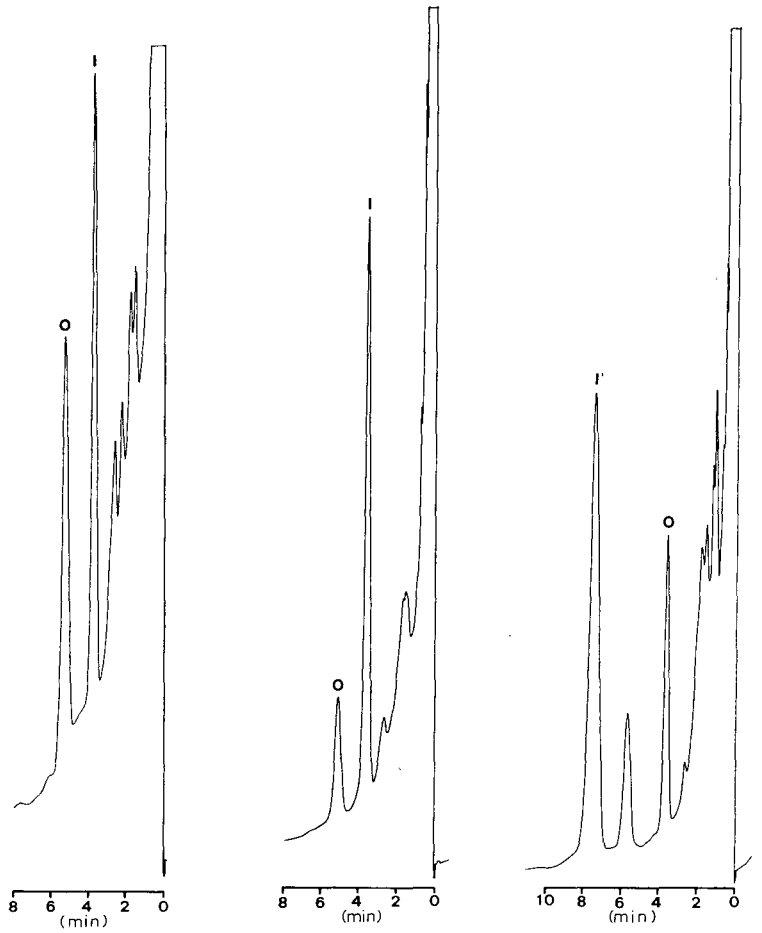

Fig. 4. Chromatogram of a rat plasma sample collected four weeks after daily oral administration of 35 mg/kg oxiconazole nitrate; oxiconazole concentration = 51 ng/ml. O = oxiconazole, I = Ro 14-1023 (internal standard); oven temperature, 270°C; range, 2; attenuation, 10.

Fig. 5. Chromatogram of a female human plasma sample collected 24 h after a single intravaginal application of 150 mg of ^{14}C-labelled oxiconazole nitrate (107 μCi); concentration of unchanged oxiconazole = 32 ng/ml. O = oxiconazole, I = Ro 14-1023 (internal standard); oven temperature, 270°C; range, 2; attenuation, 10.

Fig. 6. Chromatogram of a dog plasma sample collected three days after daily administration of 150 mg/kg oxiconazole nitrate; oxiconazole concentration = 52 ng/ml. O = oxiconazole, I' = Sgd 149-76 (internal standard); oven temperature, 280°C; range, 2; attenuation, 10.

(2) Adjustment of the amount of internal standard added to the sample to achieve reasonable values for oxiconazole/internal standard peak height ratios.

(3) Maintenance of linearity over the complete concentration range.

In each subrange an acceptable linear correlation between the peak height ratios of oxiconazole/internal standard versus concentrations of oxiconazole was observed. Table III indicates that the coefficient of determination (r^2) showed a good day-to-day reproducibility. The intercepts of the calibration curves did not differ significantly from zero.

Oxiconazole was very sensitive to adsorption sites in the chromatographic system: direct chromatography of methanolic oxiconazole solutions resulted, after a few days, in tailing peaks and non-linear calibration curves. Only in the presence of column deactivating plasma components (lipids, lecithin, etc.) were gaussian peak shapes, and thus linearity between detector response and oxiconazole concentration, obtained ("priming effect").

Even the amount of priming agents co-extracted together with oxiconazole from plasma was critical in the assay: when small plasma volumes (0.1 ml) were analysed, a non-linear standard curve was sometimes obtained when the plasma extract was reconstituted in ethyl acetate. Priming of the chromatographic system before and between the analysis by repeated injections of either lecithin [10], phospholipids [11] or plasma extracts [12] did not overcome the problem completely. Therefore, in the case of small plasma volumes, sufficient amounts of deactivating plasma components were added to the sample *after* extraction by use of a priming solution as reconstitution medium.

TABLE III

REPRODUCIBILITY OF THE COEFFICIENT OF DETERMINATION (r^2) ESTABLISHED ON FIVE DIFFERENT DAYS COVERING A PERIOD OF ABOUT SIX WEEKS

Day	Concentration range of oxiconazole		
	2.5—40 ng/ml	40—1280 ng/ml	1—8 μg/ml
1	0.9998	0.9997	0.9995
2	0.9998	0.9994	0.9998
3	0.9996	0.9999	1.0000
4	0.9987	0.9998	0.9999
5	0.9999	0.9998	0.9999
Mean ± S.D.	0.9996 ± 0.00049	0.9997 ± 0.00019	0.9998 ± 0.00019

Recovery

The recovery (extraction yield) was determined from the difference between the peak height ratio when oxiconazole was added to the plasma (the internal standard being added to the final extract) and the peak height ratio when both were added to the final extract of blank plasma.

Table IV indicates that the compound is extracted nearly quantitatively from human and dog plasma in the concentration range investigated. The extraction yield for high concentrations of oxiconazole (> 1000 ng/ml) is enhanced because in this case a 0.1-ml instead of a 1-ml plasma sample was extracted according to the extraction procedure described above (see Table II).

TABLE IV

EXTRACTION YIELD OF OXICONAZOLE FROM DOG AND HUMAN PLASMA

Concentration (ng/ml)	Human plasma (n = 3)		Dog plasma (n = 3)	
	Recovery (%)	C.V. (%)	Recovery (%)	C.V. (%)
5000	96.3	1.7	97.5	1.0
250	89.5	2.1	90.5	1.0
50	90.3	4.4	90.9	2.1
10	92.7	3.1	92.2	1.7

Stability of oxiconazole in human plasma

Oxiconazole was added to blank plasma at three different concentrations (250, 50 and 10 ng/ml) and stored at different temperatures for different time intervals (five months at −20° C, one day at +20° C). A set of five freshly prepared control samples was analysed together with five stored samples of the same concentration.

Table V illustrates that in no case was a significant difference detectable between the results of stored and control samples.

TABLE V

STABILITY OF OXICONAZOLE IN HUMAN PLASMA (n = 5)

Sample	Conc. (ng/ml)	C.V. (%)	Difference from control (%)
Control	250	1.4	
1 day, 20° C	248	0.5	−0.8
5 months, −20° C	248	2.4	−0.8
Control	50	1.9	
1 day, 20° C	50.2	3.9	+0.4
5 months, −20° C	50.3	2.8	+0.6
Control	10	3.5	
1 day, 20° C	9.74	1.4	−2.6
5 months, −20° C	9.70	5.5	−3.0

Reproducibility

The reproducibility of the internal standard method was evaluated over a concentration range of 10—5000 ng of oxiconazole per ml of plasma.

The intra-assay reproducibility was obtained by analysing five specimens from each concentration on one day. The inter-assay reproducibility was determined by analysing one specimen from each concentration over five days within a period of about six weeks.

The data presented in Table VI indicate that the precision (C.V. of replicate analyses) and accuracy (difference between found and expected concentration) were acceptable over the concentration range investigated.

TABLE VI

REPRODUCIBILITY (n = 5)

Conc. added (ng/ml)	Conc. found (ng/ml)	C.V. (%)	Difference between found and added conc. (%)
Inter-assay reproducibility			
5000	4897	1.4	−2.1
250	242.6	1.2	−3.0
50	50.7	6.8	+1.4
10	10.1	6.7	+1.0
Intra-assay reproducibility			
5000	4829	2.0	−3.4
250	246.8	4.3	−1.3
50	50.8	3.7	+1.6
10	9.6	5.5	−4.0

Application of the method to biological samples

The method described was used for the analyses of dog and rat plasma samples in 13-week tolerance studies and for the determination of the parent compound in plasma after intravaginal application of labelled oxiconazole to female human volunteers. Figs. 4—6 show typical chromatograms from these studies, demonstrating the validity of the new assay.

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CHROMBIO. 1174

ASSAY OF AN ACTIVE METABOLITE OF 6-THIOGUANINE, 6-THIOGUANOSINE 5′-MONOPHOSPHATE, IN HUMAN RED BLOOD CELLS

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SUMMARY

A flow-fluorimetric, liquid chromatographic assay for 6-thioguanosine 5′-monophosphate (TGMP) in human red blood cells (RBCs) has been developed. This compound is an active metabolite of 6-thioguanine, 6-mercaptopurine and the immunosuppressant, azathioprine.

The samples were prepared for chromatography by a novel isolation method which extracts the RBC constituents into an organic solvent and leaves the TGMP in the aqueous layer where it is oxidised to give a highly fluorescent species which is then separated by liquid chromatography.

The method is sensitive to below 200 ng ml^{-1} in RBCs which is below the levels encountered following a therapeutic dose of 6-thioguanine or azathioprine. The assay is simple and rapid enough for routine use.

INTRODUCTION

The initial step in the anabolism of the antileukaemic drug, 6-thioguanine is its conversion to 6-thioguanosine 5′-monophosphate (TGMP), which has been identified as the major metabolite accumulating in susceptible tumour and leukaemic cells [1]. This accumulation in leukaemic cells is probably important as most of the evidence indicates that 6-thioguanine exerts its cytotoxicity through the incorporation of TGMP into the cell DNA [2—4]. The mechanism of action of 6-mercaptopurine, another commonly used antileukaemic agent has also been attributed to its metabolism, via 6-thioinosine 5′-monophosphate, to TGMP, and its incorporation as such into DNA [5].

As the intracellular level of TGMP appears to be important for cytotoxic effects of both 6-thioguanine and 6-mercaptopurine, its measurement would be

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expected to yield clinically useful data concerning such factors as dosage schedules and resistance aquisition. In the absence of analytical techniques for such monitoring, valuable time may be lost by ineffective treatment through inadequate dosage or failure to identify a resistant leukaemia.

In an attempt to remedy this situation a simple, rapid assay for clinical RBC levels of TGMP has been developed using single solvent, anion-exchange liquid chromatography with flow-fluorimetric detection of the oxidised metabolite, following partial isolation from the RBC constituents by a novel solvent extraction method.

EXPERIMENTAL

Chromatography

Oxidised TGMP was separated from RBC constituents and other metabolites by anion-exchange chromatography using a Whatman Partisil 10 SAX (particle size 10 μm) strong anion-exchange column 250 mm × 4.8 mm I.D. (Jones Chromatography, Glamorgan, Great Britain), and an isocratic solvent system of methanol and 125 m*M* potassium phosphate—250 m*M* potassium chloride (1:2, v/v) at a constant flow-rate of 3 ml min^{-1} and a pressure of 200 bar using an Altex Model 110 A pump.

The separated TGMP was detected using a Schoeffel FS-970 flow spectrophotofluorimeter exciting at 330 nm, and measuring emission through a 389-nm cut-off filter. No other filters were used. The photomultiplier output was measured on a Servoscribe 1S flat bed recorder at 5 mV full scale deflection. The sample injector was a Waters Model U6K. All liquid volumes were delivered using Gilson automatic pipettes P20 to P5000.

Glass tubes used in the extraction procedures were soaked for over 15 h in 30% nitric acid and rinsed in glass-distilled water.

Chemicals and reagents

6-Thioguanosine 5'-monophosphate was a gift from Dr. R.L. Miller of the Wellcome Research Laboratories (Research Triangle Park, NC, U.S.A.). Methanol and dichloromethane were obtained from Rathburn Chemicals (Peebleshire, Great Britain). Phenyl mercury acetate (laboratory agent) was obtained from BDH (Poole, Great Britain) and a 0.3% (w/v) solution in glass distilled, deionised water was prepared weekly. Tetrabutylammonium hydroxide (AnalaR, BDH) in a 40% aqueous solution and hydrogen peroxide (AnalaR, BDH) in a 6% aqueous solution were both used as supplied. A 0.24% w/v solution of potassium permanganate (AnalaR, BDH) in glass-distilled, deionised water was prepared as required. All water used in solution preparation was glass distilled and deionised; all other reagents used were standard analytical grade.

Determination of red blood cell TGMP concentrations

A standard solution of 10 μg ml^{-1} TGMP in water was prepared. Volumes of this (2—20 μl) were added to RBCs (100 μl) to give samples for analysis in the range 200—2000 ng ml^{-1}. This method of adding varying volumes of a fixed concentration standard obviously introduces a small dilution error (less than 1%).

To each of the above samples, 40% tetrabutylammonium hydroxide (100 μl), 5 *M* sodium hydroxide (20 μl) and 0.3% phenyl mercury acetate (200 μl) were added with vortex mixing after each addition. Dichloromethane (3 ml) was added and again each tube was mixed briefly. All tubes were then mixed in a multi-tube vortex mixer for 60 sec. The addition of tetrabutylammonium hydroxide before sodium hydroxide is recommended as this prevents the formation of large pockets of cellular protein which adheres to the tube on addition of dichloromethane leading to inconsistent extractions.

After extracting with dichloromethane, all tubes were then centrifuged at 1400 *g* for 10 min leaving the precipitated protein at the interface. A portion (200 μl) of the upper aqueous layer was then taken for analysis.

To each sample, 0.24% potassium permanganate (100 μl) was added and mixed. The TGMP was completely oxidised within 5 min; however, oxidation times in excess of 10 min led to degradation of the fluorophore. Excess permanganate was destroyed by addition of 6% hydrogen peroxide (10 μl) and the precipitated manganese dioxide thus formed was removed by centrifugation at 1400 *g* for 5 min. Aliquots from the supernatant were injected into the chromatographic system (see Chromatography) using 100 μl per injection.

Oxidised TGMP showed a retention time of approximately 3.5 min.

RESULTS AND DISCUSSION

The linear relationship between peak height and RBC concentration can be seen in Fig. 1. Reproducibility was assessed by measuring duplicate samples for each point in four separate experiments. The mean standard deviation for the whole group of measurements was 6.8% with the largest individual deviation at 7.6%.

Samples from patients receiving azathioprine following renal transplantation were analysed and a typical chromatogram is shown in Fig. 2. The large hump at a retention time of approximately 30 min in the patient's sample is believed, in the absence of standards, to be 6-thioguanosine diphosphate or an unresolved mixture of this and the triphosphate.

The oxidation of TGMP gives a highly fluorescent compound, probably the 6-sulphonate [6]. Detection of this compound in RBCs is not possible by direct fluorimetry due to interference from the physiological constituents. TGMP was separated from interfering substances by a novel isolation method followed by liquid chromatography of the oxidised compound. Using this system, levels of approximately 400 n*M* (200 ng ml^{-1}) are readily measurable which has been shown to be well below those encountered in clinical samples [7].

The extraction of TGMP into non-polar, organic solvents is not possible due to the presence of highly polar groups in the molecule. Phenyl mercury acetate has been shown to react with the thiol groups in 6-mercaptopurine [8] and 6-thioguanine [9] to give non-polar complexes which can be extracted into organic solvents. It was thought that by complexing TGMP in this way and forming an ion-pair with the phosphate group and tetrabutylammonium hydroxide, this might result in a non-polar species which could be extracted in

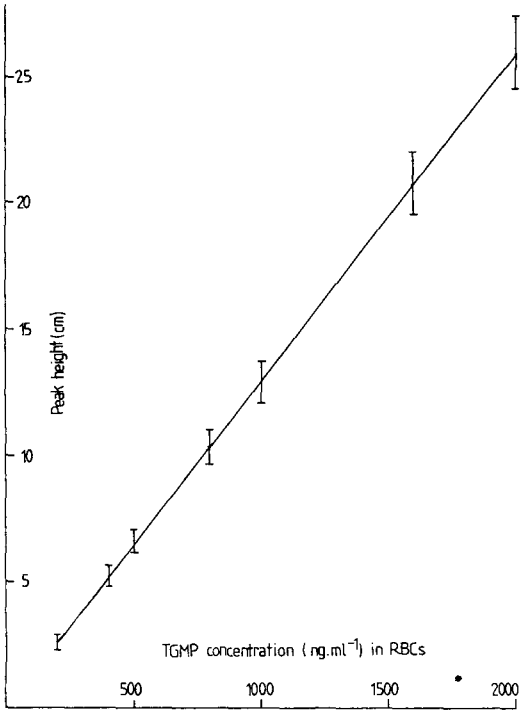

Fig. 1. Standard curve of concentration against peak height for oxidised TGMP from RBCs. Mean standard deviation for all readings was 6.8%, the largest individual deviation was 7.6%. Peak heights (± standard deviation) are converted by sensitivity factors to give readings as though they were in one instrumental sensitivity range.

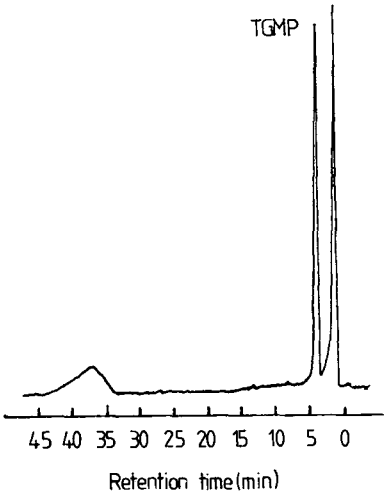

Fig. 2. Chromatogram of an extract from RBCs of a kidney transplant patient treated with azathioprine. The large hump at 30 min is believed to be the diphosphate or an unresolved mixture of the di- and triphosphate.

a similar way to 6-mercaptopurine and 6-thioguanine. However, the TGMP remained in the aqueous layer with no measurable amount in the organic phase which contained the bulk of the RBC constituents. This resulted in an excellent clean-up procedure, isolating TGMP from the RBC constituents for oxidation and chromatography.

Extraction using cold perchloric acid gave insufficient recovery (25%) from 10 μl of RBCs with 1 μg of TGMP.

Oxidation using acidic chromate was less efficient than alkaline permanganate in sample extracts.

Using slower flow-rates gave a longer retention time for TGMP with a better separation and improved sensitivity by approximately 50% (Fig. 3a—c) but it was felt that this increase in sensitivity was worth sacrificing to achieve a more rapid assay for clinical purposes.

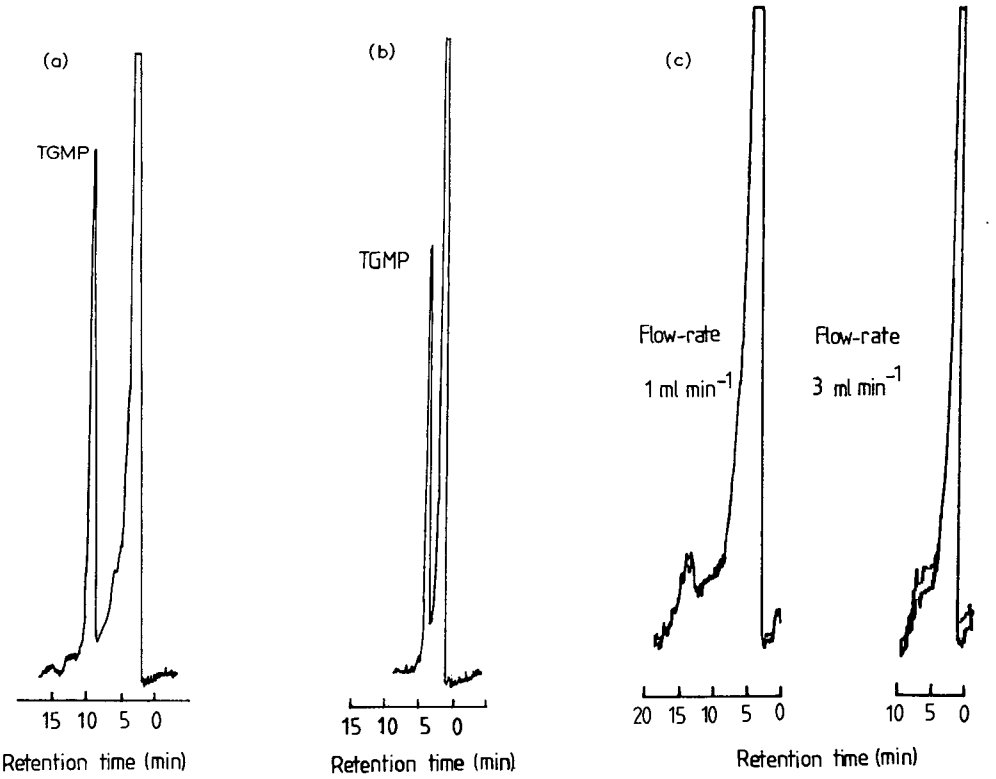

Fig. 3. Chromatograms of (a) an RBC extract using flow-fluorescent detection at a solvent flow-rate of 1 ml min^{-1}; (b) as a, except for flow-rate, 3 ml min^{-1}; (c) blank RBC extract at solvent flow-rates of 1 and 3 ml min^{-1}. Sensitivity of the fluorescence detector × 5 that in (a) and (b).

Deterioration of samples

Deterioration of the fluorophore was approximately 10% over 90 min and was not concentration dependent. For large numbers of samples, therefore, it is easier to oxidise (say) four samples shortly before injection.

Patients' samples stored at —20°C for periods from a few weeks to one year

were assayed and compared with the original results. Considerable degradation of the diphosphate to the monophosphate was seen after about three months. The monophosphate seemed to be stable up to six months.

Stock solutions in glass distilled, deionised water decayed by 10% at 4°C over a period of approximately 15 days. This was monitored and accounted for when plotting the peak height vs. RBC concentration curve by concurrently measuring an oxidised standard solution of TGMP prepared from the same stock solution used to spike the RBC samples. Regular preparation of fresh stock was impossible due to the small supply of TGMP solid.

The use of one stock solution made the monitoring of deterioration easier but resulted in a small dilution error when spiking RBC samples for analysis (see Determination of red blood cell TGMP concentrations).

With a mean standard deviation of ± 6.8% for all peak heights in the concentration range investigated (200—2000 ng ml^{-1}) reproducibility was thought to be high enough to obviate the necessity of an internal standard. By comparison with a standard solution of TGMP the isolation method was shown to be 100% efficient with no inhibition of oxidation by RBC constituents over the concentration range investigated. Factors such a injection error probably account for most of the error and to reduce this by using an internal standard would indeed improve the assay. However, after brief investigation, no suitable compound was found.

No interference was seen from oxidised 6-mercaptopurine added to TGMP extracts. Cytosine arabinoside and daunorubicin, drugs used with 6-thioguanine in leukaemia chemotherapy, do not show any measurable fluorescence at the wavelengths employed in this assay.

Excellent chromatographic separation of 6-thioguanine and its metabolites has been achieved [10] enabling the measurement of in vitro cellular levels of these compounds. Also, a highly reproducible, accurate assay for total 6-thioguanine nucleotides has been developed for the analysis of clinical RBC levels [7]. These methods however, employ solvent programming and multi-step separation techniques, respectively and for routine analysis, when time and economic considerations are important, these methods are probably unsuitable. The assay described here is sensitive and reproducible and its rapidity and simplicity of execution lend itself to routine usage.

RBCs are known to supply preformed purines to (amongst other tissues) the bone marrow [11] which is the main site of systemic toxicity of 6-thioguanine in mammals [12]. Following uptake, exogenous guanine is converted within mammalian RBCs to the nucleotide [13]. These factors make RBCs a useful model system for the design of an intracellular TGMP assay. The clinical relevance of RBC concentrations of TGMP has not been confirmed but might represent the magnitude of the supply of the active metabolite to the bone marrow cells. The measurement of leukaemic stem cell concentrations offers, perhaps, a more direct indication of drug effectiveness and indeed, clinical response has been associated with their magnitude using radiolabelled drug [14]. Studies are currently underway in our department to determine the relationship between RBC concentrations of TGMP and clinical response in leukaemic children treated with 6-mercaptopurine.

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CHROMBIO. 1178

SPECIMEN HANDLING AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF FUROSEMIDE

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SUMMARY

A simple high-performance liquid chromatographic method to measure furosemide in plasma and urine is described. Furosemide fluoresces best, but is unstable, at acidic pH and is subject to photochemical degradation. These factors were analysed and the results prompted changes in previously described methods.

All specimens were very carefully protected from light; extraction and acidification were done with acetic acid instead of hydrochloric acid. With these precautions no 4-chloro-5-sulphamoylanthranilic acid was found in biological specimens. The main metabolite was furosemide glucuronide (20% of furosemide excretion). Sensitivity was 0.1 and 0.5 μg/ml for plasma and urine, respectively. The applicability of our method for furosemide studies is demonstrated.

INTRODUCTION

Furosemide (F) is a potent short-acting loop diuretic frequently used in oedematous states. We searched for a reliable method to determine F and possible metabolites in both plasma and urine, for the purpose of pharmacokinetic and pharmacodynamic studies with F in aged patients. A reliable analytical method was deemed necessary, especially for urine samples, because the action of F seems to correlate best with urinary F levels [1—3]. Sensitive methods make use of the fluorescence of F, which is optimal at low pH (4.5), but under these acidic conditions F is rapidly hydrolysed to 4-chloro-5-sulphamoylanthranilic acid (CSA), furfuryl alcohol and other degradation products [6, 7]. This degradation may be even faster if sulphate ions are present [8] as in normal human urine. Human urine is usually weakly acidic, and may be made more acidic by the action of F [9].

Apart from this, mention has been made of photochemical degradation of F

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[6]. Several authors recommend exclusion of light [10—14], mostly without further explanation. Apparently this is necessary during preparation or storage of urine samples only [15].

Some methods of F determination are described only for plasma [16—19], and no degradation problems are mentioned in these papers. Nearly all methods of F determination so far described use hydrochloric-acid extraction (see, for example, refs. 11, 12, 14—16, 20, 21] with the risk of acid hydrolysis of F, and this probably led to the discussion in the literature about the nature of CSA (metabolite in vivo or degradation product; compare refs. 4, 11, 22, 23 with refs. 13, 14, 24). Some authors report the existence of a glucuronide as a metabolite of F [13, 24]. F had a pK_{a1} of 3.8 (pK_{a2} 7.5) and a protein-binding capacity of 95—99%. After testing some of these factors, we developed a method for the high-performance liquid chromatographic (HPLC) determination of F which involves precipitation and acidification of the samples with acetic acid, exclusion of light, extraction with a diethyl ether—*n*-hexane mixture, and the use of desmethylnaproxen as internal standard.

MATERIALS AND METHODS

Reagents

Furosemide and 4-chloro-5-sulphamoylanthranilic acid (CSA) were kindly supplied by Hoechst (Frankfurt, G.F.R.), and desmethylnaproxen by Syntex (Palo Alto, CA, U.S.A.); β-glucuronidase was obtained from Sigma (St. Louis, MO, U.S.A.). Diethyl ether, *n*-hexane, acetic acid, methanol, sodium dihydrogen phosphate, disodium hydrogen phosphate, orthophosphoric acid and sodium hydrogen carbonate were all of analytical grade (E. Merck, Darmstadt, G.F.R.); diethyl ether was distilled shortly before use.

Biological fluids

Human plasma which had been stored at −20° C was obtained from the local blood bank; human urine was collected from a male donor shortly before analysis. From one male volunteer (31 years old, 70 kg) and one patient with congestive heart failure (68 years old, 60 kg) blood and urine were collected after oral administration of a 40-mg F tablet after an overnight fast.

Sample preparation

To find a good precipitation—acidification procedure the following solutions were prepared, before extraction with diethyl ether—*n*-hexane (65:35), in subdued daylight in dark-brown test-tubes (in duplicate; see also Table I, left-hand column):

(1) 5.2 μg of furosemide in 5 ml of diethyl ether—*n*-hexane mixture;

(2) 5.2 μg of furosemide in 5 ml of diethyl ether—*n*-hexane mixture with 20 μl of 1.5 *M* hydrochloric acid;

(3) 5.1 μg of desmethylnaproxen in 5 ml of diethyl ether—*n*-hexane mixture;

(4) 5.1 μg of desmethylnaproxen in 5 ml of diethyl ether—*n*-hexane mixture with 20 μl of 1.5 *M* hydrochloric acid;

(5) 5.2 μg of furosemide and 5.1 μg of desmethylnaproxen in 5 ml of diethyl ether—*n*-hexane;

TABLE I

SAMPLE COMPOSITION AND RECOVERY RATES OF FUROSEMIDE AND DESMETHYLNAPROXEN SOLUTIONS IN DIETHYL ETHER—*n*-HEXANE, USING ACETIC ACID OR HYDROCHLORIC ACID AS ACIDIFICATION AGENT

For details of sample preparation see Materials and Methods section.

	Sample composition				Recovery (%)
	Furosemide	Desmethyl-naproxen	HCl	Acetic acid	
1	+	—	—	—	100
2	+	—	+	—	65, 52
3	—	+	—	—	100
4	—	+	+	—	8, 12
1A	+	—	—	—	100
2A	+	—	—	+	100
3A	—	+	—	—	100
4A	—	+	—	+	100
5	+	+	—	—	100
6	+	+	—	+	100

(6) 5.2 μg of furosemide and 5.1 μg of desmethylnaproxen with 20 μl of 8.5 *M* acetic acid.

The solutions were evaporated to dryness with a gentle stream of nitrogen at 30°C. The residue was dissolved in 1 ml of a methanol—0.01 *M* sodium bicarbonate mixture (3:2). The solutions were measured as described below. The same tests were repeated, using 20 μl of 8.5 *M* acetic acid instead of 20 μl of 1.5 *M* hydrochloric acid (Table I, 1A—4A).

Stability tests

The samples of F in urine obtained during a pharmacokinetic study were divided into three portions and processed immediately and after 20 and 45 days of storage at —20°C in the dark (concentration range was 3—23 μg/ml for urine and 0.1—5.6 μg/ml for plasma). Samples of F in plasma obtained during another pharmacokinetic study were measured after four days and four months of storage.

Solutions of F (130 μg/10 ml) were prepared in phosphate buffer at pH 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.3 in twilight. These solutions were exposed to standard laboratory lighting at room temperature (fluorescent lights Philips TL 40 W/34 de Luxe at 1.5 metres). At time zero, and 0.75, 1, 1.5, 2, 2.25, 3, 3.5, 4 and 5.25 h, 1-ml samples were mixed with 1 ml of methanol and measured directly by injection onto the column as described under Apparatus. The same procedure was carried out with samples of CSA (250 μg per 10 ml) and with solutions of F in human urine.

Solutions of F (130 μg per 10 ml) and CSA were made in phosphate buffer at pH 3.0, 5.5, and 8.0 in twilight. This time the solutions were protected from light; at time zero, and at 1, 2, 3 and 4 h, 1-ml samples of each solution were mixed with 1 ml of methanol and measured directly by injection onto the

column as described under Apparatus. No precautions were taken to prevent contact of the samples with air, and no efforts were made to expel oxygen from the solutions.

Determination of F glucuronide

Every aliquot of urine from a volunteer taking F and from a patient using F chronically was divided between two test-tubes: one for F determination, which was processed as described under Final procedure, and the other for determination of F and F glucuronide together.

The contents of the second test-tube were adjusted to pH 6.8 with KOH (20%) or H_3PO_4 (10%); the added volume of KOH or H_3PO_4 was negligible (maximum 40 µl to 12-ml tube). To an extraction tube containing an amount of internal standard (100 µl of a solution of 6.25 mg of desmethylnaproxen in 100 ml of methanol, evaporated to dryness) were added 0.25 ml of this urine and 0.25 ml of 0.075 *M* phosphate buffer (pH 6.8) containing 500 units of β-glucuronidase. The extraction tube was closed with a screw-cap. The mixture was incubated for 21 h at 37°C in the dark. After chilling to room temperature, 0.5 ml of 8.5 *M* acetic acid and 5 ml of the extraction mixture (diethyl ether—*n*-hexane) were added, and the sample was processed further as described under Final procedure and measured as described under Apparatus.

Final procedure

The experiments were carried out in subdued daylight. In a dark extraction tube with polytetrafluoroethylene (PTFE) screw-cap, 100 µl of internal standard (6.25 mg of desmethylnaproxen per 100 ml of methanol) were pipetted and evaporated to dryness under a gentle stream of nitrogen at room temperature. Aliquots of 0.5 ml of plasma or urine (stored in the dark), 0.5 ml of 8.5 *M* acetic acid and 5 ml of diethyl ether—*n*-hexane (65:35) were successively pipetted into the extraction tube. The extraction tube was closed with the screw-cap and shaken mechanically for 30 min, followed by centrifugation at 1300 *g* for 15 min. The organic layer was pipetted into another dark-brown test-tube and evaporated to dryness with a nitrogen stream at 30°C. The residue was dissolved in 0.6 ml of methanol (1 ml for urine). After addition of 0.4 ml of 0.01 *M* $NaHCO_3$ (1 ml for urine), 10 µl of this mixture were injected onto the column and measured under the conditions described under Apparatus. Before quantitative measurements were undertaken, an internal standard method was calibrated with the aid of a Hewlett-Packard 3353 Lab Auto System (with the option of measuring peak height instead of peak area), using a plasma calibration solution of 5 µg/ml furosemide and 12.5 µg/ml desmethylnaproxen. This was done to enable the computer to discriminate between the internal standard peak and the furosemide peak. After calibration, a standard series with the following concentrations was injected: 0.27, 0.53, 1.06, 2.12, 3.18, 4.24, 5.30 µg/ml of plasma. A basic programme calculated the regression line of the standard curve with the peak height ratio of furosemide to internal standard. The concentrations of the samples were calculated according to the regression line.

The same procedure was carried out for urine. The calibration solution for urine was 50 µg/ml furosemide and 12.5 µg/ml desmethylnaproxen. The stan-

dard series had the following concentrations: 1.04, 2.60, 5.19, 10.38, 20.76, 31.14, 51.3 μg/ml of urine.

Apparatus

A Hewlett-Packard HP1081B high-performance liquid chromatograph equipped with a variable-volume injector was used. The stainless-steel column had a length of 15 cm and an internal diameter of 4.6 mm, and was packed with LiChrosorb RP-8, particle size 5 μm (Merck). The oven temperature was 35° C and the injection volume was 10 μl. Furosemide and the internal standard desmethylnaproxen were measured with a fluorescence spectrometer Model 3000 (Perkin-Elmer); the excitation wavelength was 275 nm (for urine 235 nm), the emission wavelength was 410 nm (for urine 400 nm), and the excitation and emission slits were 10 nm.

The mobile phase, consisting of methanol—0.02 *M* phosphate buffer, pH 3.0 (1:1), was delivered at a rate of 1.0 ml/min, the resulting pressure being 240 bars.

Pharmacokinetic pilot study

A male volunteer (31 years old, 70 kg) took a standard 40-mg furosemide tablet (Lasix®) at 9.00 a.m. after an overnight fast. Blood samples were taken at time zero, and at 0.5, 1, 1.5, 2.25, 3, 5.5 and 6.5 h. A blank urine sample was voided at time zero and further samples at 0.5, 0.75, 1, 1.25, 1.5, 1.75, 2, 2.5, 3, 5, 8 and 12 h, directly into dark-brown bottles.

One male patient (68 years old, 60 kg), who during 10 days had been using furosemide for congestive heart failure, took his eleventh standard tablet of 40 mg furosemide (Lasix®) at 9.00 a.m. after an overnight fast. Blood was sampled at time zero, and at 0.5, 1, 1.5, 2, 2.5, 3 and 4 h. Urine samples were collected after spontaneous voiding, which was rather irregular. By mistake two specimens were not protected from light immediately.

RESULTS

Studies on the influence of acidification

Using hydrochloric acid (1.5 *M*) as acidification agent, we obtained a recovery of about 50% for F and 10% for the internal standard because of decomposition of F and desmethylnaproxen. Using acetic acid (8.5 *M*) as acidification agent, no degradation of F or desmethylnaproxen was observed. Details are given in the right-hand column of Table I and under sample preparation in Materials and Methods.

Stability tests: storage of urine and plasma

If the pharmacokinetic samples of urine were immediately protected from light and stored at −20° C, we found a recovery of 97 ± 7% (mean ± S.D.) after 20 days and of 94 ± 7% after 45 days. This recovery was the same with biological samples of urine of pH 5—8 and various F concentrations of 3—23 μg/ml. In the plasma samples measured after four months, we found a recovery of 102 ± 3% in the lower as well as in the higher concentration range.

Stability tests: influence of light at various pH values

If F was exposed to light (see Table II), fast hydrolysis took place which resulted in at least three compounds, highly dependent on the pH of the F solution. The degradation products had retention times of 1.93 min (F_1), 2.85 min (F_2) and 5.43 min (F_3). A degradation product of F with a retention time of 2.85 min (F_2) in the chromatographic system was much more fluorescent under the detection conditions than furosemide itself (Fig. 1). No degradation product with a retention time identical to CSA (2.1 min) was found. Decomposition of F appeared to obey first-order kinetics, and consequently a decomposition half-life of F could be calculated as shown in Table II. Apart from this we found further decomposition of pure CSA solutions, which we could not identify because the peak of the decomposition product of CSA (1.94 min) overlapped with the CSA peak (2.10 min) (see Fig. 2). When instead of water we used human urine in view of the possible influence of sulphate ions, the results were not significantly altered (nor more pronounced either). When the samples of F in 0.01 *M* phosphate buffer of pH 3.0—5.5 and 8.0 were protected from light at room temperature, there was 100% recovery after 4 h, whereas after 24 h we found only negligible decomposition peaks.

TABLE II

RECOVERY OF FUROSEMIDE (%) FROM SOLUTIONS OF DIFFERENT pH WHEN EXPOSED TO LIGHT

Time exposed to light (h)	pH									
	3.5	4.0	4.5	5.0	5.5	6.0	6.5	7.0	7.5	8.3
0	100	100	100	100	100	100	100	100	100	100
0.75	75	81	89	97						
1					95	95	96	99	100	94
1.5	46	60	81	91						
2					90	90	92	95	98	89
2.25	26	39	59	67						
3					81	84	82	84	88	84
3.5	10	24	55	62						
4					76	78	78	81	84	84
5.25	7	18	51	ND*						
$t_{½}$ decomp. (h)	1.1	1.7	4	5	10	11	11	13	16	16

*ND = not determined.

Final procedure

When we used blood bank plasma and human urine, the recovery of F in plasma was 85 ± 2% at concentration 5 μg/ml and 89 ± 3% at concentration 0.5 μg/ml; in urine the recovery was 95 ± 2% at concentration 50 μg/ml, 97 ± 3% at concentration 25 μg/ml and 95 ± 2% at concentration 2.5 μg/ml.

With plasma furosemide concentrations of 0.27—5.3 μg/ml, there is a linear relationship between the peak height ratio of furosemide to internal standard (Y) and the plasma F concentration (X), as given by the equation $Y = 0.114X$

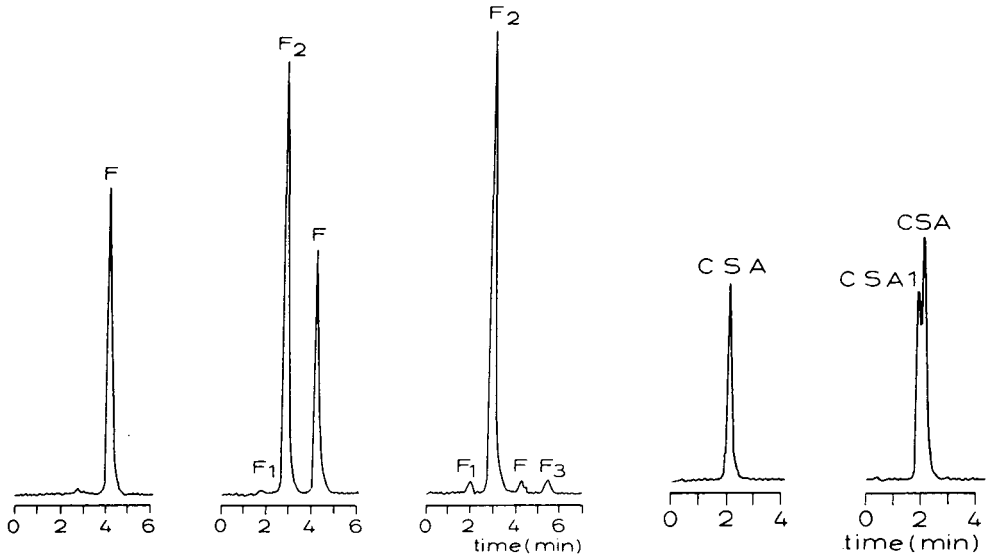

Fig. 1. Effect of light on a furosemide solution consisting of 130 μg of F dissolved in 10 ml of 0.01 *M* phosphate buffer (pH 4.0); 1-ml samples were processed and measured. Left: chromatogram of sample protected from light. Centre: chromatogram of sample processed after 0.75 h under fluorescent lights; degradation products F_1 and F_2 are visible. Right: chromatogram of sample processed after 5.25 h under fluorescent lights; degradation products F_1, F_2 and F_3 are present.

Fig. 2. Effect of light on a CSA solution formed by dissolving 250 μg of CSA in 10 ml of 0.01 *M* phosphate buffer (pH 4.5); 1-ml samples were processed and measured. Left: chromatogram of a sample protected from light. Right: chromatogram of a sample processed after 0.75 h under fluorescent lights; the overlap of degradation peak CSA_1 and original CSA peak results in an apparent rise in CSA peak height.

+ 0.0013 (r = 0.9998, n = 7). With urine concentrations of 1.04—51.9 μg/ml, there is a linear relationship between urinary F concentration (X) and peak height ratio (Y), described by the equation $Y = 0.020X - 0.0038$ (r = 0.9999, n = 7).

At a given plasma F concentration of 0.54 μg/ml, the coefficient of variation is 2.67% (n = 10): 0.5401 ± 0.0144 (range 0.514—0.560). At a given plasma F concentration of 2.04 μg/ml the coefficient of variation is 3.50% (n = 10): 2.038 ± 0.0713 (range 1.98—2.18). At a given urinary F concentration of 3.61 μg/ml, the coefficient of variation is 2.35% (n = 9): 3.614 ± 0.085 (range 3.47—3.70). At a given urinary F concentration of 21.81 μg/ml, the coefficient of variation is 1.60% (n = 10): 21.814 ± 0.3482 (range 21.36—22.26). Under the conditions described under Apparatus, the retention time of CSA was 2.1 min, that of F was 4.2 min and that of the internal standard desmethylnaproxen was 6.0 min (Figs. 3 and 4). In the case of accidental exposure to light during the procedure, peaks of degradation products appear. The lower limit of detection in plasma with spiked samples using an internal standard of 2.5 μg of desmethylnaproxen instead of 12.5 μg/ml is 0.1 μg/ml (0.1 ± 0.08, n = 10, range 0.09—0.12). The lower limit of detection in urine is 0.5 μg/ml (0.47 ± 0.02, n = 10, range 0.45—0.52). This covers very satisfactorily the drug levels at which the diuretic effects of F occur.

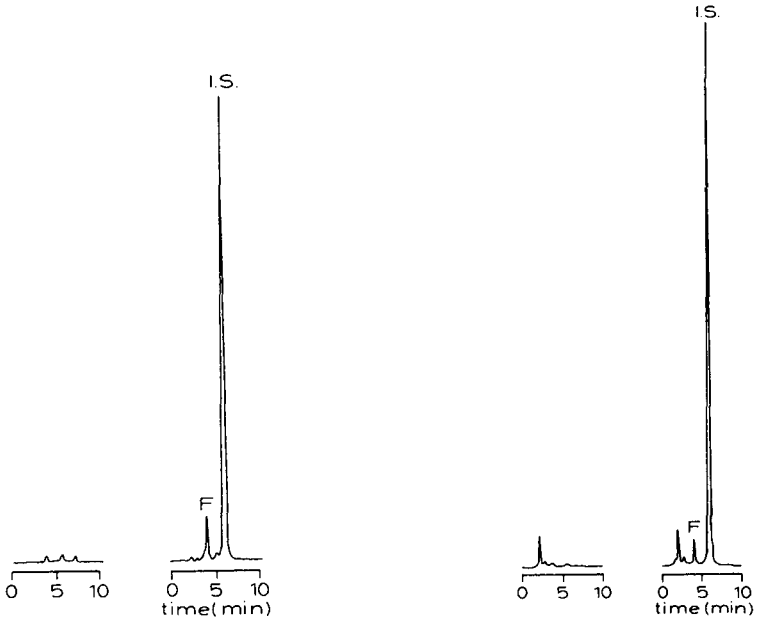

Fig. 3. Left: chromatogram of blank plasma with small peaks of endogenous fluorescent substances at 3.8 and 5.3 min. Right: chromatogram of plasma containing 1 μg/ml furosemide at 4.2 min and 25 μg/ml desmethylnaproxen (internal standard, I.S.) at 6.0 min.

Fig. 4. Chromatograms of (left) blank urine and of (right) urine containing 2.5 μg/ml furosemide and 25 μg/ml desmethylnaproxen (internal standard, I.S.).

Pilot pharmacokinetic studies

In the adult volunteer the peak plasma level was 1.8 μg/ml at 1 h. The elimination half-life was determined as 1.06 h and the area under the curve (AUC) for plasma as 3.6 mg h per l. The renal furosemide clearance was 70 ± 20 ml/min. The recovery of F in urine was 14.54 mg after 12 h; recovery of F glucuronide in urine was 3.55 mg; total recovery in urine during 12 h was 18.1 mg (45%). No trace of CSA or other metabolites was found. There was a good correlation ($r = 0.84$, $p < 0.01$) between urinary F excretion per min and urinary water excretion (as a global parameter of diuretic effect) during the first 3 h; this correlation extended also over the period 0—8 h ($p < 0.001$, $r = 0.88$) (see Fig. 5).

In the older patient, resorption was somewhat delayed: a peak plasma F level of 1.7 μg/ml was attained at 1.5 h, elimination half-life was 0.8 h and AUC for plasma was 3.1 mg h per l (renal furosemide clearance was approximately 73 ± 27 ml/min, but only four specimens were available for this estimation). The recovery of F in urine was 13.7 mg after 24 h, and recovery of F glucuronide in urine was 3.9 mg; in the two specimens exposed to light by the patient we found no CSA, but after pretreatment of the specimen with β-glucuronidase (in the dark) we found a major peak in the chromatogram with a retention time of 2.85 min (F_2, Fig. 1). No trace of this substance was found

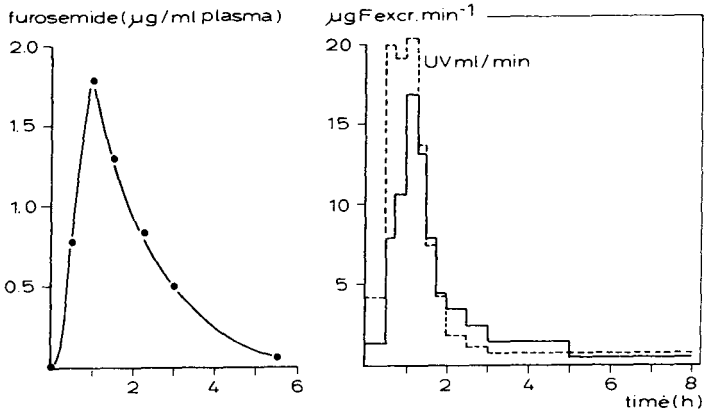

Fig. 5. Left: plasma level versus time curve after ingestion of a 40-mg furosemide tablet by a healthy volunteer. Right: urinary furosemide excretion (μg F excr.) and urinary volume (UV) versus time.

in the properly handed specimens. The patient was unable to void frequently enough to enable pharmacodynamic studies.

DISCUSSION

Various problems with other methods of F determination are described in the Introduction. Acidification of plasma samples is necessary to separate F from its protein binding and to transfer it to an organic layer. Acidification of urine samples is advisable to minimise the presence of endogenous fluorescing substances in the organic layer.

We did not use N-benzyl CSA [20] as internal standard because it has a longer retention time and is less stable under our experimental conditions than desmethylnaproxen. We did not use dichloromethane or chloroform extraction because these chemicals extract too much endogenous fluorescing substance. Our diethyl ether—*n*-hexane (65:35) extraction mixture was unsuitable if used after HCl precipitation and acidification of the F samples, probably because the organic layer contained traces of water with hydrochloric acid, and furosemide is very unstable in HCl solution, as demonstrated (see Results and Table I).

When acetic acid was used, no degradation of F could be observed. The problem seems to have been encountered before, since Steiness et al. [14] used a different acidification for urine (phosphate buffer) than for plasma (HCl).

Urine samples containing furosemide could not be kept in the deep-freezer for an indefinite time because this is likely to cause a slow decrease in recovery rate. We brought to light the degradation problems in handling furosemide. Especially acidic specimens of F should be kept in the dark from the time of taking the sample until injection onto the HPLC column; decomposition was astonishingly fast, and degradation into various products occurred. The degradation product F_1 of furosemide, with a retention time of 1.93 min in our HPLC procedure, may well be identical to degradation product CSA_1 with a retention time of 1.94 in our system. We were unable to identify CSA_1

in relation to F_1 because chromatographic separation of CSA_1 from CSA was far from complete. With proper handling of samples our method is hardly laborious. In sensitivity and reliability, our method does not differ from other published methods. In plasma samples with concentrations of 0.1—1.0 μg/ml, we now use an internal standard solution of 1.25 mg of desmethylnaproxen per 100 ml of methanol (calibrating a standard curve with 0.1, 0.2, 0.5, 1.0 and 1.5 μg/ml solutions of F). In urine, the concentration range related to the diuretic effect of F is far above our lower limit of detection; it is therefore unnecessary to reduce the amount of interal standard, although this would be possible in the same way. The preliminary studies in man produced results similar to those mentioned in other reports in the literature. Discussion about the existence of metabolites of F in man could hardly be touched in this preliminary study: we did find a glucuronide in considerable amounts; we did not find CSA itself in the specimens from the adult volunteer; from the semi-chronic user of F too few good urine specimens could be obtained to permit detailed analysis. The F_2 glucuronide we found was probably an in vitro breakdown product of F glucuronide, and the F glucuronide excretion in this patient must therefore have been even more than the 3.9 mg mentioned. Further studies are in progress.

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DETERMINATION OF URINARY HIPPURIC ACID AND *o*-CRESOL, AS INDICES OF TOLUENE EXPOSURE, BY LIQUID CHROMATOGRAPHY ON DYNAMICALLY MODIFIED SILICA

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SUMMARY

Two simple and sensitive high-performance liquid chromatographic methods for the determination of hippuric acid and *o*-cresol in urine have been developed. The chromatographic system is the same for the two methods and is based on dynamically modified silica.

The detection limits were found to be 0.05 mg/ml and 0.05 μg/ml of urine for hippuric acid and *o*-cresol, respectively, when using UV detection at 254 nm. The recovery for hippuric acid was about 100% and for *o*-cresol 33—36%. The detection limit for *o*-cresol could be lowered by a factor of ten by using fluorescence detection.

The methods were used for investigations of the urine from persons exposed to 100 ppm toluene for 6.5 h. The method for *o*-cresol may also be used for determination of other phenols in urine.

INTRODUCTION

Toluene is a solvent widely used in industry as a replacement for the carcinogenic benzene, but toluene also exhibits considerable toxicity [1]. Its main metabolite in man is benzoic acid, which is excreted as its glycine conjugate, hippuric acid. Hippuric acid is an endogenous metabolite common in human urine, but on exposure to toluene the level is enhanced [2—6]. Toluene

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is, amongst several other substances, metabolised to *o*-cresol [1, 7, 8], which until now has not been reported as a usual component in human urine. It may therefore be regarded as a more specific metabolite and thus a better index of toluene exposure.

In this paper we present a chromatographic system based on dynamically modified silica [9, 10], in which the content of both hippuric acid and *o*-cresol in human urine may be determined.

EXPERIMENTAL

Apparatus

A Waters liquid chromatograph consisting of a Model 6000A pump, a Model 710A WISP autoinjector, a Model 440 ultraviolet (UV) absorbance detector (254 nm), a Model 730 data module and a Model 720 system controller was used. The columns were thermostated in a LC 250/3 Kratos oven. A Kontron SFM 23 LC spectrophotofluorimeter was used as the fluorescence detector (excitation at 273 nm, and emission at 298 nm).

For centrifugation a Sigma 3K-1 was used at 3000 *g* in all cases.

Chemicals

Acetonitrile HPLC S grade was obtained from Rathburn Chemicals (Walkerburn, Great Britain). All other chemicals were of analytical-reagent grade and obtained from E. Merck (Darmstadt, G.F.R.).

Sample preparation

Hippuric acid. A 1-ml volume of urine was mixed with 1 ml of acetonitrile and centrifuged for 5 min; 10 μl of the supernatant were injected onto the column.

o-Cresol. To 10 ml of urine, 1 ml of internal standard (0.005% 2,5-dimethylphenol or 0.015% 2,3,5-trimethylphenol) and 1 ml of concentrated sulphuric acid were added. The mixture was placed in a boiling water bath for 1 h. After cooling, 3 ml were extracted with 7.5 ml of cyclohexane and the phases were separated by centrifugation. The cyclohexane phase was then first extracted with 2 ml of 0.2 *M* potassium phosphate buffer and finally extracted with 300 μl of 0.1 *N* sodium hydroxide; 25 μl of the sodium hydroxide phase were injected onto the column. Careful centrifugation was necessary before separating the phases.

Chromatography

The column set-up has been described previously [9, 10]. The analytical column was a Knauer column, 120 × 4.6 mm I.D., packed with LiChrosorb Si 60 (5-μm particles). The guard column (100 × 4.6 mm I.D.), situated between the pump and the autoinjector, was dry-packed with LiChroprep Si 60. Both columns were operated at 40°C. The mobile phase was acetonitrile—0.2 *M* potassium phosphate (pH 7.5)—water (30:5:65) containing 1.25 m*M* N,N,N-trimethylhexadecylammonium bromide; the flow-rate was 1.5 ml min^{-1}.

Toluene exposure

Forty-two printing workers (occupationally exposed to toluene during their daily work for ten years or more) were matched with control persons according, among others, to age and smoking habits. All persons were men. Each matched pair was randomly selected for either exposure to 100 ppm or 0 ppm of toluene in air for 6.5 h. The exposure was performed in an exposure chamber [11]. Three portions of urine were collected from each person at the following intervals: 2.5 h before the start of the experiment to time zero; from the start of the exposure to 3 h later; and from 3 to 6.5 h after the start of the exposure. The samples were stored at −20°C until analysed.

RESULTS AND DISCUSSION

Recovery, reproducibility and detection limits

Hippuric acid can be determined by injecting diluted urine onto the column. Plotting the detector response in arbitrary units of area for hippuric aicd in the concentration range 0.1—10 mg/ml gave a rectilinear standard curve with the regression equation $Y = 23237X + 3.7$ ($r = 0.999$). The reproducibility and accuracy of this simple method were determined for urine using the standard addition method. The results in Table I show a recovery close to 100% with a good precision. Typical chromatograms are shown in Fig. 1.

TABLE I

DETERMINATION OF HIPPURIC ACID IN URINE

Added (mg/ml)	Found (mg/ml)	Recovery ± R.S.D. (%) ($n = 4$)
0	0.23	—
0.20	0.44	102.7 ± 2.1
0.50	0.73	100.3 ± 1.5
1.00	1.20	96.9 ± 1.8
2.00	2.19	98.1 ± 2.0

The phenolic metabolites (*o*-cresol and *p*-cresol) of toluene are present in urine as their sulphate and glucuronide conjugates. As *p*-cresol is also an endogenous metabolite only the specific metabolite *o*-cresol is quantitated, and the first step in the procedure is cleavage of the conjugates. As the concentration range of total *o*-cresol in urine is between zero and a few ppm, the development of a sufficiently reliable method involved investigation of the pre-chromatographic extraction and concentration procedures. The lower phenols including *o*-cresol are known to be volatile, and consequently it was decided to include the concentration step in the extraction procedure.

Several organic solvents were investigated with respect to efficiency and selectivity of extraction for *o*-cresol. In Table II the recoveries are shown for three of the solvents. Mixtures of hexane and diethyl ether were also investigated but a great tendency to emulsify was observed. Furthermore, the final extracts from these extractions caused severe baseline disturbance when injected onto the column, probably due to diethyl ether dissolved in the

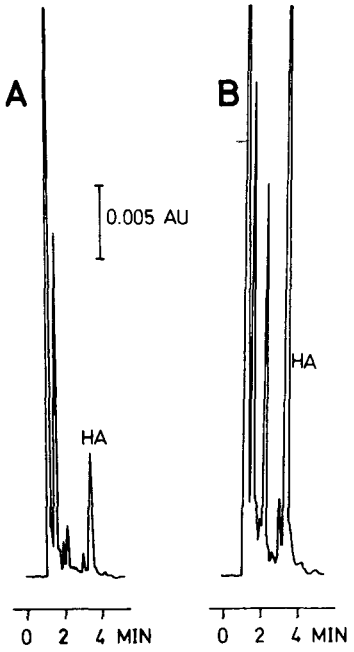

Fig. 1. Determination of urinary hippuric acid (HA). (A) Normal urine (HA = 0.29 mg/ml). (B) Urine from a person exposed to toluene (HA = 3.35 mg/ml).

TABLE II

RECOVERY OF *o*-CRESOL (1 ppm) FROM 3 ml OF 2 *M* SULPHURIC ACID EXTRACTED ONCE WITH 7.5 ml OF THE STATED SOLVENTS AND RE-EXTRACTED AS DESCRIBED IN THE PROCEDURE

Values are given in per cent (n = 4).

	Recovery	S_{rel}	S_{rel} after correction by internal standardization
Dichloromethane	72	9.6	2.6
Cyclohexane	33	12.5	2.4
Hexane	27	15.0	3.1

aqueous phase. Dichloromethane gave the highest recovery, but when using urine too many co-extractants compromised the determinations. Extraction with hexane and cyclohexane gave low recoveries with a relative standard deviation of 12—15%, but the latter problem was solved by using an internal standard. Due to the purification step with phosphate buffer pH 7.0, only a few co-extractants from urine were found in the final extract.

The recovery of phenols from urine was found to be less than from 2 *M* sulphuric acid (Table III). The relative change in partition ratio was different for the three compounds and the relative standard deviations were larger when using urine. It is therefore necessary to use urine when preparing the standard solutions. These data were reproduced several times during a three-month

TABLE III

PERCENTAGE RECOVERY OF PHENOLS FROM 2 *M* SULPHURIC ACID AND URINE USING THE METHOD DEVELOPED

Number in parentheses are the relative standard deviations in per cent (n = 4).

	2 *M* H_2SO_4	Urine
o-Cresol	35 (± 10.1)	33 (± 13.7)
Dimethylphenol	46 (± 4.1)	38 (± 9.2)
Trimethylphenol	33 (± 9.8)	24 (± 20.7)

TABLE IV

RECOVERY OF *o*-CRESOL FROM URINE USING THE METHOD DEVELOPED

All values are given in per cent (n = 4).

Amount added (ppm)	Recovery	S_{rel}	S_{rel} using internal standardization
0.1	29	20	5.5
1.0	36	11.5	4.1
5.0	33	7.2	4.1

period with different samples of urine. The recovery of *o*-cresol at different concentration levels is given in Table IV.

The detection limits for practical work with the methods developed were found to be 0.05 mg of hippuric acid per ml of urine (UV, 254 nm), 0.05 μg of *o*-cresol per ml of urine (UV, 254 nm) and 0.005 μg of *o*-cresol per ml of urine (fluorescence, excitation 273 nm, emission 298 nm). The determination of *o*-cresol was first developed for use with a UV detector, but in Fig. 2 it is seen that fluorescence detection is more selective, and it is most likely that the extraction procedure could be simplified when using fluorescence detection alone.

Occupational studies

The described methods have been used in a large investigation of workers employed in the printing industry compared with workers employed elsewhere [11].

No difference in the metabolic pattern of toluene to hippuric acid and *o*-cresol in the two groups was observed (Table V), and the excretion of urinary hippuric acid and *o*-cresol showed a linear rise during the exposure. Urinary *o*-cresol discriminated better between exposed and non-exposed persons, especially during the first 3 h of exposure. This is due to the very low concentration of *o*-cresol found in urine before exposure. The low levels of *o*-cresol found in all persons may be due to the toluene pollution in the atmosphere.

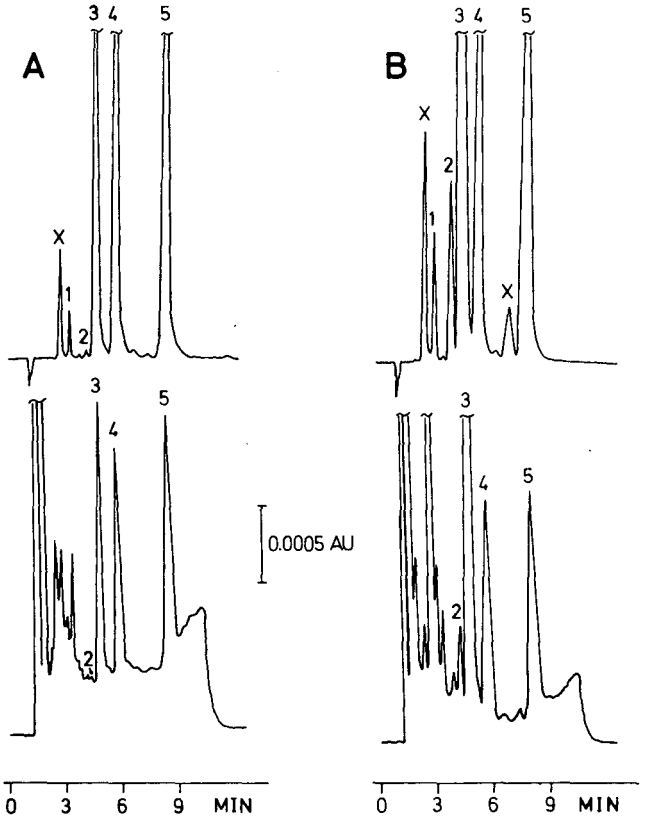

Fig. 2. Determination of urinary *o*-cresol. (A) Normal urine (0.03 μg *o*-cresol per ml). (B) Urine from a person exposed to toluene (0.9 μg *o*-cresol per ml). Upper traces: fluorescence detection (excitation 273 nm, emission 298 nm). Lower traces: UV detection at 254 nm. Peak identification: 1 = phenol, 2 = *o*-cresol, 3 = *m*- and *p*-cresol, 4 = 2,5-dimethylphenol, 5 = 2,3,5-trimethylphenol, and X = unknowns.

TABLE V

URINARY EXCRETION OF HIPPURIC ACID AND *o*-CRESOL IN 42 PRINTING WORKERS AND 43 CONTROL PERSONS DURING 6.5 h EXPOSURE TO 100 ppm OR 0 ppm TOLUENE IN AN EXPOSURE CHAMBER

	Dose of toluene (ppm)	No. of persons	Urinary hippuric acid excretion (mmol)		Urinary *o*-cresol excretion (μmol)	
			Median	(quartiles)	Median	(quartiles)
Printing workers	100	19	6.7	(6.3—7.4)	4.6	(4.0—4.9)
	0	23	1.1	(0.9—1.5)	0.2	(0.1—0.4)
Control persons	100	21	6.7	(5.5—7.7)	4.2	(3.4—5.4)
	0	22	1.5	(0.7—2.2)	0.1	(0.1—0.3)

BLE VI

INARY EXCRETION OF HIPPURIC ACID AND *o*-CRESOL IN SMOKERS AND NON-SMOKERS EXPOSED TO 0 OR 100 ppm LUENE FOR 6.5 h IN AN EXPOSURE CHAMBER

	Smokers					Non-smokers				
	No. of persons	*o*-Cresol (μmol)		Hippuric acid (mmol)		No. of persons	*o*-Cresol (μmol)		Hippuric acid (mmol)	
		Median	(quartiles)	Median	(quartiles)		Median	(quartiles)	Median	(quartiles)
fore posure .5—0 h posed to 0 ppm -6.5 h	53	0.14	(0.08—0.25)	0.87	(0.62—1.25)	31	0.04	(0.02—0.05)	0.54	(0.40—1.29)
posed to ppm -6.5 h	33	4.54	(3.68—4.92)	6.68	(5.98—7.52)	7	4.08	(3.35—6.05)	6.69	(5.68—7.18)
	20	0.38	(0.15—0.62)	1.20	(0.84—1.91)	24	0.13	(0.07—0.20)	1.13	(0.86—1.89)

Smoking was also found to have an influence on the *o*-cresol levels in the urine. Table VI shows that the levels are higher for smokers than for non-smokers during the whole experiment. Cigarette smoke is known to contain *o*-cresol [12], but smoking was not allowed during the experiment and at present no simple explanation for this observation can be given.

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A VERY PRECISE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC PROCEDURE FOR THE DETERMINATION OF CEFMENOXIME, A NEW CEPHALOSPORIN ANTIBIOTIC, IN PLASMA

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SUMMARY

A simple and very precise high-performance liquid chromatographic procedure has been developed for the determination of cefmenoxime, a new broad spectrum cephalosporin antibiotic, in plasma. The workup procedure involves ultrafiltration of samples which have been treated with sodium dodecyl sulfate to displace the drug from its binding sites on plasma proteins. The ultrafiltrates are then directly injected into a high-performance liquid chromatographic system utilizing a reversed-phase analytical column, and an ultraviolet spectrophotometric detector. The mean assay coefficient of variation over a concentration range of 0.5—200 μg/ml is slightly greater than 1% when either *p*-nitrobenzoic or *p*-anisic acid is used as the internal standard. Recoveries of drug are essentially quantitative at all levels investigated; hence the calibration curves are rectilinear from the limit of quantification (about 0.05 μg/ml) to at least 200 μg/ml.

INTRODUCTION

Cefmenoxime, 7β-[2-(2-aminothiazol-4-yl)-(*z*)-2-methoxyiminoacetamido]-3-[(1-methyl-1H-tetrazol-5-yl)thiomethyl]ceph-3-em-4-carboxylic acid, a semisynthetic cephalosporin derivative (Fig. 1) developed by Takeda Chemical Industries (Osaka, Japan), has a broad spectrum of activity against gram-positive and gram-negative organisms, including *H. influenzae*, *C. freundii*, *E. cloacae*, indole-positive *Proteus*, and *S. marcessens* [1—3]. The hemihydrochloride salt of this cephalosporin is currently under clinical evaluation by Abbott Laboratories.

Because of poor specificity, slow turn around time, and relatively poor precision, microbiological analysis was not felt to be the method of choice for determination of cefmenoxime in plasma. High-performance liquid chromatography (HPLC) is ideally suited for the analysis of these relatively polar,

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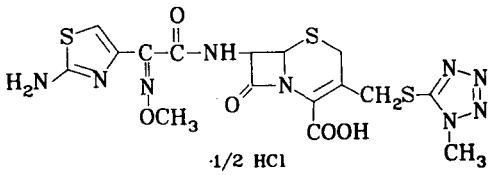

Fig. 1. Chemical structure of cefmenoxime hemihydrochloride.

nonvolatile compounds. Since the therapeutic concentrations of cephalosporins are usually in the μg/ml range, concentration techniques are usually not required; however, the majority of the high-molecular-weight proteins and fibrin must be removed from plasma samples to prevent column filter and packing bed damage. Several HPLC procedures for cephalosporins, employing classical deproteination reagents such as trichloroacetic acid [4, 5], or organic solvents [6—9], have been reported recently. The HPLC procedure described herein, involving removal of plasma proteins by ultrafiltration, offers the advantages of simplicity and high reproducibility, obviating the problems associated with the precipitation procedures (e.g. sample dilution, incomplete protein precipitation, drug co-precipitation, and acid catalyzed degradation of labile drugs). Furthermore, the ultrafiltration procedure allows the determination of the free and total drug concentrations in plasma. Cefmenoxime, as well as other protein-bound antibiotics, can be quantitatively displaced from the plasma proteins by addition of sodium dodecyl sulfate (SDS) or other highly protein-bound reagents.

EXPERIMENTAL

Chromatography

HPLC analyses were conducted using a Waters Assoc. (Milford, MA, U.S.A.) Model 6000A pump in conjunction with a spectrophotometer operated at an analytical wavelength of 254 nm. The mobile phase, consisting of approximately 13% (v/v) acetonitrile in 0.2 *M* aqueous acetate buffer at pH 5.30, was pumped at a flow-rate of 2.0 ml/min through a Waters μBondapak C_{18} analytical column (30 cm × 4 mm I.D., 10 μm particle size). Minor manipulations in the acetonitrile content and/or the pH of the mobile phase were occasionally required to accommodate column efficiency loss, or interference from atypical plasma samples.

Ultrafiltration apparatus

Plasma ultrafiltrates are prepared using the Amicon (Lexington, MA, U.S.A.) Centriflow system consisting of conical centrifuge tubes (Model CT1), conical supports (Model CS1A) and membrane cones (either Model CF25 or CF50A, with respective molecular weight cutoff values of 25,000 and 50,000). Use of the CF-25 cones was slightly favored due to their higher apparent flux and protein retentivity. The two types of membrane cones were not mixed within an analytical run.

Procedure

Calibration curves for the analysis of clinical specimens were typically prepared by supplementing blank pooled plasma with a freshly prepared aqueous solution of cefmenoxime, followed by serial dilution with pooled plasma.

Sample processing entailed ultrafiltration of an accurately measured mixture of 5 volumes of plasma (usually 1.0 ml) and 1 volume of a solution containing the internal standard and 4% (w/v) SDS. Both *p*-nitrobenzoic acid (PNBA) and *p*-anisic acid (PAA), at final concentrations greater than 1.3 μg/ml, were found to be acceptable as internal standards. All samples were centrifuged at the same relative centrifugal force (typically 450 *g*) for at least 20 min. After transfer to clean test tubes, the ultrafiltrates were refrigerated until all samples were processed. Subsequently, the ultrafiltrates could be stored frozen for several days prior to analysis. Injection of 90 μl of ultrafiltrate from a plasma sample originally containing 0.05 μg/ml of cefmenoxime was found to produce a response 2—3 times greater than background noise at a detector attenuation of 0.02 a.u.f.s.

Since cefmenoxime was found to degrade slowly in the ultrafiltrates, it was deemed advisable to allow them to stand at room temperature for no more than 2—3 h prior to analysis. For enhanced room temperature stability (e.g. overnight automatic injection), a small volume of buffer could be added to the ultrafiltrates to reduce the pH to approximately 6.

RESULTS AND DISCUSSION

Method development

Cleanup procedures. Initial attempts to prepare protein-free filtrates were largely unsuccessful. Deproteinization by addition of organic solvents (e.g. methanol. acetonitrile, dimethylformamide) caused chromatographic aberrations and apparent co-precipitation of cefmenoxime. Column chromatographic techniques (e.g. anion and cation exchange, Waters C_{18} Sep-Pak^{TM}, alumina, and silica absorption) also showed little promise.

Ultrafiltration techniques are ideally suited for the determination of the free fraction of drugs in plasma; however, pressurized ultrafiltration techniques using cells or membrane tubing are slow and cumbersome. Alternatively, ultrafiltration by centrifugation with the Centriflow apparatus is simple, fast, and highly reproducible. The apparent plasma protein binding of cefmenoxime was approximately 77% as determined by this procedure. Addition of SDS, a highly protein-bound displacing agent, to plasma samples resulted in quantitative recoveries of cefmenoxime in the filtrates. The results of the competitive binding studies of SDS with cefmenoxime, PNBA, and PAA are shown in Fig. 2. Although recoveries of cefmenoxime and of both internal standards were quantitative at or above a final SDS concentration of 0.35% (w/v), slightly higher final concentrations were employed in routine analyses to ensure quantitative recovery from samples containing high concentrations of drug or albumin.

Chromatographic conditions. With the chromatographic conditions described earlier, the following order of increasing retention was observed for

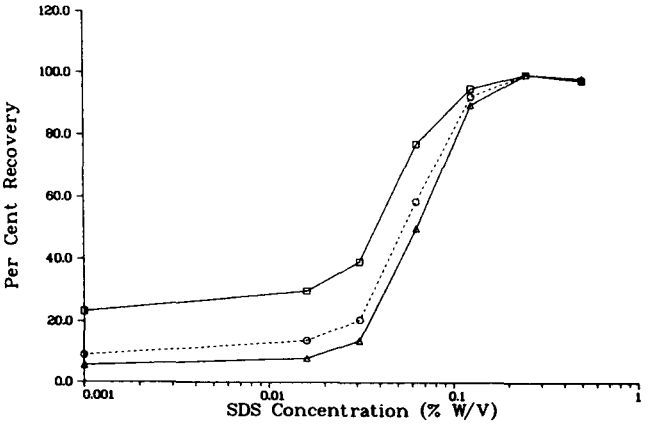

Fig. 2. Displacement of cefmenoxime (□), *p*-anisic acid (○), and *p*-nitrobenzoic acid (△) from plasma proteins by SDS.

typical plasma samples (see Fig. 3): unretained plasma constituents < PNBA < two minor plasma peaks < cefmenoxime < PAA. The relative retention volumes of these compounds varied slightly from column to column, and their resolution was moderately sensitive to pH, ionic strength, and acetonitrile content of the mobile phase.

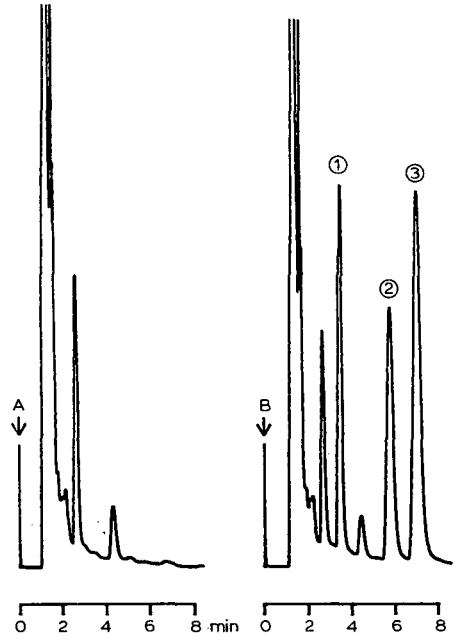

Fig. 3. Chromatograms of ultrafiltrates of (A) control plasma and (B) plasma supplemented with cefmenoxime and the proposed internal standards. Peaks: (1) *p*-nitrobenzoic acid; (2) cefmenoxime; (3) *p*-anisic acid. HPLC conditions are described in the text; mobile phase pH was 5.2. More than 2000 plasma ultrafiltrates had been injected onto this column prior to these chromatograms.

Increasing the pH of the mobile phase decreased the relative retention of both internal standards; however, near pH 5, PAA was more sensitive to pH changes, since its pK_a is higher than that of PNBA (4.5 vs. 3.4). At a mobile phase pH greater than 5.5, PAA eluted prior to cefmenoxime. Conversely, reduction of the pH to below 4.5 increases the retention of both internal standards so that the elution order is cefmenoxime < PNBA < PAA. Thus, great flexibility in the relative retentions of cefmenoxime and the internal standards was realized by pH manipulation. Generally, the pH of the mobile phase should not be reduced below 4.0, since ion-pairing interactions between SDS and cefmenoxime will occur. Manipulation of the ionic strength had less dramatic effects on the resolution of the various compounds, although better resolution was generally observed with increasing ionic strength.

Recovery

The recoveries of cefmenoxime, PNBA, and PAA in the first 50—100 μl of ultrafiltrate were slightly lower than in subsequent fractions; however, the peak height ratios were essentially independent of ultrafiltrate volume. Nevertheless, when ultrafiltrate volumes approaching or exceeding 0.5 ml were collected (e.g. 20 min centrifugation at 450 *g*), recoveries were quantitative for cefmenoxime and internal standards, providing that sufficient (>0.25%) SDS was present.

Intersubject variability in the recovery of cefmenoxime and the internal standards was assessed using blank plasma from six subjects. The chromatographic interference from compounds endogenous to plasma was negligible. The recoveries of the compounds of interest were essentially quantitative, with coefficients of variation averaging approximately 1%.

The recoveries of cefmenoxime, PNBA, and PAA were also compared using Amicon membrane filters with molecular weight cutoff values of 25,000 (CF25) and 50,000 (CF50A). The recovery of cefmenoxime was roughly 2—3% lower with the CF50A cones. Thus, it is recommended that the two types of cones not be mixed within the same assay run, if such an error is considered significant.

Precision and linearity

The precision and linearity of the procedure was assessed by quadruplicate analyses of plasma samples supplemented with cefmenoxime in the concentration range of 0.50—200 μg/ml. Aliquots of these standards (1.0 ml) were mixed with equal volumes of 1% SDS containing PNBA and PAA, and the samples were then processed by the method described above. The results of the analyses are summarized in Table I.

With no corrections made for the internal standard response, the mean assay coefficient of variation (C.V.) was 2.5 ± 3.3%. Correction of the data with the internal standard response resulted in a mean C.V. of 1.2 ± 1.0% with PNBA, and 1.3 ± 0.8% with PAA. These extremely low errors were due to the high ultrafiltration recovery and the simplicity of the workup. The use of PNBA was slightly favored over PAA because it eluted prior to cefmenoxime, this allowing a higher sample analysis rate (roughly 7 to 8 injections per hour for PNBA, compared to 6 to 7 for PAA).

TABLE I

PRECISION AND LINEARITY OF THE ANALYTICAL PROCEDURE

Samples were assayed in quadruplicate, using PNBA as the internal standard. Results for PAA as the internal standard are given in parentheses

Actual	Concentration calculated (μg/ml)	Coefficient of variation (%)
0.50	0.50 (0.50)	2.5 (2.8)
1.00	0.98 (0.99)	2.9 (2.6)
2.00	2.01 (2.01)	1.7 (1.2)
5.00	5.10 (5.15)	0.6 (1.0)
10.00	10.22 (10.22)	0.4 (0.2)
20.00	19.85 (19.92)	0.6 (1.3)
50.00	49.78 (49.37)	0.4 (0.9)
100.00	99.77 (100.46)	0.4 (0.9)
200.00	195.66 (193.22)	1.1 (1.0)

The mean data of Table I were treated by linear regression analyses using reciprocal analytical variances as the weights, thus assuring that the contribution of each value to the sum of squared deviations was proportional to its precision rather than its magnitude [10]. Since it is generally impractical to determine assay variances for each of the standards during routine analyses, the data were then refitted using 1.0, 1/concentration ($1/c$), and 1/concentration-squared ($1/c^2$) as the weighting schemes. The regression correlation coefficients, ranging from 0.9996 to 1.0000, demonstrated that analytical response was rectilinearly dependent on concentration, whereas recovery was independent of concentration. In general, the regression Y-intercepts were negligibly small and statistically insignificant. For the uncorrected data, the $1/c$ weighting scheme gave regression results most representative of the reciprocal variance weighted fit. For the data corected through the use of either PNBA or PAA as internal standard, the $1/c$ and $1/c^2$ weighting schemes both gave regression results very similar to those obtained with reciprocal variance weights. Use of either scheme would be entirely satisfactory; nonetheless, the $1/c^2$ scheme appears to be marginally more appropriate.

Stability

The stability of cefmenoxime in plasma was first assessed in room temperature incubation studies. Three sets of spiked plasma samples were studied: (1) untreated plasma, (2) plasma mixed with an equal volume of 1% SDS, and (3) plasma mixed with an equal volume of 1.0 *M* phosphate buffer, pH 6. After approximately two days at room temperature, the loss of cefmenoxime in all samples was less than 10%. After one week, drug loss was 41% in the untreated plasma, 30% in the SDS-treated plasma, and was not demonstrable in the phosphate-buffered plasma. Additionally, drug loss was not evident in the untreated or treated plasma samples which were stored frozen for one week.

Subsequently, long-term stability studies were initiated with frozen plasma

samples. Freshly collected normal human plasma was supplemented with cefmenoxime at a concentration of 10 μg/ml, and aliquants of this standard were analyzed periodically for two months. The mean recovery of drug in this experiment was 103.3 ± 1.6%. Linear regression analyses of the data failed to demonstrate a statistically significant loss of drug during the two-month period; hence, drug degradation in frozen plasma would be expected to be minimal for considerably longer periods.

The stability of cefmenoxime was also evaluated in plasma ultrafiltrates at room temperature. Plasma was supplemented with the compound, and an aliquot was ultrafiltered using the normal procedure. Another aliquant was treated with an equal volume of 0.5 *M* phosphate buffer, pH 6, containing 1% SDS. The results of the analyses of these samples are given in Table II.

TABLE II

STABILITY OF CEFMENOXIME IN PLASMA ULTRAFILTRATES AT ROOM TEMPERATURE

Time (h)	Percent remaining	
	unbuffered	buffered
0.5	97.8	101.1
1.0	98.2	98.5
2.0	98.5	98.9
3.0	98.3	100.0
4.0	99.2	99.9
5.0	98.6	100.5
6.0	97.5	99.3
7.0	99.2	100.1
22.5	89.4	98.2
24.0	89.5	98.5
27.0	86.2	97.3
30.0	84.9	97.7

Linear regression analyses of the peak height ratios vs. time data failed to show statistically significant changes during the first 7 h for either the buffered or the unbuffered ultrafiltrates; however, at the end of the 30-h incubation, the unbuffered ultrafiltrates showed a statistically significant net rate of decrease in the peak height ratios of about 0.48% per hour. The rate of decrease in the peak height ratios for buffered ultrafiltrates in the same period averaged only 0.08% per hour. It appeared that the rate of cefmenoxime loss in the unbuffered ultrafiltrate increased slightly with time or with repeated exposure to the air. This phenomenon was consistent with the general observation that plasma becomes more alkaline upon standing, and that cefmenoxime degrades more rapidly with increasing pH. Nonetheless, it would appear from these data that ultrafiltrates could be allowed to stand at room temperature for at least 7 h with no significant compromise of analytical accuracy (e.g. roughly 50 injections by an unattended automatic sample injector). Additionally, if long (>7 h) automated injection runs were desired, and maximum sensitivity were not required, 1.0 *M* phosphate buffer, pH 6, may be added

to each ultrafiltrate to improve drug stability. The internal standard contained in the ultrafiltrate would allow compensation for the dilutions. For maximum assay accuracy, particularly with manual or attended automatic sample injection, the thawed ultrafiltrates of the standards and unknowns should be treated identically. This is easily accomplished by refrigeration of the ultrafiltrates before and after injection, leaving only small groups (5—10) at room temperature (immediately prior and subsequent to injection). With this precaution, the samples may be reinjected, if necessary, at a later time for verification of any anomalous results.

Validation

During the course of routine analyses, 110 plasma samples from a clinical pharmacokinetic study were also assayed by an agar diffusion microbiological technique employing *Proteus mirabilis* (strain 13300, Takeda Chemical Industries) as the test organism. The resultant data from samples having levels quantifiable by both procedures were submitted to linear regression analysis (see Fig. 4). Differences between the calculated slope (1.023) and *Y*-intercept (0.0631) and the respective theoretical values of 1.000 and 0.000 were not statistically significant ($p = 0.05$). The correlation coefficient of the regression was 0.995.

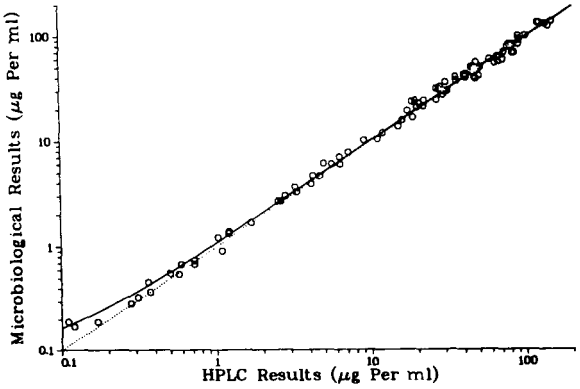

Fig. 4. Correlation of microbiological and HPLC results. Regression equation (solid line): $Y = 1.02\ (\pm 0.01)\ X + 0.06\ (\pm 0.08)$; $r = 0.995$. Dotted line = theoretical curve.

Significant chromatographic interference has not been observed in clinical studies conducted to date with cefmenoxime. Typical plasma level curves from a recent clinical study are shown in Fig. 5.

Versatility

The centrifugal ultrafiltration technique described above is an excellent alternative to classical deproteinization procedures because it is extremely simple and does not require sample adulteration. The adjunctive technique of displacement of protein-bound drugs with SDS increases the applicability range of the procedure. The ultrafiltration procedure has been successfully adapted in our laboratories for the determination of other antibiotics. Development of procedures for other similar compounds only requires selection of

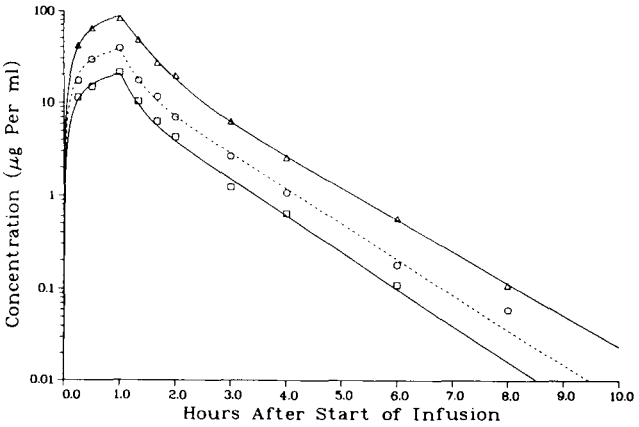

Fig. 5. Plasma level profiles, as determined by the proposed HPLC procedure for a subject receiving 500 (□), 1000 (○), and 2000 (△) mg of cefmenoxime as a 1-h intravenous infusion.

the proper chromatographic conditions to allow resolution of the drug and internal standard from compounds endogenous to plasma. Substituted aromatic acids serve well as internal standards because of their versatility. A wide range in retentivity can be realized by control of the mobile phase pH, and by choice of the substituent.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF CLIOQUINOL AND ITS CONJUGATES IN BIOLOGICAL MATERIALS

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SUMMARY

A method has been established for the determination of clioquinol (C) and its glucuronide (CG) and sulfate (CS) in biological materials. C and its internal standard were extracted with benzene—pyridine from samples. CG and CS were also hydrolyzed to C and extracted by the same method. The extracts were evaporated to dryness and redissolved in methanol. The methanol solution was subjected to HPLC using a column packed with Iatrobeads 6cp-2010 and a UV monitor (254 nm). The mobile phase was 0.1 *M* citric acid—methanol—*n*-hexane (8:86:6). The detection limit of C was 1 nmole and its recovery was above 92%.

INTRODUCTION

After the isolation of the ferric chelate of 5-chloro-7-iodo-8-hydroxyquinoline (clioquinol or chinoform, C) from the urine of a subacute myelo-optico neuropathy (SMON) [1] patient, the elucidation of the intoxication mechanism of C was one of the important goals of SMON research [2].

Some analytical methods were developed to study the metabolism and intoxication of the drug. In 1951, Haskins and Luttermoser [3] reported a spectrophotometric method for 8-hydroxyquinolines and estimated C conjugates in the urine of rabbits [4]. This method was also applied to the determination of C glucuronide in human urine [5]. However, the method could not be applied to blood samples because of its low sensitivity. Liewendahl et al. [6] investigated the metabolites in urine and plasma of man administered ^{125}I-labelled C, by radiochromatographic analysis. Tamura et al. [7] developed a sensitive method for the determination of C by gas chromatography (GC) using an electron-capture detector (ECD). Chen et al. [8] established a method of separatory determination of C and its glucuronide (CG) and sulfate (CS) in serum, urine and milk. Some modified methods for analysis of C by GC

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were also reported [9—11], and many studies on metabolism of C were reported using those methods. Although the GC method was highly sensitive to C, the procedure involved time-consuming manipulations.

Chen et al. [12] reported a method for the determination of CG and CS by high-performance liquid chromatography (HPLC) using an ion-exchange column, and applied the method to human urine. But this method could not be applied to plasma or tissues because the separation of CG and CS from large amounts of coexisting biological constituents absorbing at 254 nm was incomplete. Miura et al. [13] used HPLC in the assay of multiple enzyme activities with 8-hydroxyquinoline derivatives as substrates. However, this method could not be used to determine CG and CS in biological materials and the sensitivity was not enough to analyze micro amounts of C in blood.

We have therefore established a sensitive and simple HPLC method to determine C, CG and CS in animals.

EXPERIMENTAL

Materials

C, CG, CS (sodium salt), 5-chloro-8-hydroxyquinoline, 5,7-dichloro-8-hydroxyquinoline, 5-chloro-7-bromo-8-hydroxyquinoline and 5,7-dibromo-8-hydroxyquinoline were all kindly provided by Professor Tamura of the University of Tokyo. 8-Hydroxyquinoline and 2-methyl-8-hydroxyquinoline were purchased from Tokyo Kasei (Tokyo, Japan). 8-Hydroxyquinolines were used after recrystallization. β-Glucuronidase (13,000 Fishman units per ml) was obtained from Tokyo Zoki Co. (Tokyo, Japan). All other reagents used were of analytical grade.

Instruments for HPLC

The HPLC system used in this study comprised a Kyowa minimicro pump KSU-16H (Kyowa Seimitsu Co.), a Pyrex column (30 cm × 3 mm I.D.; Kyowa Seimitsu Co), a Mitsumi monitor SF-1205 (Mitsumi Scientific Industry Co.), equipped with a low-pressure mercury vapor lamp (254 nm), and a Toa recorder EPR-10B (Toa Electronics Ltd.). The column was packed with Iatrobeads 6cp-2010 (10 μm, polystyrene-type porous polymer; Iatron Chemical Products).

Sample preparation

To a biological sample (0.2 ml) in a glass-stoppered centrifuge tube were added a known amount of internal standard in 20 μl of methanol, 0.2 ml of 0.2 *M* EDTA (disodium salt) and 0.8 ml of distilled water. Then 4 ml of benzene—pyridine (9:1, v/v) were added to the tube, and the mixture was shaken vigorously for about a minute. After centrifugation (3500 *g*, 5 min) the organic phase collected was evaporated to dryness in vacuo and the residue was dissolved in 0.2 ml of methanol for HPLC analysis (C fraction). To remove a trace amount of C, internal standard and a fairly large amount of pyridine, the aqueous phase was washed twice with 6 ml of benzene, and the benzene was discarded. Then a known amount of internal standard in methanol, β-glucuronidase (final concentration 200 units per ml) and 0.15 ml of 1 *M* acetate

buffer (pH 5) were added to the aqueous phase, and incubation was performed with shaking at 37°C for 2 h. The liberated C and internal standard were similarly extracted and determined (CG fraction). The aqueous phase containing sulfate was similarly washed with benzene, and its acidity was adjusted to 1 *N* with 6 *N* HCl. Then a known amount of internal standard was added to the aqueous phase, and hydrolysis was carried out at 40°C for 2 h. The acid hydrolyzate, prior to extraction with benzene—pyridine, was almost neutralized with 3 *N* NaOH. The resultant extract was submitted to HPLC analysis (CS fraction). An aliquot (2—20 μl) of the methanol solution was injected into the column.

HPLC conditions

The mobile phase was 0.1 *M* citric acid—methanol—*n*-hexane (8:86:6, v/v). The flow-rate was 0.75 ml/min. The column was maintained at 37 ± 0.5°C.

RESULTS

Derivatives of 8-hydroxyquinoline gave intense absorbance at around 254 nm, which was advantageous for the microanalysis of C by HPLC with a UV detector at 254 nm. A combination of a non-polar resin with an acidic alcohol as the mobile phase was expected to give satisfactory resolution of 8-hydroxyquinolines by HPLC [12].

Therefore, resin types such as styrene—divinylbenzene and octadecylsilane-treated silica were examined for resolution of 8-hydroxyquinolines with acid—methanol solution as the mobile phase. The sample solution of 8-hydroxyquinoline, 5-chloro-8-hydroxyquinoline, 5,7-dichloro-8-hydroxyquinoline and C in methanol was used in this experiment. Both the styrene—divinylbenzene types, Iatrobeads 6cp-2010 and Hitachi 3010, could separate the four compounds described above, the former giving a better resolution and higher peaks than the latter. Iatrobeads 6cp-2010 were therefore used in the further

TABLE I

MOLAR ABSORPTIVITIES AND RETENTION TIMES OF 8-HYDROXYQUINOLINES

Compound	Absorptivity*		Retention time on HPLC** (min)
	λ_{max} (nm)	ϵ (× 10^4)	
8-Hydroxyquinoline (8HQ)	255	2.53	4.6
2-Methyl-8HQ	257	3.98	4.5
5-Chloro-8HQ	245	3.05	5.6
5,7-Dichloro-8HQ	248	3.81	6.8
5-Chloro-7-bromo-8HQ	248	3.75	7.8
5,7-Dibromo-8HQ	248	3.64	9.0
5-Chloro-7-iodo-8HQ (clioquinol)	255	3.94	9.6

*The solution was a mixture of 0.1 *M* citric acid—methanol—*n*-hexane (8:86:6).
**The conditions were as described under HPLC conditions.

study. Among the many mobile phases used, a mixture of 0.1 *M* citric acid—methanol—*n*-hexane (8:86:6) gave the best resolution of 8-hydroxyquinolines. Addition of *n*-hexane to the mobile phase at 6%, which was almost the solubility limit, reduced the retention time and made the peaks higher without affecting the resolution. The pressure in the column was lower at 37°C than at room temperature, and the resolution of the compounds was found to be reproducible at the constant temperature. The molar absorptivities and retention times of several 8-hydroxyquinolines are listed in Table I.

It was desirable to choose an internal standard for the method from among the 8-hydroxyquinolines, because it was expected to have a similar behaviour to C in the procedure. As is seen in Table I, 5,7-dichloro-8-hydroxyquinoline was separated completely from C, whereas the separation of 5-chloro-7-bromo-8-hydroxyquinoline or 5,7-dibromo-8-hydroxyquinoline from C was incomplete. The biological samples that did not contain any 8-hydroxyquinoline showed no peak at the retention time of 5,7-dichloro-8-hydroxyquinoline in the chromatogram. Thus 5,7-dichloro-8-hydroxyquinoline was chosen as internal standard. A chromatogram of C and the internal standard using Iatrobeads 6cp-2010 is shown in Fig. 1.

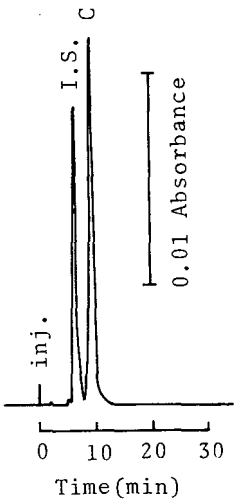

Fig. 1. Chromatogram of clioquinol (C) and 5,7-dichloro-8-hydroxyquinoline (I.S.).

C and the internal standard in biological samples showed clear peaks, whereas extracts of control biological samples free of C and internal standard, showed no peaks at their corresponding retention times in the chromatograms. CG and CS contained in biological samples were hydrolyzed to C by β-glucuronidase and HCl, according to the method by Chen et al. [8]. The extracts of these fractions also showed sharp peaks. Typical chromatograms for C in plasma, urine, bile and kidney are shown in Fig. 2.

When C and the internal standard were added to 0.2 ml of rabbit plasma, their recoveries by the preparation prior to HPLC analysis were more than 92% (Table II). The minimum detectable amount of C in methanol by the method was 20 pmoles (signal-to-noise ratio = 3). The calibration curves of

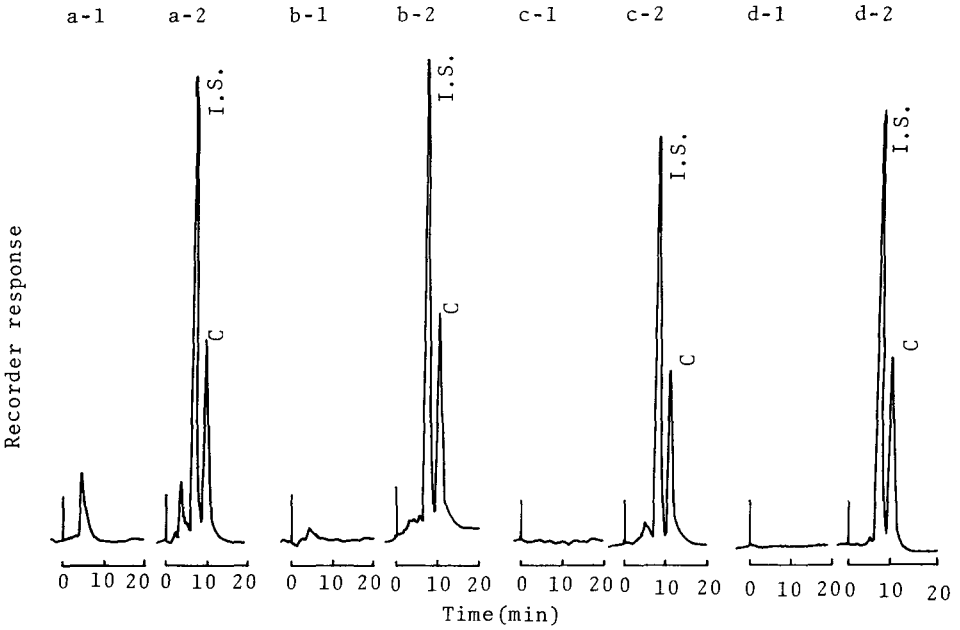

Fig. 2. Chromatograms of clioquinol (C) and 5,7-dichloro-8-hydroxyquinoline (I.S.) added in biological samples. (a-1) Extract from 0.2 ml of control plasma; (a-2) extract from 0.2 ml of plasma containing 4 nmoles of C and 12 nmoles of internal standard; (b-1) extract from 0.2 ml of control urine; (b-2) extract from 0.2 ml of urine containing 10 nmoles of C and 30 nmoles of internal standard; (c-1) extract from 0.2 ml of control bile; (c-2) extract from 0.2 ml of bile containing 10 nmoles of C and 30 nmoles of internal standard; (d-1) extract from 0.2 g of control kidney; (d-2) extract of 0.2 g of kidney containing 2 μmoles of C and 6 μmoles of internal standard.

TABLE II

RECOVERIES OF CLIOQUINOL AND 5,7-DICHLORO-8-HYDROXYQUINOLINE FROM PLASMA

Compound	Added (nmole/ml)	Recovered (nmole/ml) Mean ± S.D.	Recovery (%) Mean ± S.D.
Clioquinol	10	9.7 ± 0.13	97 ± 1.3
	100	99 ± 0.0	99 ± 0.0
5,7-Dichloro-8-hydroxy-quinoline	10	9.6 ± 0.57	96 ± 5.7
	100	92 ± 3.8	92 ± 3.8

C added to 0.2 ml of rabbit plasma showed excellent linearity in the range 2—40 nmoles/ml, when 8 nmoles of the internal standard were added.

DISCUSSION

It is well known that C is retained in the body for a long period after its administration. About 30 nmoles/ml of C were detected in the serum of a

SMON patient one month after stopping the administration of C [7], and more than 30 nmoles/g of C, CG and CS were detected in sera and several tissues of beagle dogs which showed neurological symptoms after the administration of C [14]. The proposed method was found to be effective in estimating these levels of C, CG and CS, although the GC method reported previously gave a slightly higher sensitivity than the HPLC method. Moreover, the HPLC method was found to be simpler and easier than the GC method, because the prerequisites of treatment by a Florisil column and acetylation of C in the GC method were unnecessary in the HPLC method. When both the proposed HPLC method and the GC method were applied to the analysis of C, CG and CS in plasma of a rabbit orally administered with 400 mg of C, the data obtained for the two methods were compatible (Fig. 3).

Large amounts of coexisting biological constituents that absorb at 254 nm disturbed the direct determination of CG and CS in plasma or tissues [12] as mentioned above. Improvement of the preparation was carried out, and these compounds could be almost removed from methanolic solution of C and

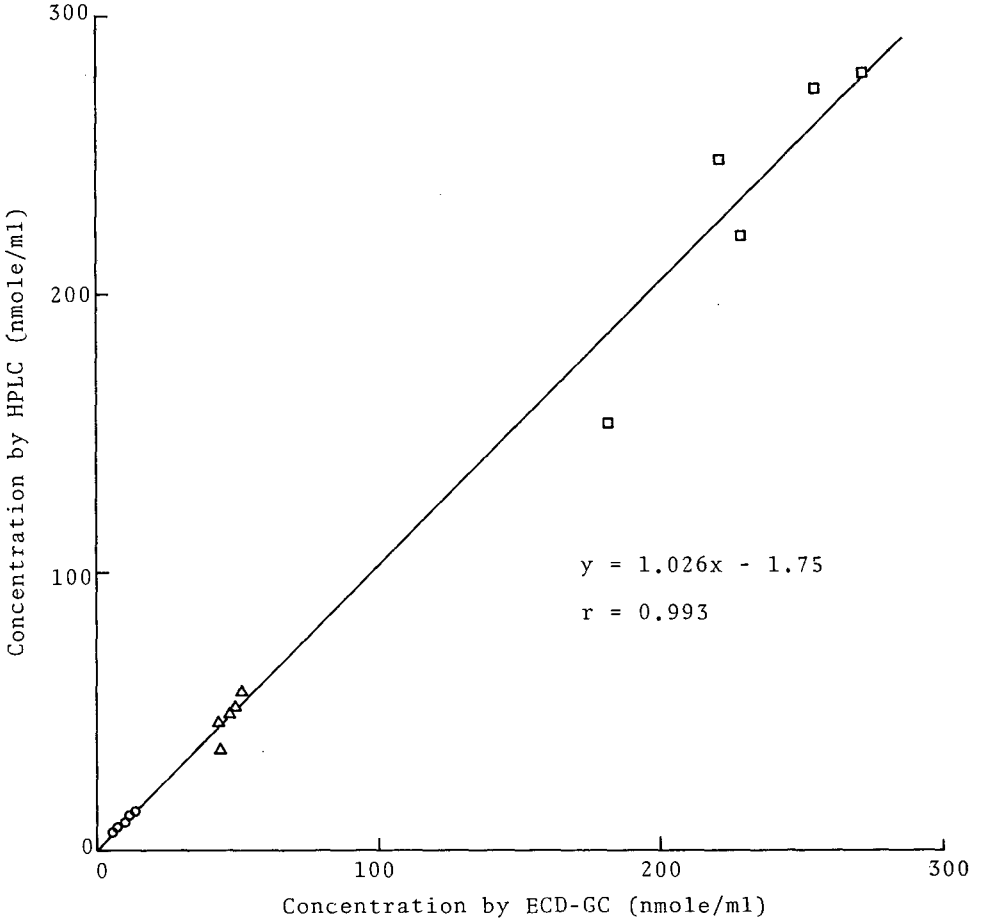

Fig. 3. Correlation between the values obtained by the HPLC method and the ECD-GC method in the analysis of C, CG and CS in plasma of a rabbit. (○), C; (□), CG; (△), CS.

internal standard by extraction with benzene—pyridine. On the other hand, the resolution of C and internal standard became worse when such metal ions as Cu^{2+}, Fe^{3+} and Zn^{2+}, which can chelate 8-hydroxyquinolines, were injected in the HPLC system. Addition of EDTA to the sample solution prevented these metal ions from being extracted into the benzene—pyridine phase and giving a sharp chromatogram of the two. Moreover, the peaks of C and internal standard were highest when *n*-hexane was added to the mobile phase near to saturation. By these improvements the sensitivity for C in the proposed method was higher than in the other HPLC method [13].

The present method should be useful in studying the metabolism and intoxication of C. Other 8-hydroxyquinoline derivatives [15] may also be detected easily by the modification of the HPLC conditions.

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DETERMINATION OF BROMO-LASALOCID IN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORIMETRIC DETECTION

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SUMMARY

A rapid, sensitive, and specific high-performance liquid chromatographic (HPLC) assay was developed for the determination of bromo-lasalocid in plasma. The compound was extracted into isooctane—ethyl acetate (90:10) from plasma saturated with potassium chloride and adjusted to strongly alkaline pH. The residue of this extract was dissolved in methanol—2-methoxyethanol (95:5) and analyzed by HPLC on a 10-μm C_{18} column [mobile phase of methanol—water—2-methoxyethanol—1 *M* potassium phosphate buffer, pH 3.0 (90:10:2.5:0.2)] using fluorescence detection with excitation at 215 nm and emission at wavelengths greater than 370 nm. The overall recovery of the assay was 65%, with a limit of sensitivity of 0.1 μg/ml. The method was used to obtain plasma concentration—time profiles in the dog following oral administration of bromo-lasalocid·ethanolate.

INTRODUCTION

Bromo-lasalocid [I] (Fig. 1), synthesized from the antibiotic X-537 A (lasalocid) [1], is presently under investigation as an ionophoric cardiovascular agent [2]. Investigation of the chemical transformations of [I] via pyrolysis and base-catalysis have been reported [3].

Analytical methods for lasalocid, the parent compound, include pyrolytic gas—liquid chromatographic—flame ionization detection (GLC—FID) determination in fermentation broths [4], bio-autoradiographic analysis in chicken tissues [5], spectrofluorometric assays in finished feeds and premixes [6] and in dog blood [7], and high-performance liquid chromatography (HPLC) with either UV [8] or fluorometric detection [9]. The GLC—FID analysis of intact lasalocid and bromo-lasalocid as their trimethylsilyl derivatives was also reported [10].

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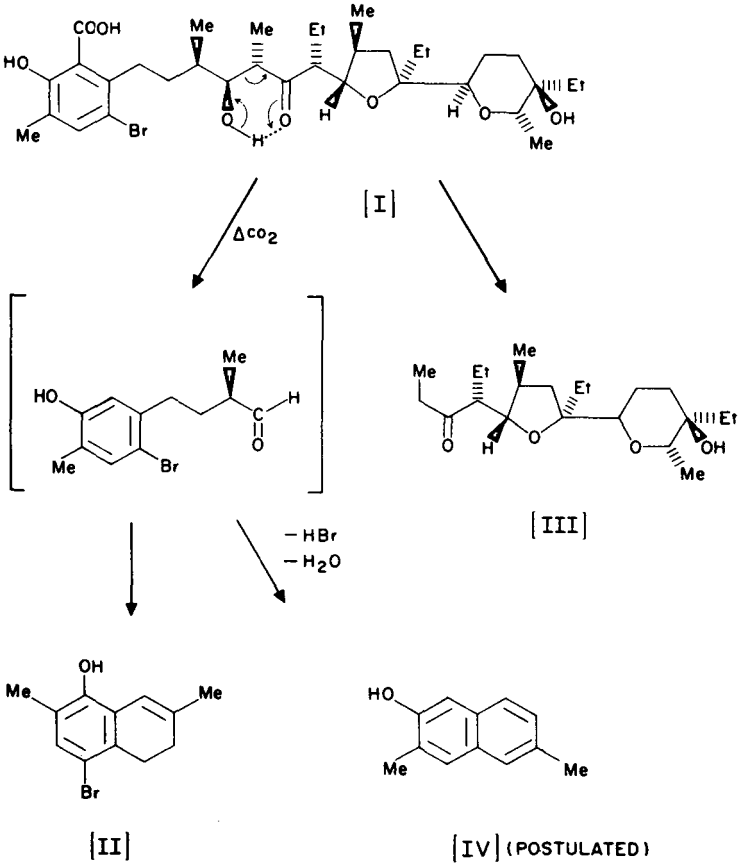


Fig. 1. GLC pyrolysis of bromo-lasalocid [4].

The present work describes a reversed-phase HPLC assay using fluorescence detection for the analysis of [I] in organic extracts of alkalinized dog plasma following intravenous and oral administration of [I]·ethanolate. The overall recovery of the assay is 65% with a limit of sensitivity of 0.1 μg/ml. In addition port. The HPLC assay is, however, preferred over the GLC—ECD assay assay is also described which utilizes the 4-bromo-5,6-dihydro-2,7-dimethyl-1-naphthol [II] (Fig. 1), produced by pyrolytic cleavage of [I] in the injection port. The HPLC assya is, however, preferred over the GLC—ECD assay due to its simplicity, higher specificity, and lower sensitivity limit.

EXPERIMENTAL

Column

The column used for reversed-phase liquid chromatography was a prepacked 30 cm × 3.9 mm I.D. stainless-steel column containing a μBondapak C-18 reversed-phase 10-μm microparticulate packing (Serial No. 115415, Waters Assoc., Milford, MA, U.S.A.).

HPLC instrumental parameters

An HPLC system (Model ALC/GPC 204/6000A, Waters Assoc.), equipped with a 400 bar (6000 p.s.i.) pump, loop injection system and UV detector with a 254-nm wavelength kit was used for chromatography. A 2-μm pre-column filter was used to improve column life. An HPLC fluorescence detector (Model FS-970, Kratos, Schoeffel Instruments, Westwood, NJ, U.S.A.),

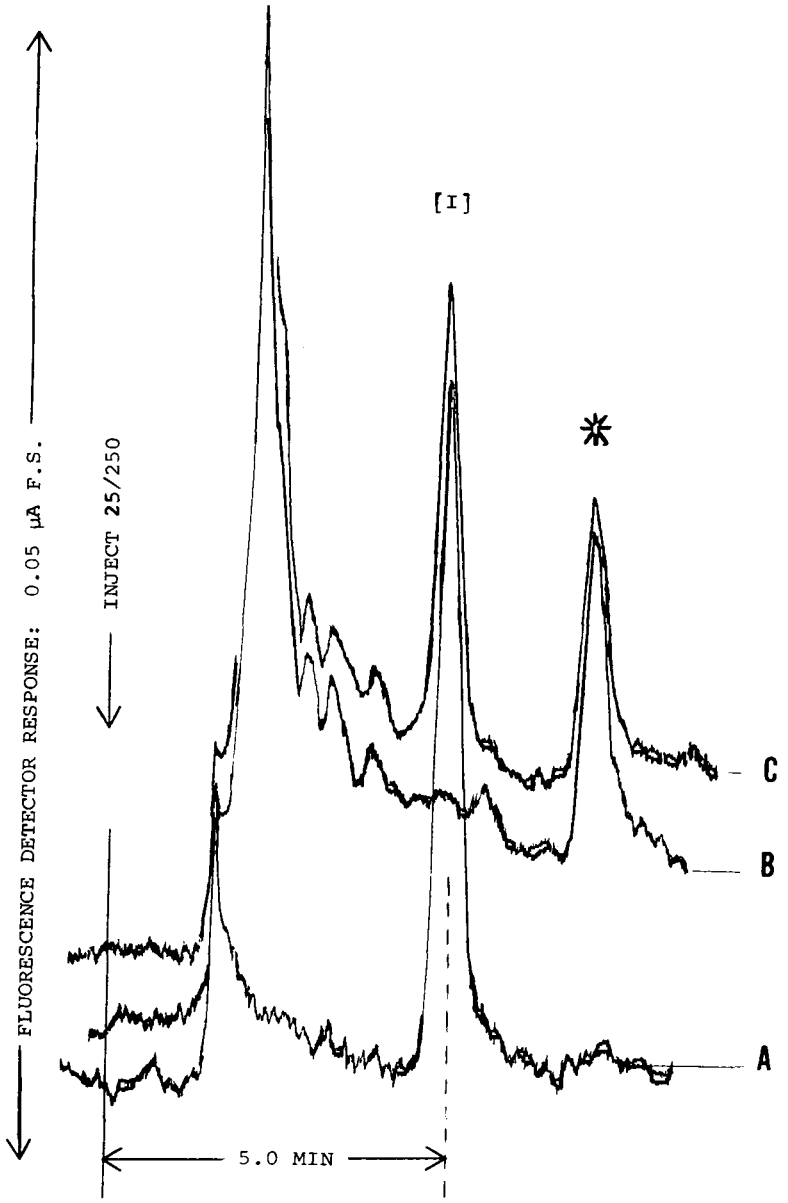


Fig. 2. HPLC separation of (A) authentic standard of 20 ng [I] injected, and extracts of 1-ml aliquots of (B) control human plasma, (C) control human plasma containing 200 ng added authentic [I] per ml. (*) Endogenous component.

operated at 215 nm for excitation with an emission filter passing wavelengths greater than 370 nm, was used for fluorometric detection. The mobile phase used for isocratic reversed-phase chromatography consisted of 900 ml of methanol, 100 ml of water, 25 ml of 2-methoxyethanol, and 2.0 ml of 1 *M* potassium phosphate buffer, pH 3.0. The solvent flow-rate was 1.5 ml/min resulting in a column head pressure of about 100 MPa. Under these conditions, the capacity factor (k') for [I] was 2.6 (retention time = 5.0 min) (Fig. 2A), and 20 ng of [I] injected gave approximately 70% full scale pen response with the detector range at 0.05 μA full scale and the photomultiplier sensitivity at 520 V. The chart speed on the dual-channel recorder (Model 7132A with option 108, Hewlett-Packard, Palo Alto, CA, U.S.A.) was 0.5 in./min.

Spectrophoto/fluorometric instrumentation

Ultraviolet absorbance spectra were recorded using a double-beam ratio-recording spectrophotometer (Coleman Model EPS-3T Hitachi Spectrometer, Coleman Instruments, Maywood, IL, U.S.A.). Corrected luminescence excitation and emission spectra (10 nm bandpass) were recorded using a spectrofluorometer equipped for direct recording of corrected excitation—emission spectra (Farrand Mark I, Farrand Optical Co., Valhalla, NY, U.S.A.). The quantum yields were determined with excitation at the absorption maxima of 310—320 nm rather than those at 209—212 nm because the diminished xenon arc lamp intensity at less than 250 nm does not yield reliable, noise-free emission spectra.

Reagents

All inorganic reagents were analytical reagent grade (ACS). All aqueous solutions were prepared with distilled, carbon-filtered, deionized water, filtered through a 0.2-μm filter (Type DC System, Hydro-Service and Supplies, Durham, NC, U.S.A.). Organic solvents suitable for spectrophotometry and liquid chromatography were purchased from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.).

Preparation of standard solutions

A stock solution of the ethanolate salt of bromo-lasalocid, $C_{34}H_{53}BrO_8 \cdot C_2H_6O$, mol. wt. = 715.75, m.p. = 110°C, was prepared by weighing 10.7 mg (equivalent to 10.0 mg of free acid) into a 10-ml amberized volumetric flask and dissolving in 10 ml of methanol—2-methoxyethanol (95:5) to give a stock solution containing 1.0 mg [I] per ml. Five working standards containing 0.1, 0.2, 0.5, 1.0, and 2.0 μg of [I] per 250 μl were prepared by serial dilution of the stock solution with methanol—2-methoxyethanol (95:5).

Aliquots (25 μl) of each working solution, equivalent to 10, 20, 50, 100, and 200 ng of [I] were injected to obtain an external calibration curve, which was used to establish the linearity and reproducibility of the HPLC system and the overall recovery of the assay.

Procedure

A 1.0-ml aliquot of each unknown plasma sample was added to separate

13 × 100 mm disposable borosilicate culture tubes (Cat. No. 14-962-10C, Fisher Scientific, Pittsburgh, PA, U.S.A.). Specimens of control plasma containing [I] were prepared by transferring 250-μl aliquots of selected working standards into separate tubes and evaporating in a stream of clean, dry nitrogen at room temperature, and then adding a 1.0-ml aliquot of control plasma to each standard and mixing well. These specimens were processed along with the unknowns as the recovery standards for the determination of [I] in the unknowns.

A 5-ml aliquot of isooctane—ethyl acetate (90:10) was added to each tube, followed by approximately 0.5 g potassium chloride and 1.0 ml 1 *N* potassium hydroxide. The tubes were stoppered with polyethylene caps (Plugtite cat. No. 127-0019-100, Elkay Products, Shrewsbury, MA, U.S.A.) and shaken for 10 min on a reciprocating shaker (Eberbach, Ann Arbor, MI, U.S.A.) at 50—80 strokes/min. The samples were centrifuged at 950 *g* for 10 min at 0—5° C in a refrigerated centrifuge (Model PR-J with a No. 253 rotor, Damon/IEC, Needham, MA, U.S.A.) and a 4.5-ml aliquot of the upper organic layer was transferred into a clean culture tube. The organic extract was evaporated to dryness at 20—25° C in a N-EVAP evaporator (Organomation Assoc., Worcester, MA, U.S.A.) under a stream of clean, dry nitrogen. The residue was dissolved in 250 μl of methanol—2-methoxyethanol (95:5) and a 25-μl aliquot was injected for HPLC analysis using reversed-phase chromatography. The Schoeffel fluorescence detector was set at 0.05 μA full scale for maximum sensitivity. Typical chromatograms are shown in Fig. 2B and C.

The concentration of [I] in each unknown was determined by interpolation from the calibration curve of the recovered standards processed with least squares regression analysis using an exponential equation.

RESULTS

Statistical validation of the method

The HPLC analysis of authentic (non-recovered) standards of [I] in the concentration range 0.01—0.20 μg injected is best described using least squares regression analysis with an exponential equation of the form $Y = mX^b$ ($Y = 2.89\ X^{0.971}$), where X = concentration injected and Y = fluorometric response (chromatographic peak height). The correlation coefficient (r) of 0.998 demonstrates the linearity and precision of the system.

The recovery of [I] from human plasma using the described assay was determined in the concentration range of 0.1—2.0 μg/ml of plasma by substitution of the fluorometric response obtained for these samples into the equation describing the calibration curve of the authentic (non-recovered) standards (see above); with the appropriate correction for aliquot injected (25/250) and for the extraction aliquot of 4.5/5.0. The mean overall recovery of bromo-lasalocid [I] (n = 14) was 64 ± 11% (S.D.).

The precision and accuracy of the method were determined by construction of a linear least squares regression calibration curve (exponential equation) from the responses of the recovered standards [$Y = 1.82\ X^{1.10}$ (r = 0.994), where Y = fluorometric response and X = concentration of [I] per ml of plasma] and substitution of the experimentally determined responses into the

TABLE I

INTRA-ASSAY STATISTICAL EVALUATION

Amount [I] added (μg/ml)	Amount [I] found ± S.D. (μg/ml)	*n*	Relative S.D. (± %)
0.1	0.088 ± 0.005	2	5.6
0.2	0.22 ± 0.02	3	10.5
0.5	0.55 ± 0.04	3	7.7
1.0	0.90 ± 0.06	3	6.8
2.0	2.03 ± 0.03	3	1.3
		Mean	6.2
$Y = mX^b = 1.82\ X^{1.16}$		$r = 0.994$	

where X = concentration of [I] in μg/ml and Y = fluorometric response (peak height)

theoretical equation to yield the amount [I] found (Table I).

The sensitivity limit of the assay was validated at 0.1 μg [I] per ml, using a 1.0-ml sample of human plasma per assay. The intra-assay mean relative standard deviation was 6.2% over the concentration range of 0.1—2.0 μg [I] per ml of plasma (Table I).

TABLE II

PLASMA CONCENTRATION OF COMPOUND [I]

Determination by HPLC—fluorometric analysis in the dog following a single oral administration of 28.57 mg [I]·ethanolate per kg (equivalent to 26.69 mg [I] per kg of body weight).

Sampling time (h)	Concentration μg [I] per ml of plasma
0.167	N.M.*
0.333	0.12
0.5	0.74
0.75	2.6
1.0	2.0
1.5	3.5
2	5.3
3	6.1
4	5.2
6	3.9
8	3.0
10	2.5
24	0.36
30	0.16
48	N.M.
72	N.M.

*N.M. = nonmeasurable, < 0.1 μg/ml of plasma.

Application of the method to biological specimens

The assay was applied to the determination of plasma concentrations of [I] in the dog following oral doses of [I]·ethanolate. Typical data are shown in Table II for the analysis of [I] in the dog following an oral dose of 28.57 mg/kg of [I]·ethanolate (equivalent to 26.69 mg of the free acid of [I] per kg of body weight) as a capsule formulation.

DISCUSSION

Gas—liquid chromatographic analysis of [I]

Pyrolysis of intact [I]. Initial development of an assay for [I] in biological fluids was based upon the pyrolytic properties of the molecule during GLC analysis [4]. The pyrolysis of [I] in the injection port during GLC analysis, monitored by a flame ionization detector, yielded three major peaks (Fig. 3). The peaks at 6.2 and 15.5 min were identified by GC—mass spectrometric analysis [11] as 4-bromo-5,6-dihydro-2,7-dimethyl-1-naphthol [II], and a retroaldol ketone [III] (Fig. 1), respectively (identical to that formed on the pyrolysis of lasalocid [3]). The peak at 2.5 min has not been identified but is

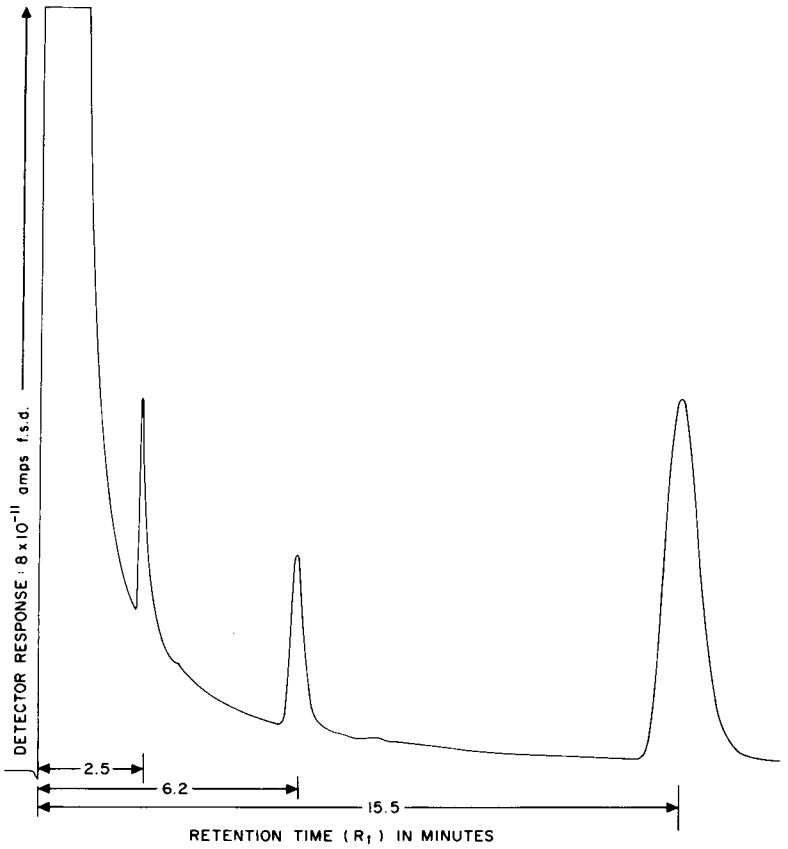


Fig. 3. GLC pyrolysis of 1 μg of [I] monitored by the flame ionization detector (GLC conditions, see Table III).

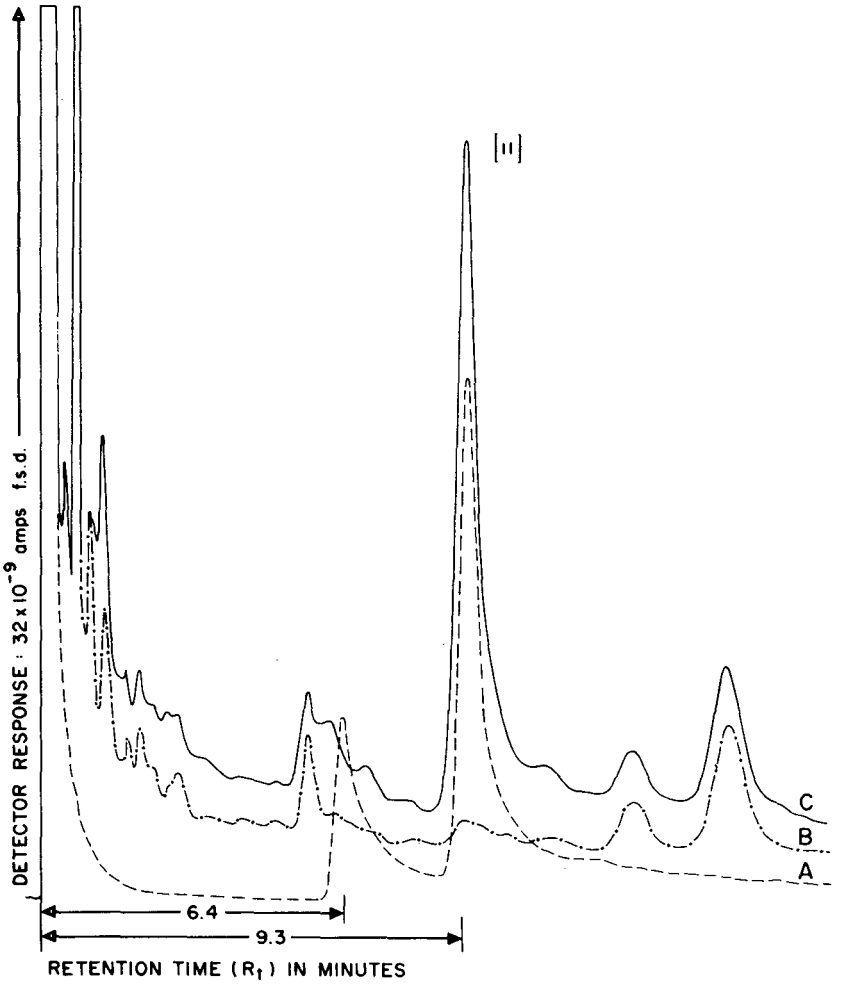


Fig. 4. Electron-capture GLC pyrolysis of (A) authentic standard, 50 ng [I] injected; (B) control blood extract; (C) extract from sample containing 750 ng of [I] per ml of blood (injection aliquot 5/50 μl) (GLC conditions, see text).

postulated to be 3,6-dimethyl-2-naphthol [IV] which is also a pyrolysis product of lasalocid (Fig. 1). The pyrolysis of [I] monitored under the same conditions with an electron-capture (EC) detector yielded two peaks (Fig. 4A), the second of which was compound [II]. This was confirmed using an authentic standard of [II] which had an identical retention time to that of the second peak monitored by both detectors. The identification was also confirmed using relative retention to 2-methylamino-5-chloro-benzophenone (MACB) (Table III) on both 3% OV-1 and 3% OV-17 column packings, using electron-capture and flame ionization detectors. The relative retention times also indicated that the first peak monitored by each detector was not the same material. Presumably, the first EC peak was a minor pyrolysis product with high sensitivity by ECD while peak 3 monitored by FID was identified as [III] by comparison to the authentic compound.

TABLE III

RELATIVE RETENTION TIMES OF PYROLYSIS PRODUCTS OF [I] TO MACB

GLC conditions: 3% phase on Gas-Chrom Q (60—80 mesh); oven temperature: 170°C; injection port: 260°C; detector temperatures: ECD, 325°C; FID, 300°C; flow-rates: ECD, 90 ml/min column flow argon—methane (90:10), 20 ml/min detector purge flow; FID, 90 ml/min nitrogen, 70 ml/min hydrogen, 570 ml/min air; samples ECD, 100 ng [I] per 10 μl injected; FID, 1 μg [I] per 10 μl injected.

Detector	OV-17 column			OV-1 column		
	Peak 1	Peak 2	Peak 3	Peak 1	Peak 2	Peak 3
FID	0.17	0.42	1.04	0.25	0.53	1.98
EC	0.30	0.43	N.D.*	0.44	0.54	N.D.

*N.D. = not detected.

The limit of sensitivity for the measurement of [II] by ECD was 10 ng injected and that for [II] and [III] by FID was 100 and 500 ng injected, respectively. The higher sensitivity of the EC detector also enabled the determination of concentrations of [I] in the dog following administration of [I]·ethanolate.

The chromatographic conditions for the analysis of [I] must be rigidly controlled to ensure reproducible pyrolysis with a linear concentration response of [II] for ECD over the concentration range of 25—100 ng injected. Variation of the injection port temperature from 225°C to 300°C demonstrated that the highest yields of [II] were obtained between 250°C and 280°C. At temperatures above 285°C larger amounts of the by-product (retention time = 6.4 min, Fig. 4A) were present than at other temperatures. The flow-rate of the argon—methane (90:10) carrier gas and the volume of sample injected are also critical for reproducible pyrolysis. Relatively slow flow-rates ($\leqslant$ 50 ml/min), and small volumes ($\leqslant$ 5 μl) gave the highest yield of [II] with small amounts of by-product. With slow flow-rates and small injection volumes, the residence time of [I] in the injection port was longer and resulted in an optimal and reproducible yield of [II].

The analysis of [I] by GLC—ECD was performed on the residue of a benzene (nanograde, Mallinckrodt, St. Louis, MO, U.S.A.) extract of blood which had been adjusted to pH 12.6 with Na_3PO_4. GLC—ECD analysis was performed on a 5-μl aliquot of the residue which was reconstituted in 50 μl of acetone. A Tracor MT-220 gas chromatograph equipped with a ^{63}Ni EC detector containing a 15 mCi ^{63}Ni β-ionization source was used. Argon—methane (9:1) (Matheson Gas Products, East Rutherford, NJ, U.S.A.) was used as the carrier gas and the column head pressure was pre-set at 40 p.s.i.g. (2.76 bar), with a column flow-rate of 50 ml/min and a detector purge of 20 ml/min. The temperature settings were as follows: oven 175°C; injection port 280°C; detector 325°C. The conditions of flow-rate and column temperature must be adjusted to obtain a retention time of 9.3 min for [II] (Fig. 4).

The solid state electrometer (Model No. 8169) input was set at 10^2, and the output attenuation was 32 giving a response of $3.2 \cdot 10^{-9}$ A for full scale deflec-

tion, the chart speed was 30 in./h, and the time constant on the 1.0-mV Honeywell recorder (Model No. 194) was 1 sec (full scale deflection). The response of the ^{63}Ni EC detector (operated in the pulsed mode) to [II] showed maximum sensitivity at 45 V d.c. at a 270-μsec pulse rate and a 4-μsec pulse width.

The column packing was a pretested phase containing 3% OV-17 on 60—80 mesh Gas-Chrom Q (Applied Science Labs., State College, PA, U.S.A.) packed in a U-shaped 1.22 m × 4 mm I.D. borosilicate glass column. The packed column was conditioned at 315°C under no-flow conditions for 4 h and then at 265°C for at least 18 h with a nitrogen flow-rate of 40 ml/min. The GLC—ECD assay for bromo-lasalocid has an overall recovery of 65 ± 15% (S.D.) in the concentration range of 0.5—10 μg/ml of dog blood. This relatively high standard deviation is directly related to the degree of cleanliness of the extracts used for analysis. The assay of these extracts, which are slightly colored and contain material which is sometimes insoluble in acetone, often yields responses (area [II]/ng [I] injected) which are lower than those obtained for the pure material. This is probably due either to the saturation of the EC detector or to the inhibition of pyrolysis. In order to obtain meaningful recovery data, it is essential that analysis of the recovery standards and the external standards be in an alternating sequence. Attempts to clean-up the samples by either formation of protein free filtrates and/or back-extraction resulted in significant losses of compound [I] with overall recovery of approximately 25%.

Derivatization reactions. Assay of intact [I] as its silyl derivative [10] was attempted; however, interferences originating from the silylation reagent made quantitation impractical. In addition, the sensitivity obtained by pyrolysis GLC—ECD was higher than that reported for the silyl derivative (300 ng). GLC—ECD examination of the pentafluorobenzyl ester and/or phenolic ether of [I] formed with pentafluorobenzyl bromide (PFBB) demonstrated that the single product formed (confirmed by thin-layer chromatography) underwent pyrolytic cleavage to yield two electron-capturing derivatives. These products were identified as having retention times of 7 and 11 min, respectively, on a 1.22 m × 2 mm glass column containing 3% OV-17 coated on 80—100 mesh Gas-Chrom Q at oven and injector temperatures of 200°C and 290°C, respectively. This approach was abandoned because reproducible pyrolysis of the pentafluorobenzyl ester/ether derivative of [I] yielding a single GLC component could not be obtained.

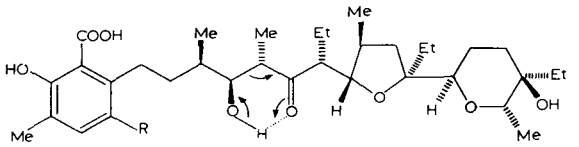
High-performance liquid chromatography

The major limitation of pyrolysis GLC—ECD analysis of [I] was the undefined specificity of the determination. The pyrolysis product, 4-bromo-5,6-dihydro-2,7-dimethyl-1-naphthol [II] constitutes only a small portion of the total bromo-lasalocid molecule [I], and as such metabolic transformation in the larger portion of the molecule would be undetected. The HPLC analysis of intact [I] was undertaken due to the undefined specificity of GLC—ECD.

Ultraviolet detection. The UV spectrum of [I] in methanol shows absorbance maxima at 212 and 315 nm, the former being approximately eightfold more intense (Table IV). Reversed-phase HPLC examination of the residue

TABLE IV

UV ABSORPTION AND FLUORESCENCE EMISSION CHARACTERISTICS OF BROMO-LASALOCID AND ITS ANALOGUES DETERMINED IN THE HPLC MOBILE PHASE



Compound (free acid)	R	UV absorption		Fluorescence	
		$\lambda_{abs.\ max}$ (nm)	Absorptivity (L/g-cm)	λ_{em} (nm)	Quantum efficiency* (Φ F)
Bromo-lasalocid	Br	212	43		
		315	5.5	425	0.014
Lasalocid	H	209	59		
		245	8.3		
		310	6.4	418	0.16
Chloro-lasalocid	Cl	210	58		
		315	6.4	423	0.22
Iodo-lasalocid	I	212	47		
		318	4.7	425	0.025

*The quantum efficiencies were determined from emission spectra obtained while exciting at the 310—320 nm maxima.

of benzene extracts of alkalinized plasma (pH 12.8, 0.7 *M* Na_3PO_4) on a μBondapak C_{18} column using a mobile phase of methanol—water—glacial acetic acid (90:10:1) showed that endogenous constituents in the control sample precluded measurement of [I] at 212 nm (variable-wavelength UV detector, Tracor Model 970A, Austin, TX, U.S.A.). However, [I] in this extract could be quantitated at 254 or 313 nm (fixed wavelength mercury-lamp detectors, Model 440, Waters Assoc.) with no interference from endogenous materials. Detection at 254 nm is preferred since the signal is 1.36 times greater with a signal-to-noise ratio two- to four-fold higher than that at 313 nm.

The HPLC—UV assay described above for [I] has an overall recovery of 65—70% in the concentration range of 1.0—5.0 μg of [I] added per ml of plasma. The data are best described by a linear regression equation of the form $Y = aX + b$ ($Y = 1.755\ X—0.19$) with a correlation coefficient (r) of 0.985 and an average deviation of 8.47%.

Fluorescence detection. The use of conventional spectrophotofluorometry [7] or fluorescence detection following HPLC separation [9] with excitation at 315 nm (as described for the high-sensitivity determination of lasalocid) is not feasible for [I] due to its ten-fold lower quantum efficiency yield (Table IV). This phenomenon is probably directly attributable to the bromine atom in the salicylic acid moiety which causes delocalization of the π electrons of the molecule.

The use of fluorescence detection with excitation at 315 nm following

HPLC did not increase the sensitivity of the analysis above that obtained using absorptiometric detection at 254 nm or 313 nm. However, the use of fluorescence detection with excitation at short UV wavelengths (215 nm) in conjunction with the use of solvents transparent at short UV wavelengths provided a five- to ten-fold increase in sensitivity beyond that obtained with UV absorption. In addition, the inherent specificity of fluorescence detection provided a much cleaner chromatographic baseline for plasma extracts than did absorptiometric detection at 215 nm.

A search for an analogue with fluorescence and HPLC characteristics similar to [I] suitable for use as an internal standard did not yield a useable compound. Although lasalocid, chloro-lasalocid, and iodo-lasalocid (Table IV) possessed UV absorbance and fluorescence emission characteristics suitable for either form of detection, they were not resolved from bromo-lasalocid under the conditions of the HPLC assay.

Compound [I] exhibits adsorption onto plasma constituents and onto glassware and stainless-steel surfaces at low concentrations. The extraction solvent was therefore changed from benzene (GLC—ECD analysis) to the more polar isooctane—ethyl acetate (90:10) which yielded more reproducible recovery at low concentrations, while 2-methoxyethanol was added to both the solvent and the mobile phase to improve the desorption and solvation of [I]. Solvent evaporation was performed at room temperature since elevated temperatures resulted in lower and more variable recovery of [I].

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY—FLUORESCENCE ANALYSIS FOR INDOMETHACIN AND METABOLITES IN BIOLOGICAL FLUIDS

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SUMMARY

A rapid and sensitive high-performance liquid chromatography—fluorescence method is described for the quantitative analysis of indomethacin and its metabolites in urine. A modified version is also shown for the detection of indomethacin in plasma. The method consists of a single extraction of an acid-buffered plasma sample or single extractions of two buffered aliquots in parallel of urine using ethyl acetate, followed by evaporation of the organic phase. Indomethacin (I) and the metabolite desmethylindomethacin (DMI) were deacylated to their fluorescent products, deschlorobenzoylindomethacin (DBI) and desmethyldeschlorobenzoylindomethacin (DMBI), respectively, prior to chromatography. The chromatographic phase utilized a reversed-phase C_{18}-bonded column with a solvent system comprised of either 22.5% or 26% acetonitrile in 0.25% acetic acid. The elution times for indomethacin metabolites ranged from 12—26 min. The total DBI (including deacylated I) and DMBI (including deacylated DMI) in the extract were each determined using fluorometric detection, with excitation at 288 nm and emission at 390 nm (370 nm cutoff filter). An internal standard of indole-3-propionic acid was used for quantitation. The lower limit of sensitivity for I in plasma was 25 ng/ml.

INTRODUCTION

Indomethacin (I) is an anti-inflammatory drug widely used in the treatment of arthritic disease [1, 2]. Recent clinical studies have demonstrated that I may also be effective as a pharmacologic alternative to surgery in closure of the patent ductus arteriosus (PDA) occurring in premature infants [3, 4]. Closure of the fetal pulmonary-aortic shunt is believed to be prevented by the dilatory effects of prostaglandin E on the smooth muscle lining the ductus [5, 6]. Indomethacin blocks the initial step in the conversion of arachidonic acid to prostaglandins through inhibition of the cyclo-oxygenase enzyme [7].

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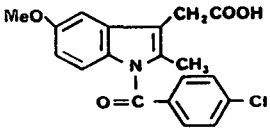
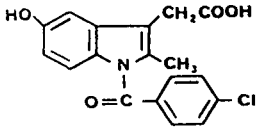
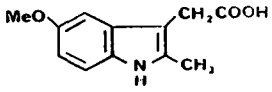


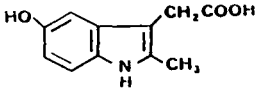
Indomethacin



DMI



DBI



DMBI

Fig. 1. The structural formulas of indomethacin and its metabolites: indomethacin (I) is 1-(*p*-chlorobenzoyl)-5-methoxy-2-methyl-indole-3-acetic acid, M.W. 358; deschlorobenzoylindomethacin (DBI), M.W. 220; desmethylindomethacin (DMI), M.W. 344; desmethyldeschlorobenzoylindomethacin (DMBI), M.W. 206.

Methods for the quantitation of I in physiologic fluids have included spectrofluorometry [8, 9], gas chromatography (GC) [10—12], GC—mass spectrometry (GC—MS) [13], high-performance liquid chromatography (HPLC) [14, 15] and radioisotope dilution [16]. The HPLC—fluorescence assay to be described provides for a sensitive determination of not only I but also its three major metabolites, deschlorobenzoylindomethacin (DBI), desmethylindomethacin (DMI) and desmethyldeschlorobenzoylindomethacin (DMBI) (Fig. 1), in urine. In addition, this technique provides a level of sensitivity approaching that of GC but without the attendant difficulties. The utility of this sensitive assay is evident when considering the restricted therapeutic range of I in neonates [17, 18]. The correlation of I levels in plasma and I plus its metabolites in urine, with administered dose and therapeutic efficacy in PDA treatment, is of current interest [19].

EXPERIMENTAL

Reagents

Indomethacin and indole-3-propionic acid (I3P, internal standard) were

purchased from Sigma (St. Louis, MO, U.S.A.). Desmethylindomethacin was a gift from Merck (West Point, PA, U.S.A.). Spectrograde ethyl acetate was supplied by Pierce (Rockford, IL, U.S.A.). Acetonitrile (UV-grade) was from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). All solutions were prepared using double-distilled water.

Protocol

(A) Urine. Analysis of I urine metabolites was based upon parallel extraction of two urine samples. One sample was used for determination of the total (I + DBI) and the total (DMI + DMBI) in the extract following alkaline hydrolysis, while the other sample extract was maintained in 100% ethanol for quantitation of directly extractable DBI and DMBI. Subtraction of the DBI found in the unhydrolyzed from that observed in the hydrolyzed sample extract provided individual values for both I and DBI. An analogous procedure was applied for quantitation of DMI and DMBI in urine.

(B) Plasma. I concentration in plasma was determined by alkaline hydrolysis of the solvent extract and quantitation of the observed DBI chromatographic peak.

Procedure

(A) Urine. Parallel 0.5-ml urine samples were buffered to pH 5.0 using 0.45 ml of 0.2 *M* sodium acetate buffer and extracted with 4.4 ml of ethyl acetate. A 50-μl aliquot of 10 μg/ml I3P (dissolved in pure ethanol) was added to the sample prior to extraction. The solvent extract in both samples was removed and evaporated to dryness using a vacuum centrifuge. One extract was redissolved in 50 μl of 0.1 *N* sodium hydroxide and allowed to stand for 15 min, then diluted with 0.4 ml of water and neutralized with 50 μl of 0.1 *N* hydrochloric acid. This sample was used for determination of total (I + DBI) and total (DMI + DMBI). The extract from the second sample was redissolved in 100 μl of pure ethanol and diluted with 0.4 ml water. This sample was used for directly extractable DBI and DMBI determinations. Approximately 50 μl from each sample were injected onto the column. Conjugated metabolites may be determined by overnight treatment with β-glucuronidase prior to extraction [16, 20].

(B) Plasma. A 100-μl aliquot of plasma was mixed with 100 μl acetate buffer (0.2 *M* sodium acetate, pH 5.0) and 50 μl of I3P (0.4 μg/ml). The mixture was extracted with 1.0 ml of ethyl acetate and the organic phase evaporated using a vacuum centrifuge. The residue was redissolved for 15 min in 40 μl of 0.1 *N* sodium hydroxide and then neutralized with 40 μl of 0.1 *N* hydrochloric acid. Approximately 60 μl from each sample was injected onto the column.

Chromatography

A Waters Assoc. (Milford, MA, U.S.A.) Model 6000A pump coupled with a Waters Model U6K injector was utilized for the solvent delivery system. Separation of the individual metabolites was performed using a Whatman (Clifton, NJ, U.S.A.) 15% C_{18}-bonded reversed-phase column (25 cm × 4.6 mm) with 10-μm particles, together with a mobile phase of either 22.5% or 26% acetonitrile—0.25% acetic acid.

Fluorescence was measured with a Schoeffel (Westwood, NJ, U.S.A.) Model FS 970 fluorometer, using sensitivities in the range 0.02—0.5 μA. The excitation wavelength was 288 nm with emission monitored at 390 nm; a 370-nm cutoff filter was used in emission detection. A Hitachi (Tokyo, Japan) Model 100-30 variable-wavelength spectrophotometer was used to measure ultraviolet absorption at 285 nm.

Mass spectrometry

A Varian MAT (Dayton, OH, U.S.A.) Model 112S mass spectrometer in electron ionization mode, with an interactive spectral data system, was used to obtain mass spectra.

Calibration

Urine samples. Peak height ratios (PHR's) were computed for a series of I and DMI standards in drug-free urine, ranging from 0.25—25.0 μg/ml, using a chromatographic solvent system comprised of 22.5% acetonitrile—0.25% acetic acid with a flow-rate of 1.5 ml/min. A DBI and DMBI series of standards were also prepared, with a range of 0.15—12.0 μg/ml, and analyzed using a 26% acetonitrile—0.25% acetic acid solvent system with 2.0 ml/min flow-rate. In both cases the internal standard (I3P) concentration was 1.0 μg/ml in the sample injected onto the column.

Plasma samples. A standard series in the range 25—200 ng/ml of I in drug-free plasma was prepared and analyzed using a 22.5% acetonitrile—0.25% acetic acid mobile phase with a flow-rate of 2.0 ml/min. Peak height ratios were calculated and plotted against I concentration based upon an I3P concentration of 250 ng/ml in the injection sample (Table II).

RESULTS AND DISCUSSION

Both DBI and DMBI are capable of fluorescing while I is devoid of such activity [8]. DMI has fluorescent properties, however, it did not appear in HPLC—fluorescence chromatograms providing separation of DBI and DMBI despite the allowance of a 30-min elution interval [8]. Therefore, it was decided to measure DMI in its deacylated state, that is, as DMBI. Deacylation of I and DMI was accomplished by exposure to dilute alkali [20, 21]. Initial studies showed that I and DMI were completely converted by 0.1 *N* sodium hydroxide to the strongly fluorescing metabolites, DBI and DMBI, respectively, within 15 min [21]. The criteria for completeness of transformation were based upon (1) HPLC with UV detection and (2) mass spectrometry.

HPLC—UV

I and DMI were dissolved in pure ethanol, evaporated to dryness, treated with either 0.1, 0.25 or 0.5 *N* sodium hydroxide for intervals varying from 2.5 to 60 min, followed by dilution with water and neutralization with equally concentrated hydrochloric acid. Samples were then introduced onto the chromatographic column, utilizing a 25% acetonitrile—0.25% acetic acid mobile phase and detected using UV absorption at 285 nm. Peak heights were observed to increase under alkali treatment for time points up to 15

min, reaching the same maximum regardless of alkali concentration. The concentration of I and DMI used was 100 μg/ml, significantly higher than levels anticipated in biologic fluids. The internal standard peak height was unaffected by sodium hydroxide.

Mass spectrometry

Samples of DBI and DMBI were prepared by dissolving I and DMI, respec-

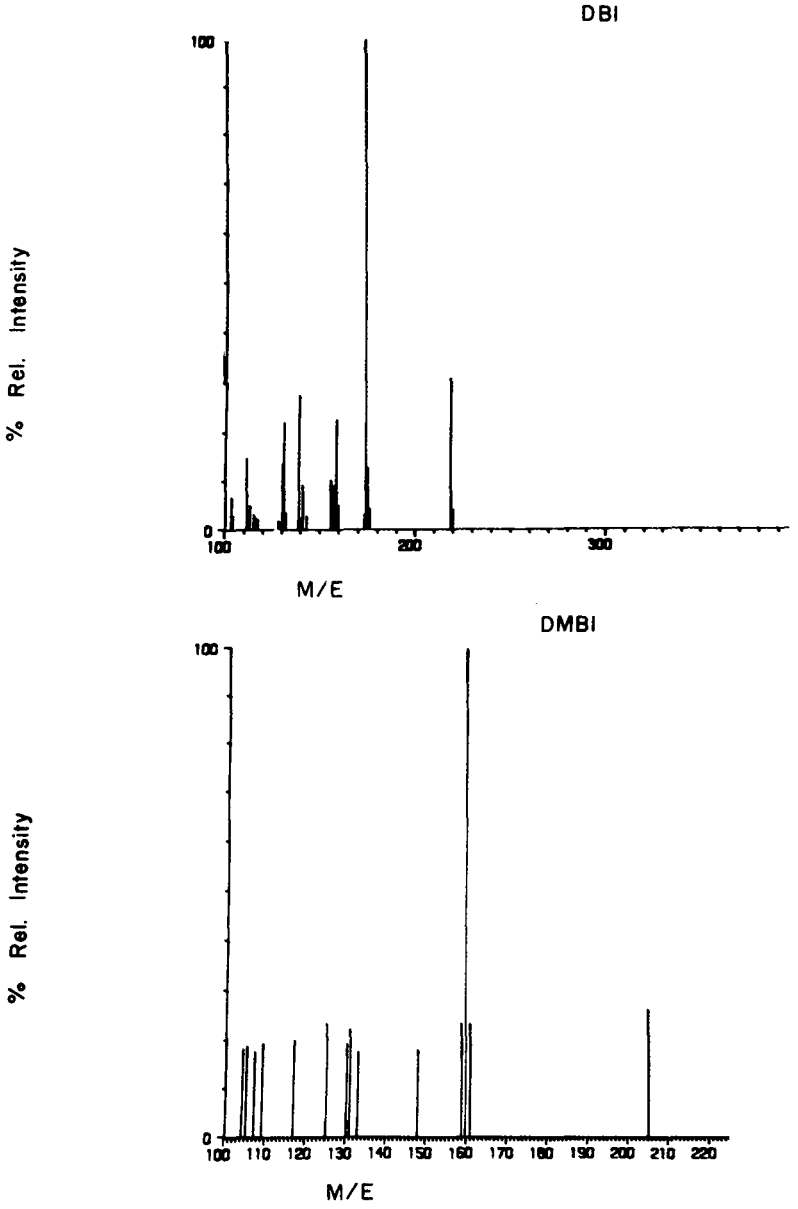


Fig. 2. Direct-probe electron ionization mass spectra of DBI and DMBI. DBI and DMBI were prepared by deacylation of I and DMI, respectively (see text).

tively, in pure ethanol and then evaporated to dryness. The residue was treated with 0.1 *N* sodium hydroxide for 15 min, diluted with water, neutralized with 0.1 *N* hydrochloric acid and extracted with ethyl acetate. The organic extract was dried and the residue placed into a crucible suitable for direct-probe MS. The alkaline-treated sample of I showed a molecular ion peak of *m/e* 219 with a base peak at *m/e* 174, corresponding to the molecular ion of DBI and its decarboxylated fragment, respectively. The alkaline-treated DMI sample exhibited a molecular ion peak of *m/e* 205 with a base peak at *m/e* 160, corresponding to the molecular ion of DMBI and its decarboxylated fragment, respectively. The mass spectra of unhydrolyzed I and DMI agreed with previously reported work [13, 22]. Mass spectra of DBI and DMBI are shown in Fig. 2.

Extraction efficiencies were determined by a comparison of peak heights, derived from HPLC—UV, of extracted versus non-extracted moieties. I and DMI were measured as DBI and DMBI, respectively. Extraction with ethyl acetate from the acetate buffer yielded efficiencies of 1.0, 1.0, 0.96, and 0.84 for I, DBI, DMI, and DMBI, respectively. Urine extractions showed somewhat lower efficiencies as summarized in Table I.

TABLE I

EXTRACTION EFFICIENCIES

I, DBI, DMI or DMBI (100 mg) was added to the sample medium, buffered to pH 5.0, extracted with ethyl acetate, dried and redissolved either in sodium hydroxide (I or DMI) or in ethanol (DBI or DMBI). An equal amount of the compound was dissolved either in sodium hydroxide or in ethanol for comparative purposes. Utilizing an HPLC solvent system of 25% acetonitrile—0.25% acetic acid with UV detection (285 nm), the mean peak height ratio of extracted/non-extracted sample for each compound was determined for extraction efficiency. Values reported are the mean of three separate extractions.

Medium	Metabolite			
	I	DBI	DMI	DMBI
0.2 *M* sodium acetate	1.0	1.0	0.96	0.84
Urine	0.94	0.92	0.92	0.75

Linear regression analyses of peak height ratios versus the concentration of compounds of interest yielded linear calibration curves. Eight concentrations each of I and DMI, in duplicate samples (with one exception) ranging from 0.25 to 25.0 μg/ml (with an internal standard of 10 μg/ml I3P), extracted from urine, provided curves with $r^2 \geqslant 0.99$. Similar analyses of DBI and DMBI, ranging from 0.15 to 12.3 μg/ml and extracted from urine, resulted in curves with $r^2 > 0.99$. Six concentrations of I, taken in triplicate or quadruplicate ($n = 21$), ranging from 25 to 200 ng/ml and extracted from plasma, yielded a calibration curve with $r^2 > 0.97$. A listing of these data is provided in Table II.

A given chromatogram (Fig. 3a) of a urine sample extract yielded three peaks of interest: (A) DMBI, (B) DBI and (C) I3P (in order of elution times). The concentrations of I and its metabolites in their unconjugated states were

TABLE II

CALIBRATION CURVE PARAMETERS

Linear regression analyses of the given data sets were carried out with Y-value being PHR (peak height of compound ÷ peak height of internal standard) and X-value being concentration (I, DMI, DBI or DMBI). n samples (including duplicate or triplicate determinations) were utilized for each calibration curve with r^2 (correlation coefficient) being a measure of linearity, b representing the Y-intercept and m indicating the slope of the regression. The consistency of data points was assessed with the mean coefficient of variation, C.V. as follows:

$$\text{C.V.}_{x_j} = \frac{\sum_{i=1}^{n} \left(\frac{Y_i - \overline{Y_{x_j}}}{n} \right)}{\overline{Y_{x_j}}} \quad (100)$$

Y_i = the PHR's corresponding to concentration x_j from which the mean PHR, $\overline{Y_{x_j}}$, has been calculated.

$$\overline{\text{C.V.}} = \frac{1}{k} \left[\sum_{j=1}^{k} \text{C.V.}_{x_j} \right]$$

Medium	Compound	Range (μg/ml)	n	b	m	r^2	$\overline{\text{C.V.}}$
Urine	I	0.25 —25.0	15	0.007	0.43	0.999	3.95
	DMI	0.25 —25.0	15	—0.06	0.32	0.999	6.19
Urine	DBI	0.15 —12.3	16	0.11	0.42	0.990	2.04
	DMBI	0.30 —12.0	14	—0.11	0.20	0.998	1.68
Plasma	I	0.025—0.20	21	—0.03	2.43	0.976	6.00

determined as follows:

(a) DBI and DMBI fluoresce and thus, were quantitated directly using peak height ratios.

(b) In a parallel sample, I and DMI were treated with sodium hydroxide, thereby converting the pair to DBI and DMBI, respectively; thus, the DBI and DMBI peaks observed in the chromatogram from hydrolyzed samples are a measure of (I + DBI) and of (DMI + DMBI), respectively.

(c) The apparent sample concentrations of I and DMI were determined by subtraction of the molar values obtained in the non-hydrolyzed samples from those of the sodium hydroxide-treated sample. However, the relative extraction efficiencies of DMI and DMBI must be considered upon calculation of the desmethyl metabolite levels; a simple subtraction is allowed only when extraction efficiencies are equal, as is the case for I and DBI.

Initial studies with plasma samples from infants receiving I failed to demonstrate the presence of metabolites. Thus, plasma samples were analyzed only for I (Fig. 3b).

The reason for the use of two different percentages of acetonitrile in the mobile phase was due to the unpredictable presence of endogenous compounds with peaks at the DMBI position of chromatographed urine samples. Similarly, a lengthy elution time is required for plasma samples due to endogenous interference with the DBI chromatographic peak. In spite of the 20 min

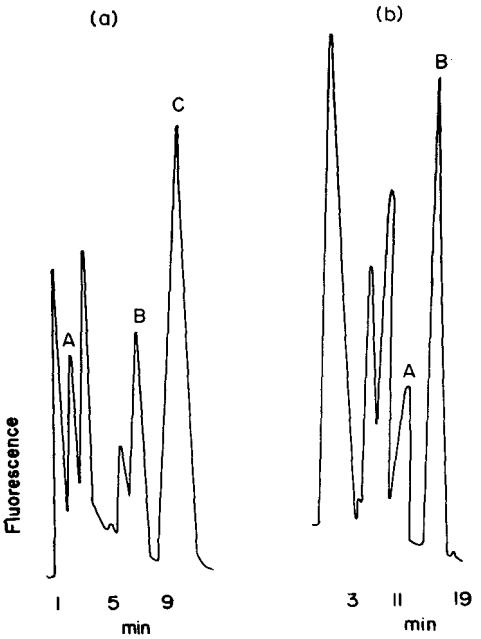


Fig. 3. HPLC chromatograms of indomethacin and two metabolites: (a) 0.60 μg/ml DMBI (A), 0.62 μg/ml DBI (B) and an internal standard, I3P (C), extracted from urine; a C_{18}-bonded reversed-phase column (25 cm × 4.6 mm) with a solvent system of 26% acetonitrile—0.25% acetic acid at 2.0 ml/min flow-rate and 0.1 μA fluorescence ($\lambda_{ex.}$ = 288 nm, $\lambda_{em.}$ = 390 nm) sensitivity was employed. (b) 100 ng/ml indomethacin (A), which has been converted to DBI (see text), with an internal standard, I3P (B), extracted from plasma: column as in (a) with a solvent system of 22.5% acetonitrile—0.25% acetic acid at 2.0 ml/min flow-rate and 0.02 μA fluorescence ($\lambda_{ex.}$ = 288 nm, $\lambda_{em.}$ = 390 nm) sensitivity was used.

allowed for plasma sample elution, endogenous plasma moieties would sometimes produce peaks coinciding with that of the internal standard. In these cases I concentration can be calculated using comparison of peak height with a standard curve. The present assay is currently being used in this laboratory for analyses of I and I metabolites in biological fluids.

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We wish to thank Louette Paul for technical assistance.

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Note

Chromatographic analysis of glutamine in plasma

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Glutamine is among the most abundant amino acids in plasma of man [1] and other mammals [2, 3]. Furthermore, it appears to play an important physiologic role in the transfer of nitrogen between muscle, kidney, liver and intestine [4]. While plasma concentrations of many amino acids are readily measured using ion-exchange chromatography, glutamine is relatively unstable at column temperatures above 40°C [5, 6]. Degradation products include pyrollidonecarboxylic acid and glutamic acid. The extent of degradation is time and temperature dependent [5]. At a column temperature of 60°C over 70% of added glutamine is lost during the 3.5 h required for elution [5]. Consequently, in many studies of amino acid metabolism glutamine was either analysed separately using enzymatic [7] or physical techniques [8] or not assayed at all.

Enzymatic analysis using either spectrophotometric [7] or fluorometric [9] techniques allows simultaneous estimation of glutamine and glutamate but other amino acids must be analysed separately. Techniques developed for measurement of amino acids using gas chromatography (GC) and high-performance liquid chromatography (HPLC) have recently been applied to the measurement of glutamine. Collins and Summer [10] demonstrated that both glutamine and glutamate form identical di-*n*-butyl glutamate derivatives during esterification, prior to GC analysis. However, glutamine also esterifies to *n*-butyl pyroglutamate which can be separately quantitated after acetylation with trifluoroacetic anhydride, and this provides an estimate of the quantity of glutamine in the original sample. A two-stage acylation procedure for simultaneous GC measurement of glutamine and glutamate has also been described [11]. In a recent study Nishimoto et al. [12] using HPLC demonstrated that glutamine can be separated from glutamate and pyrollidonecarboxylic acid and quantitatively recovered from standard samples. The appli-

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cability of either GC or HPLC to the quantitation of glutamine simultaneously with other amino acids in plasma samples has not been reported. This circumstance suggested a need to provide a chromatographic procedure which allows accurate measurement of plasma glutamine simultaneously with the measurement of other blood amino acids. In this report we describe operating methods for ion-exchange chromatographic analysis which allow rapid quantitation of glutamine and other amino acids in biological fluids. In addition, the behavior of glutamine during storage of plasma or acid extracts of plasma was examined.

MATERIALS AND METHODS

Sample preparation and storage

Crystalline glutamine (Sigma, St. Louis, MO, U.S.A.) was dissolved in sample dilution buffer (pH 2.2, Dionex, Sunnyvale, CA, U.S.A.) to a final concentration of 1 m*M*. A standard curve was constructed by serial dilution of this stock solution. These standards were either analysed immediately or stored at −20° C prior to analysis.

For plasma samples, venous blood was collected in heparinized vials from healthy young adults fasted overnight (16 h), and immediately centrifuged at 1000 *g* for 10 min at 40° C. One ml of plasma was deproteinized by addition of 1 ml of 10% sulfosalicylic acid containing lithium hydroxide and lithium chloride, pH 2.2. The deproteinizing solution also contained norleucine (400 μM) as an internal standard. Deproteinized samples were centrifuged at 10,400 *g* for 25 min at 4° C and the supernatant was either analysed immediately or stored at −20° C.

Glutamine recovery and analysis

Recovery of glutamine during chromatographic analysis was assessed using [^{14}C] glutamine (specific activity 250 mCi/nmole; New England Nuclear, Boston, MA, U.S.A.). Carrier-free [^{14}C] glutamine (5 μCi) in 50 μl of sample dilution buffer (pH 2.2) was chromatographed in an identical manner to plasma samples and unlabelled glutamine standards (see below). Fractions of column eluate were collected at 1-min intervals. A 50-μl aliquot of each fraction was dissolved in 3 ml of Aquasol and counted in a liquid scintillation counter (Searle, Chicago, IL, U.S.A.). [^{14}C] Glutamine recovery was calculated from the ratio of counts recovered in the glutamine peak to the total counts added to the column.

Glutamine standards, and deproteinized plasma samples were chromatographed under identical conditions on a D500 automated amino acid analyser (Dionex, Sunnyvale, CA, U.S.A.). The eluting buffer was 238 m*M* lithium citrate with pH adjusted to 2.75 by addition of concentrated hydrochloric acid. A microbore column (1.75 mm I.D.), packed with a 9-μm mesh cross-linked polystyrene resin (L6, Dionex), was run at a constant temperature of 40° C. The buffer flow-rate was 10.74 ml/h, and the operating pressure 175—200 bar (2600—3000 p.s.i.). The sample volume was 50 μl. Under these conditions, elution of glutamine occurs in less than 30 min.

The glutamine concentration in a plasma sample was obtained from the

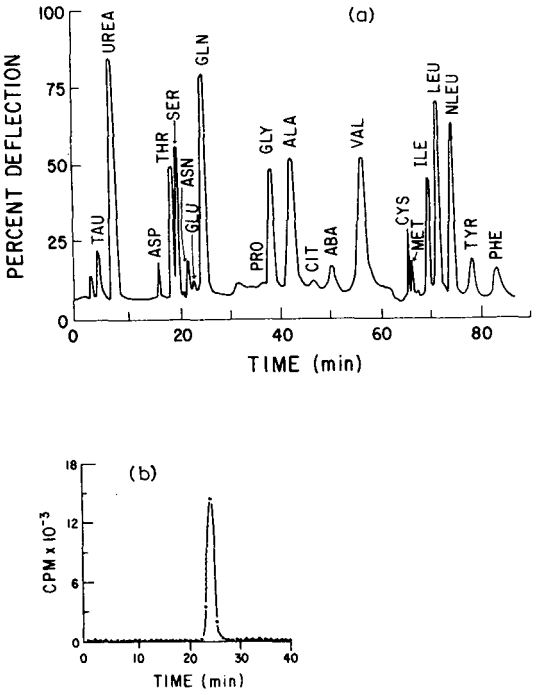
relationship:

$$[\text{Glutamine}]\ \text{plasma} = \frac{\text{glutamine area plasma}}{\text{glutamine area standard}} \times [\text{glutamine}]\ \text{standard}$$

RESULTS AND DISCUSSION

The glutamine peak in standard and plasma samples eluted with an approximate retention time of 25 min. The glutamine peak in plasma was clearly separated from glutamic acid and asparagine (Fig. 1A). [^{14}C]Glutamine chromatographed as a single peak (Fig. 1B), with a calculated recovery of 93±2% (mean ± S.D., n = 3). Approximately 3% of ^{13}C-counts added to the column eluted in a diffuse area in the pre-glutamine region, suggesting breakdown of glutamine during elution through the column. The additional 4% of added tracer was not recovered in the 40 min during which samples were collected (its nature is unknown).

The glutamine peak area increased linearly with the increasing concentrations in the standards over a range of 50—750 μM (Fig. 2). The plasma glu-



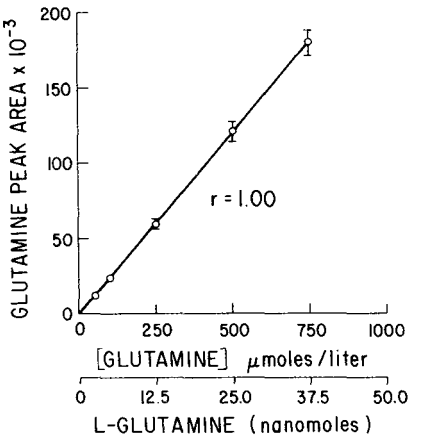


Fig. 1. (A) Typical chromatogram of a plasma sample demonstrating a large glutamine peak (GLN) and good separation of glutamine from glutamate and asparagine. (B) Elution patterns of [^{14}C]glutamine chromatographed under identical conditions to those used in analysis of plasma samples.

Fig. 2. The integrated area under the glutamine peak (ordinate) is plotted as a function of the glutamine concentration in the standard solution (abscissa). The sample size was 50 μl. Values indicate mean ± 1 S.D. (n = 4).

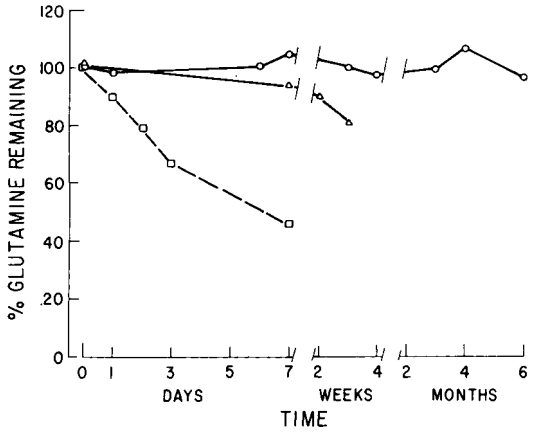


Fig. 3. The percentage of glutamine originally present in a plasma sample which remains after storage of sulfosalicylic extracts of plasma at −20°C (○——○) or 24°C (□ — — □). Sample stored as unextracted plasma at −20°C then extracted with sulfosalicylic acid immediately prior to analysis (△——△). All data points represent the mean of three samples.

tamine concentration in thirteen healthy young adults after an overnight fast was 478±37 μmol/l (mean ± S.D.). Using an enzymatic method, Stahl et al. [13] have reported venous plasma glutamine values of 493±76 μmol/l in man after an overnight fast.

The stability of glutamine upon storage at −20° C, either as plasma or deproteinized plasma, is shown in Fig. 3. There was no loss of glutamine in samples stored at −20° C as protein-free extracts for up to 6 months, while a gradual (ca. 6% per week) loss of glutamine was seen in samples stored as unextracted plasma. At room temperature, glutamine in sulfosalicylic acid extracts (Fig. 3) was rapidly degraded, with less than 50% of the original glutamine present after one week. In these samples, a decline in plasma glutamine of more than 300 μ*M* was accompanied by a rise in glutamate of only 10 μ*M*. This indicates that the glutamine loss is not secondary to its conversion to glutamate, but may occur via formation of pyrollidonecarboxylic acid, as suggested by Oreskes and Kupfer [5].

The relatively rapid degradation of glutamine at room temperature led us to estimate the degradation which might be expected during the time thawed samples remain in the storage chamber of the amino acid analyser prior to being chromatographed. In four samples analysed immediately, then 6 h and again 12 h after thawing, plasma glutamine concentration declined by 4 and 7%, respectively.

Several factors probably contribute to the improved recovery of glutamine from our plasma samples when compared with the recoveries reported by other [5, 6, 14]. As previously pointed out, the rate of glutamine degradation is temperature sensitive [5, 14]. Glutamine conversion to pyrollidonecarboxylic acid is appreciably slower at 40° C than at higher temperatures. Increasing the operating pressure above 2500 p.s.i. sufficiently accelerates glutamine elution so that exposure of glutamine to even the 40° C occurs only briefly. Yet even with this relatively rapid elution glutamine is clearly separated from both asparagine and glutamate. Finally we cannot exclude the possibility that

use of a lithium citrate rather than a sodium citrate buffer may have improved our recovery.

In conclusion, using the analyser operating conditions described here allows rapid reproducible measurement of plasma glutamine simultaneously with other plasma amino acids. Furthermore, the present results show that in properly prepared samples, glutamine is stable for extended periods of time. Finally, ion-exchange chromatography obviates the need for the time consuming derivatization procedure required for GC analysis.

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Note

Separation of peptides by cellulose-phosphate chromatography for identification of a hemoglobin variant

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This paper describes an automatic procedure of cellulose-phosphate chromatography for the separation of peptides, with an example of the application to the analysis of a human hemoglobin variant.

MATERIALS AND METHODS

Venous blood was obtained, with ethylenediaminetetraacetate (EDTA) as anticoagulant, from individuals either normal or heterozygous for a hemoglobin variant [1]. Isolation of hemoglobin components by DEAE-cellulose chromatography [2], separation and aminoethylation of α- and β-chains [3], and tryptic and chymotryptic digestion of the globins [3] were carried out using standard methods. Cellulose phosphate, 100 g, was suspended in 1500 ml of 0.5 *N* potassium hydroxide, mixed for 30 min, and then filtered through a Büchner funnel [4]. The settled cellulose phosphate was thoroughly washed with deionized water until the filtrate was near neutral; it was then resuspended in 1500 ml of 0.5 *N* hydrochloric acid, mixed for 30 min, filtered, and washed with deionized water as before. The precycling procedures with potassium hydroxide, water, hydrochloric acid and water were repeated once. The well-

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washed cellulose agent was resuspended in 2 l of 0.01 *M* ammonium acetate solution (pH 7.3), and, after standing for 30 min, the unsettled particles were decanted. The wash and decantation procedure was repeated five times. A thin slurry of cellulose phosphate was poured into a glass column, 60 cm × 0.9 cm I.D. Packing was continued under a nitrogen pressure of 0.5 kg/cm^2 until the cellulose-phosphate bed was 55 cm in height. Both equilibration in 0.01 *M* ammonium acetate (pH 7.3) and subsequent chromatography were performed at a constant flow-rate of 30 ml/h, using a liquid chromatographic pump with column pressure varying from 2 to 4 kg/cm^2. Soluble peptides, 4—15 mg, were dissolved in 0.5 ml of 0.01 *M* ammonium acetate solution and were loaded onto the column by nitrogen pressure. The chromatography was run using a linear gradient device, containing 250 ml of 0.01 *M* ammonium acetate (pH 7.3) in the mixing cylinder and 250 ml of 0.4 *M* ammonium bicarbonate (pH 9.0) in the reservoir cylinder. The column effluent was monitored continuously at 215 nm by passage through a 2-mm flow-cell, and collected in a fraction collector. Correspondence between the tubes in the fraction collector and record of the ultraviolet absorption in the effluent monitor was facilitated by the incorporation of an event marker on the side of the recorder which was activated by changes of the collector [5]. A back-pressure of up to 1 kg/cm^2 was applied to eliminate bubbles in the flow-cell. Pooled fractions were lyophilized or evaporated to dryness. Rechromatography was performed on a 50 cm × 1.0 cm I.D. column of Bio-Gel P2. The linear flow-rate of 0.01 *M* ammonium acetate (pH 7.3) was maintained at 10 ml per h per cm^2 of cross-sectional area using a peristaltic pump. The effluent was monitored as described. An aliquot of peptides purified in this manner was analysed for amino acid composition on a Model 835 analyser (Hitachi, Tokyo, Japan) equipped for single-column analysis. High-performance liquid chromatography was carried out as described previously [6].

Trypsin (Lot No. T-TCA 591) and α-chymotrypsin (3× crystallized, Lot CD1-6100-1) were obtained from Worthington Biochemical Co., Freehold, NJ, U.S.A. The cellulose-phosphate agents were from Whatman, Maidstone, Great Britain (Grade P11: Lot Nos. 611514 and 611599, with 25-μm mean fiber length and capacity of 7.4 mequiv/g), or from Serva, Heidelberg, G.F.R. (P-Cellulose: Lot No. 26076, with a capacity of 5.03 mequiv./g). Porous polyacylamidegel beads were supplied by Bio-Rad Labs., Richmond, CA, U.S.A. (Bio-Gel P-2: minus 400 mesh). A Model 034 Liquid Chromatograph Pump and UV—VIS Effluent Monitor were obtained from Hitachi. A Model P3 pump and a glass column were purchased from Phamacia, Uppsala, Sweden.

RESULTS

Electrophoresis on thin-layer starch gel or cellulose-acetate sheet detected a hemoglobin variant in hemolysate from an apparently healthy Japanese man aged 35 years. When the relative mobility was calculated by the method of Schneider and Barwick [7], the mobility of the variant was +3.0. The abnormal component comprised 35% of the total hemoglobin. The resolving power of the cellulose-phosphate chromatography and identification of the peptides are illustrated in Fig. 1. The yields of peptides from the column were essentially

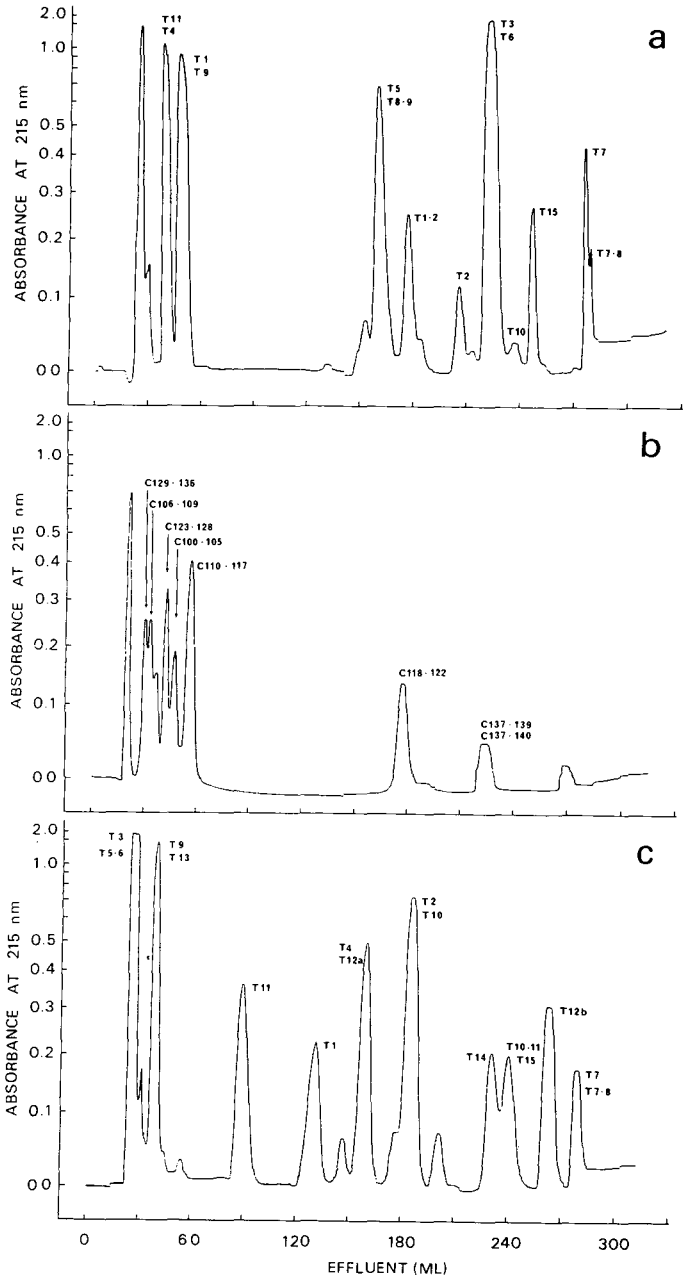

Fig. 1. The chromatographic separation of peptides produced by (a) tryptic digestion of the non-derivatized α-chains (11 mg), (b) chymotryptic digestion of the α-chain core peptide which had undergone performic-acid oxidation (4 mg), and (c) tryptic digestion of the aminoethylated β-chains (11 mg) from a hemoglobin variant. Normal βT5 peptide co-chromatographs with βT3, and the βT6 peptide with βT14. Tryptic peptides (T) are numbered in the sequence in which they occur in the polypeptide chains. Numbers beside the chymotryptic peptides (C) denote the amino- and carboxyl-terminal residues of the peptide.

TABLE I

AMINO ACID COMPOSITION OF THE β-CHAIN TRYPTIC PEPTIDES OF HEMOGLOBIN J LOME

Data are molar ratios of amino acids recovered after hydrolysis in 6 *N* HCl. The fragments are numbered in the sequence in which they occur in the polypeptide chain. Numbers in parentheses are the values expected from the normal β-chain sequence.

Amino acid	T1	T2	T3	T4	T5,6	T7	T8,9
Aspartic acid			1.97 (2)		3.94 (3)		3.20 (3)
Threonine	1.02 (1)	0.96 (1)		1.07 (1)	1.06 (1)		
Serine		0.42 (1)			1.87 (2)		1.07 (1)
Glutamic acid	2.09 (2)		2.10 (2)	1.12 (1)	1.05 (1)		
Proline	0.99 (1)			0.51 (1)	1.99 (2)		
Glycine		1.08 (1)	3.42 (3)		1.71 (2)	1.09 (1)	2.08 (2)
Alanine		2.19 (2)	1.08 (1)		1.08 (1)	0.90 (1)	1.91 (2)
Cysteine*							
Valine	0.86 (1)	1.23 (1)	3.23 (3)	2.36 (2)	1.61 (2)		0.98 (1)
Methionine					0.59 (1)		
Leucine	1.08 (1)	1.11 (1)	1.11 (1)	2.54 (2)	1.11 (1)		4.17 (4)
Tyrosine				1.04 (1)			
Phenylalanine					3.01 (3)		1.04 (1)
Tryptophan**		(+)		(+)			
Lysine	1.09 (1)	1.02 (1)			1.03 (2)	1.03 (1)	1.60 (2)
Histidine	0.87 (1)					0.98 (1)	0.94 (1)
Arginine			1.06 (1)	0.37 (1)			

*Estimated as aminoethylcysteine.
**Not quantitated.

quantitative. The fractions from the cellulose-phosphate chromatography contained only single or two major tryptic peptides, with variable degrees of contamination by minor peptides produced by incomplete or non-specific cleavage by trypsin. Gel-filtration chromatography on Bio-Gel P-2 was then applied for further purification of these fractions. Separation of two peptides from the β-chain, βT3 and βT5, was insufficient even after the rechromatography. Therefore, either high-performance liquid chromatography or paper chromatography was necessary to purify them.

The peptide composition of the α-chains from the variant was identical to that of the normal α-chains. The β-chains, on the other hand, had a different composition as compared to the normal, in that the βT5 peptide had the amino acid composition of residues 41—61 with substitution of either an aspartyl or an asparaginyl for a lysyl residue at position 59 (Table I). As a result of this substitution, the peptide bond between βT5 and βT6 (valyl-lysine) was not cleaved by trypsin, so that βT6 was absent in the chromatogram. The substitution of an asparaginyl rather than an aspartyl residue for a lysyl residue is in accord with the electrophoretic behaviour of the variant. Thus, we conclude that the variant is identical to Hemoglobin J Lome [8]. Two-dimensional paper chromatography and electrophoresis (fingerprinting) of the tryptic peptides confirmed the identification of the structure.

T10	T11	T12a	T12b	T13	T14	T15
1.20 (1)	2.06 (2)	0.95 (1)			1.11 (1)	
1.70 (2)				1.02 (1)		
1.06 (1)						
1.22 (1)	1.07 (1)			2.99 (3)		
	0.89 (1)			1.69 (2)		
0.93 (1)		1.03 (1)	1.10 (1)		1.26 (1)	
0.99 (1)		1.14 (1)	1.14 (1)	2.03 (2)	4.44 (4)	
0.96 (1)		0.42 (1)				
	1.06 (1)	2.03 (2)	1.08 (1)	1.14 (1)	2.31 (3)	
2.16 (2)	0.96 (1)	3.00 (3)	0.96 (1)		1.05 (1)	
				0.74 (1)		0.97 (1)
0.79 (1)	1.00 (1)		0.87 (1)	1.13 (1)		
0.96 (1)			0.97 (1)	1.23 (1)	0.98 (1)	
1.03 (1)	0.98 (1)		1.85 (2)		0.85 (1)	1.03 (1)
	0.98 (1)					

DISCUSSION

A combination of ion-exchange chromatography and molecular-sieve chromatography is employed for the separation of peptides from hemoglobin variants. With the use of strong cation-exchange cellulose and a very sensitive non-destructive detection system, substantial efficiency and reproducibility as well as loading capacity are achieved. The reasonable speed, versatility and inexpensive cost of performance make this technique well suited for routine assays of hemoglobin variants. Cellulose-phosphate chromatography, demonstrated to be a versatile technique for the effective separation of peptides [4, 9], has received only limited attention for solving structural variations in abnormal hemoglobins. Thus, the present study was initated as an attempt to evaluate its usefulness, introducing an element of simplicity and sensitivity to the procedure. The applicability of cellulose phosphate as the sole procedure is limited because several peptides elute together. However, molecular-sieve chromatography on microparticulate polyacrylamide gels, coupled with the same detection system, achieves the necessary repurification of peptides very efficiently. The separation of the peptides seems to be excellent sometimes but it is also often inferior to that observed by high-

performance liquid chromatographic methods [10] and by the more laborious ion-exchange resin procedures [5]. The superb resolution of high-performance liquid chromatography must be offset, in part, by the inherent disadvantages including limited loading capacity and variabel recoveries [10]. Multiple runs may be required to obtain an adequate amount of peptide for a satisfactory amino acid analysis and sequence determination, with considerable loss of peptides. On the other hand, standard procedures for peptide chromatography on ion-exchange resin columns must rely primarily on destructive postcolumn detection techniques. The use of ammonium acetate and ammonium bicarbonate in the developers, without pyridine, affords sensitive non-destructive detection at 215 nm. Neither colorimetric determination with ninhydrin reaction, nor repurification of organic chemicals is necessary in this system.

Several points germane to the general use of this technique are worth noting. Since cellulose-phosphate agents are supplied dry by the manufacturer, some ion-exchange sites become inaccessible to large molecules by the formation of new hydrogen bonds within shrunken cellulose (Whatman Bulletin No. 202). These structures may not be fully opened up by immersion of the cellulose in developer. Hence, extreme care in multistep precycling procedures is necessary to attain maximum resolution and reproducible chromatograms. Cellulose-phosphate materials obtained from Whatman appeared to give reproducible results as well as those from Serva (Lot No. 26076). However, a later supply from Serva (Lot No. 14128, with seemingly longer fiber length) was not satisfactory, in spite of the even larger capacity than that of the previous one. New developments in the elution programs, such as the use of more acidic starting buffer [9], may be possible. As shown, the difference in the peptide composition between the normal and the variant globins may be immediately clear by comparing the chromatograms. Hence, this cellulose-phosphate chromatography, designed to analyse materials of the order of 4—15 mg, may be suitable for both preparative and analytical purposes. The high recoveries, and ease of sample collection and preparation are especially suited for handling a quantity of peptides for determination of the amino acid sequence using conventional facilities. Cellulose-phosphate chromatography has been effective in determining the nature of the substitution in several other examples found in this laboratory. In any event, these procedures appear to be powerful techniques for the assay of structural variations in hemoglobins and other proteins.

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Note

Rapid method for the purification of the elastin cross-links, desmosine and isodesmosine

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Elastin is the protein component which confers unique recoil properties upon certain connective tissues. The importance of the disorganisation and degradation of the elastin architecture which occurs in atherosclerosis and pulmonary emphysema is increasingly appreciated [1]. There is also strong evidence to suggest that the corticosteroids used for the prevention of neonatal respiratory distress cause changes not only in pulmonary surfactant but also in the elastin content of the neonatal lung [2].

The identification of desmosine and isodesmosine as the major cross-links in elastin [3] has led to their acceptance as analytical markers for this connective tissue protein [4]. Since neither desmosine nor isodesmosine is absorbed from the intestinal tract [5, 6] their concentration in urine is a reliable index of elastin degradation in vivo. Thus the assay of elastin cross-links has already achieved some recognition as a potential diagnostic tool, particularly in inherited diseases [7] and in degenerative lung diseases [8].

The analytical methods for elastin employing gravimetric techniques [9] are time consuming and sometimes inappropriate [10] while the more recent methods involving amino acid analysis for desmosine and isodesmosine [4] often require preliminary purification [11, 12]. Recent radioimmunoassay techniques [8, 13, 14] have increased the speed of analysis but may also require a preliminary purification step [14]. For these reasons a rapid mini column method for isolating desmosine and isodesmosine from acid hydrolysates was developed. The method is based on the adsorption of desmosine and isodesmosine onto cellulose in organic acid—alcohol mixtures [11, 15].

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EXPERIMENTAL

Materials

n-Butanol, ethanol, acetone, pyridine, acetic acid and hydrochloric acid were of analytical grade and water was redistilled. Cellulose powder (Whatman CF_1) was obtained from W. & R. Balston (Kent, Great Britain). Silica gel GF_{254} 0.25 mm layers were from Merck (Darmstadt, G.F.R.). Isodesmosine and desmosine were purified from bovine ligamentum nuchae as described previously [15].

Mini columns were made from Samco No. 202 one-piece disposable plastic Pasteur pipettes (Saint-Amand Manufacturing Co., San Fernando, CA, U.S.A.) by cutting off the top hemisphere of the bulb (which then becomes a 4-ml solvent reservoir) and plugging the tip with a single glass bead of 3—4 mm diameter. The barrel of the pipette is conveniently clipped in a frame and 30 may be adequately managed at one time.

Methods

A slurry is prepared by mixing CF_1 cellulose (10 g) with the mobile phase, *n*-butanol—acetic acid—water (4:1:1) 200 ml in a 250-ml wide-mouth reagent bottle. The mixture is shaken to approximate homogeneity and then sonicated for 2—4 min to remove air bubbles. The columns (7 mm I.D.) are then packed to 45—50 mm height by pipetting 4—5 ml of the slurry into the bulb reservoir and allowing the cellulose to settle with occasional tapping. The column bed will not dry even if completely drained for several hours. A further 5 ml of mobile phase is added to each column to wash fine particles from the bulb reservoir.

Samples, typically 5—50 mg of sheep fetal lung tissue or 0.25 ml amniotic fluid, are hydrolysed in 6 *M* hydrochloric acid (0.5 ml) under nitrogen in sealed glass ampoules for 56 h. The vials are opened and the 6 *M* hydrochloric acid hydrolysate mixed, in order of addition, with acetic acid (0.5 ml), cellulose slurry (0.5 ml) and *n*-butanol (2 ml). During this process most of the desmosine and isodesmosine are adsorbed on the cellulose (results not shown). The slurry is transferred onto a prepared column and the ampoule washed with 1.5 ml mobile phase which is also transferred to the column. This additional slurry increases the column length by about 5 mm. The columns are then eluted with a further 15 ml mobile phase which removes hydrochloric acid and amino acids. The desmosine and isodesmosine are eluted from the column with water (5 ml) into plastic tubes. The majority of the residual butanol is displaced from the column by the first 1 ml of water and is either discarded or pipetted from the top of the aqueous fraction containing the desmosine and isodesmosine. The aqueous fraction is usually lyophilized before analysis to remove residual butanol and acetic acid and to concentrate the sample.

RESULTS

Initially all 6 *M* hydrochloric acid hydrolysates were evaporated and reconstituted in water before column separation. Later this was found to be un-

necessary as there was no difference between elution volume or recovery of isodesmosine whether the hydrochloric acid was evaporated or not. Similarly there was no effect upon either of these parameters with or without preliminary filtering of the hydrolysate.

In an experiment to monitor column performance and isodesmosine recovery, bovine serum albumin (10 mg) and gelatin (10 mg) were hydrolysed with and without the addition of isodesmosine (274 nmol) and processed as described above but with the collection of fractions (4 × 5 ml mobile phase

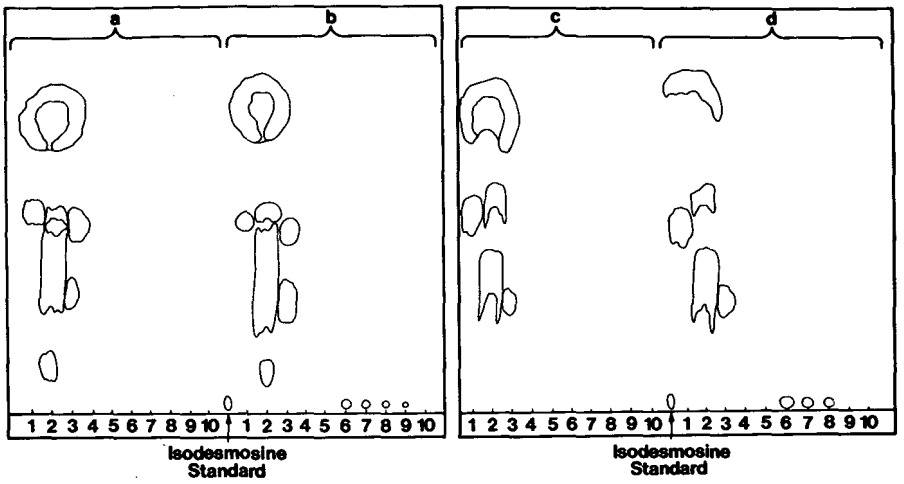

Fig. 1. The location of isodesmosine eluted from CF_1 columns by thin-layer chromatography. 1—4 are 5-ml mobile phase fractions and 5—10 are 1-ml water fractions from the CF_1 columns. Bovine serum albumin (10 mg) hydrolysates with (b) and without (a) isodesmosine (274 nmol) and gelatine (10 mg) hydrolysates with (d) and without (c) isodesmosine (274 nmol) were chromatographed as described in the text. One tenth of each CF_1 column fraction was chromatographed on silica gel GF_{254} layers by ascending development with ethanol—0.5 *M* acetic acid (1:1) to the full plate height. The photographs of the ninhydrin treated plates were pen-contrasted for clarity.

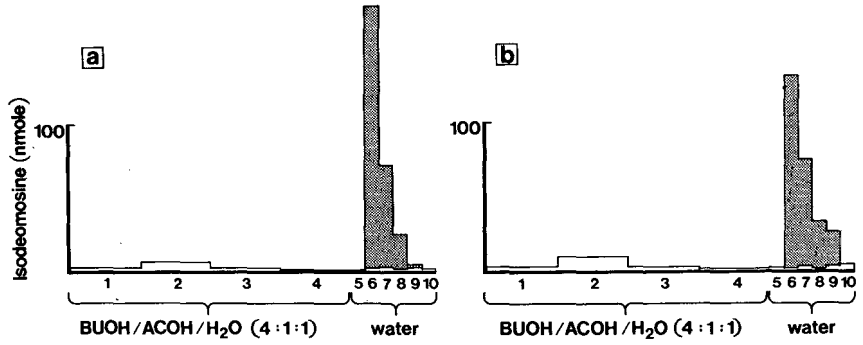

Fig. 2. Recovery of isodesmosine from CF_1 columns. The numbers of the *X* axis refer to the CF_1 column fractions. Isodesmosine was measured in aliquots (1/5000) of column fractions by radioimmunoassay. Shaded columns represent hydrolysates with isodesmosine (274 nmol) added to (a) gelatin and (b) bovine serum albumin. Unshaded areas represent material cross-reacting in the immunoassay which is present in the protein hydrolysates without added isodesmosine. BUOH = *n*-butanol, ACOH = acetic acid.

and 6 × 1 ml water). These fractions were lyophilized, the residues dissolved in water (100 μl) and an aliquot (10 μl) chromatographed on silica gel GF_{254} layers developed with ethanol—0.5 *M* acetic acid (1:1). The isodesmosine was located under ultraviolet light and, as other amino acids, with ninhydrin in acetone—pyridine (50:1). In this chromatographic system all the amino acids have R_F values $\geqslant$ 0.1 whereas the desmosine and isodesmosine remain close to the origin (Fig. 1). The isodesmosine was eluted in the water fractions, principally in fractions 6—9 (Fig. 1b and d), while all other amino acids were eluted with mobile phase, fractions 1—3. There was no appreciable ninhydrin-positive material in the water fractions from the chromatographed hydrolysates without added isodesmosine (Fig. 1a and c). The isodesmosine contents of the fractions were measured by radioimmunoassay [16] of an aliquot of the recovered fractions (Fig. 2). Summation of the quantities of isodesmosine measured by radioimmunoassay in column fractions 6—9 accounts for 99—102% of the 274 nmol isodesmosine added to the hydrolysates (Fig. 2). The small quantity of immunoreactive material in the mobile phase washes (mainly in the second fraction) is all accountable as non-specific cross-reacting material present in bovine serum albumin and gelatin.

DISCUSSION

The method presented appears to be effective in removing contaminating amino acids and leaving desmosine and isodesmosine in relative purity (Fig. 1). The material isolated has been found to be suitable for radioimmunoassay and is completely recovered (Fig. 2). Increments of isodesmosine (0—60 pmol) added to gelatine samples (5 mg) before hydrolysis were also measured, after column purification, with acceptable accuracy (r^2 = 0.992, data not shown) suggesting that the method is sufficiently reliable for routine use.

An equivalent method [11] using paper chromatography requires the 6 *M* hydrochloric acid protein hydrolysate to be filtered, evaporated, reconstituted in water, streaked on Whatman 3M chromatography paper and developed for 48 h, after which the desired material is eluted from the origin with water.

With this method the time from unsealing the 6 *M* hydrochloric acid hydrolysates to the end of the collection of the desmosine/isodesmosine fractions is 2—3 h for 30 or more samples. The only apparent source of error arose from gross inhomogeneity of the column bed due to air bubbles. This problem was eliminated by prior sonication of the cellulose slurry and minimal care in column packing.

The procedure is economical of both cost (*n*-butanol, cellulose and disposable pipette <20 cents/sample) and time. Evaporating water containing traces of acetic acid and *n*-butanol is less demanding on equipment than evaporating 6 *M* hydrochloric acid.

With increasing interest in elastin biochemistry [1], particularly in degenerative lung diseases [8], and atherosclerosis [17], this method may find wide application.

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Note

Rapid separation of nucleotides from granulocytes by isocratic, reversed-phase high-performance liquid chromatography

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The nucleotide profiles of different cells are characteristic for each tissue [1, 2]. Blood cells contain substantial quantities of adenine nucleotides. The levels of different nucleotides and the energy charge available have been related to the metabolic and physiological requirements of blood cell function [3—6]. In the past, blood cell nucleotides have been assayed in several different ways [7—11], including techniques employing firefly luciferase [12—14], an enzymatic method [15], a fluorimetric procedure [16], column chromatography [17] and high-performance liquid chromatography (HPLC) [18—21]. However, a simple, rapid and efficient method for the analysis of neutrophil nucleotides is still unavailable. We have recently developed a rapid method for the separation and evaluation of adenine and guanine nucleotides (AN and GN) from human blood platelets [21]. In the present paper, we have applied

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this procedure for complete separation of granulocyte nucleotides with a minimum of elution time and maximum efficiency.

MATERIALS AND METHODS

Blood for these studies was obtained from normal subjects. It was immediately mixed with trisodium citrate—citric acid—dextrose (CCD) buffer, (citrate 0.1 *M*, citric acid 7 m*M*, dextrose 0.14 *M*, pH 6.5), in a ratio of 9 parts of blood to 1 part anticoagulant [22]. To obtain leukocyte rich plasma (LRP) free of erythrocytes 1 part of 5% dextran (molecular weight 282,000) in saline was added to 3 parts of whole blood, mixed and allowed to sediment spontaneously by placing tubes at a 45° angle for 20—40 min. LRP separated from red blood cells was layered over a double Ficoll—Hypaque gradient [23, 24], and centrifuged at 1650 *g* for 15 min. The neutrophils were harvested from the lower interface, checked for purity (greater than 95% neutrophils), washed twice and resuspended with saline to the desired concentration. Cell counts were determined using a Royco particle counter. The suspension containing neutrophils was centrifuged for 5 min at 180 *g*. Supernatant was discarded and the neutrophil pellet resuspended in 1.5 ml of Hanks Balanced Salt Solution (HBSS). Samples were transferred in 2-ml volume centrifuge tubes and spun for 1.5 min in a Beckman microfuge. After removing the clear supernatant the pellet of neutrophils was precipitated with 100 μl of cold 2 *N* perchloric acid. The precipitate thus formed was sonicated in perchloric acid at low temperature (4° C) and the samples again centrifuged in a microfuge for 1.5 min. The clear supernatant containing neutrophil nucleotides was separated and neutralized with 5 *N* potassium hydroxide to a pH of 5.5—8.0. The neutralized samples were subjected to a freeze—thaw cycle to achieve complete precipitation of the salt. To sediment the salt generated during neutralization all the samples were centrifuged one more time in a microfuge for 1.5 min. The clear neutral extracts were separated and subjected to HPLC for the separation of nucleotides.

Separation of nucleotides

A Waters Assoc. (Milford, MA, U.S.A.) Model 204 high-performance liquid chromatograph was used for the separation of nucleotides. The chromatographic system consisted of a Model 440 fixed-wavelength (254 nm) UV detector, a 6000A solvent delivery system and a U6K universal injector. A Radial-Pak C18 (10-μm particles) with radial compression module (RCM-100) was used for the separation of nucleotides. The solvent system consisted of HPLC grade acetonitrile (Burdick and Jackson Labs, Muskegon, MI, U.S.A.), 10% (v/v), deionized distilled water of pH 7.5, 90% (v/v), and a vial of Pic A (tetrabutylammonium phosphate) (Waters Assoc.). First, Pic A was mixed with water and the pH was adjusted to 7.5 and then acetonitrile was added to make the final solvent system. The final concentration of the Pic A was 0.005 *M* per l of stock solvent used in the separation of nucleotides. Solvent flow-rate was varied depending on the degree of separation needed. Sample size was 20 μl per injection. A Hewlett-Packard 3385A automation system was used to obtain electronic integration of the peaks. Data thus

generated were further processed to obtain values in μmol/10^9 cells. Results presented are mean values for neutrophils obtained from six different normal donors.

RESULTS AND DISCUSSION

Extracts of granulocytes and an extract containing standards were analyzed using Radial-Pak™ column. Solvent elution was isocratic and flow-rate was 6 ml/min. Complete separations of all the major nucleotides from the mixtures were achieved at ambient temperatures in less than 8 min (Fig. 1). Retention times for GMP/AMP, GDP, ADP, GTP and ATP were 1.1, 2.3, 3.0, 4.9 and 7.5 min, respectively. To test the efficacy of this method, recoveries of the standards added to the neutrophil suspension were measured. The range of recovery was 94—100% for all nucleotides except for GDP (65—75%).

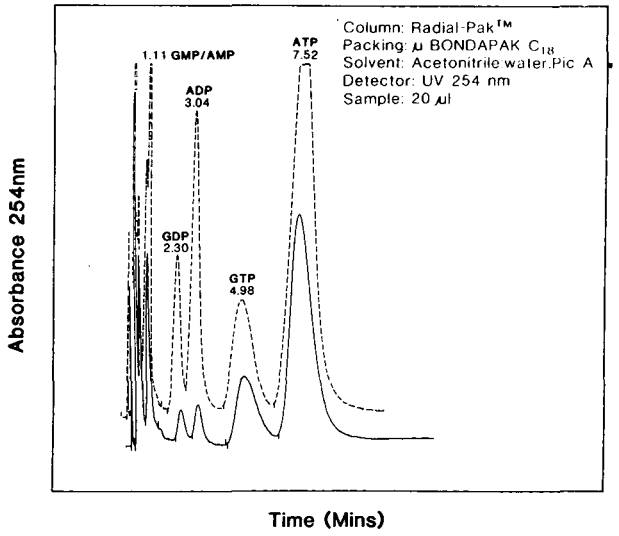

Fig. 1. Separation of adenine and guanine nucleotides by isocratic elution at ambient temperature. Chromatogram in solid lines was obtained by analyzing a granulocyte extract, whereas the one shown with dotted lines was obtained by analyzing a granulocyte extract with added adenine and guanine nucleotide standards.

Identification of individual peaks of adenine and guanine nucleotides was done by comparison of retention times of standards added to the neutrophil extract and assayed under identical conditions. In addition, a standard mixture containing both adenine and guanine nucleotides was added to a neutrophil extract and run under identical conditions as a supplementary confirmation of peaks. Enzymatic peak shift method was used to follow the loss of ATP and GTP and increases in ADP and GDP, respectively. In addition, cells were incubated with adenine and guanine and the increases in the level of phosphorylated compounds were followed. No attempts were made to identify several fast eluting compounds which may be precursors, metabolites or other UV-absorbing components.

Since in this mode of elution both GMP and AMP eluted as a single peak, the standards were run at a slower flow-rate (1 ml/min). A profile of nucleotide standards separated under such conditions is presented in Fig. 2. Under this condition, all the adenine and guanine nucleotides were completely separated. Using similar conditions, neutrophil extracts were analyzed to obtain separation of AMP and GMP (Fig. 3). There are slight differences in the retention times of individual nucleotides. However, if the runs are repeated with stan-

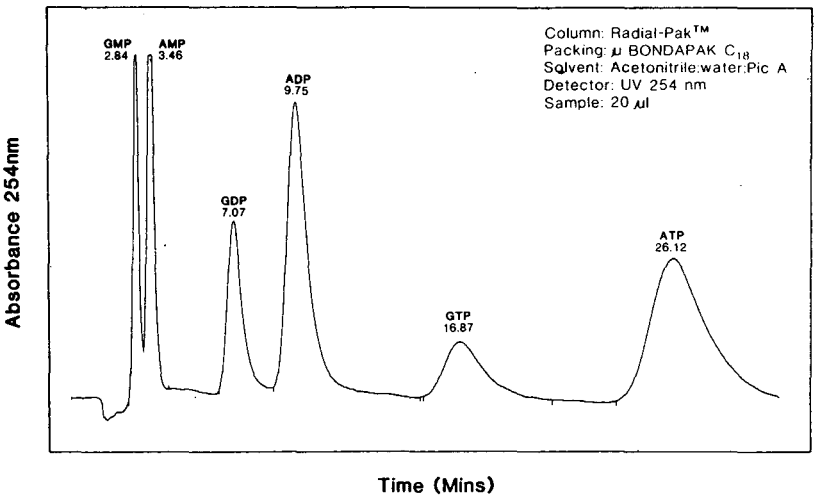

Fig. 2. Separation of various nucleotide standards from a mixture by isocratic elution. Elution time for separating all the nucleotides was less than 26 min. The range of recovery for all the nucleotides was excellent.

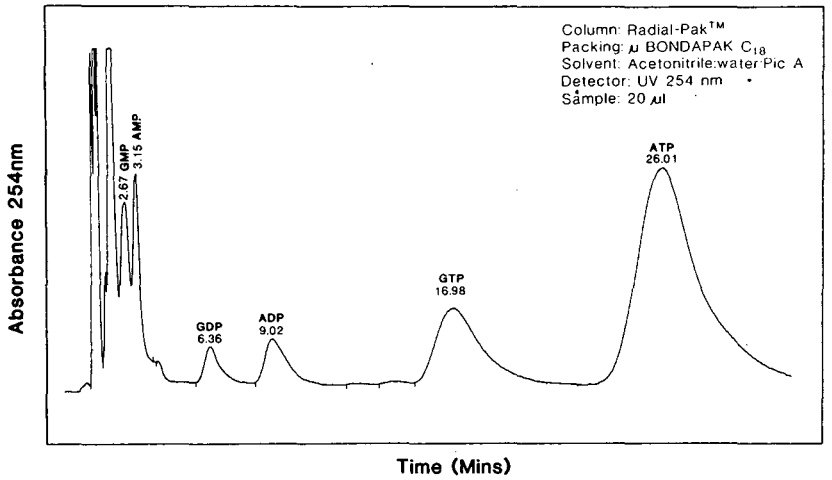

Fig. 3. Separation of granulocyte nucleotides by isocratic elution. Retention times for adenine and guanine nucleotides matched closely with those of standards obtained under identical conditions (Fig. 2). The values obtained by this method for various nucleotides were higher than those reported by others [18] using linear gradient elution to achieve complete separation of various nucleotides.

dards added to a biological extract one could get relatively matching retention times (Fig. 1). Mean values obtained for each nucleotide are presented in Table I. Values obtained for all the nucleotides were higher by this method than those reported by Scholar et al. [18] using a gradient elution technique employing ion-exchange chromatography.

The two major nucleotides, ATP and GTP, together, constituted over 84% of the total nucleotides. Since UTP, UDP and UMP are not separated from the adenine and guanine nucleotides they probably coelute with these products. However, the data from Scholar et al. [18] using an ion-exchange technique which separates all three nucleotides shows that the contribution of UTP is less than 3% towards the total nucleotides. Values obtained for ATP and GTP by reversed-phase chromatography were 40% higher than those reported by Scholar et al. [18]. Therefore, the presence of uridine nucleotides may not account for the higher values observed using our method. The reason for higher values may be better separation techniques employed for obtaining cells, method of handling cells, more complete extraction and the high resolution obtained by improved column technology. Ratios for ATP/ADP and AN/GN were 10.9 and 2.9, respectively.

In summary, this is a preliminary report on the nucleotide profiles of normal granulocyte populations. The effect of storage on the nucleotides and their function will be published elsewhere. Results of these studies show that adenine and guanine nucleotides of granulocytes could be separated rapidly and efficiently by this method.

TABLE I

NUCLEOTIDES OF HUMAN BLOOD GRANULOCYTES*

Relative values obtained for granulocyte nucleotides by isocratic reversed-phase high-performance liquid chromatography. The values obtained using this method are higher than those reported by Scholar et al. [18].

	Nucleotides found (μmol/10^9 cells)**	
	Present paper	Scholar et al. [18]
GMP	0.19 ± 0.02	—
AMP	0.10 ± 0.02	—
GDP	0.03 ± 0.01	0.012
ADP	0.11 ± 0.03	0.09
GTP	0.26 ± 0.02	0.16
ATP	1.2 ± 0.05	0.75

*UTP, UDP, and UMP are not separated from the adenine and guanine nucleotides.
**Mean ± standard error (n = 6).

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Note

Simultaneous determination of allopurinol and oxipurinol in biological fluids by mass fragmentography

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The mass fragmentographic analysis of xanthine and hypoxanthine as described previously [1] was applied to the determination of allopurinol and its major metabolite, oxipurinol. We present, in this paper, a specific and sensitive method for the simultaneous determination of xanthine, hypoxanthine, allopurinol and oxipurinol (Fig. 1) in serum and urine, and its application to studies involving patients treated daily with 200 mg of allopurinol (Zyloric®, Wellcome) for a week.

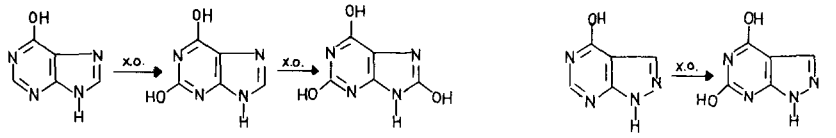

Fig. 1. Oxidative transformation of hypoxanthine to uric acid and allopurinol to oxipurinol by xanthine oxidase (X.O.).

EXPERIMENTAL

Analytical procedure

The determinations were carried out on a quadrupolar gas chromatography—mass spectrometry system (Hewlett-Packard 5985 B) in the electron-impact mode by the method of selected ion monitoring.

Each serum and urine sample was analyzed according to the procedure

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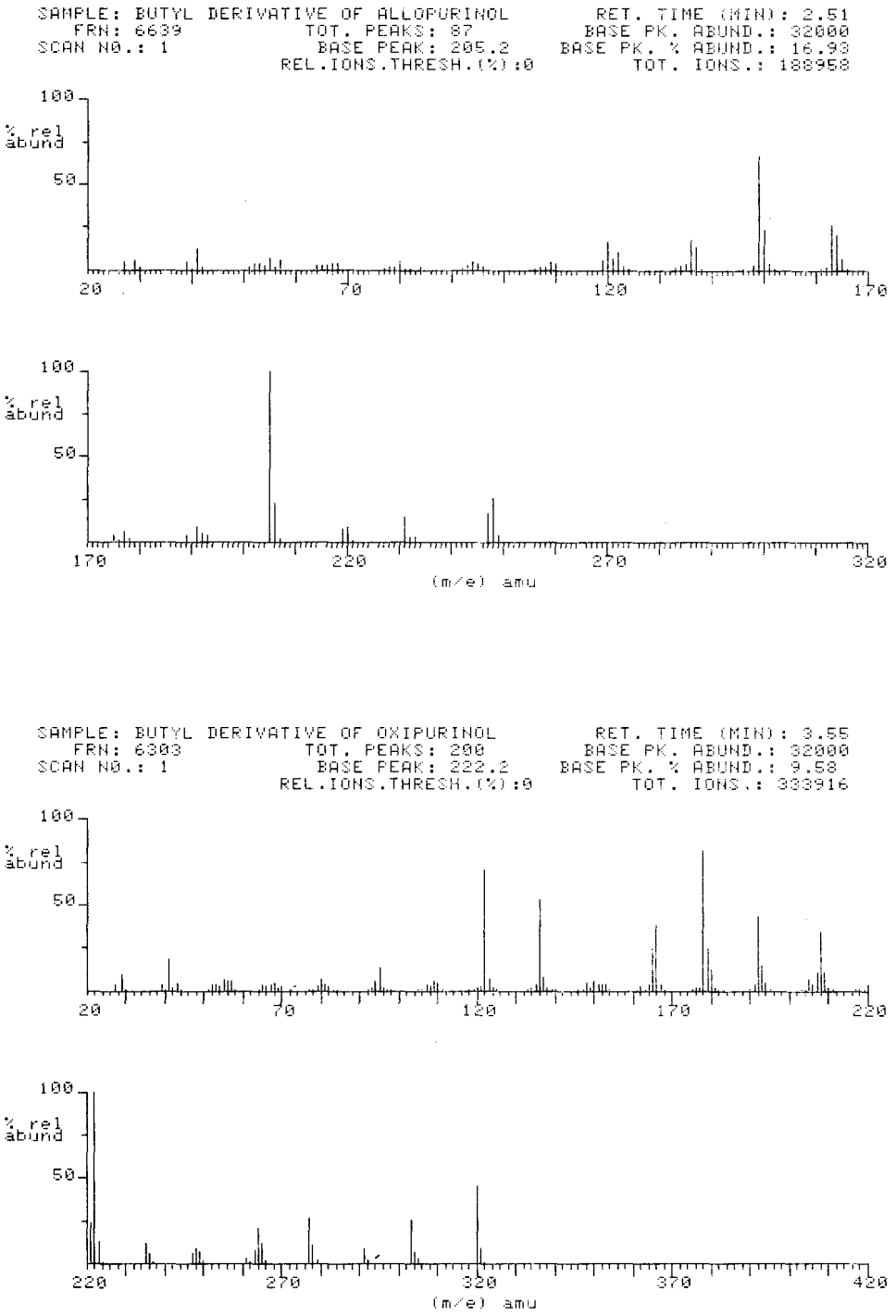

Fig. 2. Mass spectra of butylated derivatives of allopurinol and oxipurinol.

described in detail previously [1]. [7,9-^{15}N]Xanthine was added to each sample as internal standard.

Serum (0.5 ml) was deproteinized by ultrafiltration in Amicon CF 25 Centriflo cones, and was then assayed in the same way as the urine samples (0.1 ml).

After extraction by shaking with 2 ml of *n*-butanol at pH 4.2, followed by butylation according to Greeley's method [2], the chromatographic separation was carried out on a glass column (150 cm × 2 mm I.D.) packed with 3% OV-17 on Gas-Chrom Q, programmed from 190 to 260° C at 10° C/min.

Mass spectra

The electron-impact mass spectrum of the dibutylated derivative of allopurinol (Fig. 2) exhibits a slight molecular ion at *m/e* 248; so, for quantitative analysis, we selected the base peak *m/e* 205. Oxipurinol shows a fragmentation pattern (Fig. 2) similar to that of xanthine when derivatized by butylation. The molecular ion (*m/e* 320, 48%) was selected for the quantitation of oxipurinol. The ions found to be suitable for the mass fragmentographic analysis of xanthine, [7,9-^{15}N]xanthine and hypoxanthine were *m/e* 320, *m/e* 322 and *m/e* 231, respectively, as reported previously [1].

A typical mass fragmentogram from a serum extract is shown in Fig. 3.

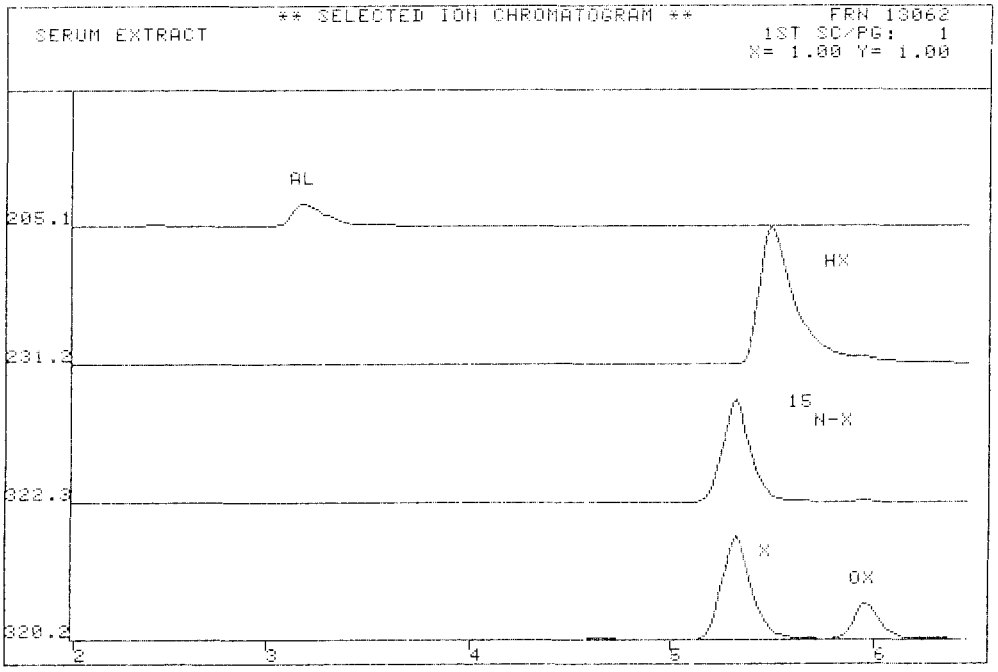

Fig. 3. Mass fragmentogram of butylated derivatives of allopurinol (AL), oxipurinol (OX), xanthine (X), [7,9-^{15}N]xanthine (^{15}N-X) and hypoxanthine (HX) in a serum extract.

Human studies

The study was conducted on 31 subjects receiving allopurinol (200 mg) orally once daily for a week, before lunch. Blood was withdrawn on the eighth day by venipuncture (20 ml in dry tubes) after overnight fasting, and urine was collected over the previous 24 h.

RESULTS AND DISCUSSION

Evaluation of the method

The limit of sensitivity was approximatively 50 ng of xanthine, hypoxanthine and oxipurinol per ml of serum and 25 ng/ml for allopurinol.

The inter-assay reproducibility of the method was determined by quadruplicate analysis of high and low standards in both serum (Table I) and urine (Table II).

TABLE I

PRECISION OF THE SIMULTANEOUS DETERMINATION OF XANTHINE, HYPOXANTHINE, ALLOPURINOL AND OXIPURINOL IN HUMAN SERUM

	Theoretical (μg/ml)	Calculated (μg/ml ± S.D.)	Coefficient of variation (%)	Mean error (%)
Hypoxanthine	5.0	4.97 ± 0.07	1.4	0.6
	2.0	2.09 ± 0.19	9.1	4.5
	0.5	0.46 ± 0.05	10.8	8.0
Xanthine	5.0	4.99 ± 0.01	0.2	0.2
	2.0	2.03 ± 0.05	2.5	1.5
	0.5	0.46 ± 0.02	4.3	8.0
Allopurinol	5.0	5.05 ± 0.06	1.2	1.0
	2.0	2.02 ± 0.21	10.4	1.0
	0.5	0.52 ± 0.06	11.5	4.0
Oxipurinol	5.0	5.00 ± 0.03	0.6	0.0
	2.0	2.07 ± 0.11	5.3	3.5
	0.5	0.50 ± 0.05	10.0	0.0

The accuracy of the method is indicated by the coefficient of variation and the mean error between the detected and the theoretical values.

The extraction recovery was found to be 65% for allopurinol at a 0.5 μg/ml level, and 62% for oxipurinol at a 5 μg/ml level, both in serum. The extraction recovery appears to be better than the xanthine recovery (55%).

Standard curves for the various compounds were linear over the concentration range investigated in both serum (Fig. 4) and urine. Correlation coefficients for serum standard curves (0.5—5.0 μg/ml) were better than 0.99. Correlation coefficients were equally good (> 0.99) for urine calibration curves for xanthine, hypoxanthine, allopurinol (2.5—25 μg/ml) and oxipurinol (10—100 μg/ml). Each sample was analyzed in duplicate and a third analysis was performed if the results differed by more than 10%.

TABLE II

PRECISION OF THE SIMULTANEOUS DETERMINATION OF XANTHINE, HYPOXANTHINE, ALLOPURINOL AND OXIPURINOL IN URINE

	Theoretical (μg/ml)	Calculated (μg/ml ± S.D.)	Coefficient of variation (%)	Mean error (%)
Hypoxanthine	25.0	25.0 ± 0.1	0.4	0.0
	10.0	10.5 ± 0.4	3.8	5.0
	2.5	2.5 ± 0.2	8.0	0.0
Xanthine	25.0	25.1 ± 0.2	0.8	0.4
	10.0	10.3 ± 0.6	5.8	3.0
	2.5	2.5 ± 0.2	8.0	0.0
Allopurinol	25.0	24.9 ± 0.1	0.4	0.4
	10.0	10.4 ± 0.5	4.8	4.0
	2.5	2.4 ± 0.2	8.3	4.0
Oxipurinol	100	100.4 ± 0.6	0.6	0.4
	50	51.2 ± 1.8	3.5	2.4
	10	10.0 ± 0.7	7.0	0.0

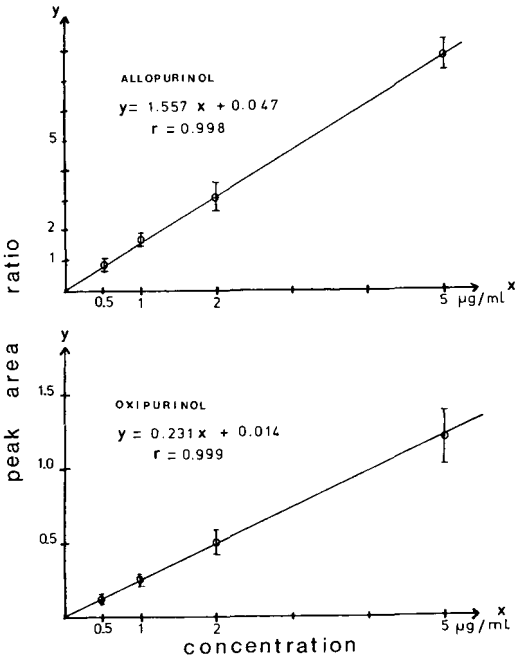

Fig. 4. Calibration curves for allopurinol and oxipurinol in serum. Each point represents the mean and standard deviation of seven determinations carried out on different days. The length of the bar corresponds to the estimated S.D.

Application of assay

Means and standard deviations for the serum concentrations and the urinary eliminations of xanthine, hypoxanthine, allopurinol and oxipurinol are listed in Table III. It can be seen that there is practically no allopurinol in the blood

TABLE III

SERUM CONCENTRATION AND URINARY ELIMINATION OF XANTHINE, HYPOXANTHINE, ALLOPURINOL AND OXIPURINOL IN 31 SUBJECTS TREATED WITH ALLOPURINOL

	Serum (μg/ml; n = 31)		Urine (mg per 24 h; n = 31)	
	Mean	S.D.	Mean	S.D.
Hypoxanthine	3.2	3.4	16.4	13.7
Xanthine	0.9	0.3	25.0	15.0
Allopurinol	0.1	0.1	7.8	5.3
Oxipurinol	3.7	1.9	122.9	48.6

samples; this conforms to our knowledge about the rapid transformation of allopurinol into oxipurinol, and is due to the important time delay between administration of the drug and the blood sampling.

The values obtained for xanthine and hypoxanthine in serum and especially in urine are higher than those observed in our first study on normal subjects, which is in agreement with the expected mechanism of action of allopurinol .

In conclusion, the method presented here is sensitive and accurate enough to allow the monitoring, in parallel to a pharmacokinetic study of allopurinol, of the influence of this drug on purine metabolism.

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Note

A time-saving method for the determination of the β_2 sympathomimetics terbutaline, salbutamol and fenoterol. Preliminary results

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Fenoterol, salbutamol and terbutaline are frequently used in asthma therapy. These compounds belong to the β_2-selective sympathomimetics and as such they show few side-effects on the heart. Although these compounds have been in clinical use for approximately a decade, the first useful methods for large-scale clinical studies were only published in 1976 for salbutamol [1] and terbutaline [2,3]. Since then, especially for terbutaline, more methods have been published [4—7]. The major disadvantage of all these methods is that they are time-consuming and specific for each drug. Some procedures involve difficult extraction steps [1,5—7] or use too large samples to allow frequent patient sampling [4]. Although the method developed by Leferink et al. [2,3] is relatively simple, it does not allow more than one injection per 20—25 min into the gas chromatography—mass spectrometry (GC—MS) system due to the disturbing influence of the ion-pair extracting agent [2,3]. An increasing number of requests for analysis of the β_2-sympathomimetics in our laboratory urged us to develop an assay that could be applied to terbutaline, salbutamol and fenoterol. In this article such a method is described comprising one extraction and derivatization procedure for these compounds and a mass-fragmentographic method for quantitation.

MATERIALS AND METHODS

Chemicals

The sympathomimetics terbutaline, salbutamol and fenoterol and the internal standard d_6-terbutaline, d_3-salbutamol and d_5-fenoterol were a gift from the respective manufacturers Draco (Lund, Sweden), Glaxo (Ware, Great Britain) and Boehringer (Ingelheim, G.F.R.). Methanol and acetonitrile were of analytical quality and used without further purification. Bondelut® C-18 cartridges (Nos. 6001 and 6003) were obtained from Analytichem International (Harbor

City, CA, U.S.A.) and Sep-Pak® C-18 cartridges were obtained from Waters Assoc. (Milford, MA, U.S.A.). The derivatizing agent, bis(trimethylsilyl)trifluoroacetamide (BSTFA), was a Pierce (Rockford, IL, U.S.A.) product. A phosphate buffer of pH 7.6 was used with a strength of 0.1 *M*.

Instrumental

A Finnigan 3200 GC—CI—MS system and a four-channel Promim® were used in the assay. The Promim channels were set according to the quasi-molecular ion masses ($M + H^+$) of the respective compounds and their standards. For terbutaline these masses were m/z 442 and 448, for salbutamol m/z 456 and 459, and for fenoterol m/z 592 and 597. The chemical-ionization reagent gas was ammonia at a source pressure gauge read-out of 800 μm. The source temperature was controlled at 140 ± 2°C. The gas chromatograph was equipped with a 20 m × 0.5 mm I.D. glass capillary column coated with CP-Sil 8. The inlet pressure for the carrier gas (helium) was 56 kPa. The connection between the capillary column and the mass spectrometer was a 60-cm fused silica capillary (0.20 mm I.D.) and an open split coupling. The interfacing capillary was kept at 310°C. The GC column was operated at 210°C for terbutaline and salbutamol and at 280°C for fenoterol. The injector remained at 300°C.

Sample preparation

Aqueous and serum samples were used in the development of the procedure. The aqueous samples contained 10 ng of the sympathomimetic in 1 ml of buffer solution.

Serum samples (1 ml) were spiked with 2—20 ng of the sympathomimetic and 1 ml of the buffer was added. These samples were used for recovery measurement. Two actual patient samples were used: one serum sample was obtained 2 h after intake of a 5-mg terbutaline sulphate tablet and one blood sample was from a case of salbutamol overdose. To these samples 10 ng of d_6- terbutaline and 20 ng of d_3-salbutamol were added as the respective internal standards: in addtion, 1 ml of buffer was added to each sample.

Method

Bondelut C-18 and Sep-Pak C-18 cartridges were pretreated as suggested by the manufacturers. The cartridges were washed with 4—5 ml of methanol, 2 ml of water and 0.5 ml of buffer. During the prewashings with water and buffer the columns were not allowed to run dry. After pretreatment the prepared samples were allowed to drain into the column and were then pressed through the column; subsequently the column was washed with 1 ml of water. Finally, the columns were eluted with 4 × 1 ml of methanol—acetonitrile (85:15, v/v). The first 150—200 μl (3—4 drops) of the eluate were discarded. The total elution time was 1 min/ml.

In the case of recovery studies 10 ng of the various internal standards were added to the combined eluate. The eluate was transferred into 1-ml Reacti® vials (Pierce) in 0.5-ml portions and blown to dryness under a nitrogen stream at 70°C. The dried residue was reconstituted and derivatized in 20 μl of BSTFA at 80°C for 10—15 min. Subsequently, 1 μl was injected onto the capillary column.

RESULTS AND DISCUSSION

Recovery measurements on the aqueous solution containing 10 ng of the sympathomimetic showed that the best recoveries are obtained with the small Bondelut columns (No. 6001). The recoveries were highest for terbutaline and fenoterol (90%) and slightly lower for salbutamol (82%). The recoveries on the larger Bondelut columns (No. 6003) were low (< 5%) using 4 ml of eluent. Even after elution with 12 ml of eluent only 50% was recovered. Sep-Pak cartridges showed recoveries between 70 and 75% for the three sympathomimetics. From these experiments it seemed that the sympathomimetics bind strongly to the C-18 cartridge packing. It was also clear that for this reason more eluent was required to elute the compound than recommended by the manufacturers for general extraction purposes.

Recovery measurements on spiked serum samples revealed no differences between the Bondelut and Sep-Pak cartridges. Again the highest recoveries were found for terbutaline and fenoterol (95%) and a lower recovery for salbutamol (80%). However, the percentage recovery was unaffected by the concentration of the sympathomimetic. At concentrations of 2, 5 and 20 ng/ml

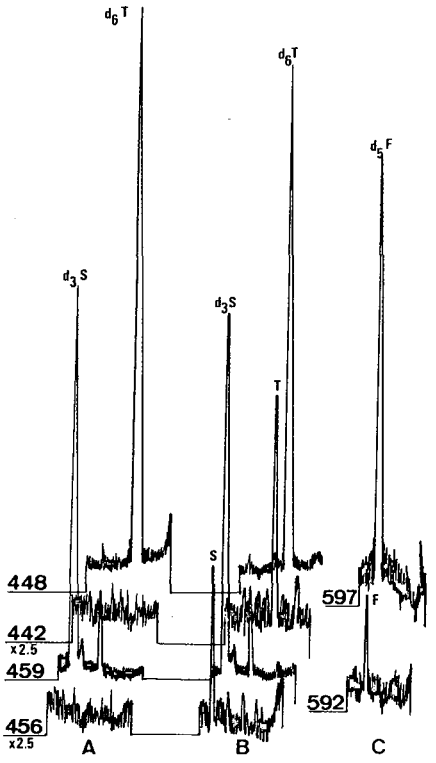

Fig. 1. Mass fragmentograms of (A) blank serum spiked with 10 ng of d_3-salbutamol and 10 ng of d_6-terbutaline, (retention times were 4.38 and 3.45 min, respectively); (B) as (A) but also spiked with 2 ng of salbutamol and 2 ng of terbutaline; (C) serum spiked with 10 ng of d_5-fenoterol and 2 ng of fenoterol (retention time 4.72 min). S = salbutamol, T = terbutaline, F = fenoterol.

the recoveries remained the same within the error in the measurement of 3% at the 20 ng/ml level to 10% at the 2 ng/ml level. Unspiked serum samples showed clean ion chromatogram recordings with no background at the retention times of the various compounds. Only in the salbutamol recordings was a major peak found at the ion trace of the internal standard (*m/z* 459). Since a capillary column was used, this coextracted impurity did not affect the accuracy of the measurement due to the large difference in retention times (Fig. 1A). Fig. 1B and C show the result of serum samples spiked with 2 ng/ml of the sympathomimetics and 10 ng of the respective internal standards and run on Sep-Pak. In this example all three sympathomimetics were added to the serum and two separate GC runs were made to obtain the results for terbutaline and salbutamol at 210°C and for fenoterol at 280°C. These data show that measurements at the 1 ng/ml level can be performed with an acceptable signal-to-noise ratio. Fig. 2A shows the recording of a serum sample taken 2 h after intake of a 5-mg terbutaline sulphate tablet. The concentration of terbutaline was calculated at 2.8 ng/ml. This value is in agreement with those obtained with the previously described method [2,3]. This experiment therefore indicates that terbutaline conjungates, which are present especially after oral intake, do not affect the measurement.

Fig. 2B represents the fragmentogram of a blood sample in a case of salbutamol overdose. The calculated concentration was 23 ng/ml of blood. The results of Fig. 2. were obtained using Bondelut (No. 6001).

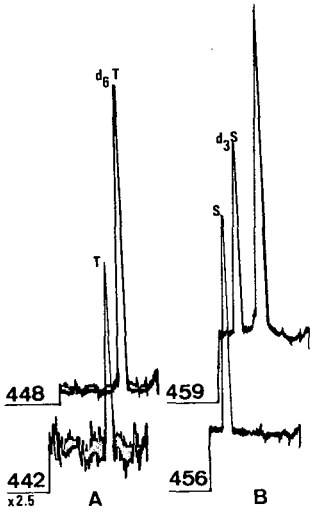

Fig. 2. (A) Patient serum sample 2 h after intake of 5 mg of terbutaline sulphate. (B) Patient blood sample after an overdose of salbutamol.

CONCLUSION

From the reported experiments it is obvious that this extraction method, using either Bondelut (No. 6001) or Sep-Pak cartridges, shows a large improvement over previously published methods in terms of time and simplicity. Futhermore, its uniformity is advantageous; in fact, on adding all three internal

standards to the biological sample routinely, one can simply screen the sympathomimetics quantitatively. One warning should be issued at this stage: although the detection limits for the three compounds are similar (around 1 ng/ml) it is not possible to measure fenoterol at the average therapeutic level. This level is <300 pg/ml as established with tritiated compounds [8]. The detection limit is sufficient, however, to measure levels in overdose cases. The therapeutic ranges for salbutamol and terbutaline are well within the range of the method.

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The authors would like to thank the drug companies Boehringer, Draco and Glaxo for their generous gifts of the sympathomimetics and the deuterated standards. Part of this work has been made possible by a grant (No. 269) from the Dutch Asthma Foundation.

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Note

Micromethod for automated identification and quantitation of fifteen barbiturates in plasma by gas—liquid chromatography

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A great number of methods for the determination of barbiturates in blood have been published the last years, essentially by gas—liquid chromatography (GLC) [1—8]. We ourselves have described the measurement of fifteen barbiturates in biological fluids using two columns of different polarity [10, 11].

Taking advantage of some new improvements in GLC such as specific detection by nitrogen—phosphorus detector, fused-silica capillary column, automation and data computer, we present an optimized technique for the identification and quantitation of fifteen barbiturates whose structures are given in Table I.

EXPERIMENTAL

Reagents and chemicals

The following reagents were used: methanol—water solution (90:10, v/v) saturated with potassium carbonate; dimethylsulfate (Normapur; Prolabo, Paris, France); hexane (Normapur; Prolabo); sodium acetate buffer, 0.2 *M*, pH 6.0.

Methohexital (Lilly, Basingstoke, Great Britain), was used as internal standard, 0.1 mg/ml in water. Stock barbiturate solutions are 1 mg/ml in methanol. The working standard is a drug-free plasma spiked with the most commonly encountered barbiturates (amobarbital, pentobarbital and secobarbital at 12.5 mg/l, phenobarbital at 25 mg/l).

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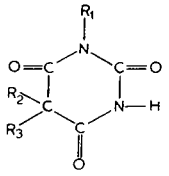
TABLE I

BARBITURATE FORMULA, RETENTION TIME AND RELATIVE RETENTION INDEX



No.	Name	R_1	R_2	R_3	Retention time (min)	Relative retention index
1	Barbital	H	Ethyl	Ethyl	4.72	0.385
2	Butalbital	H	Allyl	1-Methylpropyl	7.28	0.594
3	Butobarbital	H	Ethyl	Butyl	7.47	0.610
4	Amobarbital	H	Ethyl	3-Methylbutyl	8.70	0.710
5	Pentobarbital	H	Ethyl	1-Methylbutyl	9.54	0.779
6	Vinylbital	H	Vinyl	1-Methylbutyl	9.71	0.793
7	Vinbarbital	H	Ethyl	1-Methyl-1-butenyl	10.38	0.847
8	Secobarbital	H	Allyl	1-Methylbutyl	10.80	0.882
9	Brallobarbital	H	Allyl	2-Bromoallyl	11.79	0.962
10	Methohexital	CH_3	Allyl	1-Methyl-2-pentynyl	12.25	1.000
11	Hexobarbital	CH_3	Methyl	1-Cyclohexen-1-yl	14.14	1.154
12	Phenobarbital	H	Ethyl	Phenyl	14.84	1.211
13	Cyclobarbital	H	Ethyl	1-Cyclohexene-1-yl	15.14	1.236
14	Heptabarbital	H	Ethyl	1-Cyclohepten-1-yl	16.78	1.370
15	Reposal	H	Ethyl	Bicyclo[3.2.1.] oct-2-en-3-yl	18.07	1.475

Apparatus

An HP 5880 gas chromatograph (Hewlett-Packard, Les Ulis, France) equipped with an HP 7671 automatic sampler and nitrogen–phosphorus detector was used. Qualitative and quantitative data were given by an HP 5880 GC terminal (level four).

The column was a flexible fused-silica capillary column (12 m × 0.2 mm I.D.) coated with dimethylsilicone fluid (SP 2100, Hewlett-Packard).

Operating conditions were: injection port temperature in splitless conformation, 300°C; detector temperature, 300°C; oven temperature programmed from 50°C (initial time 0.5 min) to 140°C (final time 5 min) at 30°C/min and then from 140°C to 190°C (final time 5 min) at 5°C/min; carrier gas (helium), 1.5 ml/min.

Procedure

To 100 µl of plasma in polypropylene Eppendorf microtubes (Roucaire, Paris, France) were added 25 µl of the internal standard solution, 250 µl of the methanolic solution and 10 µl of dimethylsulfate. The stoppered tubes were shaken and placed in a water-bath at 50°C for 5 min. Then, 250 µl of sodium acetate buffer and 600 µl of hexane were added and mixed for 30 sec. After brief centrifugation, the hexane layer was transferred to automatic sampler vials and 1 µl was injected.

Quantitation

Barbiturate determination was carried out using methohexital as internal standard. Calibration curves were obtained by spiking plasma with variable

amounts of stock solutions (5, 10, 20, 30, 60 mg/l), and a constant amount of internal standard (25 mg/l). Two working standards were run with each set of analyses. Quantitation was done by the peak area ratio method.

Within-run variation was determined for two concentrations with ten spiked plasma samples. Day-to-day precision was studied using working standards.

RESULTS AND DISCUSSION

The retention times of the fifteen barbiturates and typical chromatograms of standard, blank and patient samples are shown, respectively, in Table I and Figs. 1—3. The linearity of the method is very good within the concentration range encountered in plasma and up to 100 mg/l, with correlation coefficients of 0.9995 or more. Within-run variation and day-to-day reproducibility are presented in Table II. For levels as low as 1.25 mg/l for amobarbital, pentobarbital, secobarbital and 2.5 mg/l for phenobarbital, the coefficient of variation is below 5%. Concentrations of 0.5 mg/l can be easily detected, but minor concentrations could be measured after solvent concentration.

The specificity of the method has been widely tested in this laboratory. More than 350 plasma samples from patients suspected of intoxication have been analyzed simultaneously by this method and by another method which permits identification and quantitation of about 100 drugs acting on the central nervous system [9]. No interference has been encountered.

This method presents many advantages compared to the technique previously described [10, 11], which required the use of two packed columns of different polarity (SE-30 and NPGA) and 3 ml of blood. Here, the separation of

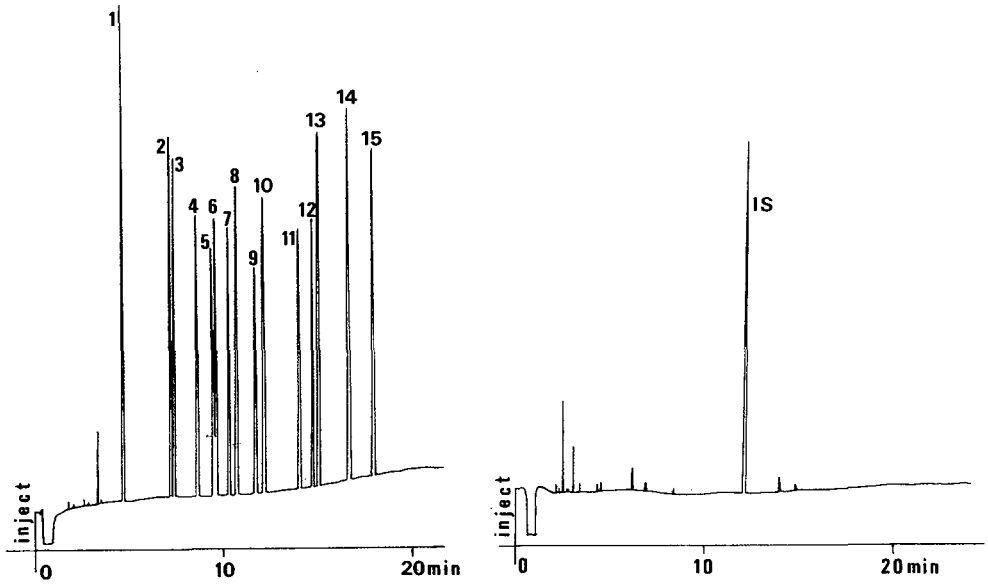


Fig. 1. Separation of a mixture of pure barbiturates. Peak numbers correspond to those of the compounds in Table I.

Fig. 2. Chromatogram of blank plasma. IS = internal standard.

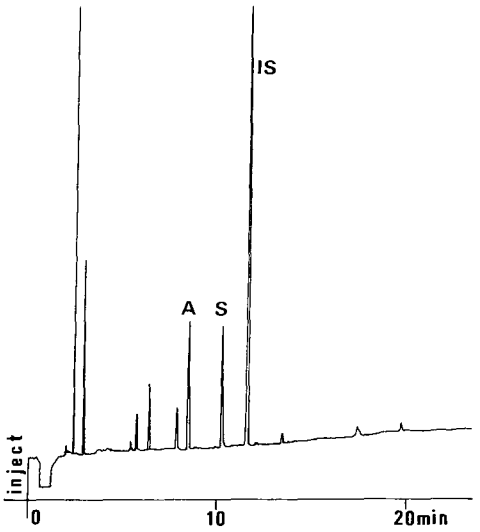


Fig. 3. Chromatogram of patient plasma containing amobarbital (A) (5.1 mg/l) and secobarbital (S) (4.6 mg/l).

TABLE II

WITHIN-RUN AND DAY-TO-DAY VARIABILITY OF THE METHOD

	Within-run			Day-to-day		
	Amount added (mg/l)	Amount found ($\bar{x}$ ± S.D., n = 10)	C.V.* (%)	Amount added (mg/l)	Amount found ($\bar{x}$ ± S.D., n = 34)	C.V.* (%)
Amobarbital	1.25	1.29 ± 0.04	3.1	12.50	12.55 ± 0.62	4.9
	10.00	9.81 ± 0.17	1.7			
Pentobarbital	1.25	1.29 ± 0.04	3.1	12.50	12.47 ± 0.62	4.9
	10.00	9.69 ± 0.19	2.0			
Secobarbital	1.25	1.29 ± 0.04	3.1	12.50	12.48 ± 0.38	3.0
	10.00	9.70 ± 0.15	1.6			
Phenobarbital	2.50	2.69 ± 0.12	4.5	25.00	25.10 ± 0.91	3.6
	20.00	20.73 ± 0.40	2.0			

*C.V. = coefficient of variation = (S.D./$\bar{x}$) × 100.

the fifteen barbiturates studied is performed on one flexible fused-silica capillary column which also presents the advantage of being less fragile and easier to connect to the injector and detector fittings than conventional glass capillary columns. This point is not negligible when routine analysis is concerned. More than 1000 analyses have been performed with only minor loss of resolution. The nitrogen—phosphorus detector is more specific and sensitive than a flame ionization detector, so a more simple purification procedure with only 100 μl of plasma is possible. As a matter of fact, methylation can occur directly in plasma or in other biological fluids without any prior purification, and hexane extraction is performed in the same tube without

further evaporation. The analysis time for one sample including preparation of plasma and chromatographic run is only 30 min, which is very interesting especially in the case of intoxication. Moreover, the rapidity and simplicity of sample preparation permit the rapid preparation of large series of samples during therapeutic control and to perform the chromatographic analysis overnight using the automatic sampler. The data system gives, after each run, a report including the name and concentration of any of the fifteen barbiturates present in the plasma and also the name of the patient.

In conclusion, this technique, involving a small quantity of plasma, minimal sample pretreatment and automated gas chromatography, is highly suitable for routine analysis.

ACKNOWLEDGEMENTS

We thank Fondation Langlois (Rennes, France) for its grant and M. Laisne for her secretarial assistance.

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Note

Determination of moroxydine in biological fluids by electron-capture gas chromatography

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For the analysis of the biguanides buformin, metformin and phenformin, gas chromatographic methods have been used in the last few years [1—5]. They are all based on the cyclization of the biguanide by a suitable anhydride. High detection sensitivity has been achieved with electron capture [2] or with mass fragmentography [4]. Recently, an alkali-flame detector in the nitrogen mode was used and even that had the capability of detecting nanogram amounts of the drug [5].

Moroxydine has previously been analyzed by non-chromatographic methods, but recently a mass fragmentographic procedure was presented [6].

In this report a gas chromatographic method is described for the determination of moroxydine in biological fluids. The method is based on isolation by ion-pair extraction, formation of a triazine derivative by cyclization with chlorodifluoroacetic anhydride and gas chromatography with electron-capture detection. Emphasis is placed on the isolation of the rather hydrophilic moroxydine.

EXPERIMENTAL

Apparatus

A Varian 1400 gas chromatograph, equipped with a scandium ^{3}H electron-capture detector, was used with a 1.5 m × 1.8 mm glass column filled with 2% OV-225 on Chromosorb G. The column was conditioned at 300° C for 3 h and operated at 220° C with a nitrogen flow-rate of 30 ml/min. The detector and injector temperatures were 280° C and 230° C, respectively.

A Multi-Temp-Blok (Lab-Line Instruments) was used for the derivatization at 50° C.

Reagents

Trichloroacetic acid, hydrochloric acid 37% min., sodium dihydrogen phosphate, disodium hydrogen phosphate and toluene were all of analytical purity grade and obtained from E. Merck (Darmstadt, G.F.R.). Sodium hydroxide (analytical purity grade) was purchased from EKA (Bohus, Sweden). Tetrabutylammonium hydrogen sulphate (purum quality) was obtained from Fluka (Buchs, Switzerland). Methylene chloride (analytical purity grade) was obtained from Fisher (Fair Lawn, NJ, U.S.A.) and chlorodifluoroacetic anhydride from Bristol Organics (Hotwells Road, Bristol, Great Britain).

Bromothymol blue (BTB) indicator was obtained from Merck and purified by extraction at pH 8.5 with tetrabutylammonium hydrogen sulphate according to principles presented by Borg et al. [7].

Moroxydine hydrochloride was synthesized at KabiVitrum and found to be more than 99.0% pure as determined by titration and high-voltage paper electrophoresis. N-Aminoiminomethyl-1-piperidinecarboximidamide dihydrochloride was synthesized at KabiVitrum and used as internal standard.

[U-^{14}C]Moroxydine hydrochloride was obtained from the Radiochemical Centre, Amersham, Great Britain, and had a specific activity of 8.0 mCi/mmol (38.6 μCi/mg) and a stated purity of > 97%.

Method

Plasma and serum samples. A maximum 0.5 ml of the sample was mixed with 25—100 μl of internal standard solution containing 0.72 mg/l. The proteins were precipitated by mixing with 0.5 ml of 0.6 *M* trichloroacetic acid in 1 *M* hydrochloric acid. After centrifugation the supernatant was transferred to a centrifuge tube containing 1 ml of a BTB solution (8×10^{-3} *M* in phosphate buffer, pH 7.5, ionic strength 1). Sodium hydroxide (10 *M*) was added until the colour changed to green. The mixture was extracted with 8 ml of methylene chloride for 15 min and then centrifuged. The organic layer was filtered through a plug of silanized glass wool and evaporated to dryness on a boiling water bath. After cooling, 2 ml of methylene chloride and 20 μl of chlorodifluoroacetic anhydride were added and, after mixing, the solution was evaporated to dryness at 50° C. The residue was dissolved in 2 ml of toluene; 2 ml of 1 *M* sodium hydroxide were added and the mixture was extracted. After centrifugation 3—5 μl of the organic phase were injected into the gas chromatograph.

Urine samples. Urine samples were treated in the same way as plasma and

serum omitting the precipitation step. Samples containing low levels of moroxydine were purified by mixing with phosphate buffer (pH 7.5, ionic strength 1) and, prior to the addition of BTB, extracted with 8 ml of methylene chloride which was then discarded.

Preparation of standard curves. Standard curves were prepared by the addition of appropriate amounts of standard and internal standard to the same volume of blank serum, plasma or water (for urines) as the samples. The standard curves obtained by peak height measurements were linear between 0 and 1.5 mg/l moroxydine. From these standard curves the concentrations of the unknown samples were calculated.

RESULTS AND DISCUSSION

Derivatization

The structures of the *s*-triazine derivatives of moroxydine and internal standard with chlorodifluoroacetic anhydride, as shown in Fig. 1, were verified by gas chromatography—mass spectrometry. The preparation of the derivatives was easily performed during the evaporation procedure at 50° C. Between 1 and 100 µl of the anhydride gave a quantitative yield. Twenty microliters of the anhydride were used and found to be sufficient, even in the presence of co-extracted biological material.

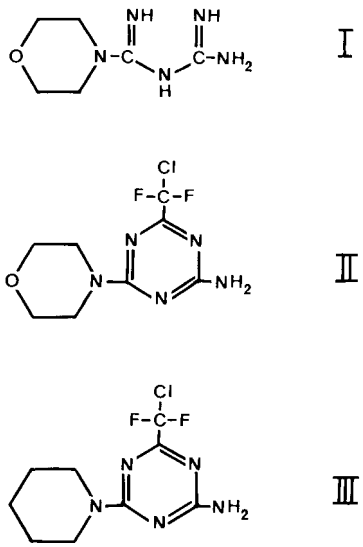

Fig. 1. Structure of moroxydine (I) and the derivatives of moroxydine (II) and of the internal standard (III).

Gas chromatography

The derivatives of moroxydine and the internal standard were run on several phases and it was found that non-polar phases like OV-1 gave tailing peaks. On OV-17, a moderately polar phase, the derivatives did not separate. The

best separation was achieved on a 2% OV-225 column, where the moroxydine derivative had a retention time of 5 min at 220° C. The relative retention of the internal standard derivative was 0.8. A gas chromatogram is shown in Fig. 2.

The derivative of moroxydine had an excellent electron-capture response. The minimum detectable concentration with a detector temperature of 280° C was 8×10^{-16} mole/sec, which means that about 17 pg of the injected derivative can be distinguished from the noise level.

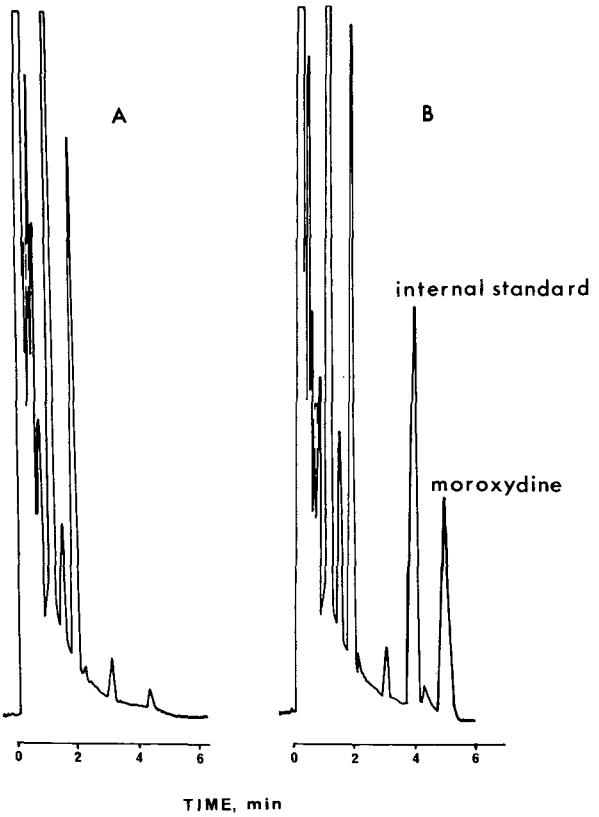

Fig. 2. Gas chromatograms from 100 μl serum. (A) Blank serum. (B) Serum containing 645 μg/l moroxydine. The injected amount of moroxydine derivative is 150 pg in 3 μl of solvent.

Extraction conditions

Garrett and co-workers [8, 9] studied the extraction conditions for three common biguanides as 1:1 ion pairs with BTB. The extraction of moroxydine as BTB ion pair was reported by Schill [10].

Initial experiments revealed that the quantitative extraction of moroxydine as base was not possible even when such highly polar solvents as 1-pentanol or 3-pentanol were used. The use of an aqueous phase with high ionic strength improved the extraction yields somewhat, but the extracts could not easily be derivatized. Furthermore, decomposition of moroxydine occurs in a strong-

ly alkaline medium, which is why such extraction conditions should be avoided.

Schill [10] determined the extraction properties of the complex between moroxydine and BTB as the monovalent anion (HB^-) and with methylene chloride as organic phase. The extraction constant was $10^{3.75}$ at pH 7.5, giving an extraction of 69% with a concentration of HB^- of 10^{-4} *M* (total concentration of BTB = 3×10^{-4} *M*), the ratio between volume of organic phase and volume of aqueous phase being 4:1. Enhancement of the degree of extraction by increasing the total concentration of BTB is limited due to the formation of dimers and tetramers of the monovalent anion (HB^-) [11].

According to the method, the BTB concentration is about 5×10^{-3} *M* (concentration of monovalent anion, HB^-, is about 10^{-3} *M*) and the phase volume ratio 5:1 in the extraction step. Calculations according to Schill [10], who used an ionic strength of 0.1, give a degree of extraction of 97%. However, the actual ionic strength was 1.0, which is why its influence on the extraction had to be elucidated. High blank values made photometric determinations impossible; thus radioactivity measurements using [U-^{14}C]moroxydine were performed. An extraction of 80% was obtained with a moroxydine concentration of 16.5 mg/l.

The internal standard is more hydrophobic (due to the lack of the ether bond) than moroxydine and has an extraction constant of $10^{4.58}$. This corresponds to an extraction of 94% under the conditions given by Schill [10].

To increase the yield of moroxydine, a second extraction could be made. However, as samples analyzed with only one extraction gave the same results as those samples extracted twice, the more practical single-extraction procedure was preferred.

The absolute recovery including extraction and derivatization steps was about 70%, determined with radioactive moroxydine in a concentration of 58 μg/l.

Extraction from plasma and serum

Initial experiments showed that precipitation of the proteins with trichloroacetic acid in hydrochloric acid [2] gave higher recoveries than dilution with buffer. No interfering peaks originating from the serum were observed in the chromatogram. However, in some cases disturbances were noted which could be attributed to BTB.

Radioactivity measurements were used in some precipitation experiments, where it could be shown that sample volumes of 0.1 ml gave about 90% recovery of moroxydine in the supernatant, whereas 0.5 ml gave about 70% recovery.

As mentioned above, the absolute recovery from an aqueous solution was about 70%. When the precipitation losses are included the recovery from 0.5 ml of serum will be reduced to about 50%. However, these losses do not influence the final results as the internal standard compensates for some of the losses and the standard curves were prepared under identical conditions.

Extraction from urine

Urine with low levels of moroxydine contained some compounds that in-

terfered in the gas chromatogram. They could be removed if the urine sample at pH 7.5 was extracted with methylene chloride prior to the addition of BTB.

Recovery and precision

When known amounts of moroxydine were added to serum in a concentration of 505 μg/l and 0.15 ml was taken for analysis, the relative standard deviation was 2.9% (n = 8). Analysis of 0.5 ml of serum containing 21 μg/l gave a relative standard deviation of 9.3% (n = 8). The higher standard deviation reflects mainly the problems encountered in the precipitation of the proteins in the larger sample volume.

Applications to biological samples

The method has been applied to samples from biopharmaceutical and toxicological studies. An example of the time course curve from an oral dose of 800 mg of moroxydine hydrochloride is given in Fig. 3.

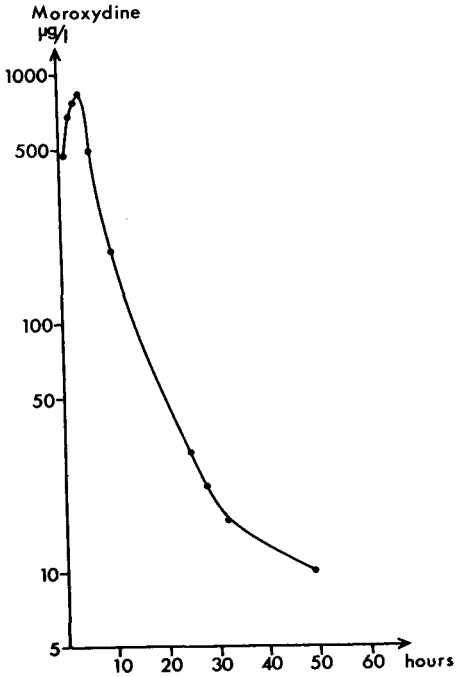

Fig. 3. Serum levels of moroxydine in a human volunteer after oral administration of 800 mg of moroxydine hydrochloride.

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Note

Rapid estimation of diflunisal in plasma and urine by high-performance liquid chromatography and a comparison with a fluorometric method

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Diflunisal (2′,4′-difluoro-4-hydroxy-3-biphenyl-carboxylic acid) is a recently introduced derivative of salicylic acid with similar analgesic and anti-inflammatory properties [1, 2].

Gas chromatographic, radioisotopic and fluorometric methods have been used for quantitation of diflunisal [3], but all have disadvantages. The gas chromatographic assay necessitates lengthy sample preparation (extraction, evaporation and derivatisation), while the radioisotopic and fluorometric methods are non-specific.

Since the present work was started, high-performance liquid chromatographic (HPLC) methods for the determination of diflunisal in plasma have been reported [4, 5]. In both, diflunisal and naproxen (internal standard) were extracted from plasma into organic solvents which were evaporated to dryness. In one report [4] the chromatography was inefficient as judged by broad tailing peaks and the limit of detection was only 5 μg/ml.

The present simple method does not require extraction and can be completed in a fraction of the time with a detection limit of 0.5—1.0 μg/ml.

EXPERIMENTAL

Materials

Diflunisal was obtained from Thomas Morson Pharmaceuticals (Division of Merck Sharp & Dohme, Hoddesdon, Great Britain) and flufenamic acid (the internal standard) from Parke Davis & Company (Pontypool, Great Britain). All solvents and reagents were of Analar grade and obtained commercially.

Standard solutions

Diflunisal (50 mg) was dissolved in 1.0 ml of methanol in a 100-ml volumetric flask and brought to volume with 1/15 *M* phosphate buffer (pH 7.2). Further dilution was made with water, urine or plasma to produce final concentrations of 1—10 and 10—100 μg/ml. Flufenamic acid (50 mg) was dissolved in 1.0 ml methanol in a 100-ml volumetric flask and made up to volume with 0.001 *M* sodium bicarbonate. The stock solutions could be stored for at least eight weeks at 4° C.

Chromatography

The HPLC system consisted of a Pye Unicam Model LC3 variable-wavelength UV detector set at 251 nm, an Orlita DMP AE 10.4 pump, a loop injector (Rheodyne Model 7120) and a recorder (Bryans Model 28000). The column was 150 × 4.5 mm I.D. internally polished stainless steel, slurry packed with 5-μm Hypersil ODS (Shandon, Runcorn, Great Britain).

The mobile phase was a mixture of 0.08 *M* potassium nitrate in 2% acetic acid—isopropanol—ethyl acetate (55:25:20) which was degassed under reduced pressure prior to use. The solvent flow-rate was 1.3 ml/min at room temperature with a working pump pressure of 110 bar (1600 p.s.i.). The detector sensitivities were 0.08 and 0.02 a.u.f.s. for the plasma and urine assays, respectively.

Estimation of diflunisal in plasma

To 0.5 ml of plasma containing 10—100 μg/ml of diflunisal in a disposable polypropylene tube was added 0.5 ml of flufenamic acid solution (250 μg) followed by 0.5 ml of acetone to precipitate the proteins. After mixing, the sample was centrifuged and 25-μl aliquots of the clear supernatant injected directly into the chromatograph. For lower concentrations, 50 μl of the flufenamic acid solution was used. Plasma containing diflunisal in concentrations of more than 100 μg/ml was diluted appropriately with 0.9% saline (dilution with saline or blank plasma gave similar results). Two standards of diflunisal (5 and 50 μg/ml) in plasma were assayed with each set of unknown samples. Diflunisal concentrations were calculated from the regression of these standards using the peak-height response ratio of diflunisal to internal standard.

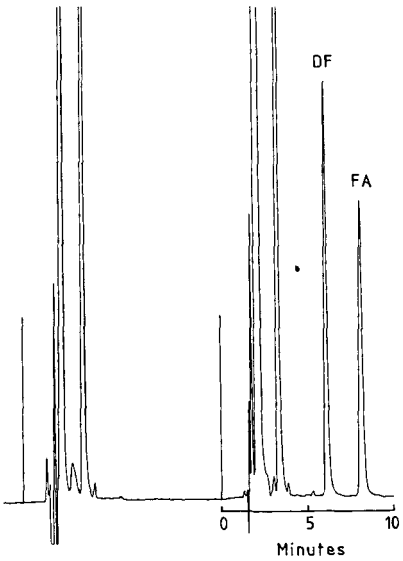
Estimation of diflunisal in urine

To 1.0 ml of urine containing 1—10 μg/ml diflunisal in a disposable polypropylene tube were added 100 μl of flufenamic acid solution (500 μg/ml). After mixing, 25 μl were injected directly into the chromatograph. Urine containing more than 10 μg/ml of diflunisal was diluted appropriately with distilled water.

Two standards of diflunisal (1 and 10 μg/ml) in urine were assayed with each set of unknown samples. Diflunisal concentrations in urine were calculated as described above for plasma.

RESULTS AND DISCUSSION

Typical chromatograms of plasma and urine obtained from a healthy volunteer 3 h after ingestion of 750 mg of diflunisal (three Dolobid tablets) are illustrated in Figs. 1 and 2. The retention time of diflunisal was 6 min in both assays and samples could be injected every 9 min. No other interfering peaks were observed.



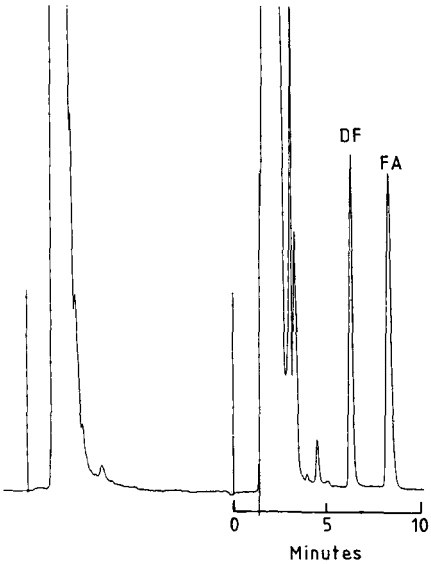


Fig. 1. Chromatogram of plasma from a healthy volunteer before (left) and 3 h after ingestion of 750 mg of diflunisal (right). Peaks: DF = diflunisal, FA = flufenamic acid (internal standard).

Fig. 2. Chromatogram of urine from a healthy volunteer before (left) and 3 h after ingestion of 750 mg of diflunisal (right). Peaks: DF = diflunisal, FA = flufenamic acid (internal standard).

The standard calibration graphs for both assays were linear and passed through the origin [6]. The precision and reproducibility of the assays are shown in Table I as the result of five replicate analyses of diflunisal in plasma and urine. The overall recovery (drug found) of diflunisal was 100.4 ± 3.6% (S.D.) for plasma and 100.2 ± 5.3% (S.D.) for urine. The respective limits of detection were 1.0 and 0.5 μg/ml. Diflunisal was stable in plasma and urine at −20° C for more than 6 months and at −4° C for at least 2 months.

The results of analyses of 17 plasma and 13 urine samples from five patients with diflunisal overdosage are given in Table II and mean plasma diflunisal concentrations in six healthy volunteers following a single oral dose of 750 mg are shown in Fig. 3.

Naproxen was initially chosen as the internal standard because it eluted before diflunisal with good separation. However, an interfering peak appeared in the urine of healthy volunteers following ingestion of diflunisal. The peak did not change after hydrolysis of urine with β-glucuronidase and arylsul-

TABLE I

REPLICATE ANALYSES OF DIFLUNISAL

Five replicate analyses were carried out at each concentration.

Plasma			Urine		
Drug added (μg/ml)	Mean concentration found (μg/ml)	Coefficient of variation (%)	Drug added (μg/ml)	Mean concentration found (μg/ml)	Coefficient of variation (%)
10	10.2	7.3	1	1.06	9.4
20	20.2	2.1	2	1.94	4.6
40	40.5	2.4	4	4.11	3.9
60	58.6	2.5	6	5.91	5.1
80	80.0	1.8	8	7.87	1.6
100	100.6	1.0	10	10.09	2.3

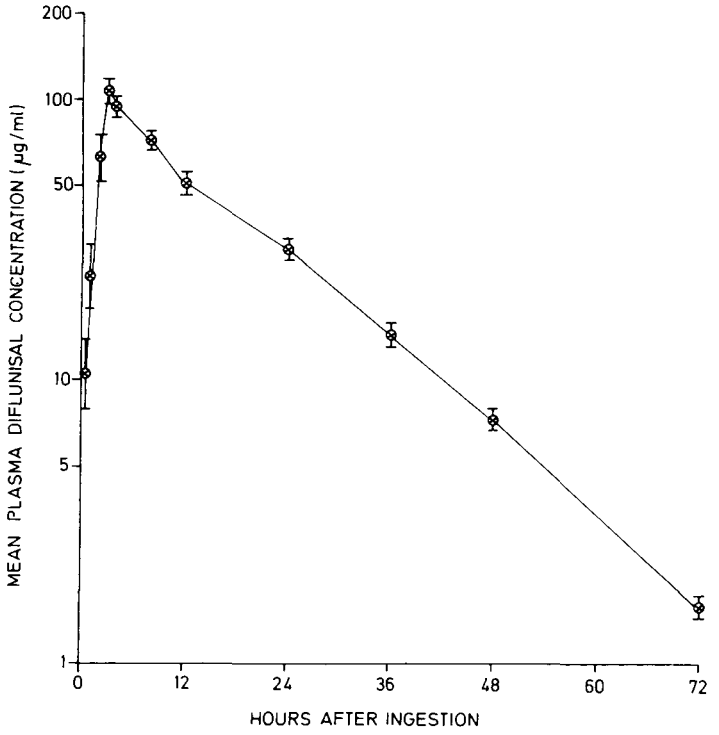


Fig. 3. Plasma concentrations of diflunisal in six healthy volunteers following an oral dose of 750 mg (means ± S.E.).

TABLE II

PLASMA AND URINE CONCENTRATIONS FOLLOWING DIFLUNISAL OVERDOSAGE MEASURED BY THE HPLC METHOD

Patient	Alleged number of Dolobid tablets	Other drugs taken	Time after ingestion (h)	Plasma diflunisal concentration (μg/ml)	Time after ingestion (h)	Urine diflunisal concentration (μg/ml)
RH	50	30 prochlorperazine	3	348	N.A.*	2.2
		30—40 dihydrocodeine	7	251	N.A.	0.9
			26	123	N.A.	0
SG	30	20 aspirin/codeine	3	103	—	—
			25	39	—	—
AD	70	—	37	173	37	57
ML	30	—	12	131	13—19	82
			18	103	19—25	50
			24	80	23—31	38
			30	70	31—43	26
			36	44	43—55	30
			48	19	55—67	12
			60	6	67—79	5
			72	2	79—81	2
			84	trace	81—93	trace
JL	?	? paracetamol plus	?	134	—	—
		d-propoxyphene	?+8	300	—	—

*N.A. = not available.

phatase whereas thin-layer chromatography showed that the glucuronide conjugates of diflunisal had disappeared. This interfering peak appeared to be an unknown metabolite of diflunisal.

Comparison of fluorometric and HPLC methods

The HPLC method was compared with the following modification of the fluorometric assay described by Tocco et al. [3].

To 100 μl of plasma containing 1—30 μg/ml diflunisal in a 15-ml round-bottomed glass tube were added 900 μl of pooled blank plasma. Appropriate dilutions were made for higher diflunisal concentrations. After mixing, 1 ml of 5 *N* hydrochloric acid was added and the drug extracted into 5 ml of chloroform. Following centrifugation, 2 ml of the chloroform phase was extracted with 3 ml of 0.1 *M* phosphate buffer (pH 8.0). The fluorescence of the aqueous phase was measured in an Aminco-Bowman spectrophotofluorometer set at 260 nm (activation) and 425 nm (emission).

Concentrations were determined by reference to a previously constructed calibration graph of per cent transmission minus the blank value plotted

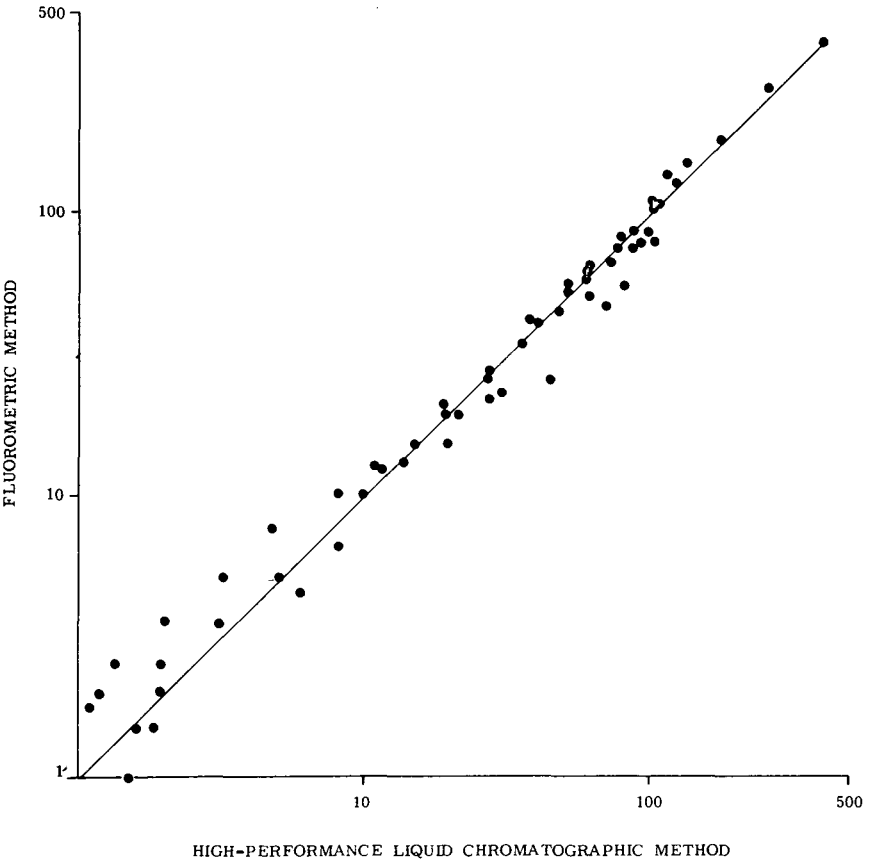


Fig. 4. Comparison of HPLC and fluorometric methods for the estimation of diflunisal in plasma. Individual measurements (μg/ml) were plotted for 59 samples and the line of identity is shown.

against diflunisal concentrations obtained from five different sets of plasma standards taken through the procedure.

Unchanged diflunisal cannot be measured in the urine by this method because of interference from the glucuronide conjugates. However, total unconjugated and conjugated diflunisal can be measured after hydrolysis with perchloric acid [3].

The plots of percentage transmission (minus the blank value) versus plasma concentrations of diflunisal passed through the origin and were linear up to 30 μg/ml. The limit of sensitivity was about 1.0 μg/ml.

Plasma concentrations in three healthy volunteers after oral administration of 750 mg diflunisal and three patients with diflunisal overdosage were measured by both methods. There was excellent agreement with concentrations above 10 μg/ml (Fig. 4).

CONCLUSION

Both the HPLC and fluorometric methods are suitable for the estimation of diflunisal in plasma. However, the HPLC method has the advantages of simplicity, specificity and may also be used to measure diflunisal in urine.

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Note

Determination of chloroquine and its desethyl metabolite in plasma, red blood cells and urine by liquid chromatography

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Chloroquine (Fig. 1) is used for prophylaxis and treatment of malaria and also in rheumatoid arthritis and systemic lupus. Determination of chloroquine in biological material is generally accomplished with the aid of spectrophotofluorometry. Several methods using this technique have been published [1—3]. These methods lack sensitivity and also specificity due to interference of metabolites such as desethylchloroquine (Fig. 1). Moreover, the extraction procedures used in these methods do not completely separate chloroquine from its main metabolite.

Recently a liquid chromatographic method using fluorimetric detection was published [4]. This utilizes a reversed-phase column and an aqueous eluent

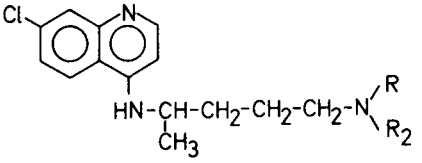

Fig. 1. R = R_2 = ethyl: chloroquine. R = H, R_2 = ethyl: desethylchloroquine. R = R_2 = methyl: internal standard.

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which was mixed with base after separation on the column, thereby generating fluorescence of the eluting chloroquine and its metabolite.

The purpose of the present method was to enable the determination of chloroquine and its desethyl metabolite, retaining the specificity and sensitivity of the latter method but using a simpler system. This was achieved by using a silica column that was eluted with a non-aqueous solvent mixture containing diethylamine, and sufficient fluorescence of chloroquine and its metabolite was generated to obtain a high sensitivity.

Several substances with a structure similar to chloroquine were synthezised and tested for use as internal standard. 7-Chloro-4-(1-dimethylamino-4-pentylamino)quinoline had the best properties of these substances.

The method was used to assay samples from healthy volunteers who had been given chloroquine equal to 300 mg of the base orally and by infusion.

MATERIALS AND METHODS

Instrumental

The liquid chromatographic system consisted of an M45 pump, a U6K injector (Waters Assoc., Milford, MA, U.S.A.) and a 970 fluorescence detector (Spectra-Physics, Santa Clara, CA, U.S.A.)

The excitation wavelength was 335 nm and a 370-nm filter was used. The column (0.15 m × 4.6 mm I.D.) was slurry-packed with LiChrosorb Si 60, 5 μm (Merck, Darmstadt, G.F.R.) and eluted with acetonitrile—methanol—diethylamine (80:19.5:0.5). The flow-rate of the eluent was 1 ml/min. A Model 204 spectrofluorometer (Perkin-Elmer, Norwalk, CT, U.S.A.) was used to obtain the fluorescence spectra.

Standards and solvents

Several 4-substituted 7-chloroquinolines were synthezised by reacting 1-dimethyl-amino-3-aminopropane, 1-diethyl-amino-3-aminopropane, 1-dipropyl-amino-3-aminopropane [5], 1-dibutyl-amino-3-aminopropane and 1-dimethyl-amino-4-aminopentane with 4,7-dichloroquinoline [6]. The products were purified on preparative silica plates developed with acetonitrile—methanol diethylamine (60:39.5:0.5).

1-Dimethyl-4-aminopentanone was prepared in a way similar to the diethyl analogue [7] by heating 1-chloro-4-pentanone (2 g) with dimethylamine (1.5 g) in a screw-capped tube for 4 h at 70°C. After distillation under vacuum, the oxime was prepared and reduced with sodium in ethanol [8]. After addition of water and extraction with diethyl ether, the organic phase was dried (sodium sulphate) and the solvent evaporated, after which the product could be used directly.

The diethyl ether used for extraction was of analytical grade and was distilled to remove stabilizers which interfered with the chromatogram. Methanol and acetonitrile were of high-performance liquid chromatography (HPLC) grade; the water content of the acetonitrile was less than 0.01%.

Plasma assay

In a 10-ml screw-capped tube, 1 ml of plasma, 1 ml of 1 *M* sodium

hydroxide, 100 μl of internal standard solution and 6 ml of diethyl ether were shaken for 15 min on a shake-board. The concentration of the internal standard solution was adjusted so that the detector signal corresponded to 0.5, 1 and 6 μg/ml chloroquine for the concentration ranges 2—25, 25—200 and 200—1000 ng/ml, respectively. The extracted sample was centrifuged for 5 min at 500 *g* (Wifug X1) and the ethereal phase was transferred to a new tube and dried (0.2—0.3 g sodium sulphate). The ether was removed after centrifugation and evaporated in a new tube by a stream of nitrogen (30° C). The residue was dissolved in 200 μl of the eluent and, after passage through a pasteur pipette closed with fine glass-wool, 100 μl were injected in the chromatograph. When plasma levels of chloroquine were below 25 ng, 2 ml of plasma were used. To the sample were then added 2 ml of 1 *M* sodium hydroxide, whereupon the whole mixture was extracted with 7 ml of diethyl ether in a 15-ml screw-capped tube. Apart from that, the procedure was the same as described for a 1-ml sample.

Red blood cell assay

Frozen and thawed red blood cells (2 ml) were diluted three times with distilled water, and 1 ml of the mixture was treated as described above.

Urine assay

Urine samples (100 μl) were diluted to 1 ml with water and then extracted as described above for the plasma samples.

Clinical samples

Blood samples were collected in heparinized Venoject^{R} tubes, cooled and centrifuged. The plasma and red blood cells were frozen separately and stored at −20° C until analysis.

RESULTS AND DISCUSSION

Sample handling

Since red blood cells of a chloroquine-treated subject have a 3—4 times higher concentration of the drug than the surrounding plasma [9], it is important that no hemolysis of erythrocytes occurs to increase the original plasma level. This is especially important with malaria plasmodium infected cells since there is an even greater proportion of chloroquine inside the red blood cells [10].

Duplicates of plasma samples that were analysed within a two-months interval did not show any variation in the measured concentrations of chloroquine that could be detected with the method of determination used. Between determinations the plasma was stored in a freezer at −20° C.

Extraction

The absolute recovery of chloroquine (and desethylchoroquine) from plasma, red blood cells and urine was 88%, 80% and 88%, respectively, with the methods described here. Drying of the ether extract with anhydrous sodium sulphate prior to evaporation was performed in order to decrease the humidity,

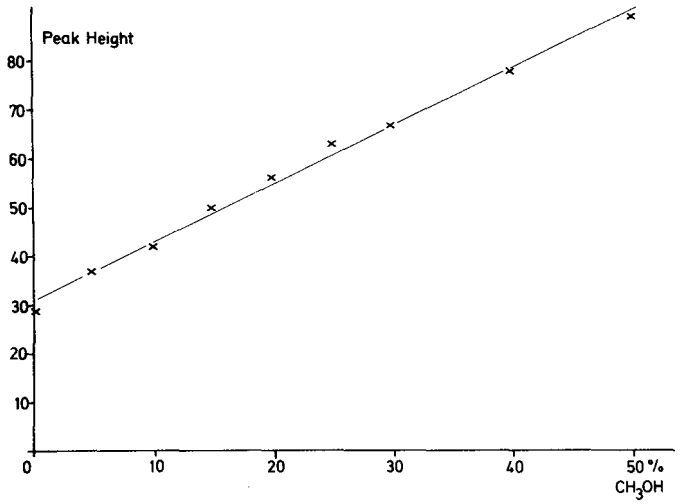
which would be detrimental to the column in the presence of diethylamine. After injection of about 100 undried samples, the back-pressure of the column had increased to an unacceptable level. No such increase could be registered after injection of several hundred dried samples.

Evaporation of the ether phase had to be performed by venting the tubes with a stream of nitrogen gas, since it was found that the use of vacuum evaporation, even at moderate temperatures (20—25° C), caused a substantial decrease in the recovery of chloroquine.

Liquid chromatography and internal standard

The water content of the eluent was found to be critical for the resolution of internal standard and desethylchloroquine. It could also cause the back-pressure on the column to increase. However, most batches of commercially available acetonitrile and methanol (HPLC grade) could be used without extra drying. If the performance of the column deteriorated it was conditioned with hexane—acetic acid—2,2-dimethoxypropane (180:20:5) [11]. The eluent was composed to give good separation of the compounds of interest and low absorption at the excitation wavelength used. However, a higher content of methanol increased the fluorescence of chloroquine (Fig. 2). With the eluent composition described here, chloroquine had 43% of the fluorescence resulting from measurement in pH 9 borate buffer [4]. The desethyl metabolite of chloroquine, however, produced the same fluorescence as the mother compound in borate buffer at equimolar concentrations in both solvent systems. Diethylamine in the eluent increased the fluorescence of chloroquine twice compared to the same amount of a 35% ammonia solution.

Among the substances synthesized for use as internal standard, 7-chloro-4-(1-dimethylamino-4-pentylamino)quinoline was selected because of its great similarity to chloroquine and suitable retention in the chromatographic system.



Fir. 2. Fluorescence response of chloroquine in mixtures of acetonitrile and methanol with 0.75% diethylamine added. The excitation and emission wavelengths were 355 nm and 390 nm, respectively.

7-Chloro-4-(1-diethylamino-3-propylamino)quinoline was an alternative, but was considered to elute too close in front of the chloroquine peak. The three other substances tested for use as internal standard eluted either too close to the solvent front or fused with the chloroquine peak.

Capability of the method

The limit of detection of the method described was 1 ng/ml of plasma for chloroquine and 0.5 ng/ml for desethylchloroquine. The precision of the method was 3.5% (n = 10) at 50 ng/ml plasma (or urine) for chloroquine and 5% (n = 10) at the 25 ng/ml for desethylchloroquine. The corresponding values when analysing red blood cells were 4% and 17% for chloroquine and desethylchloroquine, respectively.

Typical chromatograms resulting from the analysis of plasma samples are shown in Fig. 3. No interfering peaks could be detected in the chromatogram. Chromatograms obtained on analysis of red blood cells and urine samples had a similar appearance.

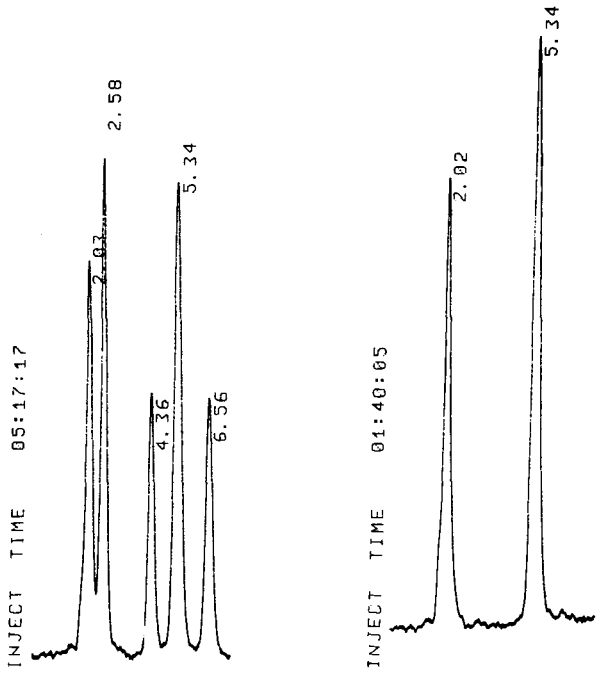


Fig. 3. Chromatograms of plasma samples. Left: sample containing 45 ng/ml chloroquine (t = 4.36), internal standard (t = 5.34) and 20 ng/ml desethylchloroquine (t = 6.56). Right: blank sample with internal standard.

The calibration curves were constructed by analysing samples spiked with chloroquine and metabolite and plotting the resulting peak height ratios (chloroquine or metabolite/internal standard) against concentration. The curves were linear and had the same slope regardless of whether plasma or urine was used. The corresponding curve for red blood cell concentrations of chloroquine had a slightly different slope.

Clinical application

Fig. 4 shows plasma concentration curves of chloroquine obtained by analysing samples from two healthy subjects according to the described method. The dose was equal to 300 mg of the base given orally and by infusion during 20 min.

An accurate chloroquine assay is much needed to elucidate the complex pharmacokinetics of this drug. There is also a great need for such an assay in field conditions where supposed resistance of the plasmodia to chloroquine has to be assessed by measuring the drug in samples from patients. Lack of compliance with the dosage prescription is a common cause for failure of malaria prophylaxis. Monitoring of drug concentrations in the treatment of malaria and rheumatic diseases is also a possibility after appropriate studies of concentration—effect relationships have been made.

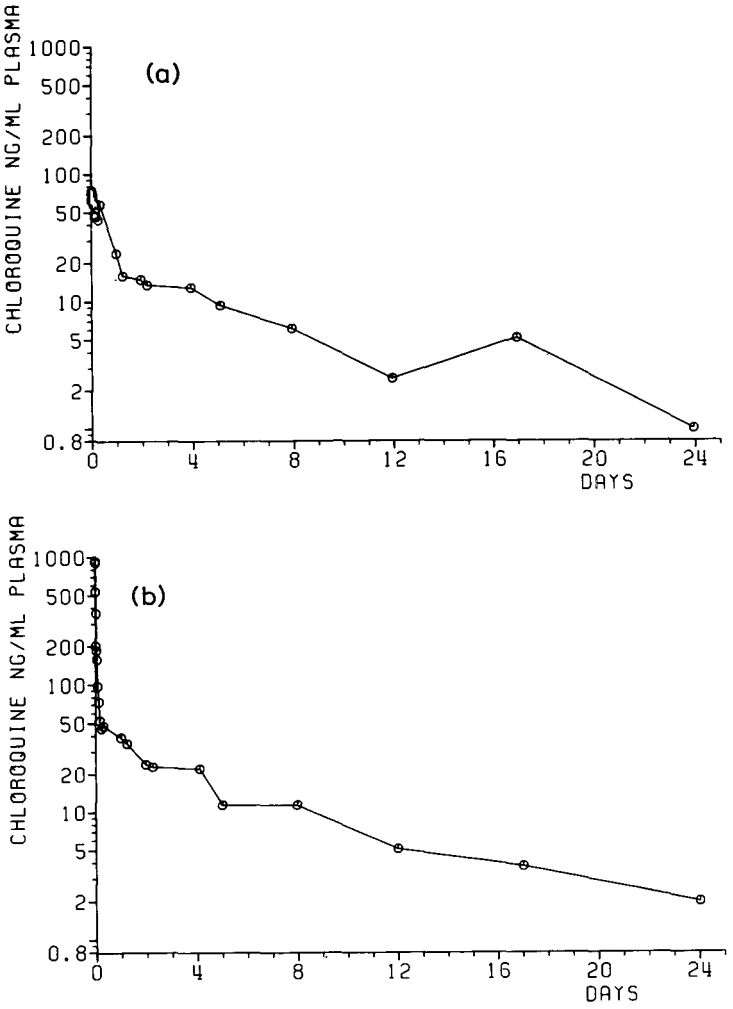


Fig. 4. Plasma concentration curves obtained from two subjects who had been given chloroquine orally (a) or intravenously (b) in doses equal to 300 mg of the base.

ACKNOWLEDGEMENT

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Note

Determination of chloroquine and its de-ethylated metabolites in human plasma by ion-pair high-performance liquid chromatography

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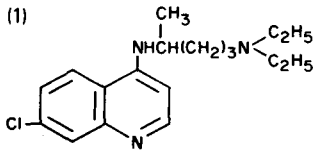
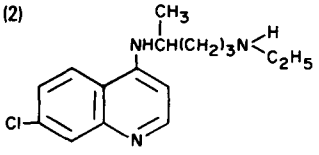
During the past 35 years, a prodigious amount of research has been conducted on the compound chloroquine [1—6] in an attempt to better understand the pharmacokinetic actions of this drug as a therapeutic modality in treating malaria in human subjects. Although much of this work has been performed on the modes of action of chloroquine in various biological systems, many questions remain unanswered.

The introduction of high-performance liquid chromatography (HPLC) has played a great part in enhancing specificity. Bergqvist and Frisk-Holmberg [7] and Staiger et al. [8] demonstrated the practicability of HPLC in assaying chloroquine and some of its metabolites in physiological fluids.

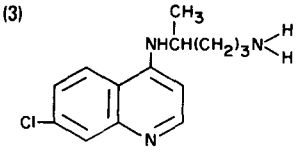
In this report, we describe a simple, specific, and sensitive method, using an ion-pair reversed-phase HPLC procedure for separating and quantifying chloroquine, desethylchloroquine, and bidesethylchloroquine in plasma samples (Fig. 1). A pretreated organic extract of plasma is required before performing the assay. Amounts as low as 5 ng on column of each compound can be quantified, and analysis time is only 12 min per sample. The use of an isocratic system minimizes analysis time by not having to equilibrate the column after each assay.



CHLOROQUINE



DESETHYLCHLOROQUINE



BIDESETHYLCHLOROQUINE

Fig. 1. Free base structural formulae of (1) chloroquine, (2) desethylchloroquine, and (3) bidesethylchloroquine.

The employment of this new method is applicable in both the research and clinical laboratories.

EXPERIMENTAL*

Apparatus

The method was developed using a Waters Model ALC/GPC-204 liquid chromatograph. The total system consisted of two Model 6000A high-pressure pumps, a 660 solvent programmer, a U6K loop injector, a Model 440 absorbance detector, set at 340 nm, a Houston Instrument Omni-Scribe A5000 dual-pen recorder, and a Columbia Scientific Industries Supergrator-3 integrator.

Reagents

Spectroquality acetonitrile (Burdick and Jackson Labs., Muskegon, MI, U.S.A.) mixed with PIC B-7 reagent (1-heptanesulfonic acid, Waters Assoc., Milford, MA, U.S.A.) was used as the mobile phase. Chloroquine diphosphate (Sigma, St. Louis, MO, U.S.A.), desethylchloroquine sesquioxalate and bidesethylchloroquine hydrobromide (Walter Reed Army Institute of Research's Inventory, Washington, DC, U.S.A.) were used to prepare all working standards. Stock solutions containing 100 ng/μl of each compound were prepared

*The manufacturers' names and products are given as scientific information only and do not constitute an endorsement by the United States Government.

in glass-distilled water. Working standards were prepared from the stock standards.

Procedure

A 300 mm × 3.9 mm I.D., 10 μm μBondapak C_{18} column (Waters Assoc.) was used to chromatograph chloroquine and its metabolites in standard solutions and experimental plasma specimens. The mobile phase consisted of 0.02 *M* 1-heptanesulfonic acid and acetonitrile. PIC B-7 reagent was prepared by dissolving 40 ml of the pre-packaged reagent into 460 ml of glass-distilled water. The pH of the solution was 3.4. A pumping ratio of 66:34 of PIC B-7 to acetonitrile was used in an isocratic mode to separate each compound. Flow-rate was 1.0 ml/min. Column pressures ranged between 62 and 76 bar. A 10-μl volume of a methanolic phosphoric acid extract of plasma was introduced onto the column through a continuous flow loop injector. Peak areas and heights were measured and computed with an on-line integrator.

Samples

Plasma specimens were collected from two normal subjects. Each volunteer received a single 300-mg dose of chloroquine diphosphate. Plasma samples were taken before dosing (control) and 12 and 24 h after dosage. A 1-ml sample of plasma and an equal volume of 1 *N* sodium hydroxide were pipetted into a 50-ml screw-capped polypropylene tube. *n*-Heptane (30 ml) was added to the mixture and the tubes were shaken for 30 min. After the extraction, 25 ml of the organic phase, containing chloroquine and its metabolites were placed into a similar type tube and the solution was blown to dryness with streams of nitrogen gas in a 30°C water bath. The residue remaining within the tubes was redissolved into 1 ml of methanol—0.1 *M* phosphoric acid (1:1). The dissolved samples were then transferred to a 3-ml polypropylene screw-capped tube for further concentration. The dried specimens were finally reconstituted with the methanol—phosphoric acid solution to a volume of 100 μl. The samples were analyzed using the previously described HPLC method. An 85% recovery of chloroquine and its de-ethylated metabolites was obtained from spiked specimens. The extraction method used for our sample preparation is a modification of the procedure described by Vogel and Konigk [9].

RESULTS AND DISCUSSION

In recent years, HPLC has proven to be an effective technique for studying the pharmacokinetic actions of various drugs in both man and animal [10,11]. The results of these efforts are demonstrated in the ability of newly developed procedures to enhance knowledge of drug metabolism in various biological systems, based on the utilization of highly specific and sensitive methods. For certain drugs, where the biotransformations of the parent compounds are subtle, previous analytical methodologies were incapable of distinguishing the small differences among chemical structures.

Chloroquine diphosphate is an example of a drug in which its metabolism in man is not completely understood, due to the non-specificity of past method-

ologies. Because of this deficiency, we designed an experiment using a new analytical method in which the metabolic fate of chloroquine could be studied in human subjects. Chloroquine, desethylchloroquine and bidesethylchloroquine were separated without interferences from endogenous compounds present in plasma. The metabolite hydroxychloroquine, which is present in urine, can also be separated using this method.

The application of the method is demonstrated in a series of chromatograms taken from the separation of standard solutions and experimental samples. Fig. 2 represents the separation of a standard containing bidesethylchloroquine, desethylchloroquine, and chloroquine, detected at 0.005 a.u.f.s. Linearity was observed for all concentrations used in this study (5—200 ng). Correlation coefficients for bidesethylchloroquine, desethylchloroquine, and chloroquine were 0.992, 0.989, and 0.978, respectively.

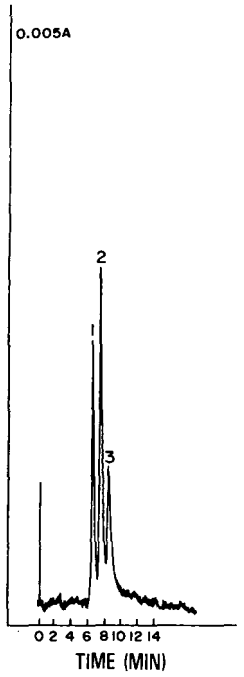


Fig. 2. Separation of a standard solution containing (1) 76 ng of bidesethylchloroquine, (2) 77 ng of desethylchloroquine, and (3) 60 ng of chloroquine. Column: 300 × 3.9 mm I.D. μBondapak C_{18}; mobile phase: 66% PIC B-7 reagent (0.02 *M*) and 34% acetonitrile; flow-rate: 1.0 ml/min; column temperature: ambient.

We analyzed and quantified a series of spiked plasma specimens containing chloroquine and its de-ethylated metabolites. Sample concentration of ten prepared specimens was 84 μg/ml for each compound. Data on within-run precision for chloroquine, desethylchloroquine, and bidesethylchloroquine were 83.2 ± 1.1 (C.V., 1.36%), 82.9 ± 1.3 (C.V., 1.55%), and 82.6 ± 1.3 (C.V., 1.62%) μg/ml, respectively. Day-to-day precision data for the ten spiked plasma specimens as evaluated during a 5-day period were 83.1 ± 1.4 (C.V., 1.42%), 82.7 ± 1.3 (C.V., 1.59%), and 82.2 ± 1.4 (C.V., 1.69%) μg/ml for

chloroquine and its mono- and di-de-ethylated metabolites, respectively. Based on the excellent resolution of the compounds present in the spiked plasma samples, experimental plasma samples collected from the two human subjects were chromatographed. Chromatograms shown in Fig. 3 and 4 depict two time frames of subject A and B. For subject A, three characteristic peaks were observed in the 12-h plasma sample (Fig. 3). Calculating their percentages, chloroquine was present in the sample at 63.1%, followed by desethylchloroquine at 28.3% and bidesethylchloroquine at 8.5%.

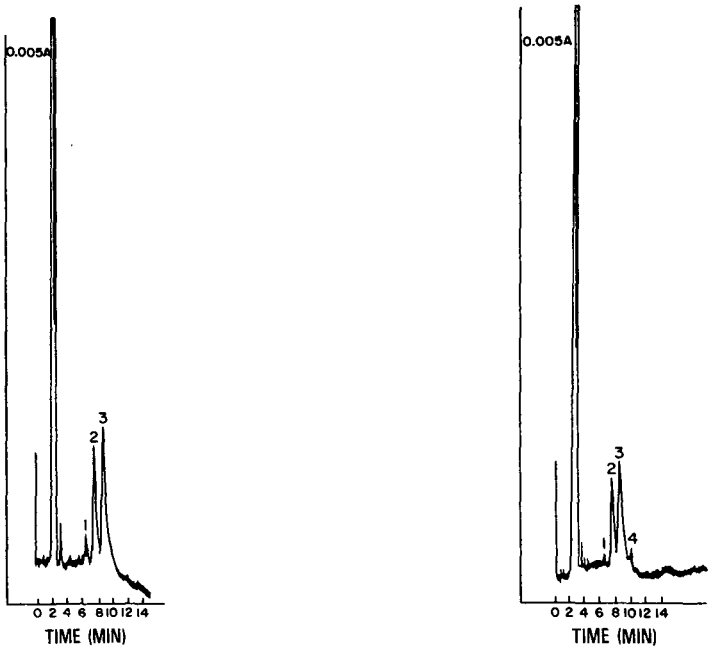


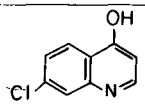
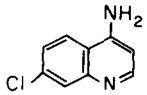
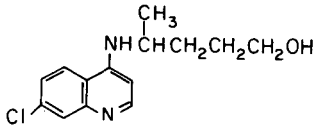
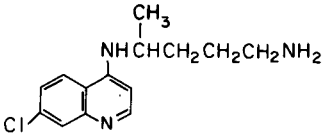
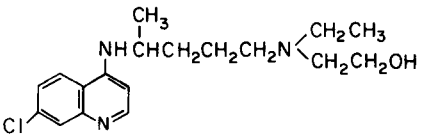
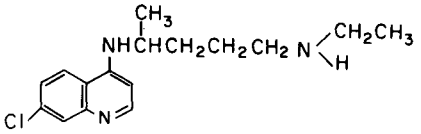
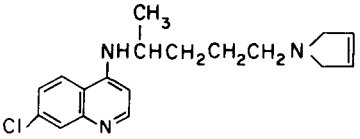
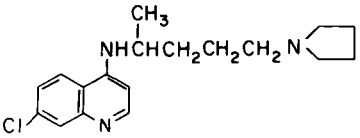
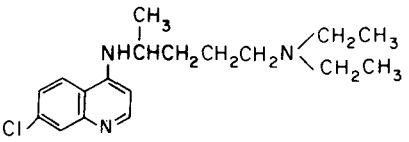
Fig. 3. Chromatogram of a 12-h plasma extract from human subject A. Peaks: (1) bidesethylchloroquine, (2) desethyl chloroquine, and (3) chloroquine. Sample volume: 10 μl; detection wavelength: 340 nm.

Fig. 4. Chromatogram of a 24-h plasma extract from human subject B. Peaks: (1) bidesethylchloroquine, (2) desethyl chloroquine, (3) chloroquine, and (4) unknown. Sample volume: 10 μl.

The chromatogram shown in Fig. 4, representing subject B, displayed a somewhat different profile. The total peak area for the same assayed volume of subject B sample was 1/3 less than that of subject A. Whereas three peaks were observed in the 12-h specimen, the 24-h specimen showed four peaks. Chloroquine again was the major compound present in the sample. The percentages for the assayed sample were 52.3% for chloroquine, 29.8% for desethylchloroquine, 2.9% for bidesethylchloroquine and 14.9% for an unknown compound. The retention time of this unknown metabolite did not match any of the retention times obtained for the 7-chloro-4-substituted derivatives shown in Table I. Additional information, using alternative analytical techniques is presently being obtained to characterize and identify the unknown metabolite.

TABLE I

7-CHLORO-4-SUBSTITUTED DERIVATIVES

Retention time (min)	Structure	Compound
4.93		7-Chloro-4-hydroxyquinoline
5.59		4-Amino-7-chloroquinoline
6.93		4-(4′-Hydroxy-1′-methylbutylamino)-7-chloroquinoline
7.17		Bidesethylchloroquine
7.94		Hydroxychloroquine
8.39		Desethylchloroquine
9.06		7-Chloro-4[4′-(3-pyrrolidino)-1-methylbutylamino]quinoline
9.47		4-(4′-Pyrrolidyl-1-methylbutylamino)-7-chloroquinoline
9.62		Chloroquine

From this study, we demonstrated the applicability of a new analytical methodology for observing the metabolic fate of chloroquine in humans. Because of the advantages of this technique, further comprehensive studies are planned using various mammalian species in order to determine similarities and differences in the metabolism of chloroquine in mammals.

ACKNOWLEDGEMENT

We thank Mr. Stewart Galloway for his excellent secretarial assistance in the preparation of this manuscript.

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CHROMBIO. 1172

Note

Improved high-performance liquid chromatographic assay for cimetidine using ranitidine as an internal standard

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(First received September 1st, 1981; revised manuscript received November 18th, 1981)

The analysis of cimetidine (CMT), an H_2-receptor antagonist, has been reported in the literature by several researchers [1—5]. All methods were minor modifications of the method of Randolph et al. [1]. The methods used a silica column, and mobile phases containing acetonitrile, methanol, water, and ammonium hydroxide. Three of the methods [1,3,4] utilized octanol as the extraction solvent, while Larsen et al. [2] used ethyl acetate, a solvent which is readily hydrolysed at the extremes of pH used during the extraction. Another method [5] used a complex precipitation procedure. All the extraction methods [1—5] are time consuming and required the salting out of cimetidine into ethanol or methylene chloride as the final step.

Randolph et al. [1] indicated that an extensive conditioning for 8 h is necessary for new silica columns. They also indicated that inlet and outlet frits must be replaced to reduce back pressure. Similar problems in these laboratories have manifested as leaks at the pump seals, probably due to the ammonia present in the mobile phase.

The existing methods are difficult to reproduce, technically fragile and require frequent instrumental maintenance.

The proposed new high-performance liquid chromatographic (HPLC) method utilizes a different column, an entirely different mobile phase, and a single-step extraction, which is both rapid and efficient.

EXPERIMENTAL

Instrumentation

Analysis was performed using a constant-flow high-pressure liquid chromatograph (Model 5000, Varian Instruments, Palo Alto, CA, U.S.A.) with a

variable-wavelength UV detector set at 228 nm (Vari-Chrom, Varian). The alkylnitrile column, Micropak CN-10 (10 μm, 3000 mm × 4 mm I.D.) was supplied by Varian. A manual injection valve with 10-μl loop was used to introduce the samples.

Reagents

The extraction solvents, methylene chloride and isopropyl alcohol were distilled in glass and of chromatographic grade (Burdick and Jackson, Labs., Muskegon, MI, U.S.A.). Acetonitrile, HPLC grade (Fisher Scientific, Fairlawn, NJ, U.S.A.), reagent grade sodium monobasic phosphate and sodium hydroxide were used without further purification. The mobile phase consisted of 50% acetonitrile and 50% 0.01 *M* NaH_2PO_4.

Standard solutions

Stock solutions of CMT (Smith Kline and French Labs., Philadelphia, PA, U.S.A.) and ranitidine (Glaxo Labs., Fort Lauderdale, FL, U.S.A.) each containing 10 mg/ml were prepared in distilled water. Aliquots of CMT stock solution were added to 1.0 ml of plasma so that concentrations of 0.2—2.0 μg/ml of plasma resulted. A 50-μl aliquot of the stock solution of ranitidine, the internal standard, was added to each 1 ml of plasma in the above solutions.

Extraction

The spiked plasma samples were vortexed for a few seconds to ensure adequate mixing. The samples were then made alkaline with 75 μl of 2.5 *N* sodium hydroxide, vortexed for a moment and allowed to stand for 1 min. The extracting solvent, consisting of 5 ml of methylene chloride—isopropanol (90:10), was then added to each test tube. The tubes were shaken 10 min on a vortex mixer then centrifuged at 2000 *g* for 10 min. The aqueous upper layer was aspirated and discarded. The organic layer was transferred to a clean test tube and evaporated to dryness with a stream of dry nitrogen in a water bath at 25°C. The samples were reconstituted with 150 μl of the methylene chloride—isopropanol mixture, vortexed for a few seconds and retained for HPLC analysis. Plasma samples containing unknown amounts of CMT were handled similarly, after spiking with 50 μl of the stock solution of ranitidine.

Chromatography

The column was conditioned by flushing firstly with acetonitrile for 15 min at 1 ml/min, then with the mobile phase for 15 min at 1 ml/min. The flow-rate was then increased to 1.6 ml/min and maintained at this rate for a few minutes until a steady baseline was obtained and the analysis performed. Samples were injected and monitored at 228 nm. The peak height ratios of CMT to ranitidine were calculated for the standards and plotted against concentration of CMT. The resulting standard curve was then used to convert peak height ratio of knowns to CMT concentration.

RESULTS AND DISCUSSION

A typical chromatogram of CMT obtained from spiked human plasma at

0.2 μg/ml is shown in Fig. 1. There is a good separation between CMT and the internal standard, ranitidine. Potential interfering peaks from plasma components are eluted in the first 3 min of the chromatogram, with the detector reaching the baseline before the peaks of interest begin to elute (see Fig. 2). Cimetidine and ranitidine have retention times of 4.0 and 5.0 min, respectively, in the system, with no interfering peaks in this region of the chromatogram. The sample is completely eluted in 6 min. The response at 0.2 μg/ml is strong and hence the limit of sensitivity for the assay is at least 0.1 μg/ml, and with care as low as 0.05 μg/ml.

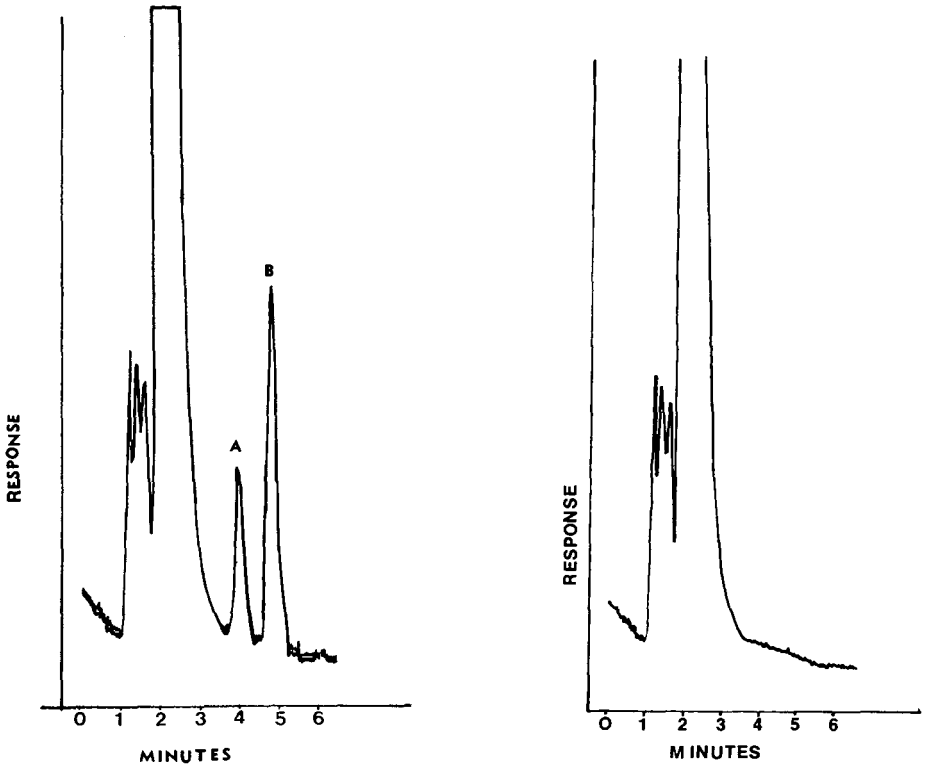

Fig. 1. Typical chromatogram for 0.2 μg/ml cimetidine in human plasma. Peaks: A = cimitidine; B = ranitidine (0.5 μg/ml).

Fig. 2. Chromatogram of blank human plasma sample.

Table I demonstrates the accuracy at each concentration with coefficients of variation of less than 6% for each of five points on the standard curve. Each point is an average of four replicates. The extraction efficiency ranges from 60 to 71% and is better than that obtained by Chiarmonte and Schentag [4]. The correlation coefficient obtained from the linear regression was 0.9953.

Table II demonstrates the inter-assay precision. Duplicate samples were prepared and run side by side, using two technicians. The coefficient of variation between run 1 and run 2 was determined to be less than 6% demonstrating that the precision of the analysis did not suffer. The correlation coefficients for runs 1 and 2 were r = 0.9985 and 0.9968, respectively.

TABLE I

ACCURACY AND EXTRACTION EFFICIENCY FOR CIMETIDINE IN HUMAN PLASMA

Concentration (μg/ml)	Peak height ratios (± S.D., $n = 4$)	C.V. (%)	Extraction efficiency (%)
0.2	0.440 ± 0.011	2.61	71.1
0.4	0.741 ± 0.028	3.79	70.6
0.8	0.160 ± 0.091	5.71	67.9
1.6	3.310 ± 0.160	4.89	70.0
2.0	3.820 ± 0.093	2.36	60.7

TABLE II

INTER-ASSAY PRECISION

Concentration (μg/ml)	Peak heights ratio		Average ± S.D.	C.V. (%)
	Run 1	Run 2		
0.2	0.474	0.492	0.482 ± 0.028	5.7
	0.442	0.518		
0.4	0.885	—		
	0.869	—		
0.8	1.651	1.852	1.803 ± 0.093	5.2
	1.814	1.897		
1.6	3.750	3.882	3.817 ± 0.067	1.8
	3.750	3.886		
2.0	4.684	4.563	4.59 ± 0.062	1.3
	4.579	4.515		

The proposed analytical procedure offers a robust method to determine plasma levels of CMT in a routine manner. The method is insensitive to small variations of pH, in the ionic strength of the NaH_2PO_4 solution, and hence in minor variations in the composition of the mobile phase. The column needs no unusual conditioning and the extraction is fast and simple. The samples must be run within a few hours after reconstitution, but may be stored dry in a freezer until needed without loss of activity. Plasma samples of cimetidine have previously been reported to be stable when stored in the frozen state [6]. Ranitidine dissolved in 10 mol/l KH_2PO_4 buffer at pH 3 is reported to be stable for several months stored at 4°C [7].

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BIOMEDICAL APPLICATIONS

NEWS SECTION

CALENDAR OF FORTHCOMING EVENTS

May 3–6, 1982
Brussels, Belgium

XXXth Annual Colloquium "Protides of the Biological Fluids"
Contact: Colloquium "Protides of the Biological Fluids", Secretariate, Institute for Medical Biology, Alsembergsesteenweg 196 Chaussée d'Alsemberg, 1180 Brussels, Belgium. Tel.: (02)344 19 50; (Int.) 32.2.344 19 50. Telex: CDHBRU 26501. (Further details published in Vol. 227, No. 1.)

May 11–14, 1982
Ghent, Belgium

4th International Symposium on Quantitative Mass Spectrometry in Life Sciences
Contact: Prof. A. De Leenheer, Symposium Chairman, Laboratoria voor Medische Biochemie en voor Klinische Analyse, De Pintelaan 135, B-9000 Ghent, Belgium. (Further details published in Vol. 226, No. 2.)

May 16–18, 1982
Indiannapolis, IN,
U.S.A.

1982 LCEC Symposium: Biomedical Applications of LCEC and Voltammetry
Contact: LCEC Symposium, P.O. Box 2206, West Lafayette, IN 47906, U.S.A. Tel.: (317) 463-2505; Telex: 276 141. (Further details published in Vol. 226, No. 1.)

June 1–4, 1982
Goslar (near Hannover),
G.F.R.

3rd International Symposium on Isotachophoresis
Contact: Dr. C.J. Holloway, ITP 82, Abteilung für klinische Biochemie, Medizinische Hochschule Hannover, Karl-Wiechert-Allee 9, D-3000 Hannover 61, G.F.R. (Further details published in Vol. 219, No. 3.)

June 6–11, 1982
Kansas City, MO,
U.S.A.

International Symposium on the Synthesis and Application of Isotopically Labeled Compounds
Contact: Dr. Alexander Susán, Scientific Secretary of the Symposium, c/o Midwest Research Institute, 425 Volker Boulevard, Kansas City, MO 64110, U.S.A. Tel: (816) 753-7600, extension 268. (Further details published in Vol. 225, No. 1.)

June 7–11, 1982
Philadelphia, PA, U.S.A.

VI International Symposium on Column Liquid Chromatography
Contact: R.A. Barford, ERRC – SEA, U.S. Department of Agriculture, 600 E. Mermaid Lane, Philadelphia, PA 19118, U.S.A. (Further details published in Vol. 211, No. 3).

June 18–21, 1982 Lund, Sweden	**Flow Analysis II** Contact: Flow Analysis II, c/o The Swedish Chemical Society, Upplandsgatan 6A, 1 tr., S-111 23 Stockholm, Sweden. (Further details published in Vol. 216.)
June 20–23, 1982 Bordighera (near San Remo), Italy	**International Symposium on Chromatography and Mass Spectrometry in Biomedical Sciences** Contact: Dr. Alberto Frigerio, c/o Istituto di Ricerche Farmacologiche "Mario Negri", Via Eritrea, 62 – 20517 Milan, Italy. Tel.: 35.54.546; Telex: 331268 NEGRI I. (Further details published in Vol. 225, No. 1.)
June 20–24, 1982 Toronto, Canada	**North American Medicinal Chemistry Symposium** Contact: Symposium Secretariat, North American Medicinal Chemistry Symposium, c/o Ayerst Laboratories, P.O. Box 6115, Montreal, Quebec H3C 3J1, Canada.
July 11–16, 1982 Washington, DC, U.S.A.	**6th International Conference on Computers in Chemical Research and Education (ICCCRE)** Contact: Dr. Stephen R. Heller, Chairman, 6th ICCCRE, EPA, MIDSD, PM–218, 401 M Street, S.W., Washington, DC 20460, U.S.A. Tel: (202) 755–4938, Telex: 89–27–58.
July 12–15, 1982 Guildford, Great Britain	**Membrane-located Receptors: 8th International Subcellular Methodology Forum** Contact: Dr. E. Reid, Wolfson Bioanalytical Unit, Robens Institute, University of Surrey, Guildford GU2 5XH, Great Britain.
July 14–17, 1982 Guildford, Great Britain	**Workshop on Methodology for Neuroreceptors** Contact: Dr. E. Reid, Wolfson Bioanalytical Unit, Robens Institute, University of Surrey, Guildford GU2 5XH, Great Britain.
Aug. 8–13, 1982 Anaheim, CA, U.S.A.	**34th Annual Meeting of the American Association for Clinical Chemistry** Contact: Michele Tuttle, Meetings Director, AACC, 1725 K Street, NW, Washington, DC 20006, U.S.A.
Aug. 15–21, 1982 Perth, Australia	**12th International Congress of Biochemistry** Contact: Brian Thorpe, Department of Biochemistry, Faculty of Science Australian National University, Canberra A.C.T. 2600, Australia.
Aug. 22–28, 1982 Vancouver, Canada	**XIth International Carbohydrate Symposium** Contact: Mr. K. Charbonneau, Executive Secretary, XIth International Carbohydrate Symposium, c/o National Research Council of Canada, Ottawa, Ontaria, Canada K1A 0R6. Tel.: (613) 993-9009; Telex: 053-3145.
Aug. 30–Sept. 3, 1982 Vienna, Austria	**9th International Mass Spectrometry Conference** Contact: Interconvention, P.O. Box 105, A-1014 Vienna, Austria. (Further details published in Vol. 206, No. 1)
Aug. 31–Sept. 2, 1982 Vienna, Austria	**5th International IUPAC Symposium on Mycotoxins and Phycotoxins** Contact: Prof. P. Krogh, Department of Veterinary Microbiology, Purdue University, West Lafayette, IN 47907, U.S.A.

Sept. 5–9, 1982 Liège, Belgium	**8th European Workshop on Drug Metabolism** Contact: Professor Jacques E. Gielen, Laboratoire de Chimie Médicale, Institut de Pathologie, Université de Liège, Bâtiment B 23, B-4000 Sart Tilman par Liège 1, Belgium. Tel.: (32-41)-56.24.80/81. (Further details published in Vol. 225, No. 2.)
Sept. 6–9, 1982 Bath, Great Britain	**4th European Symposium on Chemical Structure – Biological Activity: Quantitative Approaches** Contact: Dr. J.C. Dearden, School of Pharmacy, Liverpool Polytechnic, Byrom Street, Liverpool L3 3AF, Great Britain.(Further details published in Vol. 228.)
Sept. 6–9, 1982 Hradec Králové, Czechoslovakia	**8th International Symposium on Biomedical Applications of Chromatography** Contact: Dr. K. Macek, Institute of Physiology, Czechoslovakian Academy of Sciences, Víděňská 1083, CS-142 20, Prague 4-Krč, Czechoslovakia. (Further details published in Vol. 225, No. 2.)
Sept. 13–17, 1982 London, Great Britain	**14th International Symposium on Chromatography** Contact: The Executive Secretary, Chromatography Discussion Group, Trent Polytechnic, Burton Street, Nottingham, NG1 4BU, Great Britain. (Further details published in Vol. 211, No. 3)
Sept. 19–24, 1982 Singapore, Republic of Singapore	**2nd Asian-Pacific Congress on Clinical Biochemistry** Contact: 2nd Asian-Pacific Congress on Clinical Biochemistry, Singapore Professional Centre, 129B Block 23, Outram Park, Singapore 0316, Republic of Singapore.
Oct. 4–6, 1982 Tarrytown, NY, U.S.A.	**Capillary Chromatography '82 – An International Symposium** Contact: Professor A. Zlatkis, Chemistry Department, University of Houston, Houston, TX 77004, U.S.A. Tel.: (713) 749-2623.
Oct. 12–14, 1982 Salzburg, Austria	**DIOXIN 82, 3rd International Symposium – Workshop on Chlorinated Dioxins and Related Compounds** Contact: Dr. E. Merian, Im Kirsgarten 22, CH-4106 Therwil, Switzerland. (Further details published in Vol. 219, No. 3.)
Oct. 18–22, 1982 Columbus, OH, U.S.A.	**Symposium and Workshop on New Spectroscopic Methods for Biomedical Research** Contact: Karen L. Waite, Battelle's Columbus Laboratories, 505 King Avenue, Columbus, OH 43201, U.S.A. Tel.: (614) 424-4179
Oct. 21–22, 1982 Montreux, Switzerland	**2nd Montreux Workshop on LC–MS and MS–MS** Contact: Professor Dr. R.W. Frei, Department of Chemistry, Free University, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands. (Further details published in *J. Chromatogr.*, 251 (1982) 225.)
Dec. 6–8, 1982 Parsippany, NJ, U.S.A.	**3rd Biennial Symposium on Advances in Thin-Layer Chromatography** Contact: Dr. Touchstone, Hospital of the University of Pennsylvania, Philadelphia, PA, U.S.A. Tel.: (215) 662-2082. (Further details published in Vol. 235, No.1.)
May 2–6, 1983 Baden-Baden, G.F.R.	**VIIth International Symposium on Column Liquid Chromatography** Contact: Gesellschaft Deutscher Chemiker, Abteilung Fachgruppen, Postfach 90 04 40, Varrentrappstrasse 40–42, D-6000 Frankfurt (Main), G.F.R.

May 30–June 3, 1983 Melbourne, Australia	**International Conference on Chromatographic Detectors** Contact: The Secretary, International Conference on Chromatographic Detectors, University of Melbourne, Parkville, Victoria 3052, Australia. (Further details published in Vol. 216.)
June 4–12, 1983 Cologne, G.F.R.	**29th Congress of the International Union of Pure and Applied Chemistry (IUPAC)** Contact: Dr. M. Williams, Executive Secretary, IUPAC, Bank Court Chambers 2–3 Pound Way, Cowley Centre, Oxford OX4 3YF, Great Britain.
June 7–10, 1983 Brussels, Belgium	**1st International Symposium on Drug Analysis** Contact: Ms. C. Van Kerchove, Secretary, Société Belge des Sciences Pharmaceutiques/Belgisch Genootschap voor Pharmaceutische Wetenschappen, rue Archimedesstraat 11, B-1040 Brussels, Belgium. Tel.: (02) 733 98 20, ext. 33.
July 17–23, 1983 Edinburgh, Scotland, Great Britain	**SAC '83, International Conference and Exhibition on Analytical Chemistry** Contact: The Royal Society of Chemistry, Analytical Division, Burlington House, London W1V 0BN, Great Britain. Tel.: 01-734-9971. (Further details published in Vol. 214, No. 2.)
Aug. 28–Sept. 2, 1983 Amsterdam, The Netherlands	**9th International Symposium on Microchemical Techniques** Contact: Symposium Secretariat, c/o Municipal Congress Bureau, Oudezijds Achterburgwal 199, 1012 DK Amsterdam, The Netherlands. Tel: (020) 552 3459.
Aug. 29–Sept. 2, 1983 Bratislava, Czechoslovakia	**4th Danube Symposium on Chromatography and 7th International Symposium "Advances and Application of Chromatography in Industry"** Contact: Dr. Ján Remen, The Analytical Section of the Czechoslovak Scientific and Technical Society, Slovnaft, 823 00 Bratislava, Czechoslovakia. (Further details published in Vol. 235, No. 1.)
Oct. 1–5, 1984 Nürnberg, G.F.R.	**15th International Symposium on Chromatography** Contact: Gesellschaft Deutscher Chemiker, Abteilung Fachgruppen, Postfach 90 04 40, Varrentrappstrasse 40–42, D-6000 Frankfurt (Main), G.F.R.

NEW BOOKS

Immunochemical techniques for the identification and estimation of macromolecules (Laboratory Techniques in Biochemistry and Molecular Biology, edited by T.S. Work and E. Work, Vol. 1, Part III) by J. Clausen, Elsevier/North-Holland Biomedical Press, Amsterdam, New York, Oxford, **2nd (revised) ed.**, 1981, XIV + 387 pp., price Dfl. 61.00, US$ 29.75, (paperback) ISBN 0-444-80244-4.

Topics in pharmaceutical sciences (Proc. 41st Int. Congr. Pharmaceutical Sciences of F.I.P., Vienna, September 7–11, 1981), edited by D.D. Breimer and P. Speiser, Elsevier Biomedical Press, Amsterdam, New York, 1981, XIV + 536 pp., price Dfl. 150.00, US$ 69.75, ISBN 0-444-80403-X.

Progress in the quality control of medicines, edited by P.B. Deasy and R.F. Timoney, Elsevier Biomedical Press, Amsterdam, New York, 1982, X + 298 pp., price Dfl. 185.00, US$ 86.00, ISBN 0-444-80344-0.

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PUBLICATION SCHEDULE FOR 1982

Journal of Chromatography (incorporating *Chromatographic Reviews*) and *Journal of Chromatography, Biomedical Applications*

MONTH	J	F	M	A	M	J	J	A	S	O	N	D
Journal of Chromatography	234/1 234/2 235/1 235/2	236/1 236/2	237/1 237/2 237/3	238/1 238/2 239	240/1 240/2 241/1	The publication schedule for further issues will be published later.						
Chromatographic Reviews		251/1		251/2								
Biomedical Applications	227/1	227/2	228	229/1	229/2							

INFORMATION FOR AUTHORS

(Detailed *Instructions to Authors* were published in Vol. 209, No. 3, pp. 501–504. A free reprint can be obtained by application to the publisher.)

Types of Contributions. The following types of papers are published in the *Journal of Chromatography* and the section on *Biomedical Applications:* Regular research papers (Full-length papers), Short communications and Notes. Short communications are preliminary announcements of important new developments and will, whenever possible, be published with maximum speed. Notes are usually descriptions of short investigations and reflect the same quality of research as Full-length papers, but should preferably not exceed four printed pages. For reviews, see page 2 of cover under Submission of Papers.

Submission. Every paper must be accompanied by a letter from the senior author, stating that he is submitting the paper for publication in the *Journal of Chromatography*. Please do not send a letter signed by the director of the institute or the professor unless he is one of the authors.

Manuscripts. Manuscripts should be typed in double spacing on consecutively numbered pages of uniform size. The manuscript should be preceded by a sheet of manuscript paper carrying the title of the paper and the name and full postal address of the person to whom the proofs are to be sent. Authors of papers in French or German are requested to supply an English translation of the title of the paper. As a rule, papers should be divided into sections, headed by a caption (e.g., Summary, Introduction, Experimental, Results, Discussion, etc.). All illustrations, photographs, tables, etc., should be on separate sheets.

Introduction. Every paper must have a concise introduction mentioning what has been done before on the topic described, and stating clearly what is new in the paper now submitted.

Summary. Full-length papers and Review articles should have a summary of 50–100 words which clearly and briefly indicates what is new, different and significant. In the case of French or German articles an additional summary in English, headed by an English translation of the title, should also be provided. (Short communications and Notes are published without a summary.)

Illustrations. The figures should be submitted in a form suitable for reproduction, drawn in Indian ink on drawing or tracing paper. Each illustration should have a legend, all the *legends* being typed (with double spacing) together on a *separate sheet*. If structures are given in the text, the original drawings should be supplied. Coloured illustrations are reproduced at the author's expense, the cost being determined by the number of pages and by the number of colours needed. The written permission of the author and publisher must be obtained for the use of any figure already published. Its source must be indicated in the legend.

References. References should be numbered in the order in which they are cited in the text, and listed in numerical sequence on a separate sheet at the end of the article. Please check a recent issue for the lay-out of the reference list. Abbreviations for the titles of journals should follow the system used by *Chemical Abstracts*. Articles not yet published should be given as "in press", "submitted for publication", "in preparation" or "personal communication".

Proofs. One set of proofs will be sent to the author to be carefully checked for printer's errors. Corrections must be restricted to instances in which the proof is at variance with the manuscript. "Extra corrections" will be inserted at the author's expense.

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