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# JOURNAL OF CHROMATOGRAPHY BIOMEDICAL APPLICATIONS





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CHROMBIO. 1920

# FLUOROMETRIC HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF 9-AMINOPHENANTHRENE-DERIVATIZED FREE FATTY ACIDS

MITSUO IKEDA*, KENJI SHIMADA and TAKEICHI SAKAGUCHI

*Niigata College of Pharmacy, 5829 Kamishin'ei-cho, Niigata 950-21 (Japan)*

and

USHIHO MATSUMOTO

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

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## SUMMARY

The application of 9-aminophenanthrene (9-AP), a fluorescence-labeling reagent for free fatty acids (FFA), was examined. 9-AP dissolved in benzene was added to a benzene solution of FFA chlorides derived from FFA and oxalyl chloride. The mixture was allowed to react for 45 min at 70°C. By the method, 9-AP-tagged FFA with a strong fluorescence was formed. The materials thus obtained have a $\lambda_{max}$ at around 303 nm for excitation and 376 nm for emission. By using this derivatization method, recoveries were measured for seven kinds of FFA added to 0.5 ml of healthy human serum. Significant recoveries ranging from 96 to 107% (coefficient of variation 1.4—5.0%) were obtained for each FFA. The proposed method was clinically applied to the determination of FFA in 0.5 ml of healthy human serum, and almost satisfactory results were obtained. Detection limits of FFA by this derivatization method were 10 pmol for $C_{14:0}$, $C_{16:0}$, $C_{16:1}$, $C_{18:1}$ and $C_{18:2}$, and 15 pmol for $C_{18:0}$ and $C_{20:4}$. As a quantitative measurement of FFA, gas chromatography and high-performance liquid chromatography with fluorescence detection, which have been routinely used, were chosen for comparison with the present method.

## INTRODUCTION

In biological tissues and fluids, the amount of free fatty acids (FFA) increases or decreases according to the physiological and pathological changes in the living body. Therefore, the quantitative determination of FFA has been widely used in the search for the causes of various kinds of diseases [1—3].

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High-performance liquid chromatography (HPLC) has recently been used for the determination of FFA. Accordingly, the development of derivatization reagents with high sensitivity for FFA detection has been desired. To date several kinds of derivatization reagents have been reported. Most of the reagents are ultraviolet (UV) tagging agents for the analytes, and provide almost identical detection sensitivity in gas–liquid chromatography, with which several nanograms of FFA can be detected. Recently, fluorescence-labeling reagents for FFA have been developed, and 4-bromomethyl-7-methoxycoumarin, the first fluorescence derivatization reagent for FFA, was reported by Dünges [4]. The development of this reagent was followed by that of 9,10-diaminophenanthrene [5], 9-anthryldiazomethane (ADAM) [6, 7] and 1-bromoacetylpyrene [8]. These fluorescence-labeling reagents provided the highly sensitive detection required to permit the measurement of picomole levels of FFA.

Most of the derivatization reagents for FFA which have already been reported are newly synthesized ones [9–11] with high reactivity for FFA, and much time has been spent in their synthesis.

In previous reports [12, 13] we described the induction of 1-naphthylamine (NA) to acid chloride of FFA as a new derivatization method for FFA. In this derivatization method the presence of a primary amine makes the induction of reagents with fluorescent and UV absorption to FFA easy, with high reactivity of acid chloride and amine. From the present study we may conclude that 9-aminophenanthrene (9-AP) is useful for fluorescence-labeling of FFA.

## EXPERIMENTAL

### *Reagents*

Myristic acid ($C_{14:0}$, ML), palmitic acid ($C_{16:0}$, PT), palmitoleic acid ($C_{16:1}$, PL), stearic acid ($C_{18:0}$, ST), linoleic acid ($C_{18:2}$, Ll), arachidonic acid ($C_{20:4}$, AR) were purchased from P-L Biochemicals (Milwaukee, WI, U.S.A.). Oleic acid ($C_{18:1}$, OL) and palmitoyl chloride (PT · Cl) were purchased from Sigma (St. Louis, MO, U.S.A.). Margaric acid ($C_{17:0}$) was from Nakarai Chemicals (Kyoto, Japan). Linoleoyl chloride (Ll · Cl) and triethylamine (TEA) were from Tokyo Kasei Kogyo (Tokyo, Japan). Oxalyl chloride, $(COCl)_2$, was from Wako Pure Chemical (Osaka, Japan). 9-Aminophenanthrene (9-AP) was from Aldrich (Milwaukee, WI, U.S.A.). 9-Anthryldiazomethane (ADAM) was purchased from Funakoshi Chemical (Tokyo, Japan). The ethereal diazomethane solution was prepared by the established method [14].

### *Apparatus*

A Hitachi high-performance liquid chromatograph Model 635A equipped with a JASCO FP-110 spectrofluorometer and a Hitachi multiwavelength UV monitor was used. For measuring the infrared (IR) spectra, a JASCO A-3 IR spectrometer was used. $^1$H-NMR spectra were determined on a JEOL Fx-200 nuclear magnetic resonance (NMR) spectrometer with tetramethylsilane (TMS) as an internal standard. UV spectra and mass spectra were measured with a Shimadzu UV-210A and a Hitachi RMU-7MG spectrometer, respectively. Gas chromatographic (GC) analysis was performed on a Shimadzu GC-3BF

equipped with a flame ionization detector. Fluorescence spectra were recorded with a Hitachi fluorescence spectrometer 204-S.

*HPLC conditions*

Column: μBondapak $C_{18}$ (30 × 0.4 cm I.D., particle size 8—10 μm). Fluorescence detector: the excitation wavelength was set at 303 nm and the emission wavelength at 376 nm. Mobile phase: methanol—acetonitrile—water (53:27:20). Flow-rate: 2.0 ml/min. Column temperature: 40°C.

*Purification of 9-AP*

9-AP was purified by the method of Altiparmakian et al. [15]. In 30 ml of ethanol 50 mg of crude 9-AP were dissolved and filtered. Hydrochloric acid-saturated ether was added to the filtrate until the white precipitation no longer appeared. The precipitates were filtered, washed with ether, and dried in a vacuum desiccator. The dried crystals were dissolved in hot water, and the solution was made basic by the addition of ammonia to yield white crystals (m.p. 134°C).

*Preparation of N-palmitoyl-9-aminophenanthrene (PT · AP) and N-linoleoyl-9-aminophenanthrene (Ll · AP)*

PT · AP (0.25 mmol), 9-AP (0.25 mmol) and TEA (0.25 mmol) were dissolved in 20 ml of benzene in a reaction vial, and the solution was allowed to react by mixing for 30 min in an oil bath at 70°C. The solvent was removed at reduced pressure and the residue was recrystallized from ethanol to give PT · AP: m.p. 130.5°C. IR $\nu_{max}^{KBr}$ $cm^{-1}$: 3280 (NH), 1660 (CO). NMR ($C^2HCl_3$) δ: 0.88 (3H, t, $J$ = 6.4 Hz, 16-H), 1.26 (26H, brs), 1.81 (1H, br, NH), 2.48 (2H, brs, 2-H), 7.38—8.79 (9H, m, aromatic H). Mass spectrum $m/z$: 431 ($M^+$).

In the same way as the preparation of PT · AP, Ll · AP was obtained by recrystallization with ethanol after a 30-min reaction at 70°C in benzene solution containing Ll · Cl (0.25 mmol), 9-AP (0.25 mmol), and TEA (0.25 mmol): m.p. 95.5°C. IR $\nu_{max}^{KBr}$ $cm^{-1}$: 3250 (NH), 1640 (CO). NMR ($C^2HCl_3$) δ: 0.88 (3H, t, $J$ = 6.6 Hz, 18-H), 1.29 and 1.36 (16H, each brs, 3-H — 7-H + 15-H — 17-H), 1.85 (1H, br, NH), 2.03 (4H, 8-H + 14-H), 2.55 (2H, brs, 2-H), 2.77 (2H, t, $J$ = 5.3 Hz, 11-H), 5.36 (4H, m, 9-H, 10-H, 12-H, 13-H), 7.28—8.70 (9H, m, aromatic H). Mass spectrum $m/z$: 455 ($M^+$).

*Preparation of a 9-AP solution*

9-AP, 77.6 μmol, was dissolved in benzene to give a total volume of 10 ml. The solution, thus prepared, was kept shielded from light.

*Preparation of a 0.1% TEA solution*

TEA, 720 μmol, was dissolved in benzene to give a total volume of 10 ml.

*Preparation of a 2% oxalyl chloride solution*

Oxalyl chloride, 586 μmol, was dissolved in benzene to give a total volume of 10 ml.

*Derivatization procedures*

According to the reaction conditions which we had examined previously [13], FFA was converted to the acid chloride. FFA (1—700 nmol) dissolved in benzene (0.2—0.4 ml) was mixed with the same volume of a 2% oxalyl chloride solution, shaken sufficiently and the mixture allowed to react for 30 min in an oil bath at 70°C. After reaction, the excess oxalyl chloride and solvent were removed under a stream of nitrogen gas; 0.1 ml of a 9-AP solution and 0.1 ml of a 0.1% TEA solution were added to the preparation. The mixture was allowed to react at 70°C for 45 min, and the 9-AP derivative of FFA was obtained.

*FFA extraction from serum*

To 0.5 ml of serum were added 0.1 ml of a methanol solution containing 1 μg of margaric acid and 1.4 ml of *M*/15 phosphate buffer (pH 7.0); this mixture was shaken sufficiently and poured into a modified Extrelut column [16]. After adsorbtion for 20 min, FFA was eluted out with 10 ml of chloroform.

*Recovery of the FFA added to human serum*

To 0.5 ml of human serum were added 0.1 ml of methanol solution containing the internal standard, 5 μg of margaric acid, and the following composition of FFA : ML 2 μg, PT 20 μg, PL 4 μg, ST 4 μg, OL 40 μg, Ll 10 μg, AR 4 μg. Separation and determination of FFA were performed according to the methods described above.

*Method A (HPLC of FFA using ADAM reagent)*

FFA was allowed to react with ADAM reagent (which has been developed by Nimura and Kinoshita [6]) in methanol at room temperature. An ADAM-derivatized FFA solution was injected onto a μBondapak $C_{18}$ column, and eluted with acetonitrile—water (90:10) at a flow-rate of 1.5 ml/min. FFA derivative was detected by a fluorescence detector (excitation 365 nm, emission 412 nm).

*Method B (determination of FFA by GC)*

FFA was methylated with the ethereal diazomethane solution according to the method of Shlenk and Gellerman [17]. The excess diazomethane—ether solution was evaporated under a stream of nitrogen. Methylated FFA was dissolved in acetone and applied to GC. GC was carried out using a glass column (200 × 0.3 cm I.D.) packed with 15% diethylene glycol succinate (DEGS) on 80—100 mesh Chromosorb W AW DMCS. The injection temperature was maintained at 225°C and the column oven at 195°C. The flow-rate of nitrogen gas was 60 ml/min. The amount of each FFA was calculated from the calibration curves which were previously drawn on the basis of the peak height ratio of FFA to that of the internal standard ($C_{17:0}$).

## RESULTS AND DISCUSSION

From among the 9-AP-derivatized FFA, PT · AP and Ll · AP were selected, and their fluorescence and UV spectra were measured. Both PT · AP and

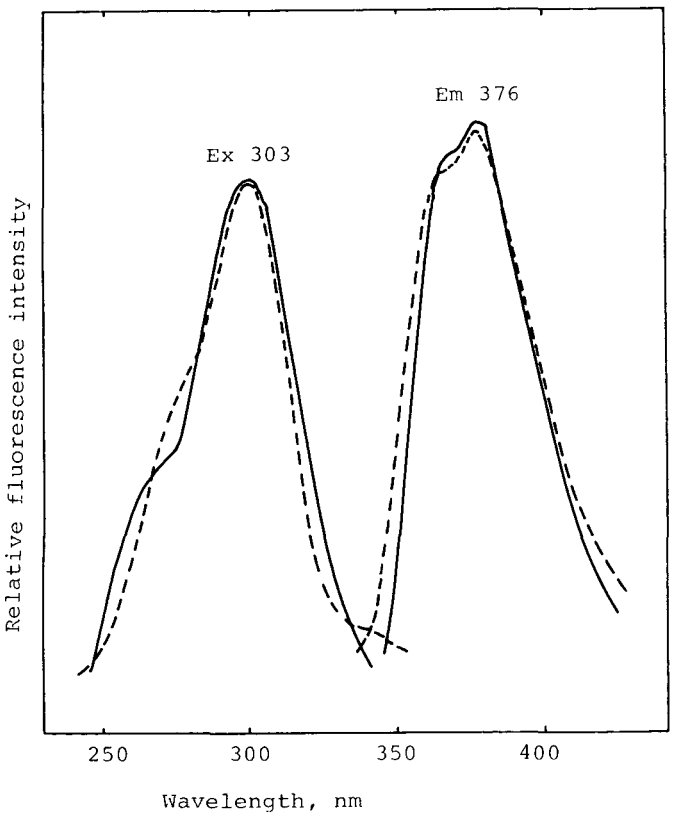


Fig. 1. Fluorescence spectra of N-palmitoyl-9-aminophenanthrene (——) and N-linoleoyl-9-aminophenanthrene (- - -) in methanol—water (81:19).

Ll · AP have a strong fluorescence around excitation wavelength of 303 nm and emission wavelength of 376 nm (Fig. 1). These derivatives also had strong absorption in the UV region, and maximal absorption at around 254 nm.

These results showed that FFA can be converted to the fluorescent FFA derivative with 9-AP. Secondly, reaction conditions necessary for the introduction of 9-AP into FFA were studied. The optimum conditions necessary for the introduction of 9-AP into an acid chloride of FFA were investigated in this paper, since the reaction conditions for the derivatization of FFA to an acid chloride form had already been evaluated [13]. In the reaction of PT · Cl and Ll · Cl with 9-AP in benzene, increase of reaction time from 15 to 30 min and to 45 min increased the production of PT · AP and Ll · AP; and increase of reaction temperature from 30°C to 50°C and 70°C also increased the production of these derivatives. The reaction for 45 min at 70°C produced a maximum amount of PT · AP and Ll · AP. The recovery rates of their derivatives were 97% in PT · AP, and approximately 100% in Ll · AP. However, it is supposed that a further increase of reaction temperature does not facilitate production, but breaks FFA down by the heat [18]. Therefore, the reaction conditions for FFA acid chloride with 9-AP were decided to be 45 min at 70°C. Under these conditions, derivatization of seven FFA ($C_{14}$—$C_{20}$) with

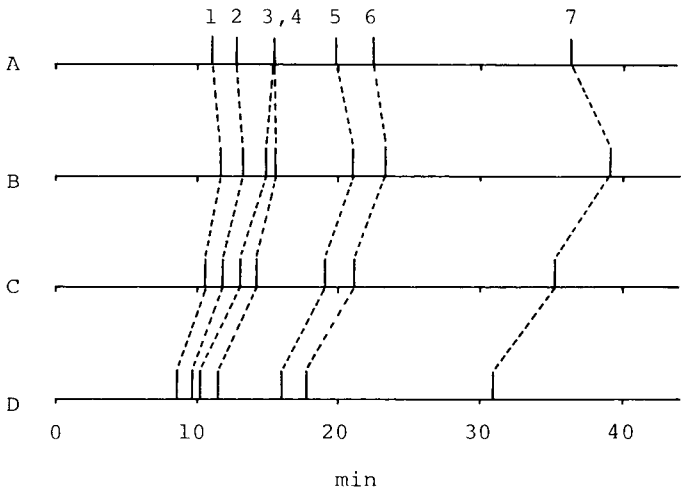


Fig. 2. Separation of a mixture of 9-AP-derivatized FFA. Column: μBondapak $C_{18}$. Flow-rate: 2.0 ml/min. Fluorescence excitation at 303 nm, fluorescence emission at 376 nm. Temperature: 40°C. 1 = $C_{14:0}$, 2 = $C_{16:1}$, 3 = $C_{20:4}$, 4 = $C_{18:2}$, 5 = $C_{16:0}$, 6 = $C_{18:1}$, 7 = $C_{18:0}$. Mobile phase composition (methanol—acetonitrile—water): (A) 80:0:20, (B) 55:25:20, (C) 53:27:20, (D) 50:30:20.

9-AP was carried out. Subsequently, HPLC separation conditions for these derivatized FFA were discussed. It was decided to use a μBondapak $C_{18}$ column at 40°C, and a flow-rate of 2.0 ml/min. Under these conditions, resolution of each derivatized FFA was studied using different eluates (Fig. 2). With methanol—water (80:20), AR and Ll remained in a same location unseparated. With an eluate of decreased methanol and acetonitrile instead of the decrease in methanol, AR and Ll were separated from each other. It was confirmed that FFA of PL, AR, and Ll were most favourably separated with methanol—acetonitrile—water (53:27:20) (Fig. 3). D'Amboise and Gendeau [19] separated phenacyl derivatives of FFA by HPLC with the solvent system methanol—acetonitrile—water on LiChrosorb RP-8 (10 μm).

For quantitative determination of FFA, calibration curves of seven kinds of saturated and unsaturated FFA ($C_{14}$—$C_{20}$) were drawn. From chromatograms obtained, the concentration of FFA was calculated from the ratio of the peak height of samples to that of the internal standard ($C_{17:0}$). All seven FFA showed straight lines in the range 10—100 ng. The detection limit of FFA, assuming a signal-to-noise ratio of 3, was 10 pmol for ML, PT, PL, OL and Ll, and 15 pmol for ST and AR. This result showed almost the same sensitivity as the other fluorescent-labeling reagents, 4-bromomethyl-7-methoxycoumarin (Br-Mmc) [20] or 9-anthryldiazomethane (ADAM) [6, 7], and showed ten times more sensitivity than that of the UV-labeling reagent, *p*-bromophenacyl bromide (PBPB) which was developed by Durst et al. [21].

Before applying the determination method of FFA to biological samples, seven kinds of FFA were added to human serum and recovered from it. The same amount as an average FFA level in 0.5 ml of normal serum was added. Each FFA was recovered 96—107%, and the coefficient of variation was 1.4—5.0% ($n$ = 4), showing a high sensitivity (Table I).

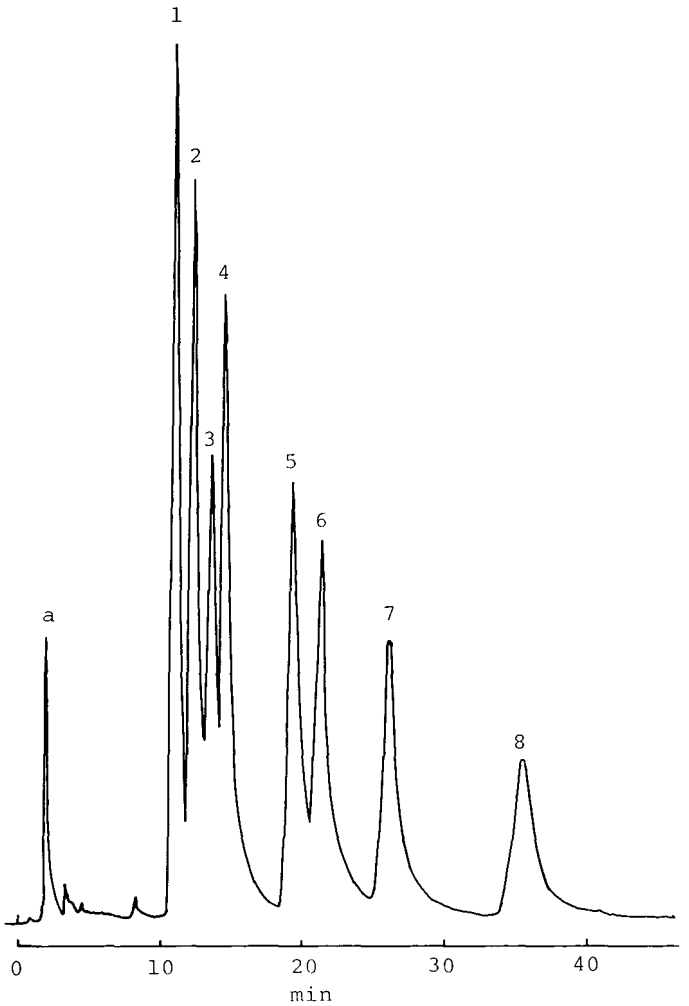


Fig. 3. Separation of the 9-AP derivatives of an FFA mixture. Mobile phase: methanol—acetonitrile—water (53:27:20). Flow-rate: 2.0 ml/min. a = 9-AP, 1 = $C_{14:0}$, 2 = $C_{16:1}$, 3 = $C_{20:4}$, 4 = $C_{18:2}$, 5 = $C_{16:0}$, 6 = $C_{18:1}$, 7 = $C_{17:0}$ (I.S.), 8 = $C_{18:0}$.

With 0.5 ml of serum separated immediately from the blood collected from six volunteers (adult, both sexes), FFA was quantitatively analysed by the present method (Table II). These results were almost the same as the average normal FFA level reported by Rogiers [22]. In order to determine the FFA level in serum with UV-labeling reagents, especially to detect PL and AR which are scarcely liberated in the blood, the extract from at least 50 $\mu$l of serum has to be injected into the HPLC column. However, the amount of extract injected into the column can be reduced to approximately one-tenth by the use of the present method. This is also good for maintaining higher column efficiency. In order to compare 9-AP with ADAM, a derivatization experiment for three kinds of FFA with the two reagents was performed. The derivatives obtained were applied to a $\mu$Bondapak $C_{18}$ column, and the recovery was measured using HPLC under optimum conditions. For comparison with the GC method, the same amounts of FFA in a specimen were measured, after

TABLE I

ANALYTICAL RECOVERY OF FFA ADDED TO HEALTHY HUMAN SERUM

The mixture of FFA was added to 0.5 ml of serum.

| Fatty acid | Added ($\mu$g) | Found* ($\mu$g) | Recovery* (%) | C.V. (%) |
|---|---|---|---|---|
| $C_{14:0}$ | 2.0 | 1.9 ± 0.1 | 96.3 ± 4.8 | 5.0 |
| $C_{16:0}$ | 20.0 | 21.5 ± 0.3 | 107.3 ± 1.5 | 1.4 |
| $C_{16:1}$ | 4.0 | 4.0 ± 0.1 | 100.0 ± 2.0 | 2.0 |
| $C_{18:0}$ | 4.0 | 3.9 ± 0.1 | 97.5 ± 3.5 | 3.6 |
| $C_{18:1}$ | 40.0 | 38.9 ± 0.2 | 97.3 ± 0.5 | 0.6 |
| $C_{18:2}$ | 10.0 | 9.7 ± 0.2 | 96.8 ± 1.7 | 1.8 |
| $C_{20:4}$ | 4.0 | 4.0 ± 0.1 | 101.0 ± 1.5 | 1.5 |

*Mean ± S.D., $n = 4$.

TABLE II

FFA CONCENTRATION IN HEALTHY HUMAN SERUM DETERMINED BY HPLC

| Volunteer | FFA ($\mu M$) | | | | | | |
|---|---|---|---|---|---|---|---|
| | $C_{14:0}$ | $C_{16:0}$ | $C_{16:1}$ | $C_{18:0}$ | $C_{18:1}$ | $C_{18:2}$ | $C_{20:4}$ |
| M.I. | 13 | 97 | 5 | 16 | 109 | 41 | 8 |
| N.E. | 9 | 40 | 3 | 10 | 48 | 36 | 3 |
| A.S. | 9 | 77 | 1 | 13 | 73 | 58 | 5 |
| Y.A. | 8 | 31 | 2 | 10 | 31 | 24 | N.D.* |
| J.I. | 16 | 68 | 6 | 26 | 72 | 36 | 5 |
| K.T. | 9 | 65 | 4 | 6 | 58 | 26 | 5 |
| Ref. 22 | 5 ± 4 | 62 ± 23 | 11 ± 7 | 26 ± 11 | 88 ± 42 | 42 ± 22 | — |

*N.D. = not detectable.

methylation, using GC with a 15% DEGS column. An average value of quadruplicate measurements suggested that in the GC method FFA was recovered almost 90—100%. However, the coefficient of varation was as large as 2.1—7.6%, and this suggested low reliability of measurement. On the other hand, two methods of fluorescence HPLC showed a small distribution in the results of quadruplicate measurements compared with that by the method of GC. Especially the 9-AP derivatization method showed a coefficient of variation of 1.4—3.4%, with high reliability (Table III).

These results showed that FFA detection at the picomole level has become possible, and that FFA in serum can be precisely assayed. With the establishment of this method, the usefulness of the primary amine has been established as a labeling preparation, and it may be used for the development of a new FFA derivatization reagent. For most FFA derivatization reagents which have been developed up to now, polar and aprotic solvents were used for reaction with FFA [21], but crown ethers were used for the reaction of higher FFA with low solubility [23—25]. Although the present derivatization method has the disadvantage that the process is slightly complicated, acid chlorides of FFA

TABLE III

COMPARISON OF 9-AP DERIVATIZATION METHOD WITH THE OTHER METHODS FOR THE DETERMINATION OF FFA

| Fatty acid | Added (ng) | Proposed method (9-AP) | | | Method A** | | | Method B*** | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Found* (ng) | Recovery* (%) | C.V. (%) | Found* (ng) | Recovery* (%) | C.V. (%) | Found* (ng) | Recovery* (%) | C.V. (%) |
| $C_{14:0}$ | 30 | 29.6 ± 0.6 | 98.6 ± 2.0 | 2.1 | 29.0 ± 1.1 | 96.8 ± 3.7 | 3.8 | 29.3 ± 1.3 | 97.5 ± 4.3 | 4.4 |
| | 50 | 46.4 ± 1.2 | 92.7 ± 2.4 | 2.6 | 44.8 ± 2.0 | 89.6 ± 4.0 | 4.5 | 48.7 ± 3.5 | 97.3 ± 7.0 | 7.2 |
| | 70 | 65.6 ± 1.6 | 93.7 ± 2.2 | 2.4 | 65.6 ± 2.0 | 93.7 ± 2.8 | 4.5 | 62.6 ± 3.3 | 89.4 ± 4.6 | 5.2 |
| | 90 | 90.3 ± 1.5 | 100.3 ± 1.7 | 1.7 | 88.3 ± 2.5 | 98.1 ± 2.8 | 2.8 | 86.6 ± 4.2 | 96.3 ± 4.5 | 4.6 |
| $C_{18:1}$ | 30 | 31.7 ± 0.8 | 105.8 ± 2.7 | 2.6 | 30.9 ± 1.1 | 103.1 ± 3.7 | 3.6 | 29.1 ± 0.6 | 97.0 ± 2.0 | 2.1 |
| | 50 | 46.4 ± 0.8 | 92.8 ± 1.5 | 1.6 | 51.8 ± 2.1 | 103.5 ± 4.2 | 4.0 | 50.5 ± 2.8 | 101.0 ± 5.9 | 5.8 |
| | 70 | 63.5 ± 1.8 | 90.6 ± 2.5 | 2.8 | 66.3 ± 1.1 | 94.8 ± 1.5 | 1.6 | 70.4 ± 4.3 | 100.5 ± 6.1 | 6.1 |
| | 90 | 90.0 ± 3.1 | 100.0 ± 3.4 | 3.4 | 86.2 ± 1.6 | 95.8 ± 1.8 | 1.9 | 89.1 ± 5.1 | 98.9 ± 5.7 | 5.8 |
| $C_{18:2}$ | 30 | 30.6 ± 0.8 | 102.1 ± 2.7 | 2.6 | 25.9 ± 0.6 | 86.4 ± 1.9 | 2.2 | 30.7 ± 0.7 | 102.2 ± 2.3 | 2.3 |
| | 50 | 46.8 ± 0.7 | 93.6 ± 1.4 | 1.5 | 46.1 ± 2.3 | 92.2 ± 4.6 | 5.0 | 48.3 ± 1.8 | 96.5 ± 3.5 | 3.7 |
| | 70 | 65.1 ± 1.1 | 92.9 ± 1.6 | 1.7 | 67.9 ± 3.3 | 97.0 ± 4.7 | 4.9 | 61.6 ± 2.7 | 88.1 ± 3.9 | 4.4 |
| | 90 | 92.9 ± 1.3 | 103.2 ± 1.4 | 1.4 | 82.8 ± 2.1 | 92.0 ± 2.3 | 2.5 | 85.6 ± 6.5 | 95.1 ± 7.2 | 7.6 |

*Mean ± S.D., $n = 4$.

**Method A: The FFA were dissolved in methanol. To the solutions 0.05% methanolic ADAM solution was added and the mixture was allowed to stand for 2 h at room temperature. The reaction mixture was injected directly into the column. The HPLC conditions are given in the experimental section.

***Method B: The FFA were methylated with the ethereal diazomethane solution. The methyl ester solution obtained was injected into the column. The GC conditions are given in the experimental section.

and primary amine react easily to produce 9-AP derivatives of FFA in high yield. As the reaction of the present method is conducted with non-polar solvents, it is convenient for the higher FFA with high solubility for such solvents. The 9-AP solution is very stable when protected from light, and can be used for more than one month.

By using the preseparation technique, it is not necessary to remove excess reagents or degradation products which may interfere with the determination.

It has been reported recently that the intake of unsaturated higher FFA which are said to be precursors of prostaglandin or thromboxane in platelets [26, 27] or vascular smooth muscle [28], has something to do with myocardial infarction or thrombosis. To clarify the mechanisms, the application of this method for FFA determination with such high sensitivty is expected to determine FFA at the cellular level and to provide much information.

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CHROMBIO. 1915

# ANALYSIS OF CORTISOL, METHYLPREDNISOLONE, AND METHYLPREDNISOLONE HEMISUCCINATE

# ABSENCE OF EFFECTS OF TROLEANDOMYCIN ON ESTER HYDROLYSIS

WILLIAM F. EBLING, STANLEY J. SZEFLER and WILLIAM J. JUSKO*

*Departments of Pharmaceutics*, Pharmacology and Therapeutics, and Pediatrics, Schools of Pharmacy and Medicine, State University of New York at Buffalo, Buffalo, NY 14260 (U.S.A.)*

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## SUMMARY

A sensitive, selective, and reproducible high-performance liquid chromatographic assay for the simultaneous measurement of cortisol and methylprednisolone using dexamethasone as the internal standard is presented. Samples are extracted with methylene chloride, washed with sodium hydroxide and then water, and chromatographed on a microparticle silica gel column with ultraviolet detection at 254 nm. Sensitivity is greater than 10 ng/ml and the intra-day coefficient of variation is less than 5% for both steroids. The use of porcine liver esterase allows the quantitation of the hemisuccinate ester of methylprednisolone. This assay has been applied in pharmacokinetic studies including investigations of troleandomycin—methylprednisolone interactions. A typical plasma concentration—time profile for methylprednisolone and its ester prodrug is presented for one subject before and after receiving troleandomycin therapy. Although methylprednisolone elimination is reduced in the presence of troleandomycin therapy, there is no effect on the pharmacokinetics of methylprednisolone sodium succinate.

## INTRODUCTION

Methylprednisolone is an important glucocorticoid for the treatment of various diseases. This steroid has particular utility in therapy of acute bronchospasm (status asthmaticus) when it is administered as its water-soluble sodium succinate ester [1—4]. Inter-patient variability in the therapeutic response to methylprednisolone therapy and the occurrence of unexplained adverse effects warrant the examination of methylprednisolone disposition in selected

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patients. Thus, assays for methylprednisolone are needed for use in pharmacokinetic studies including bioavailability assessment and the examination of altered steroid disposition in the presence of disease or as a result of drug—steroid interactions [4]. An assay which can simultaneously measure endogenous cortisol in addition to serum methylprednisolone may further aid in the characterization of the therapeutic response to and side effects from methylprednisolone therapy. The measurement of serum cortisol has the added advantage of monitoring the recovery of the adrenal-pituitary axis during withdrawal of steroid therapy.

Because the active steroids are of primary interest, the analysis of the ester prodrug should not diminish the sensitivity, specificity or reliability of the methylprednisolone and cortisol assay. Analytic techniques for the simultaneous measurement of methylprednisolone and its ester prodrug have been reported [5, 6]; however, they are either non-specific or do not have the ability to simultaneously analyze cortisol and methylprednisolone. Other normal-phase high-performance liquid chromatographic (HPLC) assays for methylprednisolone and cortisol have been presented [7, 8]. In addition to assaying the hemisuccinate ester, this assay has an improved prechromatography extraction procedure, has obviated some potential problems with the internal standards, and has allowed further in vitro and in vivo studies of the drug interaction between methylprednisolone and troleandomycin [4].

## EXPERIMENTAL

### *Materials*

The HPLC system consisted of a continuous flow, constant volume Model 6000A solvent delivery system and a Model 440 UV absorbance detector (Waters Assoc., Milford, MA, U.S.A.). Injections were made onto the system with a Model 7125 universal loop injector (Rheodyne, Berkeley, CA, U.S.A.). The UV absorbance of all steroids was measured at 254 nm. A Zorbax SIL (Dupont, Wilmington, DE, U.S.A.) column (25 cm × 4.6 mm I.D., 5—6 $\mu$m particle size) equipped with a 70 × 6 mm stainless-steel Whatman precolumn was used to separate the compounds. The precolumn consisted of HC-Pellocil (37—53 $\mu$m particles) (Whatman, Clifton, NJ, U.S.A.). The methylene chloride and hexane used in the extraction procedure and mobile phase were purchased from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). The pure ethanol (U.S.P.) employed in the mobile phase was obtained from U.S. International Chemicals (New York, NY, U.S.A.). The glacial acetic acid, also used in the mobile phase, was purchased from J.T. Baker (Phillipsburg, NJ, U.S.A.).

The methylprednisolone and methylprednisone were gifts of UpJohn (Kalamazoo, MI, U.S.A.). Methylprednisolone hemisuccinate was purchased from Steraloids (Wilton, NH, U.S.A.). Cortisol, dexamethasone, and carboxylic ester hydrolase (EC 3.1.1.1) Type II were obtained from Sigma (St. Louis, MO, U.S.A.). Pharmaceutical grade decolorizing carbon, neutral, was purchased from Amend Drug and Chemical (Irving, NJ, U.S.A.). Anhydrous sodium sulfate, 1 *M* sodium hydroxide solution, sodium phosphate monobasic and sodium phosphate dibasic were obtained from Fisher Scientific (Fairlawn, NJ, U.S.A.).

*Standard preparation*

Decolorizing carbon (12.5 g) was added to 240 ml of pooled human plasma and the mixture stirred for 2 h at room temperature. The carbon was then removed by centrifugation for 6 h at 17,000 *g* at 4°C. Finally, the plasma was filtered through a 0.45-μm Millipore filter to remove carbon fines. To this cortisol-stripped plasma, standards of cortisol and methylprednisolone in acetonitrile—methanol (1:1) were added to provide concentrations of 25—500 ng/ml.

*Extraction procedure*

Plasma samples (up to 1 ml) were added to acid-washed glass extraction tubes with PTFE-lined screw caps (20 × 150 mm). If plasma samples were less than 1 ml, sufficient distilled water was added to bring the total sample volume to 1 ml. The internal standard, dexamethasone (250 ng in 80 μl of methanol) was mixed with the sample, 15 ml of methylene chloride were added, and the tubes were shaken for 20 min. The tubes were centrifuged and the aqueous layer and creamy interface aspirated. The organic phase was then washed with 1 ml of 0.1 *M* sodium hydroxide and subsequently with 1 ml of distilled water. After aspirating the aqueous phase, 1 g of anhydrous sodium sulfate was added to dry the methylene chloride. The latter was evaporated to dryness at 45°C under a nitrogen gas stream.

*Chromatography*

The residue was reconstituted with approximately 200 μl of mobile phase for injection. The mobile phase consisted of a hexane—methylene chloride—ethanol—acetic acid (26:69:3.4:1) mixture and the flow-rate was maintained at 2 ml/min.

*Steroid recovery*

The assay recovery of each steroid was assessed at 50 and 500 ng/ml in the following manner. Ten plasma samples (1 ml) containing each steroid were extracted and injected onto the chromatograph. Ten injections of the same amount of steroid (e.g. 50 and 500 ng) in mobile phase were directly injected. The peak heights of the steroids in both sets of samples were measured. The assay recovery of each steroid was computed using the following equation:

$$\text{Percent recovery} = \frac{\text{Peak height, extracted drug}}{\text{Mean peak height, direct injection}} \times 100$$

*Methylprednisolone hemisuccinate hydrolysis*

A hydrolysis study was performed to assess the time required for complete hydrolysis of the hemisuccinate to methylprednisolone. Human plasma (8 ml) was spiked with methylprednisolone hemisuccinate at a concentration of 1000 ng/ml, 72 units of carboxylic-ester hydrolase were then added to the plasma and 1-ml samples were obtained over a 50-min period. The hydrolysis reaction was terminated by immediately extracting the samples with methylene chloride.

*Quantitation of methylprednisolone hemisuccinate*

Prior to the addition of the internal standard in the extraction procedure, 80 $\mu$l containing 9 units of carboxylic-ester hydrolase in 0.5 *M* sodium phosphate buffer, pH 7.4 were added to the samples. The samples were incubated with the enzyme for 30 min at 37° C. After incubation, the internal standard was added and the samples were carried through the extraction procedure. The concentration of methylprednisolone hemisuccinate was calculated as the difference between methylprednisolone concentrations from hydrolyzed and unhydrolyzed samples.

## REPRODUCIBILITY

The within-day and between-day reproducibility of the assay was assessed for methylprednisolone and cortisol at 50 and 500 ng/ml concentrations. The intra-day coefficient of variation for measurement of methylprednisolone hemisuccinate was also determined at these concentrations in the presence of 500 ng/ml methylprednisolone.

*Patient studies*

The following procedure was utilized to evaluate the effect of troleandomycin therapy on methylprednisolone sodium succinate hydrolysis. Corticosteroid pharmacokinetics were studied in an 11-year-old female asthmatic patient before and one week after initiating a course of troleandomycin therapy. The study dose consisted of 40 mg methylprednisolone sodium succinate (Solu-Medrol, Upjohn) administered over 2 min via an intravenous catheter. The study conditions were identical except for the oral administration of troleandomycin 250-mg capsules (TAO, Roerig) every 6 h for one week before and on the day of the second corticosteroid study. Blood samples were obtained from another intravenous catheter prior to and at selected times following the intravenous corticosteroid dose. This catheter was placed on the arm contralateral to that of the injection site. Blood samples were immediately centrifuged and plasma stored at −20° C until analysis. Since the patient was receiving steroid therapy for the treatment of her disease, endogenous cortisol secretion was suppressed resulting in plasma cortisol concentrations below the detection limits of our assay. To illustrate the ability of this assay to simultaneously measure methylprednisolone and cortisol, corticosteroid pharmacokinetics were examined after the administration of 20 mg of methylprednisolone sodium succinate (Solu-Medrol, Upjohn) to a 31-year-old normal male volunteer at 9 a.m. Steroid administration and sample collection were the same as the above patient study.

## RESULTS

A chromatogram resulting from the HPLC analysis of natural human plasma is presented in Fig. 1a. Endogenous cortisol elutes as the last peak. Fig. 1b illustrates the response to steroid concentrations of approximately 250 ng/ml in charcoal-stripped human plasma from which endogeneous steroids were removed. Each steroid eluted with sharp peaks and distinct separation at base-

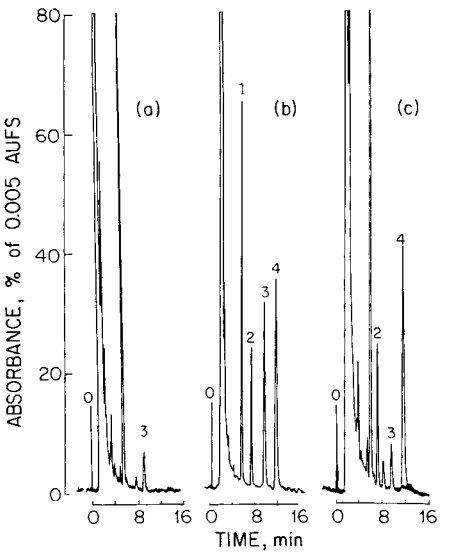


Fig. 1. Chromatograms of (a) blank plasma extract; (b) cortisol-stripped plasma spiked with 250 ng of steroids; (c) plasma extract obtained 0.5 h after a single 20-mg intravenous dose of methylprednisolone hemisuccinate. Peaks: (1) methylprednisone; (2) internal standard, dexamethasone: (3) cortisol; (4) methylprednisolone. The symbol 0 designates the injection point.

line. A chromatogram resulting from the HPLC analysis of plasma obtained 0.5 h after the intravenous injection of 20 mg of methylprednisolone hemisuccinate to a human subject is shown in Fig. 1c. This chromatogram represents concentrations of 103 ng/ml for cortisol and 398 ng/ml for methylprednisolone. Each steroid of interest is defined clearly. An endogenous compound which elutes between dexamethasone and cortisol is prominent in natural plasma, but does not interfere.

The steroid extraction recoveries at two concentrations and sensitivity limits of the assay are presented in Table I. The mean assay recoveries of methylprednisolone, cortisol, and dexamethasone were about 62% and were independent of concentration. The recovery of each of these steroids is also independent of the amount of plasma contained in the initial aqueous phase. Thus, diluted plasma samples may be assayed along with undiluted samples utilizing the same standard curve. Such dilution of plasma is desirable to bring the peak height ratio within the calibration plot range for samples of high concentrations. This dilution is also necessary if the initial aqueous:organic phase ratio is to be maintained when limited volumes of plasma are available. The equivalent extraction recovery of dexamethasone compared with cortisol and methylprednisolone substantiates the suitability of this steroid as an internal standard in this assay procedure.

The apparent minimum quantitation limit for both methylprednisolone and cortisol is 10 ng/ml. The minimum detection limit for these steroids is appproximately 2 ng/ml. The latter concentration cannot be quantified, but reflects the lower limit of steroid concentration producing a detector response.

Calibration curves of peak height ratio versus steroid concentration are linear

TABLE I

STEROID EXTRACTION RECOVERIES AND ASSAY SENSITIVITY LIMITS

| Steroid | Percent recovery | | | Minimum limits (ng/ml) | |
|---|---|---|---|---|---|
| | 50 ng/ml | 500 ng/ml | Mean | Quantitation** | Detection |
| Methylprednisolone | 62.6 (4.5)* | 61.3 (5.7) | 62.0 | 10 | 2 |
| Cortisol | 62.8 (4.5) | 59.8 (5.3) | 61.2 | 10 | 2 |
| Dexamethasone | 64.2 (4.7) | 60.4 (5.5) | 62.3 | — | — |

*Mean (S.D.).
**Based on a signal-to-noise ratio of 2.5.

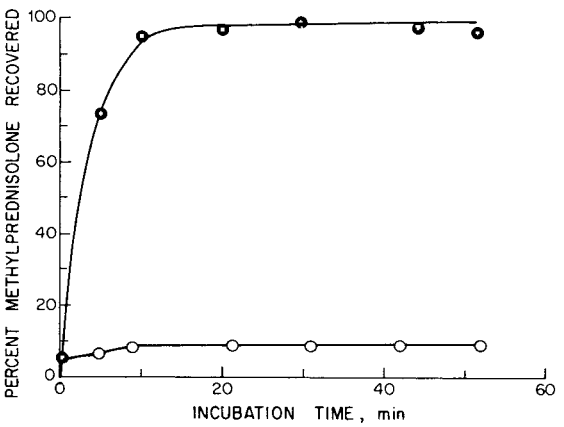


Fig. 2. Time course of hydrolysis of methylprednisolone hemisuccinate in the presence (•) and absence (○) of carboxylic ester hydrolase.

over the range 10—1000 ng/ml for both cortisol and methylprednisolone. However, cortisol produces a slightly greater detector response than does methylprednisolone.

The hydrolysis rate of methylprednisolone hemisuccinate is shown in Fig. 2. Methylprednisolone hemisuccinate is hydrolysed rapidly and completely to its free alcohol within the incubation time for this assay. The lack of progressive hydrolysis of the ester in the absence of carboxylic ester hydrolase demonstrates the stability of the ester in plasma. Similar results are observed if the hydrolysis is performed in whole blood in the absence of the esterase. Neither troleandomycin nor oleandomycin in plasma samples interfere with the rate and extent of hydrolysis catalyzed by this enzyme.

The intra-day and inter-day variability of the assay for methylprednisolone and cortisol are presented for high and low steroid concentrations in Table II. Both coefficients of variation are less than 5% for the two steroids. Since the hemisuccinate ester is quantified by difference, intra-day variability of the assay for this ester was determined in the presence of 500 ng/ml methylprednisolone. Under these conditions, the variability in determination of the steroid ester is comparable with the free alcohol. The relatively high variability

TABLE II

INTRA-DAY AND INTER-DAY COEFFICIENTS OF VARIATION

All variability statistics are based on ten measurements.

| Steroid | Intra-day coefficient of variation (%) | | Inter-day coefficient of variation (%) | |
|---|---|---|---|---|
| | 50 ng/ml | 500 ng/ml | 50 ng/ml | 500 ng/ml |
| Methylprednisolone | 1.6 | 3.7 | 3.5 | 1.6 |
| Cortisol | 2.2 | 1.9 | 4.7 | 4.2 |
| Methylprednisolone hemisuccinate* | 17.3 | 3.6 | — | — |

*In the presence of methylprednisolone, 500 ng/ml.

TABLE III

RELATIVE RETENTION TIMES OF SELECTED GLUCOCORTICOIDS

Retention times relative to methylprednisolone, corrected for column hold-up time.

| Steroid | Relative retention time (min) |
|---|---|
| Fluocinonide | 0.29 |
| Cortisone | 0.34 |
| Corticosterone | 0.35 |
| Methylprednisone | 0.37 |
| Prednisone | 0.44 |
| Beclomethasone | 0.51 |
| Dexamethasone | 0.54 |
| Betamethasone | 0.63 |
| Cortisol | 0.77 |
| Methylprednisolone | 1.00 |
| Prednisolone | 1.03 |

at the 50 ng/ml concentration is expected since the difference between the hydrolysed and unhydrolysed concentration is less than 10%.

The assay was examined for selectivity by injecting other endogenous and synthetic glucocorticoids onto the chromatographic system. The relative retention times of these materials are listed in Table III. The assay method clearly separates this series of closely related compounds. Many other conjugated and oxidized metabolites of these steroids probably do not warrant concern as the initial organic extraction step excludes the lipid-insoluble biotransformation products from the HPLC column. This assay has been utilized for the determination of methylprednisolone concentrations in patients receiving other therapeutic agents [4]. These drugs include: theophylline, terbutaline, isoproterenol, epinephrine, phenobarbital, phenytoin, troleandomycin, erythromycin, and hydroxyzine. None of these drugs or their metabolites interfered with this assay.

The conversion of methylprednisolone hemisuccinate to its free alcohol is rapid with maximal concentrations of the latter found at 30 min (Fig. 3A

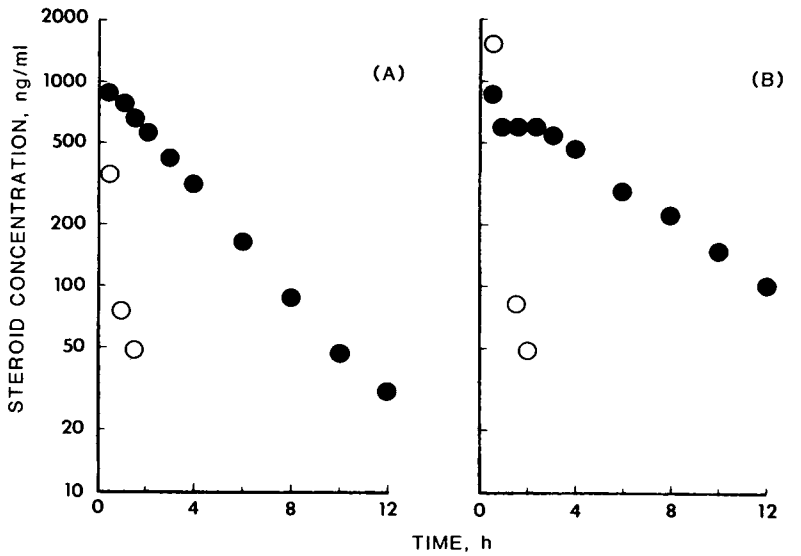


Fig. 3. Plasma methylprednisolone (•) and methylprednisolone hemisuccinate (○) concentrations after a single intravenous dose of 40 mg of methylprednisolone hemisuccinate given to an 11-year-old asthma patient before (A) and after (B) administration of 250 mg of TAO every 6 h for one week.

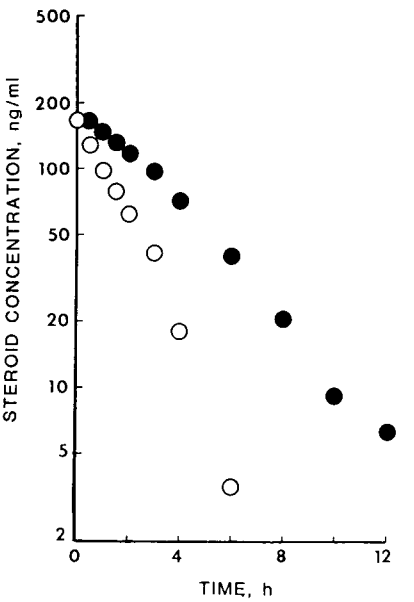


Fig. 4. Plasma methylprednisolone (•) and endogenous cortisol (○) concentrations after a single intravenous dose of 20 mg of methylprednisolone hemisuccinate given to a 31-year-old normal male volunteer at 9 a.m.

and B). When TAO is co-administered, the ester behaves similarly, although inhibition of methylprednisolone disposition was found. In the baseline study methylprednisolone is eliminated with a half-life of 2.3 h and plasma clearance

of 358 ml/min per 1.73 $m^2$. While this patient was receiving TAO, the elimination half-life and plasma clearance were 3.3 h and 248 ml/min/1.73 $m^2$.

The simultaneous measurement of cortisol and methylprednisolone is illustrated in Fig. 4. The zero h (9 a.m.) cortisol concentration was 180 ng and rapidly fell below assay detection limit by 4 p.m. (7 h). Although the 9-a.m. value is within normal limits (60 to 260 ng/ml) the 4-p.m. value in normal subjects is typically between 20 and 180 ng/ml. This marked decline in cortisol concentrations is indicative of suppression of cortisol secretion by methylprednisolone.

## DISCUSSION

The simultaneous measurement of methylprednisolone and cortisol by this HPLC method is efficient, precise, sensitive and selective. To date, over 2000 samples have been analyzed by this method in studies of the disposition kinetics of methylprednisolone and cortisol. These compounds are stable in frozen serum for extended periods. Some serum samples have been repeatedly assayed at time intervals of twelve months or more and yield identical steroid concentrations.

Incubation of samples with carboxylesterase allows determination of the hemisuccinate ester of methylprednisolone by the difference of results with and without use of the enzyme. A lower quantitation limit for the analysis of the ester has not been presented since this limit is determined primarily by the relative difference in methylprednisolone and total methylprednisolone concentrations. With this method, methylprednisolone hemisuccinate concentrations are considered non-quantifiable if the relative difference is less than 10%.

The hemisuccinate ester does not partition into methylene chloride because the extraction of plasma is carried out under neutral pH conditions. This affords subsequent treatment of the methylene chloride extract with acidic or basic washes without having the assay confounded by hydrolysis of the ester to the active steroid. Although we have not found any interference from co-administered drugs or their metabolites, the ability to rigorously wash the extract provides a method of circumventing interference if the problem arises.

Extractability of the internal standard from aqueous solutions is identical with the steroids of interest. Other published HPLC assays for methylprednisolone do not share this feature. One reversed-phase assay has been reported which utilizes an endogenous glucocorticoid, cortexolone, as an internal standard [5]. However, cortexolone extraction recovery was lower than that of methylprednisolone. This assay method also does not allow simultaneous measurement of methylprednisolone and cortisol. A normal-phase assay has been reported which employs the acetate ester of methylprednisolone as the internal standard [7]. Although the extractability of this ester was not provided, the water—octanol and water—diethyl ether partition coefficients of this ester are probably ten-fold greater than methylprednisolone or cortisol [9]. Additionally, because this internal standard is an ester, the extraction process and subsequent treatment of the extract may require mild conditions to prevent its hydrolysis to methylprednisolone. Another assay for methylprednisolone utilizes an internal standard which is added after extraction [8].

Much of the utility of the internal standard in correcting for recovery and other sources of variation is lost at that stage of the procedure.

Pharmacokinetic studies of methylprednisolone have been carried out using a radioimmunoassay technique [6, 10]. Cross-reactivity with the major metabolite of this steroid, methylprednisone, was reported and may complicate the results as patients attain appreciable plasma concentrations of methylprednisone [4]. The radioimmunoassay method also does not permit concomitant assay for cortisol. While the present assay is unable to resolve methylprednisone, it has a shorter retention time and does not interfere with measurement of cortisol or methylprednisolone.

The diminished apparent clearance and prolonged half-life of methylprednisolone during co-administration of troleandomycin have been reported [4]. This drug combination is often beneficial in treatment of patients with severe steroid-dependent asthma [1—3]. However, mechanisms responsible for this drug interaction have not been fully elucidated. Since methylprednisolone must be cleaved from its ester prodrug before it appears in plasma, alterations in ester disposition were of concern. TAO is also converted to active forms through de-esterification and competition with hydrolysis of methylprednisolone hemisuccinate require consideration. The determination of plasma concentrations of methylprednisolone hemisuccinate along with the active steroid (Fig. 3) indicates that the in vivo disposition of the ester appears to be unaltered in the presence of TAO. The in vitro hydrolysis of the ester was similarly unaffected by the presence of the antibiotic. Thus, the inhibition of methylprednisolone elimination is unlikely to be due to altered hydrolysis of the ester.

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CHROMBIO. 1935

# HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSER FOR GUANIDINO COMPOUNDS USING BENZOIN AS A FLUOROGENIC REAGENT

YIAU-LIN HUNG, MASAAKI KAI, HITOSHI NOHTA and YOSUKE OHKURA*

*Faculty of Pharmaceutical Sciences, Kyushu University 62, Maidashi, Higashi-ku, Fukuoka 812 (Japan)*

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## SUMMARY

An automatic analyser based on high-performance liquid chromatography has been developed for the quantification of biogenic guanidino compounds in human physiological fluids. Fourteen guanidino compounds are mutually separated within 35 min on a cation-exchange column with a stepwise gradient elution of pH and/or ionic strength in the mobile phase and then converted automatically to their fluorescent derivatives with benzoin. The method in this system is simple, rapid and sensitive; the lower limits of detection are 5—50 pmol for monosubstituted guanidino compounds, 1 nmol for creatine and 20 nmol for creatinine in 100 μl of injection volume.

## INTRODUCTION

Guanidino compounds such as methylguanidine and guanidinosuccinic acid have been implicated as uremic toxins in uremic syndrome because these compounds accumulate in body fluids of uremic patients [1—3] and give rise to a symptom complex similar to uremia [4, 5].

Among the current analytical methods for guanidino compounds, ion-exchange chromatography coupled with automatic colorimetric or fluorimetric detection, including high-performance liquid chromatography (HPLC), is the most popular because of its simplicity in operation, though other chromatographic methods based on paper [6, 7], thin-layer [8—10] and gas chromatography [11—13] have been reported. However, colorimetric detection of the compounds by means of the Sakaguchi or Voges-Proskauer reaction in ion-exchange chromatography has a limited sensitivity and thus necessitates a large amount of sample [14—16]. On the other hand, fluorimetric detection based

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on post-column derivatization after HPLC, using ninhydrin or 9,10-phenanthraquinone as fluorogenic reagent can offer a method sensitive enough to measure the compounds at the picomole level [17—19].

Recently we have developed a manual fluorimetric method for the selective determination of guanidino compounds using benzoin [20, 21]. This reagent is a non-fluorescent material, but reacts with the guanidino moiety of the compounds in an alkaline medium and gives highly fluorescent derivatives — 2-substituted amino-4,5-diphenylimidazoles [22]. Therefore this reaction is applicable to the fluorimetric detection of guanidino compounds as both pre- and post-column derivatives in HPLC. The application of the reaction to pre-column derivatization of the compounds in HPLC has been described in a separate paper [23]; this method is most sensitive and useful for the determination of guanidino compounds at the femtomole level; however, the limited separation of the fluorescent derivatives did not permit the assay of biogenic guanidino compounds without a clean-up procedure for removal of oligopeptides in biological samples.

In this paper we describe the HPLC conditions for the rapid simultaneous separation of guanidino compounds on a cation-exchange column and the application of the benzoin reaction to the post-column fluorescence derivatization of the compounds in order to assemble an automatic analyser for routine assay of biogenic guanidino compounds in human urine and serum from normal subjects or uremic patients. The following fourteen guanidino compounds were used as representative compounds for the investigations; taurocyamine, TC; guanidinosuccinic acid, GSA; creatine, CR; guanidinoacetic acid, GAA; $N^{\alpha}$-acetylarginine, AcARG; argininic acid, ARA; guanidinopropionic acid, GPA; creatinine, CRN; guanidinobutyric acid, GBA; arginine, ARG; phenylguanidine, PG; guanidine, G; methylguanidine, MG; agmatine, AGM.

## EXPERIMENTAL

### *Chemicals and solutions*

All chemicals were of analytical reagent grade, unless otherwise noted. Distilled water was used. Benzoin (Wako, Osaka, Japan) was recrystallized from absolute methanol. Tris(hydroxymethyl)aminomethane (Tris) (Wako, Osaka, Japan) was recrystallised from 60% (v/v) aqueous methanol to remove fluorescent impurities. Standard solutions of the guanidino compounds were prepared in 0.05 *M* hydrochloric acid.

*Mobile phase for HPLC.* Three aqueous eluents are required for separation of the guanidino compounds. Buffer A (pH 3.5): dissolve 5.25 g of trisodium citrate dihydrate, 2.7 g of sodium chloride, 8.1 g of citric acid and 7.2 mg of sodium pentachlorophenol as a preservative in approximately 400 ml of water and dilute with water to 500 ml (the final concentration of each component is 36, 92, 77 and 0.05 m*M*, respectively). Buffer B (pH 5.0): dissolve 13.35 g of trisodium citrate dihydrate, 20 g of sodium chloride, 3.05 g of citric acid and 7.2 mg of sodium pentachlorophenol in approximately 400 ml of water, and dilute with water to 500 ml (the final concentration of each component is 91, 684, 29 and 0.05 m*M*, respectively). Potassium hydroxide solution (1.0 *M*):

dissolve 28.05 g of potassium hydroxide in about 400 ml of water and dilute with water to 500 ml. The solutions were thoroughly degassed in the usual manner before use.

*Reagents for post-column derivatization.* Benzoin solution (4.0 m*M*): dissolve 0.425 g of benzoin in 500 ml of a mixture of methylcellosolve and water (6:4, v/v). Potassium hydroxide solution (4.0 *M*): dissolve 122.2 g of potassium hydroxide in about 400 ml of water and dilute with water to 500 ml. Sodium dihydrogen phosphate (1.6 *M*)—Tris (1.0 *M*) mixture: dissolve 110.5 g of sodium dihydrogen phosphate and 60.55 g of Tris in about 400 ml of water and dilute with water to 500 ml. The solutions were degassed before use.

*Chromatographic system and its operation*

Fig. 1 is a schematic diagram of the HPLC analyser constructed for analysis of the guanidino compounds.

A cation-exchange column (80 mm × 4 mm I.D.; packing material Hitachi 2619 resin; particle size 5 μm) was used in the HPLC with a stepwise gradient elution of pH and/or ion strength by using buffers A and B, and 1.0 *M* potassium hydroxide. The temperature of the column with a water-jacket was kept at 80°C by an Hitachi constant-temperature circulator. The mobile phase (buffers A and B, and 1.0 *M* potassium hydroxide) was pumped at a flow-rate of 0.5 ml/min by an Hitachi 638-30 high-performance liquid chromatograph which had a programming controller of the electronic valves placed prior to

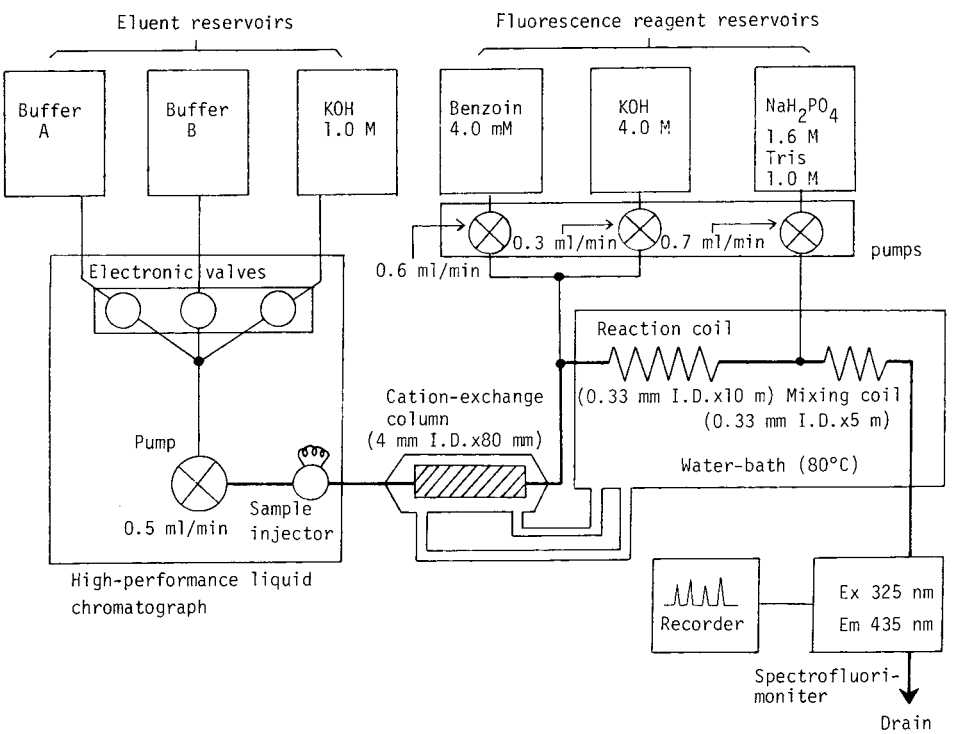


Fig. 1. Schematic diagram of HPLC analyser for guanidino compounds.

the pump inlet for various gradient elutions. Buffer A was first run into the column for 2 min, then a mixture of buffers A and B (1:1, v/v) for 2 min, then buffer B for 11 min and 1.0 *M* potassium hydroxide for 10 min to separate the guanidino compounds; then successively the column was equilibrated with buffer A for 20 min before the start of the next sample (Fig. 2). The above change of eluents was automatically controlled with the electronic programmer of the chromatograph.

The eluate from the column was conducted to the fluorescence reactor system through a PTFE tube (20 cm × 0.33 mm I.D.). All the coils in the reaction system are made of PTFE. Benzoin (4.0 m*M*) and potassium hydroxide (4.0 *M*) solutions were first added to the eluate stream at a tee-connector by an Hitachi reagent-delivery pump for amino acid analyser and an Hitachi 663-C chemical pump at flow-rates of 0.6 and 0.3 ml/min, respectively, and then the mixture was passed through a reaction coil (10 m × 0.33 mm I.D.) immersed in an 80°C water-bath. After the fluorescence reaction, sodium dihydrogen phosphate (1.6 *M*)—Tris (1.0 *M*) mixture was added to the reaction mixture at a flow-rate of 0.7 ml/min by an Hitachi reagent-delivery pump for amino acid analyser and the mixture was passed through a mixing coil (5 m × 0.33 mm I.D.). The fluorescence intensity from each guanidino compound in the last eluate was monitored at 435 nm emission against 325 nm excitation (both slit-widths 5 nm) by an Hitachi 650-10LC spectrofluorimeter equipped with a flow cell (18 $\mu$l) and a xenon lamp.

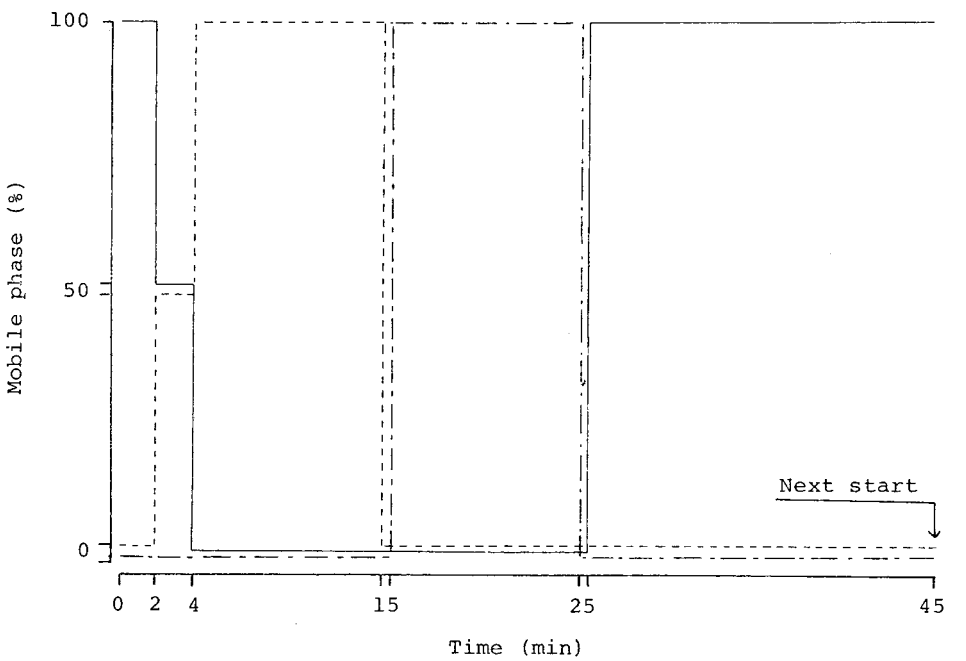


Fig. 2. Elution mode of the stepwise gradient. (——), buffer A; (- - -), buffer B; (- — -), potassium hydroxide.

*Preparation of physiological fluids*

Urine and serum specimens were obtained from healthy volunteers in our laboratory and from patients with chronic renal failure being hemodialysed at Japan Red Cross Fukuoka Hospital (Fukuoka, Japan).

*Urine samples.* To 100 $\mu$l of urine centrifuged at approximately 1000 *g* for 5 min, were added 800 $\mu$l of 7.5 nmol/ml phenylguanidine. An aliquot (100 $\mu$l) of the mixture was applied to the HPLC analyser.

*Serum samples.* A 100-$\mu$l aliquot of serum was mixed with 50 $\mu$l of 1.8 *M* perchloric acid and 25 $\mu$l of 25 nmol/ml phenylguanidine. The mixture was centrifuged at 1500 *g* for 10 min. A 100-$\mu$l aliquot of supernatant was neutralized by adding 50 $\mu$l of 0.6 *M* potassium carbonate and then the potassium perchlorate formed was removed to avoid the precipitation of the salt in the lines of the HPLC analyser. The pH of the resultant supernatant was adjusted to approximately 1.7 with about 20 $\mu$l of 0.7 *M* hydrochloric acid. An aliquot (100 $\mu$l) of the final mixture was applied to the HPLC analyser.

## RESULTS AND DISCUSSION

*Separation of guanidino compounds*

The conventional separation of guanidino compounds has been performed by chromatography using a strong cation-exchange resin, based on the separation technique for basic amino acids [24]. A resin of this type was used in our investigations. The resin particles were small and arranged to be of the same diameter (5 $\mu$m) since well-regulated small particles of resin provide an

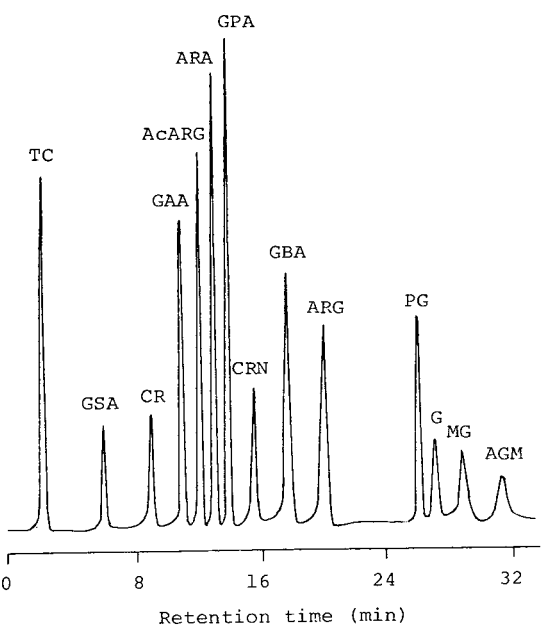


Fig. 3. Chromatogram of a standard mixture of guanidino compounds, each at the amount of 800 pmol, subjected to HPLC (CR, 40 nmol; CRN, 0.8 $\mu$mol). For the separation and detection conditions of the HPLC analyser, see text.

increased theoretical plate number [25]. Thus the resin packed in a short column (80 mm × 4 mm I.D.) gave a high resolution of the guanidino compounds in HPLC. Fig. 3 shows a chromatogram of a standard mixture of fourteen guanidino compounds obtained with the HPLC analyser. Complete separation of the compounds on the column is achieved within 35 min in a single run with a stepwise gradient elution (the operational procedure is described in Experimental).

The guanidino compounds tested, except for the strongly basic compounds such as PG, G, MG and AGM, were retained on the column and resolved using a sodium citrate buffer (0.1 *M*, pH 4.0) with isocratic elution; their retention times were affected by the pH and ionic strength in the buffer. The ionic strength was controlled by the addition of sodium chloride to the buffer. The rise of pH and/or ionic strength in the buffer resulted in an early elution of the guanidino compounds. On the other hand, G, MG, PG and AGM were strongly retained on the column and not eluted with the acidic buffer. However, with an alkaline solution such as 1.0 *M* potassium hydroxide, they were resolved with reasonable retention times. From the above preliminary studies on the separation of the guanidino compounds, the combination of three eluents — buffer A (pH 3.5), buffer B (pH 5.0) and 1.0 *M* potassium hydroxide — as mobile phase was employed for HPLC (the constituent of each eluent and the elution mode for the stepwise gradient are described in Experimental). Buffer A is mainly used for the separation of TC and GSA, buffer B for CR, GAA, AcARG, ARA, GPA, CRN, GBA and ARG, and 1.0 *M* potassium hydroxide for PG, G, MG and AGM.

The retention times of the guanidino compounds are also influenced by the column temperature. Increased temperature facilitates early elution of the guanidino compounds, especially late-eluting compounds such as G, MG and AGM, without deterioration of resolution and peak shape. For example, when the column was operated at ambient temperature (23°C), MG eluted late at about 44 min, but at 80°C this compound was eluted at 29.2 min. Consequently the column was operated at 80°C not only to shorten the analytical time but also to obtain a definite retention time of each guanidino compound. Good durability of the column was also observed; the column can be used for more than 2000 analyses.

*Fluorescence reactor and detection*

In the manual method [21], the fluorescence reaction of the guanidino compounds with benzoin needs a strongly alkaline medium and heating conditions to minimize the reaction time; also the fluorescent derivatives produced fluoresce most intensely in a weakly alkaline solution (pH 8.5—10.5). Thus the eluate from the column was first mixed with benzoin and potassium hydroxide, and then the mixture was heated at 80°C through a reaction coil for approximately 45 sec and made weakly alkaline by adding a mixture of sodium dihydrogen phosphate and Tris. The excitation and emission maxima of the fluorescence from all the guanidino compounds tested, obtained with this detection system, were around 325 and 435 nm, respectively. These data agreed with those obtained with the manual method previously described [21].

Reaction conditions of the post-column fluorescence derivatization were

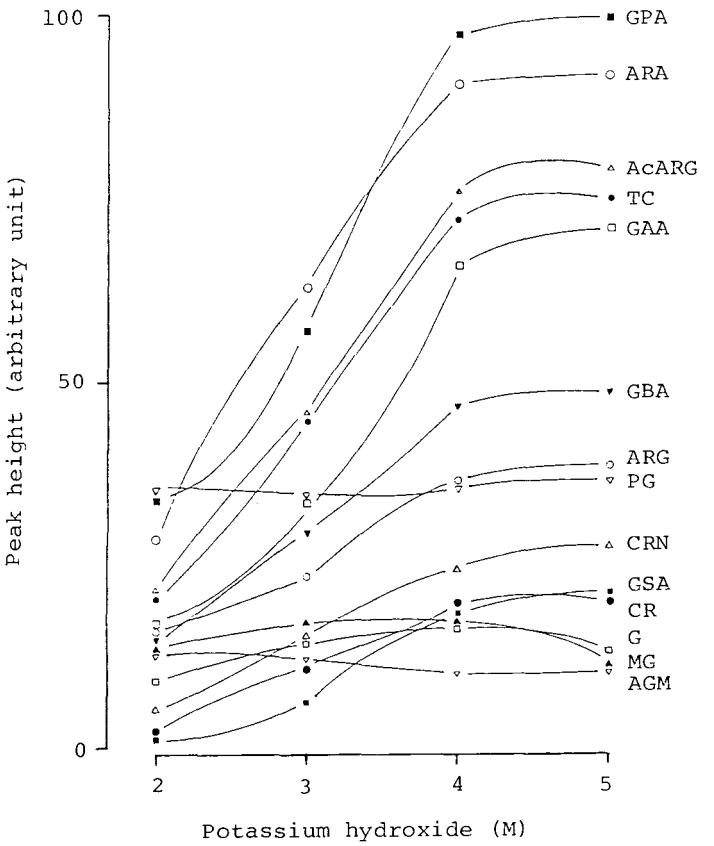


Fig. 4. Effect of potassium hydroxide concentration on the fluorescence development of guanidino compounds. The separation conditions and the amounts of the guanidino compounds are the same as in Fig. 3.

examined to construct the fluorescence reactor system. A potassium hydroxide solution in the concentration range 4.0—5.0 *M* is required to obtain a maximum fluorescence intensity, corresponding to the peak height of each guanidino compound, as shown in Fig. 4; 4.0 *M* potassium hydroxide was used in the reactor system. The benzoin concentration also influences development of the fluorescence from each guanidino compound. With increasing concentration of benzoin in the range 2.0—8.0 m*M*, the fluorescence intensities of the compounds other than CR and CRN decrease slightly, but the intensities of both CR and CRN increase (Fig. 5). In the system, 4.0 m*M* benzoin was selected to obtain fairly large fluorescence intensities from the guanidino compounds except for CR and CRN, because concentrations of CR and CRN in human urine and serum are much higher than those of the other guanidino compounds. An elevated reaction temperature is required for development of the fluorescence from all the guanidino compounds tested, as shown in Fig. 6. When the reaction temperature was higher than 80°C, an irregular baseline frequently occurred on the chromatogram; this may be caused by air bubbles generated in the reaction coil. Thus a compromise temperature of 80°C was

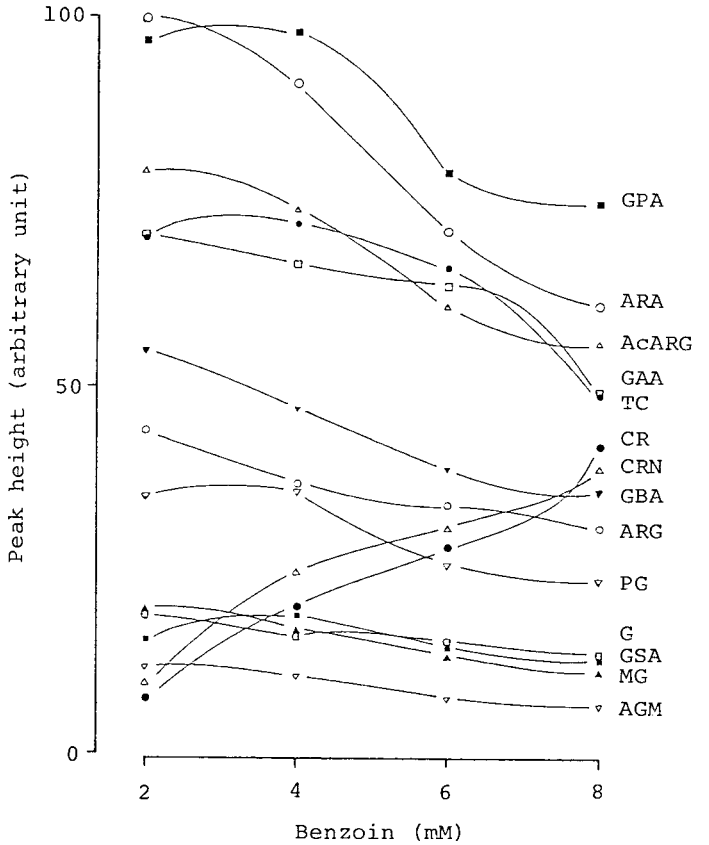


Fig. 5. Effect of benzoin concentration on the fluorescence development of guanidino compounds. The separation conditions and the amounts of the guanidino compounds are the same as in Fig. 3.

used for the reaction in the reactor system, and this temperature was maintained by using the same water-bath as that used for the column. In order to attain intense fluorescence of the benzoin derivatives, it was necessary to adjust the pH of the mixture after the benzoin reaction to a weakly alkaline pH by adding a pertinent acidic salt. When a mixture of sodium dihydrogen phosphate (1.6 *M*) and Tris (1.0 *M*) was added to the reaction mixture, the pH of the final eluate was sufficiently lowered and ranged from 8.5 to 10.7 during the operation of the HPLC analyser, even with stepwise gradient elution of the pH in the mobile phase. At these pH values the derivatives of all the guanidino compounds tested fluoresce at least five times as strongly as those without adjustment of the pH.

A linear relationship was observed between the peak height and the amount of each guanidino compound in the injection volume (100 $\mu$l) up to at least 8 nmol for monosubstituted guanidino compounds, 100 nmol for CR and 8 $\mu$mol for CRN under the established conditions of the fluorescence reactor system. The lower limits of detection are 5 pmol for ARA and GPA, 8 pmol for TC, GAA and AcARG, 12 pmol for GBA, ARG and PG, 30 pmol for GSA,

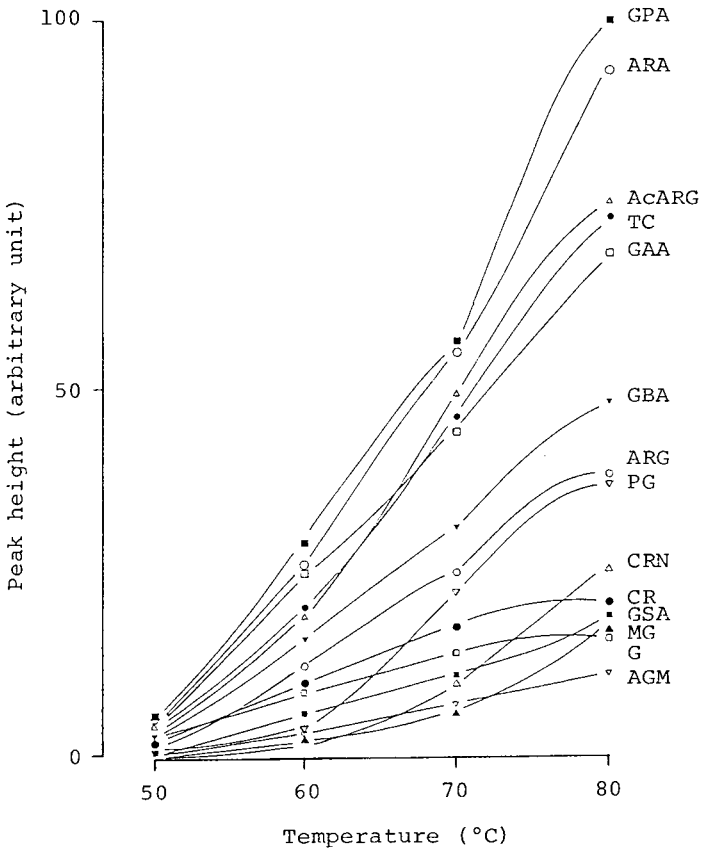


**Fig. 6. Effect of reaction temperature on the fluorescence development of guanidino compounds. The separation conditions and the amounts of the guanidino compounds are the same as in Fig. 3.**

G and MG, 50 pmol for AGM, 1 nmol for CR, and 20 nmol for CRN. The limit is defined as the amount in 100 $\mu$l of injection volume giving a peak height of twice the noise level.

*Analysis of biogenic guanidino compounds in human urine and serum*

Typical chromatograms obtained by the HPLC analyser are shown in Fig. 7 for a normal urine and in Fig. 8 for serum from a normal subject and from a patient with chronic renal failure. The guanidino compounds in the samples were identified on the basis of their retention times in comparison with standard compounds and also by co-chromatography of the standards and samples with different elution of the mobile phase, i.e. using a lower pH and/or ionic strength than those used for the recommended procedure. This elution provided better separation of the guanidino compounds but their elution was delayed. In addition, their retention times were not affected by the biological matrix of the samples. Unidentified peaks were also observed in the chromatograms, though the benzoin reaction works only on compounds with a guanidino moiety [20, 21]. The peaks are probably from native fluorescent

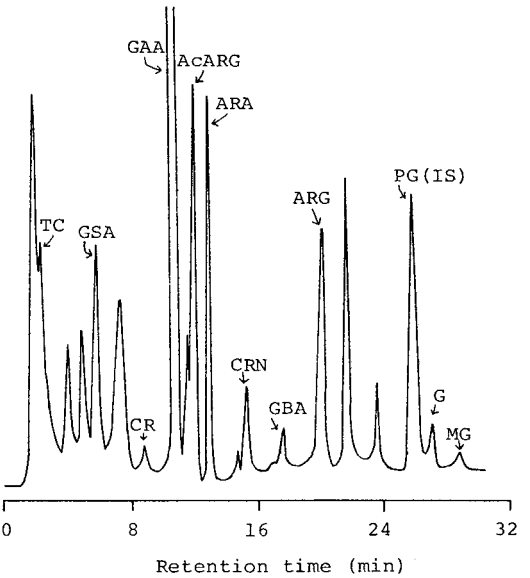


Fig. 7. Chromatogram of guanidino compounds in urine from a healthy man. Phenyl guanidine (6 nmol) was spiked in 100 $\mu$l of urine as an internal standard and the sample was treated as described in Experimental.

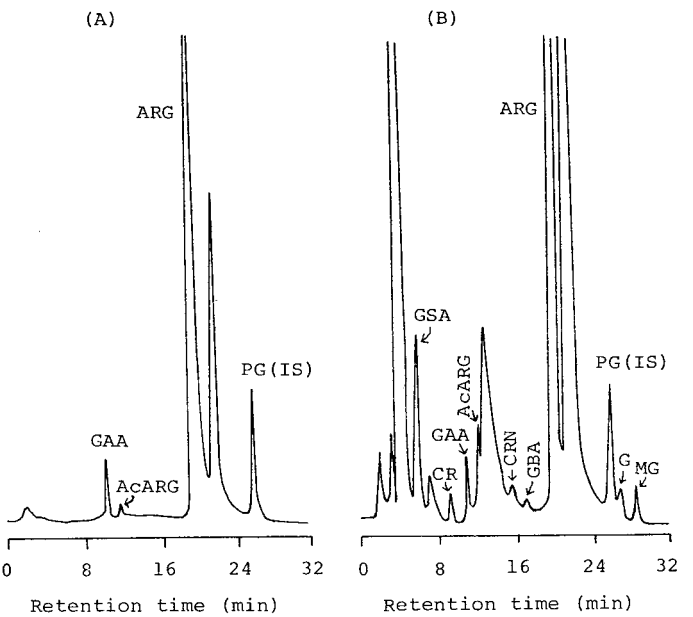


Fig. 8. Chromatograms of guanidino compounds in sera from (A) a healthy man, and (B) a uremic patient. Phenylguanidine (625 pmol) was spiked in 100 $\mu$l of each serum and the samples were treated as described in Experimental.

substances (including drugs administered to the patient during therapy), and/or unknown guanidino compounds and/or peptides with an arginyl residue which were not removed by deproteinization. Oligopeptides with one or two arginyl residues such as tuftsin, angiotensins I, II and III, kallidin, bradykinin, luteinizing-hormone releasing hormone, substance P and neurotensin, were examined for their retention times in HPLC. These peptides were co-eluted at a retention time of approximately 22 min next to the elution of ARG. Therefore, in the chromatogram of the patient serum, one of the unkown big peaks around 22 min may be ascribed to some peptides characteristic of uremia since several peptides such as middle molecule substances also have been suspected of contributing to the toxic manifestation in uremic syndrome [26, 27]. Other unidentified peaks in the chromatograms were not studied.

For a precise and facile quantification of the guanidino compounds, phenylguanidine was used as an internal standard. The calibration curves for both urine and serum, which were made by plotting the ratios of net peak heights of the spiked guanidino compounds against the peak height of the internal standard, were linear in the relationship between the ratios and amounts of the guanidino compounds added to urine or serum. The calibration curves are

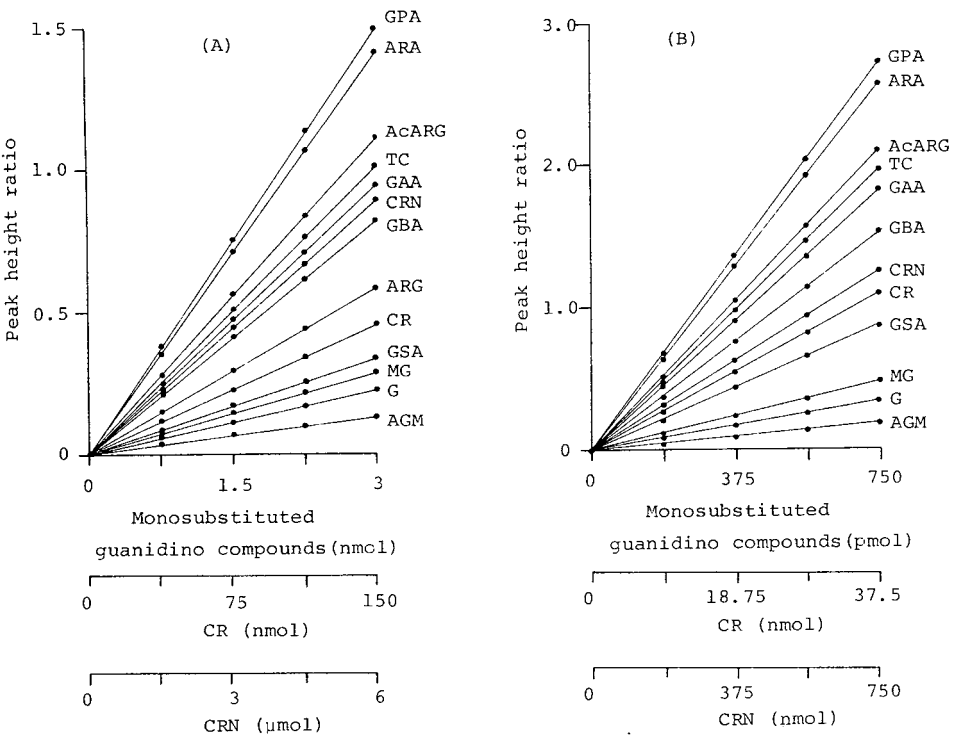


Fig. 9. Calibration curves for guanidino compounds in (A) urine, and (B) serum. (A) To 100-μl portions of a pooled urine, 0—3 nmol of the monosubstituted guanidino compounds, 0—150 nmol of CR, 0—6 μmol of CRN and 6 nmol of phenylguanidine (IS) were added. (B) To 100-μl portions of a pooled serum, 0—750 pmol of the monosubstituted guanidino compounds, 0—37.5 nmol of CR, 0—750 nmol of CRN and 625 pmol of phenylguanidine (IS) were added. The curve for ARG was not constructed because the peak of ARG in serum was too high compared with that of the internal standard.

TABLE I

CONCENTRATIONS OF GUANIDINO COMPOUNDS* IN URINE FROM HEALTHY MEN

| Age | Guanidino compound (μmol/day) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | TC | GSA | CR | GAA | AcARG | ARA | CRN | GBA | ARG | G | MG |
| 26 | 11.34 | 24.94 | N.D.** | 323.00 | 17.51 | 8.50 | 11333 | 7.36 | 13.04 | 11.34 | N.D. |
| 25 | 6.66 | 28.34 | 250.00 | 516.25 | 15.00 | 15.84 | 15834 | 17.50 | 30.84 | 18.34 | 10.00 |
| 23 | N.D. | 29.41 | N.D. | 340.00 | 25.50 | 7.94 | 17000 | 14.74 | 17.00 | 18.14 | 6.80 |
| 23 | 16.15 | 22.80 | N.D. | 544.67 | 10.45 | 6.65 | 13300 | 8.55 | 17.10 | 15.20 | 5.70 |
| 24 | N.D. | 22.40 | 210.00 | 583.00 | 26.60 | 12.60 | 13066 | 7.94 | 27.02 | 15.82 | 6.54 |
| 28 | N.D. | 16.44 | 316.60 | 437.00 | 11.40 | 5.39 | 8866 | 4.75 | 16.15 | 8.24 | 3.80 |
| 34 | N.D. | 42.54 | 513.34 | 366.63 | 21.26 | 23.46 | 24934 | 5.14 | 28.60 | 16.14 | 8.80 |
| 28 | N.D. | 38.00 | 570.00 | 709.30 | 25.33 | 11.40 | 17732 | 6.33 | 30.40 | 17.73 | 7.60 |
| 29 | N.D. | 9.37 | N.D. | 405.30 | 3.87 | 8.70 | 11600 | 5.80 | 7.73 | 17.40 | 9.67 |
| Mean ± S.D. | 11.38 ±4.75 | 26.06 ±10.10 | 371.98 ±160.76 | 477.48 ±135.00 | 17.42 ±7.90 | 11.16 ±5.61 | 14851 ±4742 | 8.67 ±4.45 | 20.87 ±8.46 | 15.37 ±3.42 | 7.36 ±2.09 |

*Other guanidino compounds could not be successfully determined because of their minute amounts.
**N.D. = not detected.

TABLE II

CONCENTRATIONS OF GUANIDINO COMPOUNDS* IN SERA FROM HEALTHY MEN

| Age | Guanidino compounds (nmol/ml) | | | | |
|---|---|---|---|---|---|
| | CR | GAA | AcARG | G | MG |
| 26 | 11.22 | 1.61 | 0.92 | N.D.** | N.D. |
| 29 | 23.21 | 2.18 | 1.02 | N.D. | N.D. |
| 34 | 18.03 | 2.45 | 0.80 | N.D. | N.D. |
| 22 | 32.86 | 2.50 | 0.92 | N.D. | 0.46 |
| 24 | 16.43 | 3.24 | 1.14 | N.D. | N.D. |
| 25 | 8.19 | 3.17 | 0.72 | 0.69 | 0.46 |
| 24 | 19.18 | 2.73 | 0.98 | N.D. | 0.38 |
| 26 | 9.63 | 2.51 | 0.79 | 0.31 | 0.77 |
| Mean ±S.D. | 17.34 ±8.11 | 2.55 ±0.52 | 0.91 ±0.14 | 0.50 ±0.27 | 0.52 ±0.17 |

*Other guanidino compounds could not be successfully determined because of their minute amounts, except for ARG.
**N.D. = not detected.

shown in Fig. 9. The correlation coefficients ($r$) of all the curves were more than 0.997 and no change of the slopes in the graphs was observed depending on the urine or serum used.

The recovery of each guanidino compound added to 100 μl of urine in amounts of 3—6 nmol for monosubstituted guanidino compounds, 300 nmol for CR, and 6 μmol for CRN was in the range 95—105% (C.V. 6.2%); when the compounds were added to 100 μl of serum in the amounts of 0.75 nmol for monosubstituted guanidino compounds, 37.5 nmol for CR and 750 nmol for CRN, the recoveries were in the range 97—104% (C.V. 6.3%). The values are the average of ten independent analyses.

TABLE III

CONCENTRATIONS OF GUANIDINO COMPOUNDS* IN SERA FROM PATIENTS WITH CHRONIC RENAL FAILURE

| | Guanidino compounds (nmol/ml) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | GSA | CR | GAA | AcARG | CRN | GBA | G | MG |
| 1 | 4.64 | 40.50 | 3.05 | 2.56 | 325.87 | 0.19 | 2.25 | 2.82 |
| 2 | 13.90 | 31.25 | 2.07 | 1.71 | 506.75 | N.D.** | 2.25 | 5.19 |
| 3 | 22.21 | 53.47 | 1.92 | 1.65 | 451.34 | N.D. | 2.46 | 6.66 |
| 4 | 6.95 | 121.42 | 1.97 | 1.57 | 377.45 | N.D. | 1.97 | 3.03 |
| 5 | 15.53 | 80.21 | 1.60 | 1.75 | 599.00 | N.D. | 2.66 | 7.21 |
| 6 | 4.13 | 104.25 | 1.06 | 2.27 | 585.03 | N.D. | 2.41 | 2.89 |
| 7 | 25.20 | 182.03 | 2.12 | N.D. | 677.22 | N.D. | 2.62 | 3.06 |
| 8 | 3.39 | 64.21 | 2.18 | 0.57 | 200.00 | 0.40 | 2.07 | 2.18 |
| 9 | 25.51 | 64.67 | 2.39 | 1.80 | 646.67 | 0.30 | 3.03 | 5.17 |
| 10 | 20.05 | 86.02 | 2.58 | 2.34 | 860.21 | 0.41 | 2.21 | 3.69 |
| Mean | 14.15 | 82.80 | 2.09 | 1.80 | 522.95 | 0.33 | 2.39 | 4.19 |
| ±S.D. | ±8.89 | ±44.46 | ±0.54 | ±0.58 | ±192.12 | ±0.10 | ±0.31 | ±1.75 |

*Other guanidino compounds could not be successfully determined because of their minute amounts, except for ARG.
**N.D. = not detected.

The precision of the method for the determination of the biogenic guanidino compounds was also examined by performing five analyses separately on pooled urine and serum. The coefficients of variation for the asay of the compounds [the compound and concentration (nmol/ml) in parentheses] in urine were 5.3% (GSA, 15.6), 6.0% (CR, 451.0), 8.1% (GAA, 312.0), 3.1% (AcARG, 10.9), 4.6% (ARA, 6.6), 9.1% (CRN, 6393.2), 1.1% (GBA, 5.4), 3.1% (ARG, 18.5), 3.1% (G, 10.2) and 3.5% (MG, 3.1), and for the assay of the compounds in serum were 3.9% (GSA, 7.0), 5.8% (CR, 121.4), 8.4% (GAA, 2.0), 9.6% (AcARG, 1.6), 3.2% (CRN, 377.5), 3.6% (G, 2.0) and 3.8% (MG, 3.0).

The concentrations of the guanidino compounds in urine and serum samples from healthy men, and sera from patients with chronic renal failure in maintenance with hemodialysis, were determined by this method (Tables I—III). Relatively many guanidino compounds were identified in the urine compared with those in sera from healthy men. Higher concentrations of GSA, CR, CRN, G and MG than those in the normal sera were observed in the patient sera. GAA and AcARG were present in the normal sera but at levels not significantly different from those in the patient sera. The mean values for the individual guanidino compounds are in good agreement with published data [17, 19].

The present method for the automatic determination of guanidino compounds is rapid and gives satisfactory sensitivity in the analysis of physiological fluids; the sensitivity permits use of less than 100 $\mu$l of urine and serum from normal subjects or uremic patients, and this HPLC analyser will be an invaluable tool in clinical studies of guanidino compounds in uremic syndrome.

ACKNOWLEDGEMENTS

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# THE USE OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY IN THE ISOLATION AND CHARACTERISATION OF MOUSE AND RAT EPIDERMAL GROWTH FACTORS AND EXAMINATION OF APPARENT HETEROGENEITY

JOHN A. SMITH*, JACK HAM*, DEREK P. WINSLOW, MICHAEL J. O'HARE and PHILIP S. RUDLAND

*Ludwig Institute for Cancer Research (London Branch), Royal Marsden Hospital, Sutton, Surrey SM2 5PX (U.K.)*

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## SUMMARY

Various epidermal growth factor preparations obtained from the mouse submaxillary gland (mEGF), have been separated into a number of components by reversed-phase high-performance liquid chromatography (HPLC). It is shown here, however, that when the mEGF is isolated rapidly, using only reversed-phase HPLC for trace enrichment and high-resolution fractionation, it is a single molecular species as determined with several ion-pairing solvent systems, provided that proteolysis is inhibited in the original extracts. This indicates that the minor components of mEGF that have been reported are artefacts formed during the isolation procedure, and are of no biological significance. The products of deliberate mild degradation of mEGF are shown to produce similar chromatographic profiles to those observed in samples of mEGF prepared in the absence of proteolytic inhibitors. Rat EGF has been isolated in a similar manner, and is shown to share many of the properties of the major tryptic digestion product of mEGF.

## INTRODUCTION

Since first being isolated [1] epidermal growth factor (EGF) has been shown to have effects on a wide variety of cells in culture [2]. In spite of its importance in the study of control of cell proliferation, it is only readily available from the mouse [3], although lengthy schemes for purification have been published for the rat [4] and human [5] factors. Mouse EGF (mEGF)

*Present address: Department of Peptide Chemistry, National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, U.K.

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has previously been shown to chromatograph on a reversed-phase high-performance liquid chromatographic (HPLC) system, which is capable of a high degree of resolution [6] and has been separated into a number of components [7—9]. However, since HPLC is sufficiently efficient to separate oxidation products of peptides [10], it is necessary to distinguish between naturally occurring variants of a protein, which might be of biological interest, and artefactually generated heterogeneity, which appears to be the cause of relaxin variants [11].

We have examined the heterogeneity of mEGF preparations, and show that mouse and rat EGFs each comprise a single molecular species when prepared rapidly.

## EXPERIMENTAL

### *Materials*

Epidermal growth factor was receptor-grade material from Collaborative Research (Waltham, MA, U.S.A.). Pepstatin A was obtained from Sigma (St. Louis, MO, U.S.A.), bovine serum albumin (BSA) was Fr V reagent grade from Miles Labs. (Stoke Poges, U.K.). Acetonitrile was HPLC S-grade from Rathburn Chemicals (Walkerburn, U.K.).

### *Animals*

Submaxillary glands were obtained from adult male C57 black mice, or from adult male Sprague—Dawley rats weighing between 150 and 250 g which were stimulated with testosterone as described by Moore [4].

Submaxillary glands were extracted in 0.05 *M* acetic acid [3] or with 0.155 *M* sodium chloride made to pH 2.1 with hydrochloric acid, supplemented with 10 μg/ml Pepstatin A. The extract was frozen and thawed, and centrifuged at 60,000 *g* for 1 h at 4° C. The resulting supernatant was collected by decantation through glass wool to remove floating fat.

### *Radioimmunoassay of mEGF*

For the preparation of $^{125}$I-labelled EGF 10 μg of mEGF (Collaborative Research) was labelled with 0.5 mCi of $Na^{125}I$ to a specific radioactivity of between 30 and 40 μCi/μg by the method of Hunter and Greenwood [12] using 50 μg of chloramine T as oxidant and a reaction time of 1 min. The active, labelled hormone was eluted from a column (12 ml) of Sephadex G-25 equilibrated in 0.05 *M* phosphate buffer, pH 7.5, containing 0.5% (w/v) BSA.

The method of radioimmunoassay used was as previously described [13], except that the 0.2 ml of 25% (v/v) lamb serum in phosphate-buffered saline (PBS) was added post-incubation. The incubation period was 60—70 h at 4° C. The antiserum used was obtained commercially (Collaborative Research) and used at a final dilution of 1:15,000.

### *Receptor-binding assay for mEGF*

The procedure used was as previously described [13]. The target cells were a squamous carcinoma head and neck cell line LICR-LON-HN-1 [14] which has been shown to possess membrane EGF receptors, having $7.5 \cdot 10^5$ sites per cell [15].

*Bioassay*

Rama 27, a clonal line of cells from the stroma of normal rat mammary gland [16] was used to assay the ability of EGF to increase DNA synthesis. Rama 27 cells were plated at a density of $2 \cdot 10^5$ cells per well in Sterilin 6-well plates in Dulbecco's Modified Eagles Medium (MEM) supplemented with 5% Fetal Calf Serum. After 24 h they were washed twice and refed with Dulbecco's MEM supplemented with 250 $\mu$g/ml BSA, 48 h later they were again refed with Dulbecco's MEM supplemented with 250 $\mu$g/ml BSA, and growth factors were added. After a further 18 h, [$^3$H] thymidine was added at a concentration of 0.4 $\mu$Ci/ml (1.5 $\mu M$) and incubated for 1 h. The cells were then washed twice with PBS ($Ca^{2+}$- and $Mg^{2+}$-free), twice with ice cold 5% trichloroacetic acid and twice with cold ethanol. They were allowed to dry, incubated with 0.1 *M* sodium hydroxide at 37°C for 30 min, aliquots were taken, and counted in Instagel (Packard) in a Packard liquid scintillation counter.

*HPLC methods*

Initial fractionation using a bulk trace-enrichment technique was performed on a column 7.5 cm × 4.6 mm I.D. of Partisil 10 ODS (Whatman, Maidstone, U.K.) as described by Nice et al. [17].

Gradient reversed-phase HPLC was carried out on ODS Hypersil ($C_{18}$ bonded phase, 5 $\mu$m particle size, 9 nm pore size, Shandon Southern) or Ultrapore RPSC ($C_3$ bonded phase, 5 $\mu$m particle size, 30 nm pore size, Altex Scientific) as indicated, on a column 15 cm × 4.6 mm I.D. using 0.155 *M* sodium chloride adjusted to pH 2.1 with hydrochloric acid as primary solvent, and acetonitrile as secondary solvent, using an Altex 324-40 system.

Analytical reversed-phase HPLC was also performed, where indicated, using 0.1% (w/v) trifluoroacetic acid in water as primary solvent, and 0.1% trifluoroacetic acid in acetonitrile as secondary solvent. Retention times on the gradient reversed-phase HPLC system can vary with the age of the column and with protein load. The relative positions of standard proteins do not alter, however, so each column was regularly standardised with a number of peptides and proteins.

Unless otherwise stated, gradient reversed-phase HPLC was performed using the following programme: primary solvent alone, 5 min; gradient of 2% per min secondary solvent, 5 min; then a gradient of 0.75% per min secondary solvent. Isocratic reversed-phase HPLC by the method of Matrisian et al. [9] used a solvent system of 0.05 *M* acetic acid brought to pH 5.6 with triethylamine, with 26% acetonitrile (v/v). Flow-rates of 1 ml/min at 45°C were used for all reversed-phase HPLC separations.

Size exclusion chromatography was performed on an Altex Spherogel TSK 3000 SW column, 30 cm × 7.5 mm I.D., at a flow-rate of 0.5 ml/min in 0.1 *M* phosphate buffer, pH 6.5, containing 0.3 *M* sodium chloride and 20% (v/v) acetonitrile. The acetonitrile was used to avoid hydrophobic interactions with the stationary phase [18]. Ion-exchange HPLC was performed on an Altex Spherogel-TSK IEX-545 DEAE column, 15 cm × 6.0 mm I.D., using 0.05 *M* Tris · HCl, pH 8, in 20% acetonitrile, as primary solvent and a gradient of 10 m*M* per min of sodium chloride. Size exclusion and ion-exchange chromatography were carried out at ambient temperature. Eluted materials were

detected by UV absorbance (LDC Spectromonitor III) or endogenous tryptophan fluorescence (254/340 nm, Schoeffel FS-970).

*Modification of growth factors*

Oxidation was carried out in halide-free solution with a 1:1000 dilution of fresh 30 vol hydrogen peroxide for 30 min to 2 h at room temperature. Under these conditions, methionine residues are oxidised to the sulphoxide form [19]. Deamidation was attempted by incubation of EGF in 0.01 *M* hydrochloric acid at 37°C for 1—2 days, conditions which result in deamidation of the N-terminal asparagine in the insulin A chain [20]. Digestion of EGF with trypsin and with chymotrypsin was performed as described by Savage et al. [21]. Amino acid analyses were performed on a Biotronik LC 2000 analyser.

## RESULTS

*Rapid isolation of EGF*

The crude extract of mouse submaxillary gland was first fractionated using a previously developed trace enrichment technique described above. The EGF activity partitioned cleanly into the cut between 20% and 60% acetonitrile (Fig. 1). The acetonitrile was evaporated in a stream of nitrogen, and the active fraction applied to the 5-$\mu$m $C_{18}$ column. On eluting with a gradient of acetonitrile, well defined UV absorbing peaks, with a single major peak of EGF

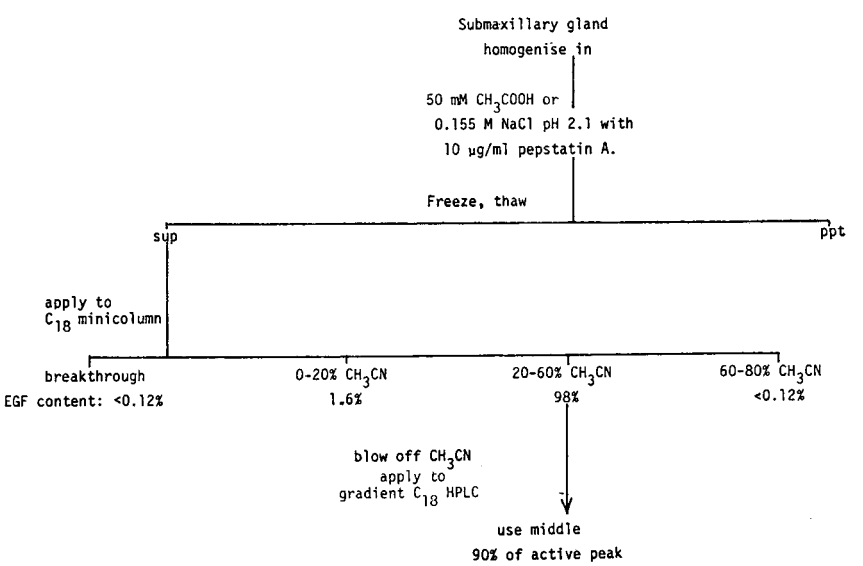


Fig. 1. Procedure for preparation of EGF. The initial homogenate was frozen, and thawed, and centrifuged at 60,000 *g* for 1 h at 4°C. The supernatant was decanted through glass wool to remove floating fat, before application to the minicolumn. Of the EGF present in the original extract 98% was recovered in the fraction retained in the presence of 0.155 *M* sodium chloride, pH 2.1, 20% acetonitrile, but eluted by 0.155 *M* sodium chloride, pH 2.1, containing 60% acetonitrile. This fraction, after removal of the acetonitrile, was subjected to reversed-phase HPLC, as shown in Figs. 2 and 3, to yield pure EGF.

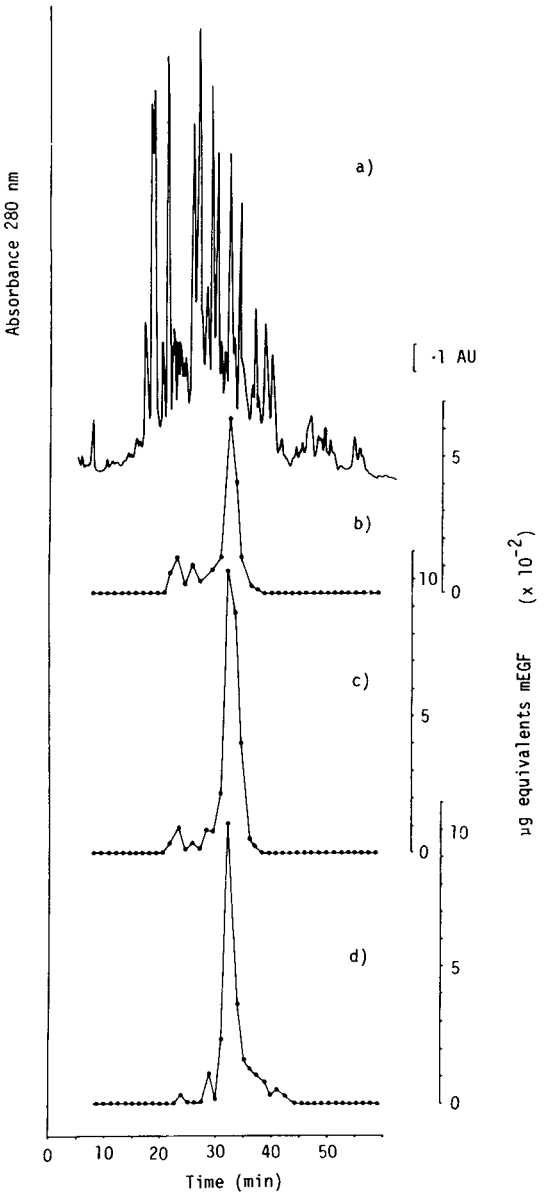


Fig. 2. Elution of mouse EGF (20 to 60% acetonitrile minicolumn cut) from $C_{18}$ reversed-phase column. A sample containing 20 mg of protein was obtained by elution of an acetic acid mouse submaxillary gland extract from a $C_{18}$ minicolumn as described in Fig. 1. The acetonitrile content of the sample was reduced by passing a stream of nitrogen over it prior to loading on a $C_{18}$ reversed-phase gradient HPLC column. Elution was carried out using an acetonitrile gradient of 0.75% per min, and a flow-rate of 1 ml/min (gradient shape is the same as that shown in Fig. 4a). Fractions (1 ml) were collected. Aliquots of 10 $\mu$l were taken from each fraction and diluted as required for assay; (a) absorbance at 280 nm; (b) radioimmunoassay; (c) radioreceptor assay; (d) bioassay. The combined results are shown for a single chromatogram.

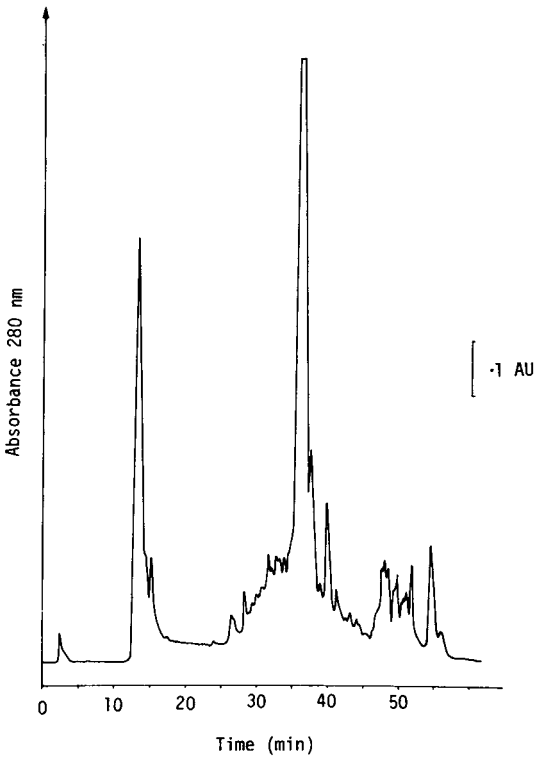


Fig. 3. Absorbance profile of a 20 to 60% acetonitrile minicolumn cut of mouse EGF eluted from $C_{18}$ reversed-phase column. Conditions were as described in Fig. 2, except that the original extraction was performed with 0.155 *M* sodium chloride at pH 2.1. The major UV-absorbing peak contained the EGF activity.

activity as assayed by radioimmunoassay, by radioreceptor assay, or by bioassay on a mammary stromal cell line were observed (Fig. 2). A much cleaner extract was obtained if the initial extraction was performed with 0.155 *M* sodium chloride, adjusted to pH 2.1 with hydrochloric acid, and pepstatin added, rather than with 0.05 *M* acetic acid (Fig. 3). A similar result was obtained for the rat submaxillary gland, although the active peak eluted somewhat earlier (Fig. 4). The active fraction was reapplied to the column, and eluted with a shallower gradient (Fig. 4c). A number of minor UV absorbing contaminants were removed from the active peak at this stage. The overall recovery of highly purified EGF from the original extract was approximately 90%. Much of the remaining 10% could be recovered by rerunning fractions at the edges of the active peak, but this was not really worthwhile. Provided some purified material was available to act as a chromatographic standard, the whole procedure, involving the purification of EGF from 20 g of submaxillary gland could be completed within a working day.

*Identification of mEGF*

The mouse EGF, prepared as above, co-chromatographs with mouse EGF purchased from Collaborative Research on both $C_{18}$ (ODS) and $C_3$ (RPSC)

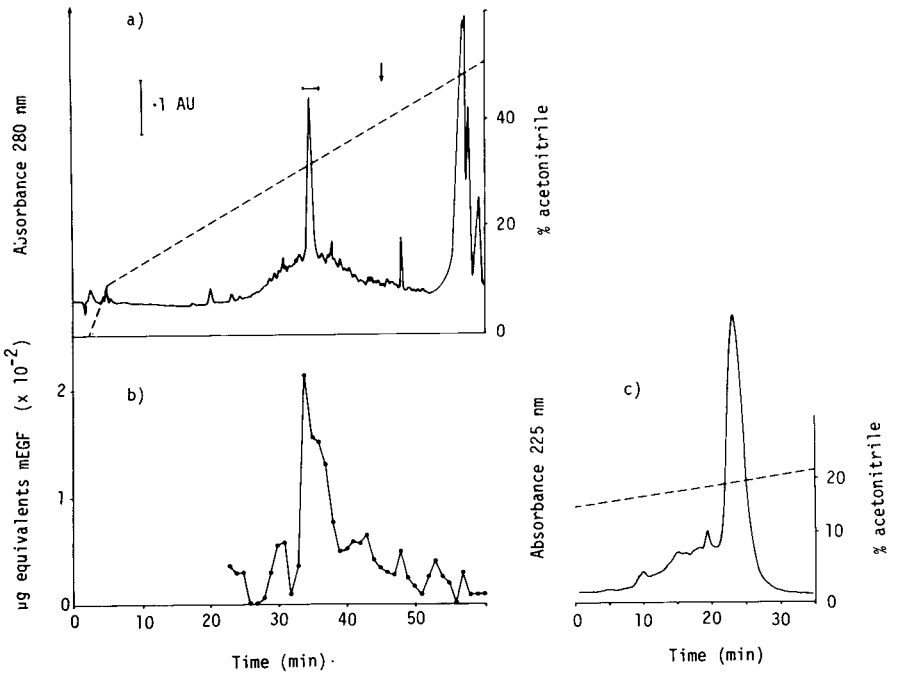


Fig. 4. Elution of rat EGF from $C_{18}$ reversed-phase column. Conditions were as described in Fig. 2 except that the rat preparation was eluted from the minicolumn in a cut of 15 to 40% acetonitrile before application to the gradient column. Arrow shows elution position of mouse EGF. (a) Absorbance profile at 280 nm and (b) radioimmunoassay are shown. (c) The active fraction from (a) and (b) was rechromatographed with a shallower gradient of acetonitrile 0.33% (v/v) per min and flow-rate of 1 ml/min. Absorbance profile at 280 nm is shown, and the major peak contained all EGF immunoactivity. The dashed lines show the gradient of acetonitrile applied to the column.

reversed-phase systems, by ion-exchange HPLC, and by gel chromatography on TSK 3000 SW. The HPLC-produced material and the commercial (conventionally purified) EGF also yielded the same peptides on digestion with trypsin, and on digestion of the performic acid-oxidised tryptic fragment with chymotrypsin, and had the same amino acid composition.

On all high-performance chromatographic systems, our HPLC-purified mouse EGF ran as a single peak unlike material prepared by conventional chromatographic methods [7, 9]. It chromatographed as $\alpha$ EGF on the systems of Matrisian et al. [9] and Burgess et al. [8] and was at least 99% homogeneous (Fig. 5 a and b). Peaks corresponding to the $\beta$-form described by these workers (and other components of the conventionally purified materials) were not seen (Fig. 5a). A small amount of a new product is, however, produced on prolonged storage. This elutes nearly 2 min earlier on the reversed-phase systems, and its content could be increased by treatment with an oxidizing agent (Fig. 5c). The single oxidation product was compatible with the existence of a single methionine residue per molecule. No separate products were observed under conditions which we know to be effective at engendering deamidated products in other proteins, although degradation of the molecule was observed on prolonged treatment with acid.

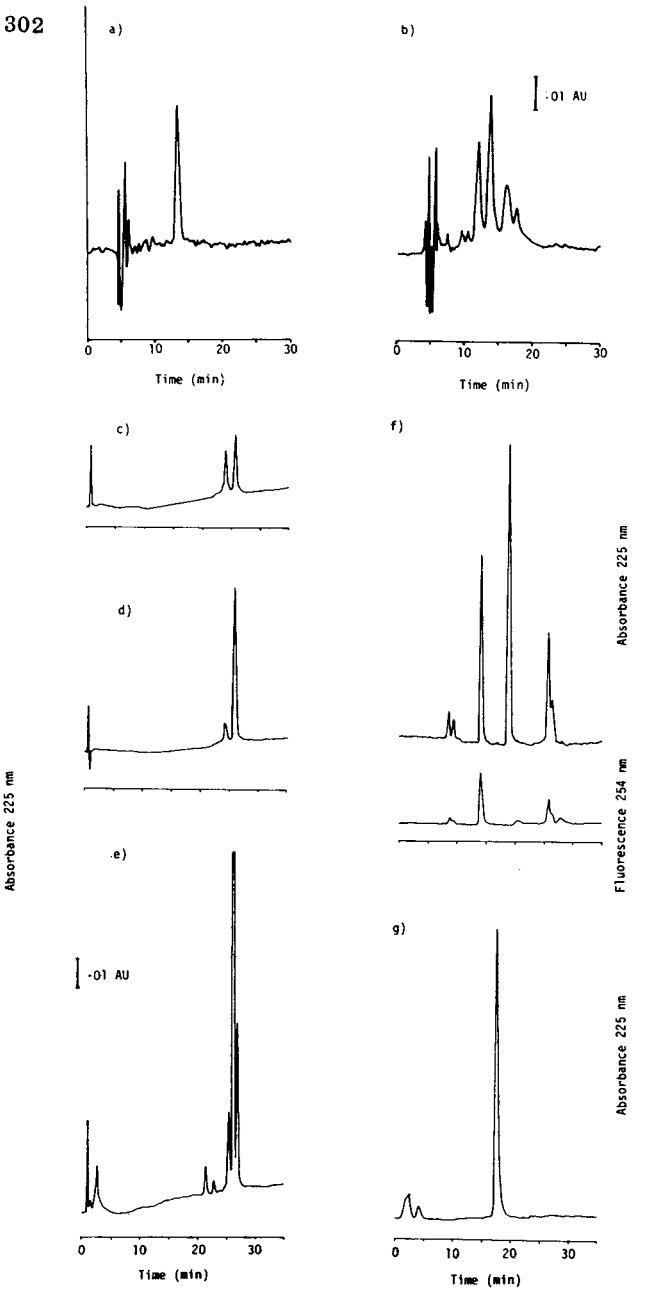


Fig. 5. Isocratic $C_{18}$ reversed-phase chromatograhy, according to the method of Matrisian et al. [9] of (a) HPLC-prepared mouse EGF and (b) culture grade mouse EGF from Collaborative Research. The first peak of (b) eluting at 12 min is absent from receptor-grade EGF. (c—g) $C_3$ Reversed-phase chromatography of (c) mouse EGF oxidised as described in Experimental; (d) mouse EGF as found after preparation by HPLC and prolonged storage; (e) mouse EGF after limited digestion with chymotrypsin; (f) mouse EGF after digestion with trypsin; lower trace shows intrinsic fluorescence due to tryptophan (measured at 254 nm), which is present in the C-terminal pentapeptide, eluting at 14 min, and in the remaining intact EGF eluting at 26 min, but is absent from the T-EGF, eluting at 18 min; (g) rat EGF. Chromatography was performed using a gradient of acetonitrile in the presence of 0.1% (w/v) trifluoroacetic acid. Chymotrypsin digestion was performed as described by Savage et al. [21] for 1 h at 37°C, but using intact mouse EGF.

A complex pattern similar to that seen in conventional preparations [7, 9] was observed if intact HPLC-purified EGF was briefly digested with chymotrypsin. These products were all eluted within 1 min of the major peak, with gradient elution (Fig. 5e); they may be due to the generation of molecules of EGF nicked at different sites by the protease, but still held together by disulphide bridges.

*Isolation of rat EGF*

Rat EGF was prepared in a similar manner to mouse EGF, except that the initial minicolumn cut was 15 to 40% in acetonitrile, as the rat activity eluted earlier from the reversed-phase column (at 20% acetonitrile compared with 26% acetonitrile). The lower hydrophobicity of the rat molecule is primarily due to its lack of tryptophan, as indicated by the absence of intrinsic fluorescence at 254 nm. In this respect, the rat EGF resembled T-EGF, the tryptic digestion product of mouse EGF, in which the C-terminal pentapeptide, containing both tryptophan residues, is absent [21]. Indeed, T-EGF chromatographed close to rat EGF on the reversed-phase HPLC (Fig. 5f and g). Mouse EGF, rat EGF and T-EGF were found to have a similar molecular weight by gel filtration (Fig. 6). However, if the organic modifier (acetonitrile) was omitted, the mouse EGF

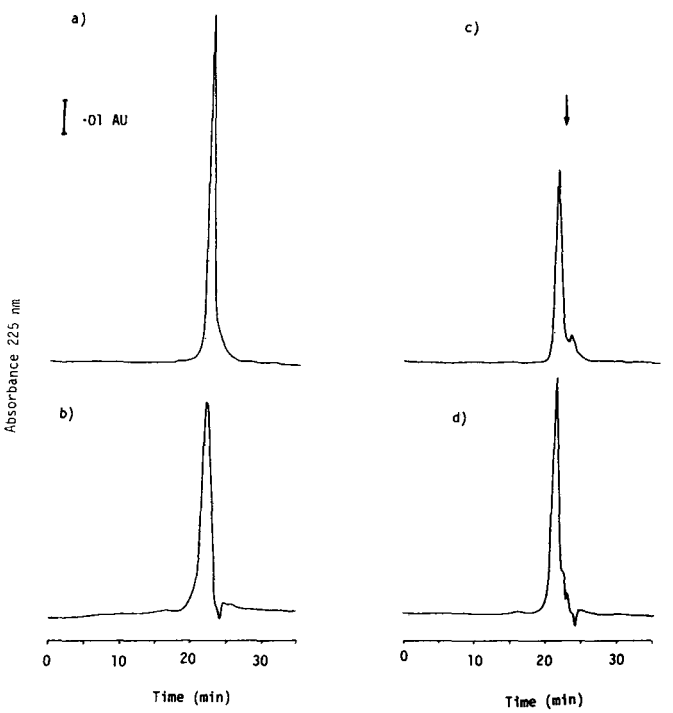


Fig. 6. Size exclusion chromatography on TSK 3000 SW of (a) mouse EGF prepared by HPLC; (b) mouse EGF obtained commercially; (c) rat EGF; and (d) T-EGF, obtained by $C_3$ reversed-phase chromatography of a trypsin digest of mouse EGF. Conditions were as described in Experimental: flow-rate was 0.5 ml/min. Protein load was 5 $\mu$g. Arrow in (c) shows elution position of mouse EGF. Standard proteins eluted as follows: bovine serum albumin (68 Kd) 15.9 min; $\alpha$-lactalbumin (14.2 Kd) 19.2 min; cytochrome C (11.7 Kd) 21.1 min; human calcitonin (3.4 Kd) 22.1 min; *l*-tryptophan (0.2 Kd) 25.6 min.

TABLE I

AMINO ACID ANALYSES OF EGF

Quantities are presented as number of residues per molecule EGF; the new data is normalised on the basis of Arg = 4.0. No correction has been made for losses of serine and threonine during hydrolysis, or for oxidation of the sulphur-containing amino acids.

| | Mouse* | Mouse* sequence | Mouse (found) | Rat (found) | Rat** | Mouse* T-EGF |
|---|---|---|---|---|---|---|
| Asp | 8—9 | 7 | 6.3 | 6.4 | 7 | 7 |
| Thr | 2 | 2 | 1.4 | 1.5 | 3 | 2 |
| Ser | 6—7 | 6 | 5.1 | 3.8 | 5 | 6 |
| Glu | 3—4 | 3 | 2.8 | 3.4 | 5 | 2 |
| Pro | 2 | 2 | 1.4 | 1.3 | 2 | 2 |
| Gly | 6—7 | 6 | 6.7 | 8.4 | | 6 |
| Ala | 0 | 0 | 0 | 0 | 1—2 | 0 |
| Cys*** | 6 | 6 | 4.2 | 4.0 | 6 | 6 |
| Val | 2 | 2 | 2.4 | 1.1 | 2 | 2 |
| Met | 1 | 1 | 0.9 | 0.5 | 1 | 1 |
| Ile | 2 | 2 | 1.8 | 1.3 | 2 | 2 |
| Leu | 4 | 4 | 3.8 | 3.0 | 4 | 3 |
| Tyr | 5 | 5 | 5.3 | 2.4 | 3 | 5 |
| Phe | 0 | 0 | 0 | 0 | 0—1 | 0 |
| Lys | 0 | 0 | 0 | 0 | 2—3 | 0 |
| His | 1 | 1 | 0.9 | 0.9 | 1—2 | 1 |
| Arg | 4 | 4 | 4.0 | 4.0 | 4 | 3 |
| Tryp§ | 2 | 2 | 2 | 0 | 2 | 0 |

*From ref. 21.
**From ref. 4.
***Cysteine content was measured as cysteic acid.
§Tryptophan content was calculated from the intrinsic fluorescence at 254 nm.

was retained by the column, eluting near tryptophan (data not shown), while the rat EGF and T-EGF are unaffected; this was assumed to be due to hydrophobic interactions of the C-terminal end of the mouse EGF with the column packing in the absence of the organic solvent.

The amino acid analysis of the rat EGF showed it to be related to mouse EGF, notably by the absence of phenylalanine, alanine and lysine (Table I). This is in contrast with an earlier report of the composition of rat EGF [4], as is the observation, referred to above, of its lack of tryptophan. The contents of the other amino acids are similar in rat and mouse EGF, and to previously reported results; rat EGF has a significantly reduced amount of tyrosine. However, ion-exchange HPLC shows the rat EGF to be significantly less acidic than either mouse EGF, or its tryptic residue (Fig. 7). Similarly, chromatography of the chymotryptic digestion products of rat EGF and T-EGF did not show great homology (Fig. 8), probably because of the lower tyrosine content of the former. In its activity, however, the rat EGF is identical to mouse EGF, whether measured by stimulation of thymidine into DNA in rat mammary fibroblasts, by receptor binding, or by radioimmunoassay.

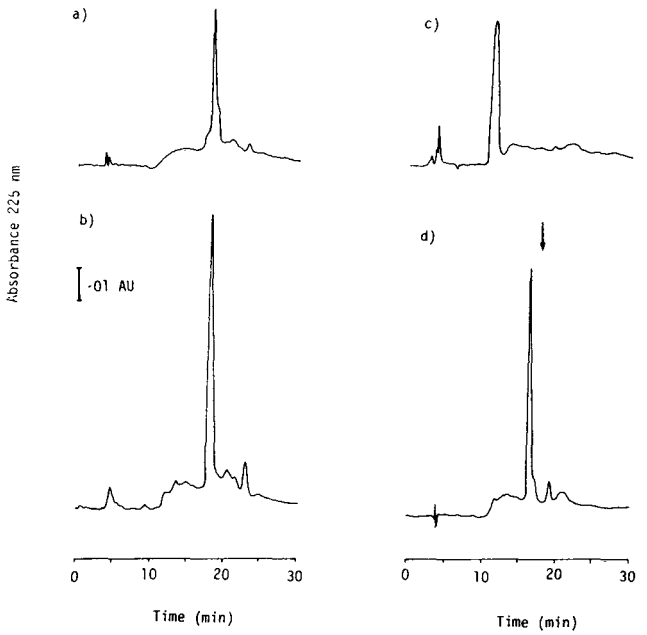


Fig. 7. Elution by ion-exchange chromatography (DEAE-TSK) HPLC of (a) mouse EGF obtained commercially; (b) mouse EGF prepared by HPLC; (c) rat EGF; and (d) T-EGF. Conditions were as described in Experimental, salt gradient was 10 m*M* per min, flow-rate 1 ml/min, protein load was 5 μg. No attempt has been made to correct for the variable background absorbance at 225 nm which is invariably encountered in the system at this sensitivity. Arrow in (d) shows elution position of intact mouse EGF.

## DISCUSSION

The existence of more than one species of active mEGF has been known for some time; a well characterised proteolytic derivative was described by Savage and Cohen [3]. Reversed-phase HPLC revealed in addition a derivative which is separable by any of the systems used here [7], which appears to be due to the oxidation of the methionine residue, and a number of other derivatives which do not separate with 0.155 *M* sodium chloride—hydrochloric acid but which are resolved in the presence of the ion-pairing reagents trifluoroacetic acid and heptafluorobutyric acid [7, 8] and with a triethylamine—acetic acid solvent system [9]. These latter components separate within a narrow concentration range of organic modifier (0.75% acetonitrile), and behave similarly to derivatives generated by the action of chymotrypsin. Since they are not found in the mEGF prepared rapidly in the presence of pepstatin, the suggestion is that, when present, they have been formed by protease action during isolation. It is interesting to note in this regard that when electrophoresed under denaturing conditions the β EGF of Matrisian et al. [9] runs with a smaller apparent molecular weight than the α form. It appears, then, that only a single form of mEGF exists in the submaxillary gland in vivo.

Similarly, only one form of EGF was obtained from the rat submaxillary gland. The question remained whether the rat EGF as described above was the form found in vivo, or whether it was a natural digestion product related to

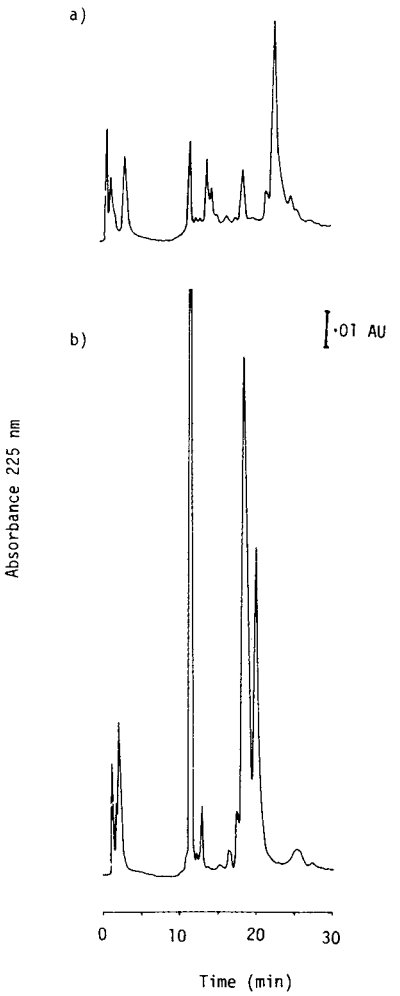


Fig. 8. Elution by $C_3$ reversed-phase HPLC of chymotryptic peptides of (a) rat EGF oxidised by performic acid; and (b) T-EGF oxidised by performic acid. Samples were prepared as described by Savage et al. [21]. Elution conditions were as described in Fig. 2. The following peptide allocations for the mouse were deduced from amino acid composition data; 2 min, mEGF residues 38—48; 11 min, mEGF residues 1—10; 18 min, residues 11—37 min; 20 min, mEGF residues 11—29.

mouse T-EGF. No separate peak of activity from the rat preparations was found to elute at a time corresponding to greater hydrophobicity, and although the submaxillary gland is a rich source of proteases, the mouse EGF was not degraded under the conditions of isolation used here. Similar experience has been reported in the preparation of relaxin, where a family of molecules is obtained by the usual methods, but a single species is obtained when a reversed-phase system is used [11].

The rat EGF was obtained with a yield of 2 mg per 20 g of submaxillary gland from testosterone-treated males or females, and from untreated males

(untreated females were not used). The yield was comparable to that reported by Moore [4], and although substantially less than that obtained from mice, nevertheless represented an easy source of the growth factor. Previously, its purification has been lengthy, as the Biogel method used for mouse EGF was not appropriate, relying as it did on the hydrophobicity of the mouse factor. The present method allowed preparation of the EGF from either species in high yield and purity within a working day.

It is noteworthy that the apparently harsh conditions of reversed-phase HPLC did not inactivate EGF, which once purified, remained biologically and immunologically active on storage in the eluent solvent for more than 1 year at −20°C. A number of other growth factor activities have been chromatographed on the $C_3$ reversed-phase system to assess the general applicability of the method for their identification and purification. Thus fibroblast growth factor, ovarian growth factor, platelet-derived growth factor, and multiplication stimulating activity [22] chromatographed successfully, and had retention times of 3 min, 0 min, −2 and 1 min, and 0.5 min, respectively, relative to that of mouse EGF. In all of these cases activity was recovered without significant loss.

This makes it seem likely that HLPC methods, with their high resolving power for many polypeptides and small proteins [6], may be useful in the purification of novel growth factors, where their very low abundance precludes purification by conventional means alone. However, care will be required to ensure that they are not simply artefactual derivatives of existing growth factors. Of particular relevance, is the ability to compare and distinguish the EGF-related growth factors identified in cells from a number of normal and neoplastic tissues. They might be expected to be of interest in elucidating the relationship between tumours and their environment in host tissues. Regarding EGF itself, in addition to its proliferative action in vivo on the epidermis and related epithelial tissues, it also has an inhibitory effect on gastric acid secretion, and thus promotes ulcer healing [23]. Any clinical application of this effect to use EGF as an anti-ulcer agent, however, will require milligram quantities of EGF in a pure form. Reversed-phase HPLC as described here is a suitable means of producing such material.

ACKNOWLEDGEMENTS

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CHROMBIO. 1927

# SCREENING PROCEDURE FOR DETECTION OF ANTIDEPRESSANTS AND THEIR METABOLITES IN URINE USING A COMPUTERIZED GAS CHROMATOGRAPHIC—MASS SPECTROMETRIC TECHNIQUE*

HANS MAURER and KARL PFLEGER*

*Institut für Pharmakologie und Toxikologie der Universität des Saarlandes, D-6650 Homburg/Saar (F.R.G.)*

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## SUMMARY

A method for the identification of antidepressants and their metabolites in urine after acid hydrolysis is described. The acetylated extract is analysed by computerized gas chromatography—mass spectrometry. An on-line computer allows rapid detection using mass fragmentography with the masses 58, 84, 86, 100, 191, 193, 194, 205. The identity of positive signals in the reconstructed mass fragmentogram is established by a comparison of the entire mass spectra with those of standards. The mass fragmentogram, the underlying mass spectra and the gas chromatographic retention indices (OV-101) are documented.

## INTRODUCTION

Within the scope of a screening procedure for detection of psychotropic and addictive drugs and their metabolites in urine [1, 2], screening for benzodiazepines [3], butyrophenone and bisfluorophenyl neurolectics [4, 5], anti-inflammatory analgesics [6] and opioids and other potent analgesics [7] has been described; screening for phenothiazine and analogous neuroleptics and antiparkinsonian drugs is in preparation [8]. Screening for tri- and tetracyclic antidepressants is described below. Such a screening is necessary in analytical toxicology to diagnose a probable intoxication. Furthermore, antidepressants are encountered frequently in analysis, when monitoring patients who may have taken addictive drugs and simultaneously taken antidepressants therapeutically. Only a gas chromatographic—mass spectrometric technique (GC—MS) detecting a few of these drugs as parent compounds in gastric

*These results were reported in part at the Jahrestagung der Deutschen Gesellschaft für klinische Chemie, Stuttgart, F.R.G., September 23th and 24th, 1982.

0378-4347/84/$03.00 

contents or plasma after intoxications has been documented [9]. The following procedure allows rapid identification and differentiation of 21 antidepressants and their metabolites in urine after therapeutic dosage. If necessary, plasma levels of the identified drugs can be determined using a procedure (e.g. gas—liquid chromatography, high-performance liquid chromatography, radioimmunoassay) described and cited in the review article of Scoggins et al. [10].

## EXPERIMENTAL

Apparatus, sample preparation (acid hydrolysis, extraction, acetylation) and the GC—MS technique used for this study have been previously described [3, 5, 6, 11].

## RESULTS AND DISCUSSION

All investigations were carried out using the urine of man with the exception of dimetacrine, melitracene, noxiptyline, protriptyline and trazodone, which were detected (in the absence of human samples) in the urine of rats. Some of the antidepressants are excreted in urine completely metabolized and conjugated. Therefore, the conjugates were decomposed by acid hydrolysis, which can be completed more quickly than enzymatic hydrolysis. To improve their GC characteristics hydroxy and amino groups were acetylated.

The results of our investigations are shown in Table I. The mass fragmentogram with the eight proposed masses allows the detection of 21 antidepressants and their metabolites. Some of these compounds are acetylated. The retention indices were determined using a gas chromatograph combined with a flame ionisation detector and a nitrogen-sensitive flame ionisation detector with a temperature programme [3]. In our experience retention indices give preliminary indications and may be useful to gas chromatographers without a GC—MS facility and so they are given here. Furthermore, they allow one to distinguish between the isomeric hydroxy metabolites which give the same mass spectra (mass spectra Nos. 9, 24 and 26 in Fig. 1). All of the mass spectra are shown in Fig. 1 for the precise identification of the compounds. Formulae are proposed for probable structures of metabolites.

Only those metabolites that were usually found are listed. Because of individual differences in metabolism, time elapsed after administration and different routes of administration, not all metabolites given in Table I were detected in each sample. Further metabolites can be found. The mass spectra of those will be included in a forthcoming handbook [12].

Dimetacrine and its metabolites are completely decomposed by the acid hydrolysis. Thus, they must be identified in a direct extract [11] of urine.

Amitriptyline, amitriptyline-N-oxide and nortriptyline lead to common metabolites. If amitriptyline-N-oxide was taken, amitriptyline and noramitriptyline (mass spectra Nos. 1 and 4) cannot be detected. If nortriptyline was taken, amitriptyline (mass spectra Nos. 1, 2 and 3) cannot be detected.

Desipramine, imipramine and lofepramine also lead to common metabolites. If desipramine or lofepramine were taken, imipramine and hydroxyimipramine (mass spectra Nos. 27 and 29) cannot be detected. Desipramine and lofe-

TABLE I

MONITORING PROGRAMME FOR ANTIDEPRESSANTS AND THEIR METABOLITES

| Mass spectrum No. | $M^+$ | Drug/metabolite (M) | *m/e* (relative intensities) | | | | | | | | Retention index (OV-101) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 58 | 84 | 86 | 100 | 191 | 193 | 194 | 205 | |
| 01 | 277 | Amitriptyline | +++ | | | | | | | | 2204 |
| 02 | 230 | M (HO-N-oxide) | | | | | | | | | 2000 FID |
| 03 | 275 | M (HO-) | +++ | | | | | | | | 2236 |
| 04 | 305 | M (Nor-) | | | + | | | | | | 2660 |
| 05 | 303 | M (Nor-HO-) | | | + | | | | | | 2670 |
| 06 | 361 | M (Nor-di-HO-) | | | + | | | | | | 2800 |
| 07 | 232 | Amitriptyline-N-oxide | | | | | | | | | 1976 FID |
| 02 | 230 | M (HO-) | | | | | | | | | 2000 FID |
| 03 | 275 | M (Desoxo-HO-) | +++ | | | | | | | | 2236 |
| 05 | 303 | M (Desoxo-nor-HO-) | | | + | | | | | | 2670 |
| 08 | 314 | Clomipramine | +++ | | | | | | | | 2457 |
| 09 | 372 | M (HO-) 1st isomer | +++ | | | | | | | | 2806 |
| 09 | 372 | M (HO-) 2nd isomer | +++ | | | | | | | | 2906 |
| 10 | 342 | M (Nor-) | | | + | + | | | | + | 2994 |
| 11 | 400 | M (Nor-HO-) | | | + | + | | | | | 3205 |
| 12 | 308 | Desipramine | + | | + | + | + | ++ | + | | 2669 |
| 13 | 195 | M (ring) | | | | | + | + | ++ | | 1931 |
| 14 | 366 | M (HO-) | | | + | + | | + | | | 3066 |
| 15 | 295* | Dibenzepin | +++ | | | | | | | | 2465 |
| 16 | 323 | M (Nor-) | | | | + | + | + | + | | 2800 |
| 17 | 295 | M (Ter-nor-) | | | | | | + | + | | 2824 |
| 18 | 309 | M (Bis-nor-) | + | | | ++ | | | + | + | 2869 |
| 19 | 294 | Dimetacrine | ++ | | +++ | | | | + | | 2313 |
| 20 | 209 | M (ring) | | | | | + | + | +++ | | 1906 |
| 21 | 249 | M (N-oxide) | | | | | + | ++ | ++ | | 2020 |

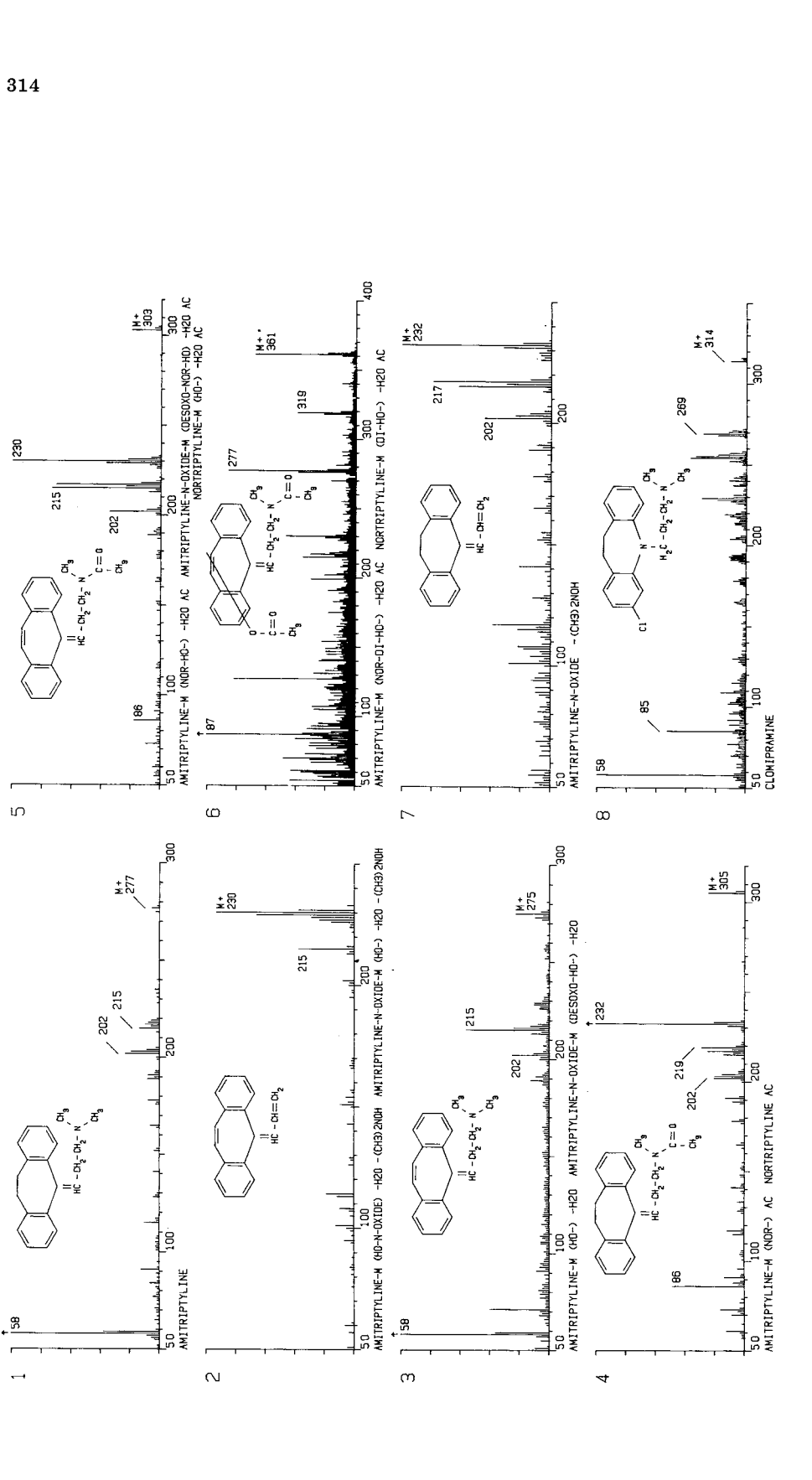
(Continued on p. 312)

TABLE I (continued)

| Mass spectrum No. | $M^+$ | Drug/metabolite (M) | *m/e* (relative intensities) | | | | | | | | Retention index (OV-101) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 58 | 84 | 86 | 100 | 191 | 193 | 194 | 205 | |
| 22 | 279 | Doxepin | +++ | | | | | | | + | 2242 |
| 23 | 234 | M (N-oxide) | | | | | + | | | + | 1970 FID |
| 24 | 337 | M (HO-) 1st isomer | +++ | | | | | | | | 2540 |
| 24 | 337 | M (HO-) 2nd isomer | +++ | | | | | | | | 2583 |
| 25 | 307 | M (Nor-) | | | ++ | | | | | + | 2700 |
| 26 | 365 | M (Nor-HO-) 1st isomer | | | +++ | | | | | | 2987 |
| 26 | 365 | M (Nor-HO-) 2nd isomer | | | +++ | | | | | | 3036 |
| 27 | 280 | Imipramine | +++ | + | | | + | ++ | | + | 2214 |
| 13 | 195 | M (ring) | | | | | + | + | ++ | | 1931 |
| 28 | 193 | M (Dehydro ring) | | | | | + | +++ | + | | 1981 |
| 29 | 338 | M (HO-) | +++ | + | | | | | | | 2610 |
| 12 | 308 | M (Nor-) | + | | + | + | + | ++ | + | | 2669 |
| 14 | 366 | M (Nor-HO-) | | | + | + | | + | | | 3066 |
| 00 | 000 | Lofepramine | | | | | | | | | 0000 |
| 13 | 195 | M (ring) | | | | | + | + | ++ | | 1931 |
| 12 | 308 | M (Desalkyl-) | + | | + | + | + | ++ | + | | 2669 |
| 14 | 366 | M (Desalkyl-HO-) | | | + | + | | + | | | 3066 |
| 30 | 319 | Maprotiline | + | | + | ++ | + | | | + | 2800 |
| 31 | 306 | M (HO-propyl-) | | | | | ++ | | | | 2426 FID |
| 32 | 305 | M (Nor-) | | | | | ++ | | | | 2765 |
| 33 | 364 | M (Di-HO-) | | | | | | | | | 2820 FID |
| 34 | 377 | M (HO-ethanediyl-) | + | | + | ++ | ++ | | | | 2996 |
| 35 | 377 | M (HO-anthryl-) | | | | ++ | + | | | | 3096 |
| 36 | 363 | M (Nor-HO-) | | | | | | | | | 3150 |
| 37 | 422 | M (Tri-HO-) | | | | | | | | | 3200 FID |
| 38 | 291 | Melitracene | +++ | | | | | | | | 2285 |
| 39 | 319 | M (Nor-) | + | | + | | + | + | | | 2760 |
| 40 | 379 | M (Nor-HO-dihydro-) | + | | + | | + | + | | | 3028 |
| 41 | 264 | Mianserin | + | | | | | +++ | | | 2208 |
| 42 | 322 | M (HO-) | + | | | | | | | | 2581 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 43 | 292 | M (Nor-) | | | | +++ | | ++ | | 2595 |
| 44 | 350 | M (Nor-HO-) | | | | +++ | | | | 3005 |
| 45 | 280 | Nomifensine | | | | | | | + | 2470 |
| 46 | 338 | M (HO-) 1st isomer | | | | | | | | 2850 |
| 47 | 338 | M (HO-) 2nd isomer | | | | | | | ++ | 2880 |
| 48 | 368 | M (HO-methoxy-) | | | | | | | | 2970 |
| 04 | 305 | Nortripytline | | | + | | | | | 2660 |
| 05 | 303 | M (HO-) | | | + | | | | | 2670 |
| 06 | 361 | M (Di-HO-) | | | +++ | | | | | 2800 |
| 49 | 294* | Noxiptyline | +++ | | | | | | | 2269 |
| 50 | 320 | M (Nor-HO-) | + | | + | ++ | | | ++ | 2750 |
| 51 | 378 | M (Nor-di-HO-) | + | | + | +++ | + | ++ | + | 3020 |
| 52 | 405 | Opipramol | + | | | | + | +++ | + | 3171 |
| 28 | 193 | M (ring) | | | | | + | +++ | + | 1980 |
| 53 | 305 | Proptriptyline | | | | | +++ | | | 2688 |
| 54 | 291 | M (Nor-) | | | + | + | +++ | + | + | + 2780 |
| 55 | 363 | M (HO-) | | | | | | | | 2895 |
| 56 | 175 | Tranylcypromine | | ++ | | | | | | 1636 |
| 57 | 371 | Trazodone | | | | | | | | +++ 3345 |
| 58 | 429 | M (HO-) | | | | | | | | +++ 3380 |
| 59 | 294 | Trimipramine | +++ | | | | | + | + | 2224 |
| 13 | 195 | M (ring) | | | | | + | + | ++ | 1931 |
| 60 | 352 | M (HO-) | +++ | + | | | | | | 2652 |
| 61 | 380 | M (Nor-HO-) | | | + | | | | | 3153 |
| 62 | 438 | M (Nor-di-HO-) | | | + | | | | | 3556 |
| 63 | 279 | Viloxazine | | | ++ | +++ | | | | 2219 |
| 64 | 337 | M (HO-) | | | + | +++ | | | | 2610 |

*Not detectable.


1
AMITRIPTYLINE
2
AMITRIPTYLINE-M (NO-N-OXIDE) -H2O -(CH3)2NOH AMITRIPTYLINE-N-OXIDE-M (HO-) -H2O -(CH3)2NOH
3
AMITRIPTYLINE-M (HO-) -H2O AMITRIPTYLINE-N-OXIDE-M (DESOXO-HO-) -H2O
4
AMITRIPTYLINE-M (NOR-) AC NORTRIPTYLINE AC
5
AMITRIPTYLINE-M (NOR-HO-) -H2O AC AMITRIPTYLINE-N-OXIDE-M (DESOXO-NOR-HO) -H2O AC
NORTRIPTYLINE-M (HO-) -H2O AC
6
AMITRIPTYLINE-M (NOR-DI-HO-) -H2O AC NORTRIPTYLINE-M (DI-HO-) -H2O AC
7
AMITRIPTYLINE-N-OXIDE -(CH3)2NOH
8
CLOMIPRAMINE

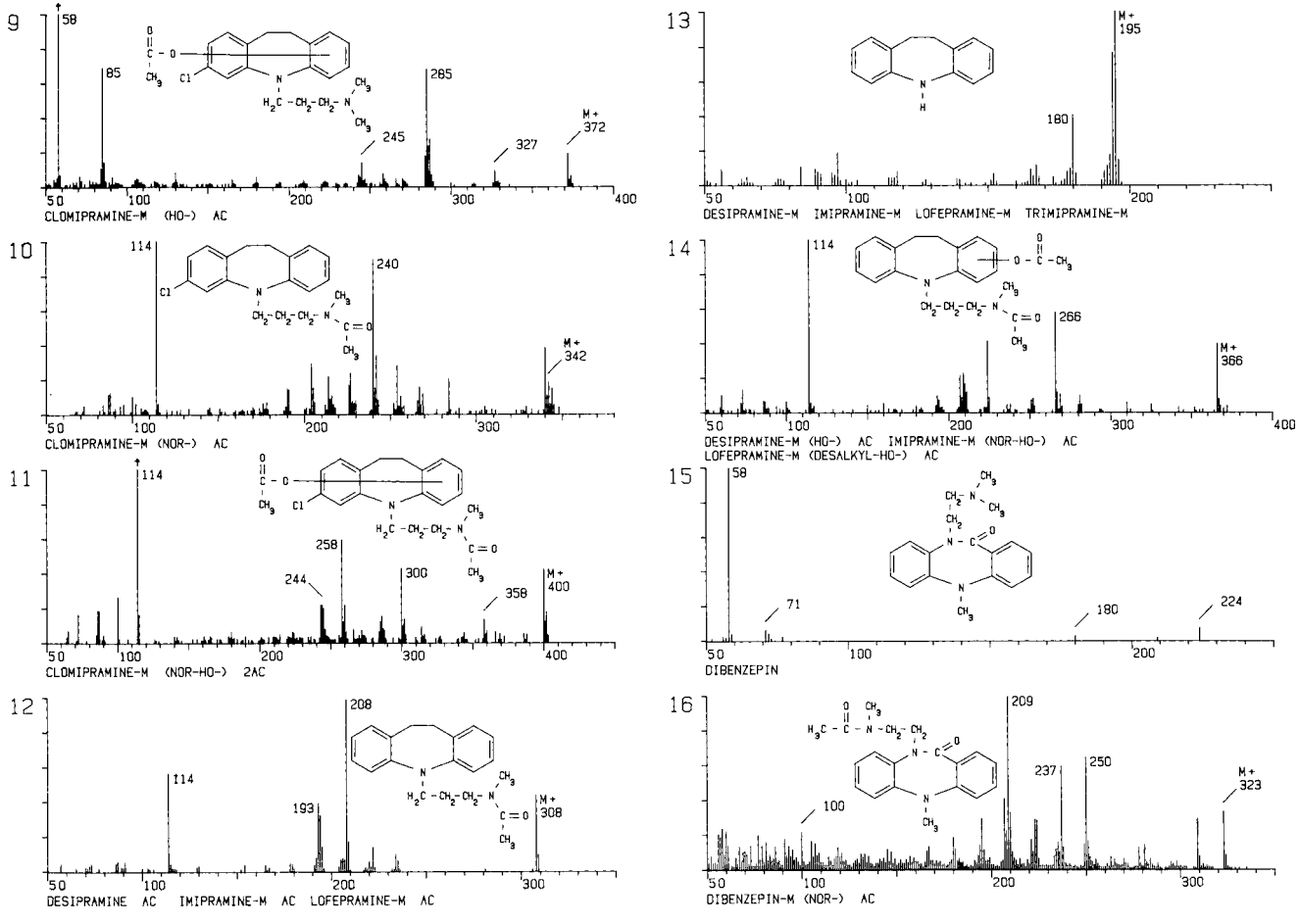
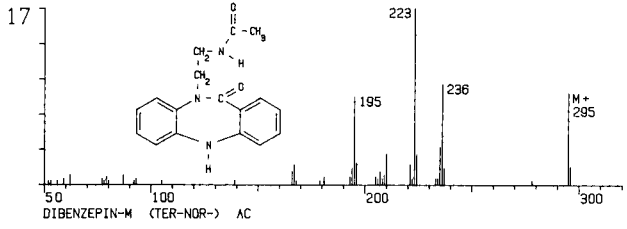
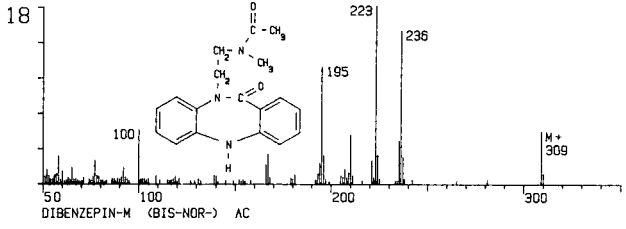
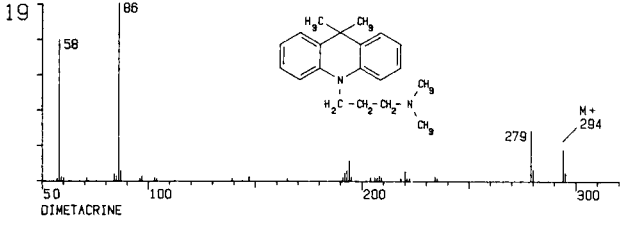


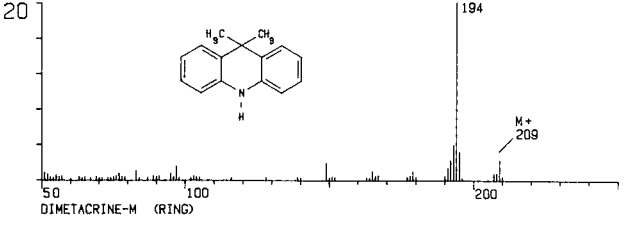

Fig. 1.

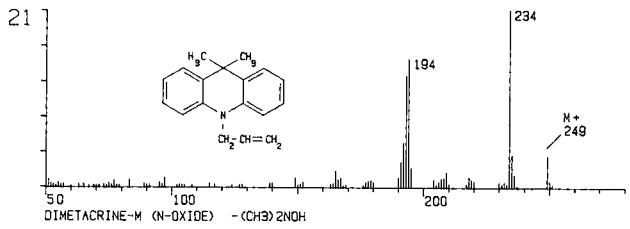
(Continued on p. 316)

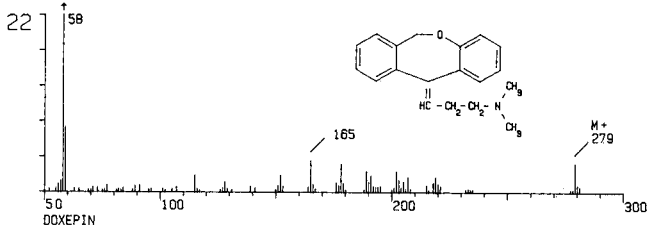

17
223
195
236
M+
295
50
100
200
300
DIBENZEPIN-M (TER-NOR-) AC

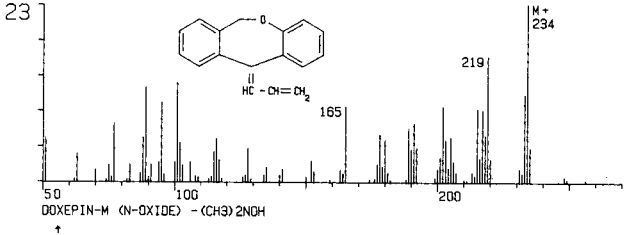

18
223
236
195
100
M+
309
50
100
200
300
DIBENZEPIN-M (BIS-NOR-) AC


19
86
58
279
M+
294
50
100
200
300
DIMETACRINE


20
194
M+
209
50
100
200
DIMETACRINE-M (RING)


21
234
194
M+
249
50
100
200
DIMETACRINE-M (N-OXIDE) -(CH3)2NOH


22
58
165
M+
279
50
100
200
300
DOXEPIN


23
M+
234
219
165
50
100
200
DOXEPIN-M (N-OXIDE) -(CH3)2NOH


24
58
257
M+
337
50
100
200
300
DOXEPIN-M (HO-) AC

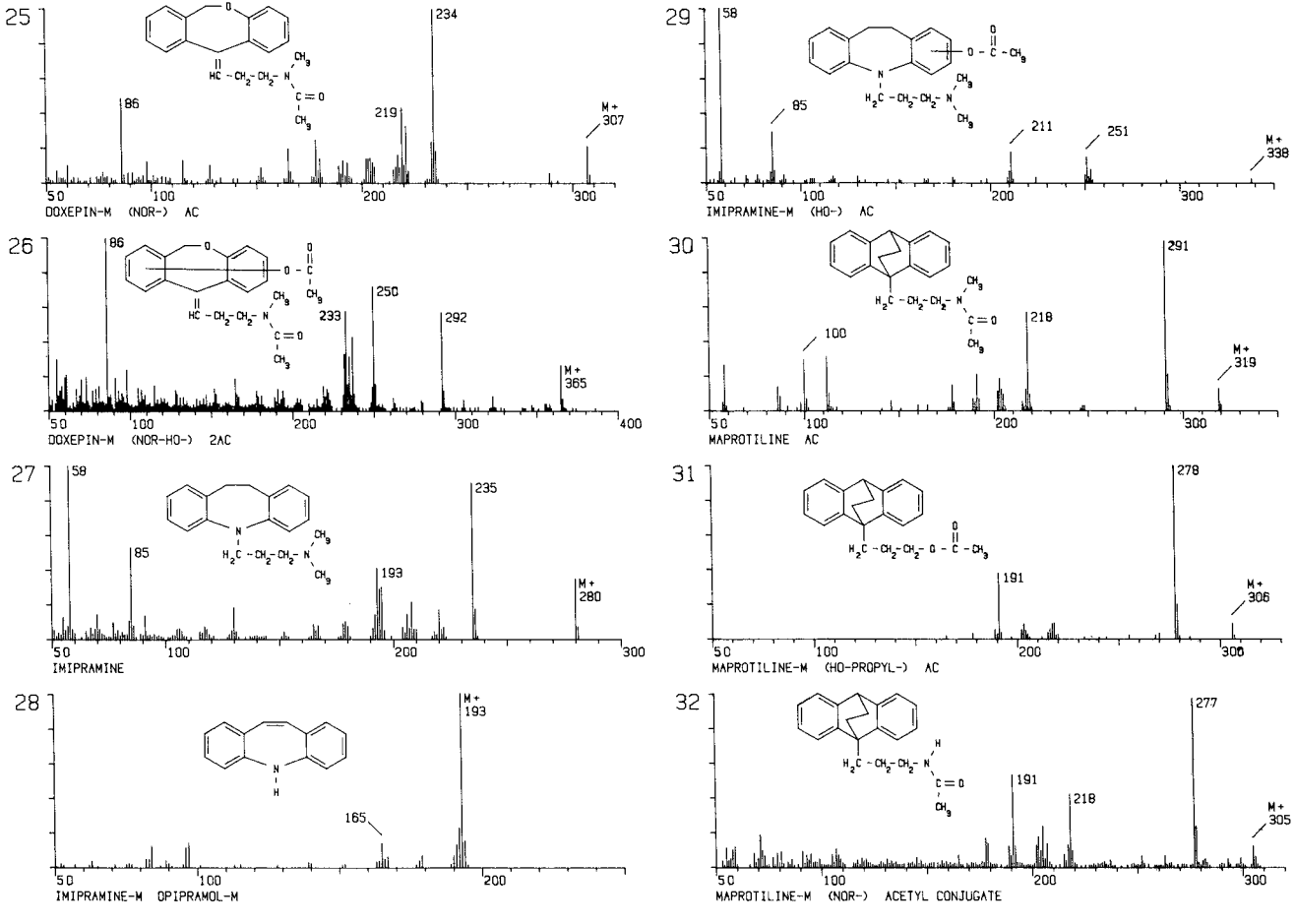
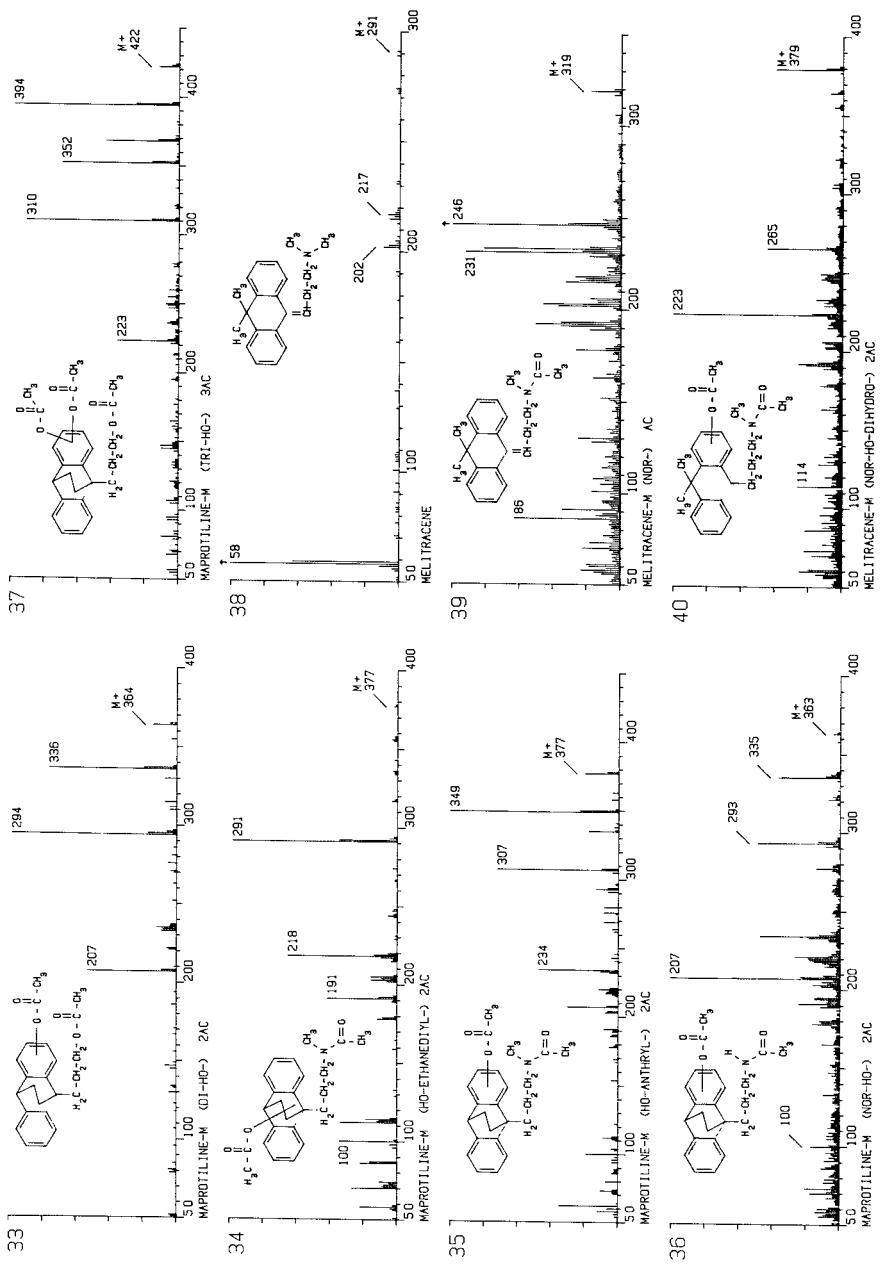
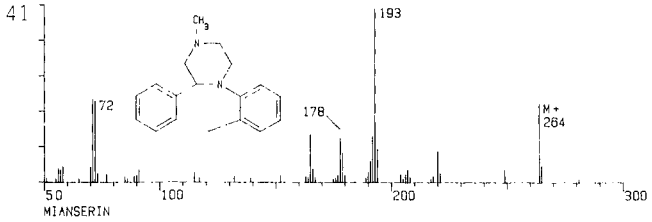
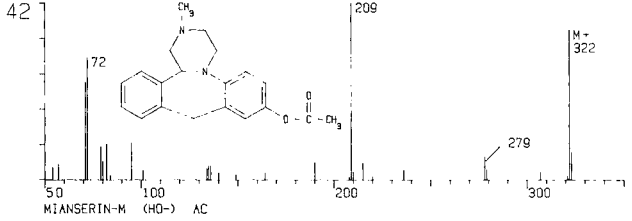


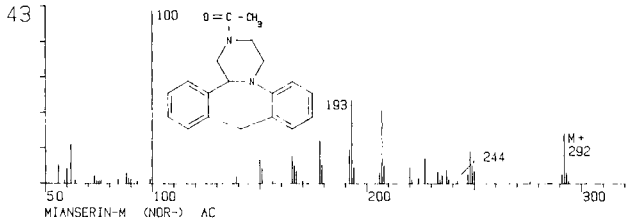

Fig. 1.

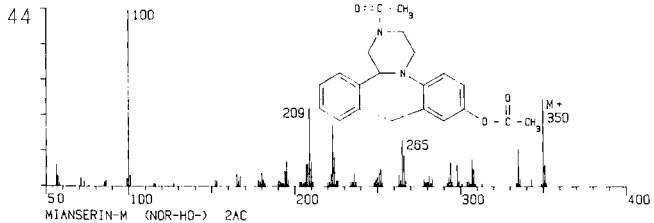
(Continued on p. 318)

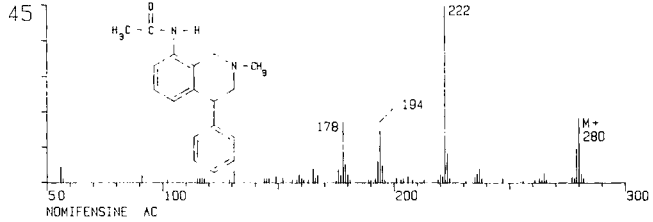

33
MAPROTILINE-M (DI-HO-) 2AC
34
MAPROTILINE-M (HO-ETHANEDIYL-) 2AC
35
MAPROTILINE-M (HO-ANTHRYL-) 2AC
36
MAPROTILINE-M (NOR-HO-) 2AC
37
MAPROTILINE-M (TRI-HO-) 3AC
38
MELITRACENE
39
MELITRACENE-M (NOR-) AC
40
MELITRACENE-M (NOR-HO-DIHYDRO-) 2AC

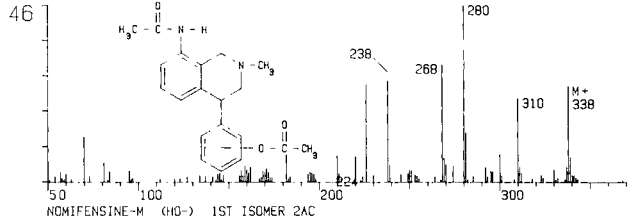


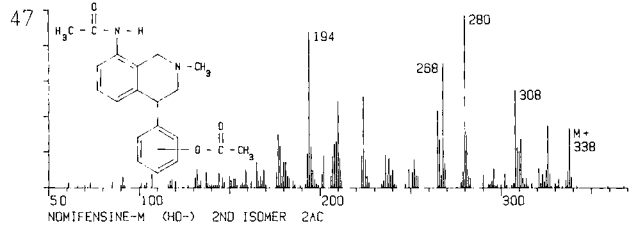



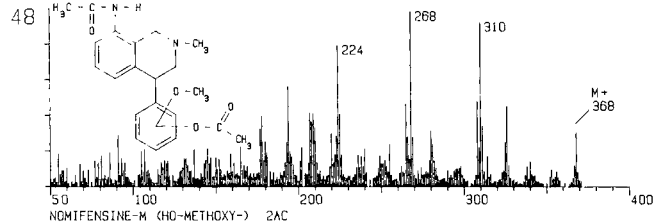
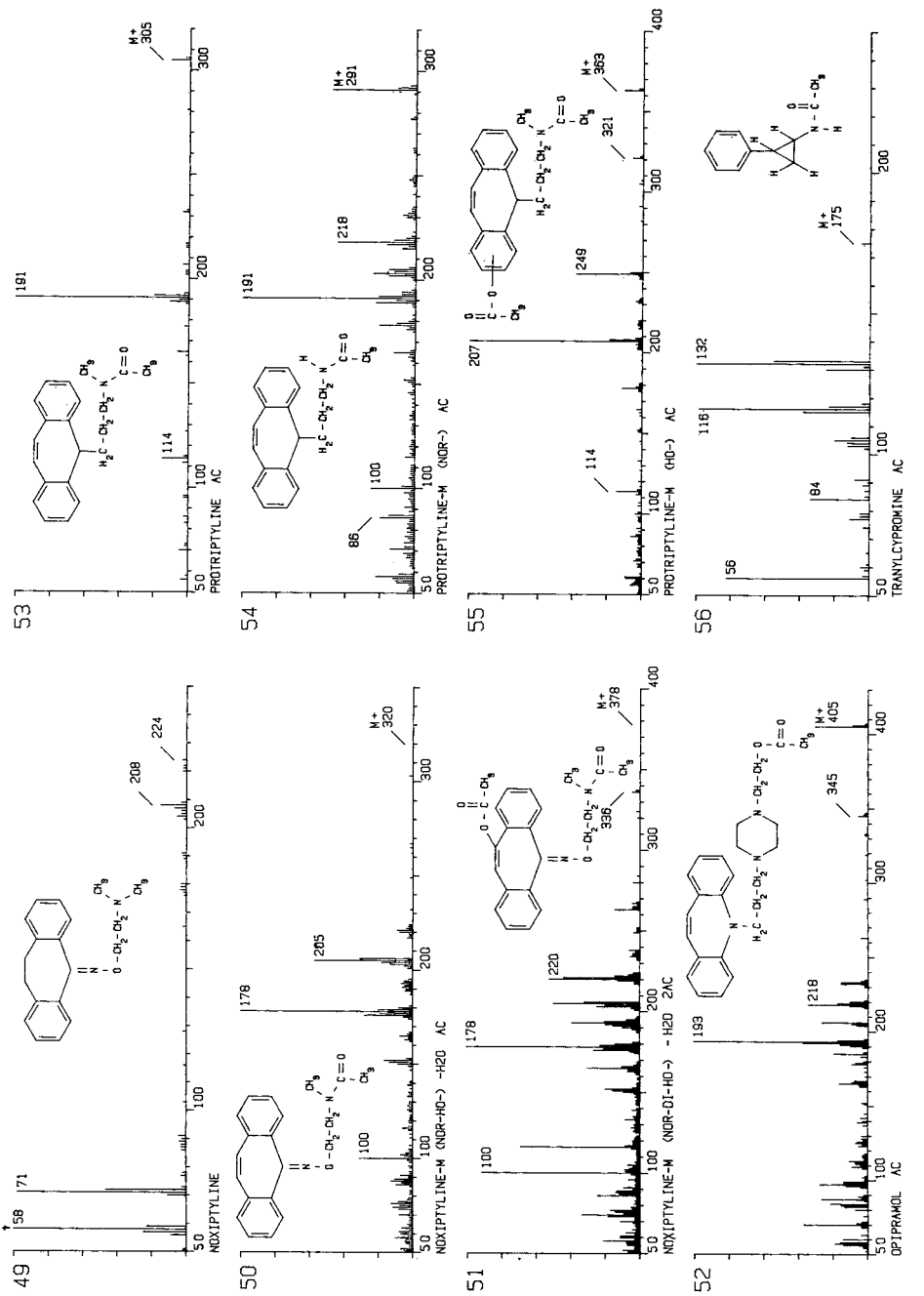




Fig. 1.

(Continued on p. 320)


49
NOXIPTYLINE
50
NOXIPTYLINE-M (NOR-HO-) -H20 AC
51
NOXIPTYLINE-M (NOR-DI-HO-) - H2O 2AC
52
OPIPRAMOL AC
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PROTRIPTYLINE AC
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PROTRIPTYLINE-M (NOR-) AC
55
PROTRIPTYLINE-M (HO-) AC
56
TRANYLCYPROMINE AC

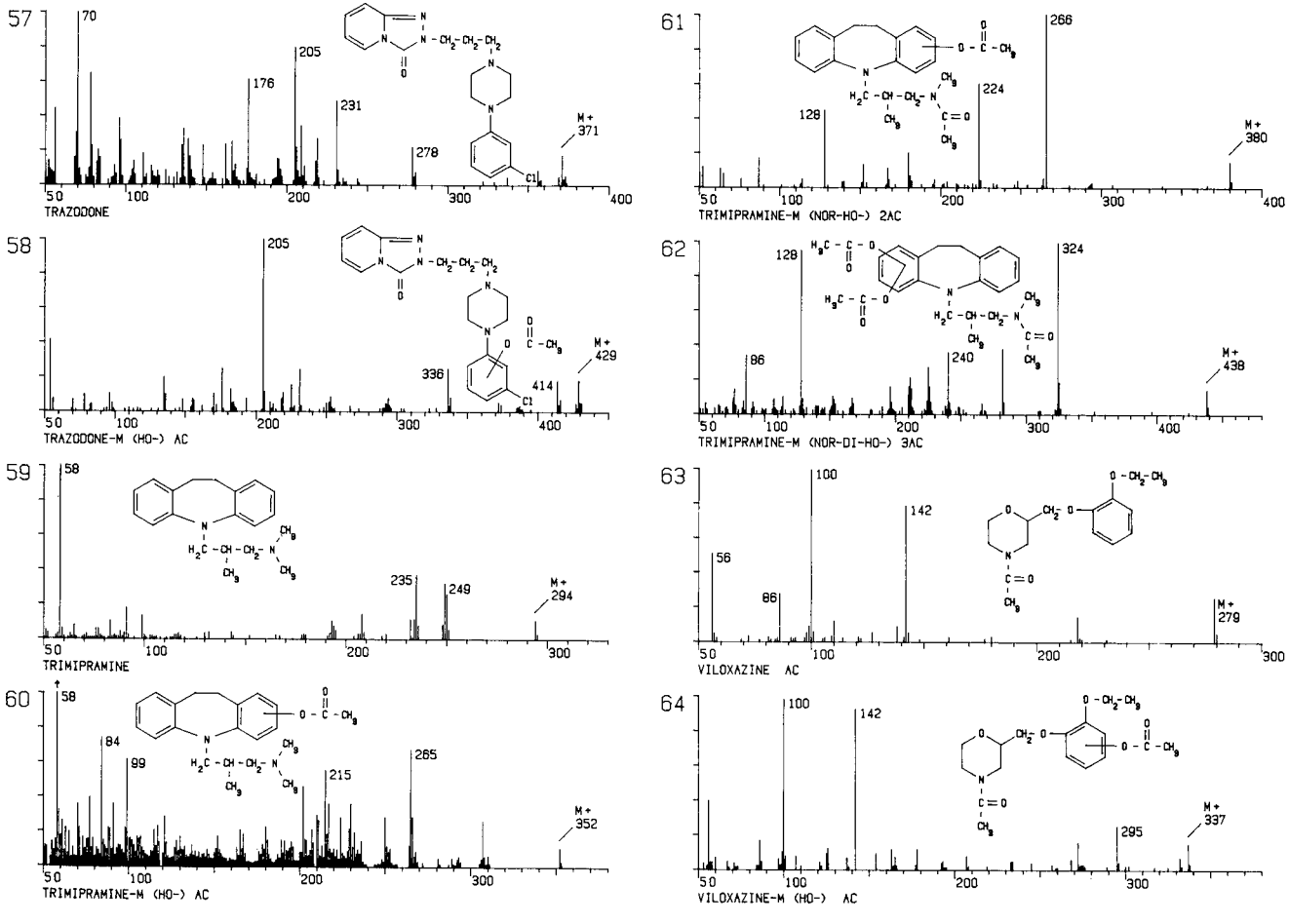


Fig. 1. Mass spectra of antidepressants and their metabolites identified in urine after hydrolysis, extraction and acetylation.

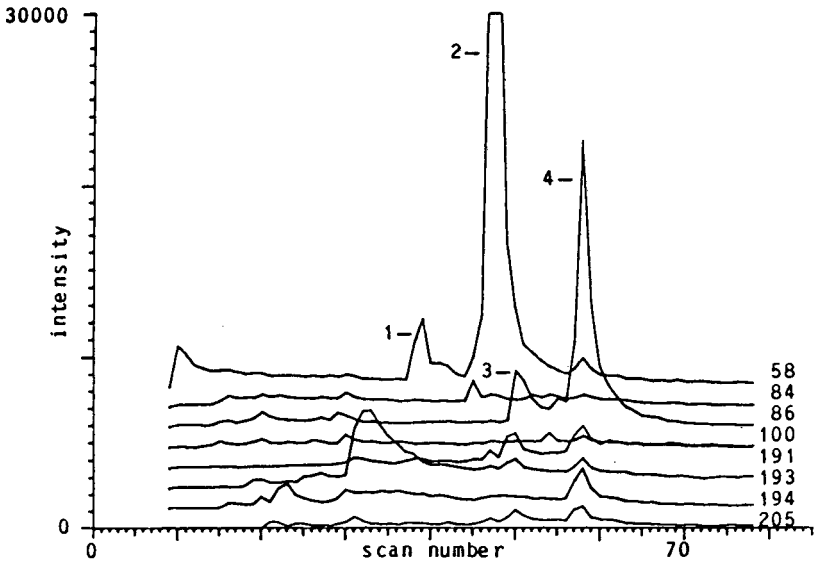


Fig. 2. Mass fragmentogram of doxepin and its metabolites. For identification of peaks, see text.

pramine cannot be differentiated by this screening procedure because lofepramine is not volatile under the GC conditions used. If necessary, the ingestion of lofepramine can be detected by identifying *p*-chlorobenzoic acid after a sample preparation described in the literature [13].

Two kinds of artifacts result from the analytical procedure used. Metabolites with alcoholic hydroxy groups eliminate water, and N-oxides undergo the Cope eliminaton reaction [14]. The mass spectra of these artifacts are included in Fig. 1 (mass spectra Nos. 2, 3, 5, 6, 50 and 51, and Nos. 2, 7, 21 and 23, respectively).

Because all compounds possibly indicated by the mass fragmentogram can be precisely differentiated by comparison of the underlying mass spectra with those of standards (Fig. 1), interference by other drugs is impossible. Furthermore, if the resolution of the peaks is imperfect, a temperature programme with a lower rate or an isothermic procedure can be used. Where there is still doubt a capillary column can help to obtain sufficient resolution.

To illustrate the method, a mass fragmentogram from a psychiatric patient is shown in Fig. 2. Peak 1 indicates doxepin (mass spectrum No. 22), peak 2 the two isomers of hydroxydoxepin (mass spectrum No. 24), peak 3 nordoxepin (mass spectrum No. 25) and peak 4 the two isomers of norhydroxydoxepin (mass spectrum No. 26). This example shows that the presented screening procedure allows a rapid and exact identification and differentiation of antidepressants and their metabolites in urine.

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# ELECTRON-CAPTURE GAS CHROMATOGRAPHIC ASSAYS OF 5-NITROIMIDAZOLE CLASS OF ANTIMICROBIALS IN BLOOD*

S.C. BHATIA* and V.D. SHANBHAG

*Hindustan Ciba-Geigy Limited, Research Centre, Goregaon, P.O. Box 9002, Bombay 400 063 (India)*

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## SUMMARY

Sensitive and selective electron-capture gas chromatographic methods for the determination of N-1-substituted 5-nitroimidazole class of antiprotozoals from blood are described. Metronidazole, secnidazole and ornidazole having a hydroxyl function in the N-1 substitution, were converted to their respective trimethylsilyl derivatives before chromatography on an OV-11 column. Tinidazole and satranidazole, devoid of the hydroxy group but containing a sulphur atom in the molecule, were chromatographed as such on the same stationary phase. Blood levels as low as 50 ng/ml for all the 5-nitroimidazoles have been measured with good precision. The methods can be readily utilized for pharmacokinetic studies.

## INTRODUCTION

The advent of metronidazole and other N-1-substituted 5-nitroimidazoles like secnidazole, ornidazole, tinidazole and satranidazole** (Fig. 1) has revolutionized the chemotherapy of protozoal and anaerobic bacterial infections [1]. Barring their broad similarity, the compounds differ markedly with respect to their pharmacokinetic characteristics and, hence, in dosage regimens. However, different analytical methods have been employed for comparative studies. These include bioassay [2], absorptiometry [3], polarography [4], "flying-spot" thin-layer chromatographic (TLC) densitometry [5], high-performance liquid chromatography [6—9] and gas chromatography with flame ionization detection [10—12].

In this paper we report two sensitive and selective electron-capture gas chro-

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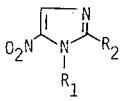
**International non-proprietary name for Go 10213.

0378-4347/84/$03.00 



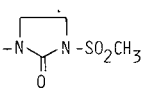
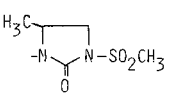
| | | $R_1$ | $R_2$ |
|---|---|---|---|
| I | METRONIDAZOLE | $-CH_2CH_2OH$ | $-CH_3$ |
| | SECNIDAZOLE | $-CH_2CHOHCH_3$ | $-CH_3$ |
| | ORNIDAZOLE | $-CH_2CHOHCH_2Cl$ | $-CH_3$ |
| II | TINIDAZOLE | $-CH_2CH_2SO_2CH_2CH_3$ | $-CH_3$ |
| | SATRANIDAZOLE | $-CH_3$ |  |
| | GO 11386 | $-CH_3$ |  |

Fig. 1. Structural formulae of N-1-substituted 5-nitroimidazoles assayed with (I) and without (II) derivatization procedure.

matographic procedures: one for the analysis of 5-nitroimidazoles with a hydroxyl function in the N-1 substitution (metronidazole, secnidazole and ornidazole), and the other for compounds without the hydroxy group but containing a sulphur atom in the molecule (tinidazole and satranidazole). Both methods are devoid of elaborate extractions, wash procedures and TLC clean-up steps, and utilize a single column and liquid stationary phase. The methods are easily reproducible and hence suitable for routine analysis of blood for pharmacokinetic studies following therapeutic doses of 5-nitroimidazoles.

## EXPERIMENTAL

### *Chemicals and reagents*

Spectroscopic grade methanol (Uvasol) and reagent grade methylene chloride, toluene and cyclohexane were obtained from E. Merck (Bombay, India). Diethyl ether was obtained from Hyderabad Chemicals and Pharmaceuticals (Hyderabad, India). Methylene chloride and diethyl ether were freshly distilled on a 120-cm Vigreux column before use. Toluene and cyclohexane were thoroughly washed successively with concentrated sulphuric acid, water, 1 *M* sodium hydroxide and water, dried over calcium chloride and distilled on a 120-cm Vigreux column.

The derivatization reagent, N,O-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) was obtained from Pierce Chemical Company (Rockford, IL, U.S.A.), and the stationary phase (3% OV-11 on 100—120 mesh Supelcoport) was from

Supelco (Bellefonte, PA, U.S.A.). Ultra pure nitrogen (Iolar-2) from Indian Oxygen (Bombay, India), served as the carrier gas.

All the 5-nitroimidazoles mentioned in this study were synthesized in-house.

*Synthesis of trimethylsilyl derivatives*

A mixture of 1-(hydroxyalkyl)-2-methyl-5-nitroimidazole (3 mmol) and N,O-bis-(trimethylsilyl)-trifluoroacetamide (1 ml) was stirred at 25—27°C for 10 h under anhydrous conditions. Excess reagent was then evaporated off by heating at 60—70°C at 2—3 mm Hg for 3 h to afford the trimethylsilyl (TMS) derivative. TLC (silica; chloroform—methanol, 96:4, v/v) comparison with starting material showed complete conversion.

*TMS derivative of metronidazole.* Analysis: found C, 44.41; H, 7.25; N, 17.58. $C_9H_{17}N_3O_3Si$ (mol. wt. 243) requires C, 44.42; H, 7.04; N, 17.27. $^1$H-NMR* ($C^2HCl_3$): δ 7.97 (1H, s, C-4H), 4.47 (2H, t, N-$CH_2$), 3.90 (2H, t, O-$CH_2$), 2.50 (3H, s, 2-$CH_3$) and 0 ppm [9H, s, Si$(CH_3)_3$]. MS: 243 ($M^+$ at *m/z*).

*TMS derivative of secnidazole.* Analysis: found C, 46.96; H, 7.71; N, 16.73. $C_{10}H_{19}N_3O_3Si$ (mol. wt. 257) requires C, 46.67; H, 7.44; N, 16.33. $^1$H-NMR ($C^2HCl_3$): δ 8.05 (1H, s, C-4H), 4.60 (1H, d with fine structure, 1H of N-$CH_2$), 3.9—4.2 (2H, complex m, C*H*$CH_3$ + 1H of N-$CH_2$), 2.62 (3H, s, 2-$CH_3$), 1.37 (3H, d, CHC*H*$_3$) and 0 ppm [9H, s, Si$(CH_3)_3$]. MS: 257 ($M^+$ at *m/z*).

*TMS derivative of ornidazole.* m.p. 47—50°C. Analysis: found C, 41.42; H, 6.53; N, 14.06. $C_{10}H_{18}ClN_3O_3Si$ (mol. wt. 293) requires C, 41.16; H, 6.21; N, 14.40. $^1$H-NMR ($C^2HCl_3$): δ 8.05 (1H, s, C-4H), 4.80 (1H, d with fine structure, 1H of N-$CH_2$), 4.13—4.60 (2H, complex m, C*H*$CH_2Cl$ + 1H of N-$CH_2$), 3.65 (2H, d, $CH_2Cl$), 2.63 (3H, s, 2-$CH_3$) and 0 ppm [9H, s, Si$(CH_3)_3$]. MS: 293 ($M^+$ at *m/z*).

*Internal standards*

In procedure I, ornidazole served as the internal standard for both metronidazole and secnidazole, while metronidazole was used as the internal standard for ornidazole assays. In procedure II, Go 11386 served as the internal standard for the assays of both tinidazole and satranidazole.

*Procedure I (metronidazole, secnidazole and ornidazole)*

One milligram each of metronidazole, secnidazole and ornidazole was dissolved separately in 100 ml of methanol to yield stock solutions of 10 μg/ml concentration. Aliquots corresponding to 100, 200, 300 and 400 ng of each compound and 200 ng of appropriate internal standard were pipetted into 15-ml standard joint silanized glass tubes; 1.0 ml of human blood was added and the tubes were vortexed and allowed to stand for 5 min at room temperature for equilibration. Then 0.1 ml of 1 *M* sodium hydroxide was added and the tubes vortexed again; 2 ml of a mixture of methylene chloride—diethyl ether (11:14, v/v) were added, the tubes were stoppered with a water seal and extraction was carried out on a reciprocal shaker for 5 min at full speed. The tubes

*NMR chemical shifts are with reference to TMS as the external standard.

were centrifuged at 4000 *g* for 5 min and the organic layer was transferred to a 10-ml conical glass tube. The extraction was repeated and the organic layers pooled and evaporated to dryness at 37°C under a gentle stream of nitrogen. The sides of the tubes were rinsed with 0.5 ml of the solvent mixture and re-evaporated to near dryness.

Derivatization was now carried out by adding 20 μl* of BSTFA reagent; the tubes were gently swirled and allowed to stand at room temperature (26°C) for 1 h. Excess reagent was then evaporated to dryness at 37°C under nitrogen and the TMS ester reconstituted in 1 ml of cyclohexane; 2—3 μl were injected into the gas chromatograph.

*Procedure II (tinidazole and satranidazole)*

Stock standard solutions of tinidazole, satranidazole and Go 11386 (internal standard) were prepared in methanol to yield concentrations of 10 μg/ml. Working standards (2 μg/ml) of tinidazole and satranidazole were prepared by a five-fold dilution of the stock solution, while that of Go 11386 (1 μg/ml) was prepared by a ten-fold dilution with methanol. Aliquots corresponding to 100, 200, 300 and 400 ng of tinidazole or satranidazole and 50 ng of internal standard were pipetted into 15 ml-standard joint silanized glass tubes; 1.0 ml of human blood was added and the tubes were vortexed and allowed to stand for 5 min. Then 0.1 ml of 1 *M* sodium hydroxide was added and tubes were vortexed again. A single extraction with 4 ml of methylene chlorode—diethyl ether (11:14, v/v) mixture was carried out on a reciprocal shaker for 5 min. The tubes were centrifuged at 4000 *g* for 5 min. The organic phase was separated, evaporated to near dryness under nitrogen and reconstituted in 0.5 ml of distilled toluene; 2—3 μl of the solvent were injected directly into the gas chromatograph.

*Gas chromatography*

The gas chromatograph consisted of a Pye-Unicam Model 204 instrument equipped with a 10-mCi $^{63}Ni$ electron-capture detector. For all the 5-nitroimidazoles in the two procedures, chromatography was carried out on a pre-

TABLE I

CHROMATOGRAPHIC CONDITIONS

| Compound | Column | Gas flow-rate (ml/min) | Temperature (°C) | | |
|---|---|---|---|---|---|
| | | | Column | Injector | Detector |
| Metronidazole, Secnidazole, Ornidazole | 3% OV-11 | 50 | 180 | 150 | 250 |
| Tinidazole | 3% OV-11 | 45 | 260 | 200 | 300 |
| Satranidazole | 3% OV-11 | 40 | 280 | 220 | 300 |

*50 μl for secnidazole concentrations of 300 ng/ml and above.

conditioned 150 cm × 4 mm I.D. glass column packed with 3% OV-11 on 100—120 mesh Supelcoport. Other chromatographic conditions are summarized in Table I.

All injections were performed on-column using a 10-$\mu$l Hamilton microsyringe with a 10-cm needle. The detector attenuation was set at 64 × 5 and chromatograms were recorded on an Omniscribe recorder (10 mV) at a chart-speed of 0.5 cm/min. Peak heights were measured and standard/internal standard height ratios were used for calibration and quantitation.

## RESULTS

### *Resolution*

Under the chromatographic conditions described in Table I, TMS derivatives of metronidazole and secnidazole were well resolved from that of ornidazole — the internal standard. Tinidazole and satranidazole were also well separated from the internal standard Go 11386. The chromatograms of synthetic TMS esters of metronidazole, secnidazole and ornidazole and of standards of tinidazole, satranidazole and Go 11386 are presented in Fig. 2. The retention times of all the 5-nitroimidazoles are listed in Table II. The internal standard Go 11386 exhibits a retention time of 5.0 min when used with tinidazole and 2.8 min under the conditions of satranidazole assay.

### *Reaction kinetics and recoveries*

The optimal conditions for derivatization were determined by incubating BSTFA reagent with an extract of blood spiked with 200 ng/ml of

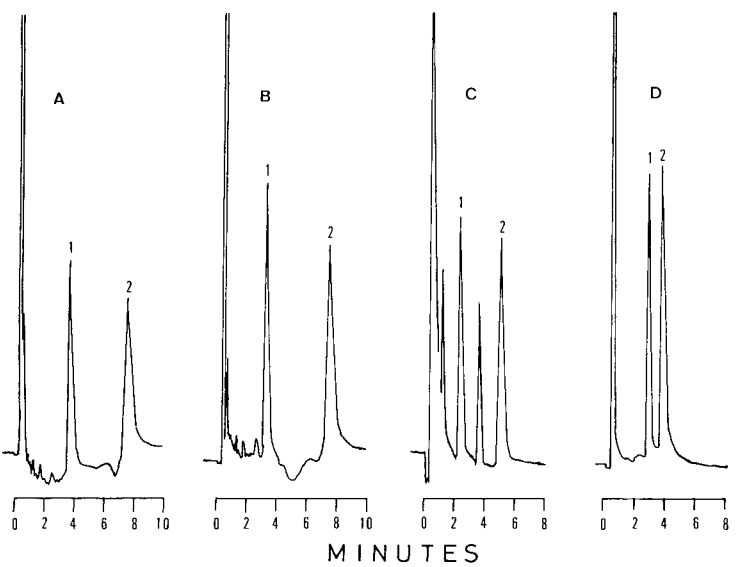



Fig. 2. Chromatograms of synthetic standards of: (A) 400 pg of TMS-metronidazole (1) and 400 pg of TMS-ornidazole (2); (B) 500 pg of TMS-secnidazole (1) and 500 pg of TMS-ornidazole (2); (C) 800 pg of tinidazole (1) and 400 pg of Go 11386 (2); and (D) 200 pg of Go 11386 (1) and 500 pg of satranidazole (2).

TABLE II

RETENTION TIMES OF 5-NITROIMIDAZOLES ON 3% OV-11 COLUMN

Chromatographic conditions are given in Table I.

| Compound | Retention time (min) |
|---|---|
| Metronidazole | 3.6 |
| Secnidazole | 3.4 |
| Ornidazole | 7.8 |
| Tinidazole | 2.4 |
| Satranidazole | 3.8 |
| Go 11386 | 2.8*, 5.0** |

*Under the conditions of satranidazole assay.
**Under the conditions of tinidazole assay.

TABLE III

CHROMATOGRAPHIC PRECISION AND RECOVERIES FROM SPIKED BLOOD SAMPLES

Results are based upon six within-day replicate injections.

| Compound | Spiked concentration (ng/ml) | Precision (C.V., %) | Recovery (%) |
|---|---|---|---|
| Metronidazole | 200 | 3.2 | 82 |
| Secnidazole | 200 | 5.7 | 73 |
| Ornidazole | 200 | 3.1 | 81 |
| Tinidazole | 250 | 2.2 | 81 |
| Satranidazole | 250 | 1.8 | 97 |
| Go 11386 | 100 | — | 89 |

hydroxylated nitroimidazoles for 0.5—1.5 h at room temperature or 50°C and comparing peak heights with those of synthetic TMS ester standards. Reactions were generally complete by 1.0 h at room temperature for all the three nitroimidazoles. Prolonged incubation times under our conditions (relative humidity > 60%) resulted in lower recoveries, especially of secnidazole. TMS esters are well known to be unstable in the presence of moisture.

Recoveries for the entire procedure, with a 1-h derivatization period, were 82, 73 and 81% for metronidazole, secnidazole and ornidazole, respectively. In procedure II, where only a single extraction step is involved, the recoveries for tinidazole, satranidazole and Go 11386 were 81, 97 and 89%, respectively (Table III).

*Chromatographic precision*

Six replicate injections of spiked blood samples submitted to the full procedures were carried out to check within-day chromatographic precision. The results are recorded in Table III. The coefficient of variation (C.V.) was within 5.7% for all the compounds.

*Linearity*

The linearity of the methods was established with human blood calibration curves in the concentration range 100—400 ng/ml based on a 1-ml sample volume. Table IV lists the regression equations and correlation coefficients obtained with 6–8 replicate analyses for each concentration. Correlation coefficients were obtained in the range 0.9951—0.9998, indicating excellent linearity of the two procedures for all the 5-nitroimidazoles tested. Typical chromatograms are shown in Figs. 3 and 4.

*Accuracy and precision*

The methods were applied to determine concentrations of spiked blood samples in the range of 50—450 ng/ml for assessing the accuracy and precision of the procedures. Table V records the mean values and C.V. Results indicate

TABLE IV

REGRESSION EQUATIONS FOR HUMAN BLOOD CALIBRATION CURVES

| Compound | Concentration range (ng/ml) | $n$* | Equation ($Y = a + bX$) | Correlation coefficient ($r$) |
|---|---|---|---|---|
| Metronidazole | 100—400 | 8 | $Y = -0.055 + 0.0071X$ | 0.9998 |
| Secnidazole | 100—400 | 8 | $Y = 0.095 + 0.0074X$ | 0.9951 |
| Ornidazole | 100—400 | 8 | $Y = 0.070 + 0.0027X$ | 0.9988 |
| Tinidazole | 100—400 | 8 | $Y = 0.035 + 0.0062X$ | 0.9993 |
| Satranidazole | 100—400 | 6 | $Y = -0.080 + 0.0059X$ | 0.9979 |

*Number of analyses per concentration.

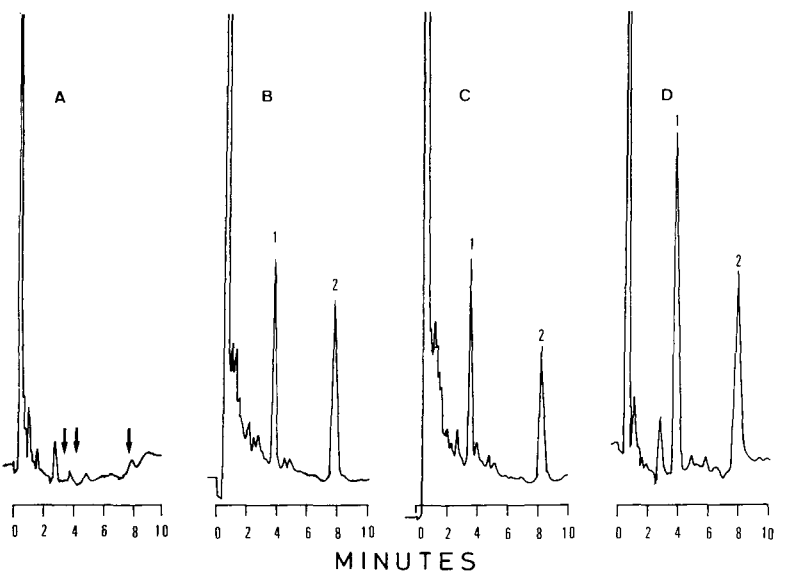



Fig. 3. Chromatograms of extracts: (A) human blood blank; (B) blood spiked with 200 ng of metronidazole (1) and 200 ng of ornidazole (2); (C) blood spiked with 200 ng of secnidazole (1) and 200 ng of ornidazole (2); and (D) blood sample of a volunteer receiving 200 mg of metronidazole per os; metronidazole 4.61 μg/ml (1) and ornidazole (2).

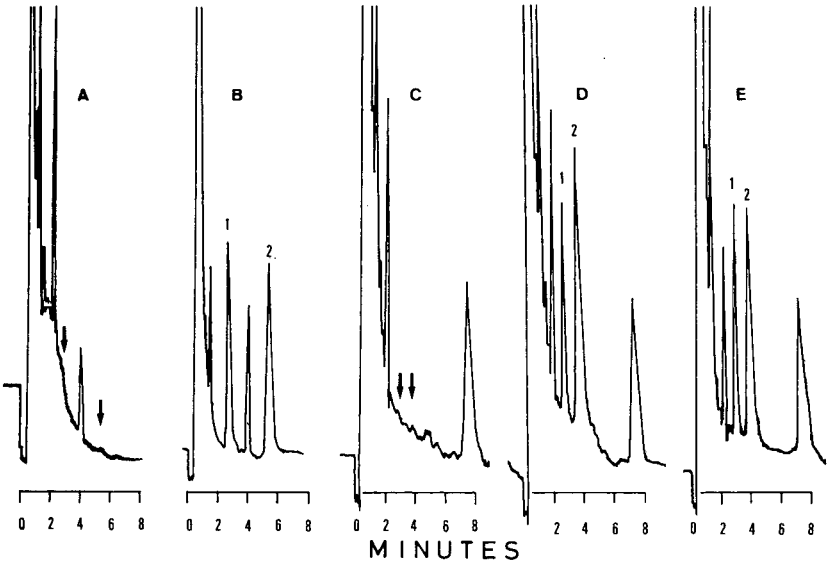



Fig. 4. Chromatograms of extracts of: (A) human blood blank; (B) blood spiked with 200 ng of tinidazole (1) and 100 ng of Go 11386 (2); (C) human blood blank (for satranidazole assay); (D) blood spiked with 50 ng of Go 11386 (1) and 200 ng of satranidazole (2); and (E) blood sample of a volunteer receiving 200 mg of satranidazole per os; Go 11386 (1) and satranidazole 4 μg/ml (2).

TABLE V

PRECISION AND ACCURACY OF THE PROCEDURES APPLIED TO SPIKED HUMAN BLOOD SAMPLES

Data represent concentrations found (ng/ml) ± C.V. (%). Each value represents the mean of four determinations.

| Compound | Concentration spiked (ng/ml) | | | |
|---|---|---|---|---|
| | 50 | 150 | 250 | 450 |
| Metronidazole | 53.7 ± 9.4 | 160.3 ± 5.7 | 255.2 ± 3.3 | 445.2 ± 2.6 |
| Secnidazole | 52.3 ± 5.1 | 148.6 ± 4.1 | 241.0 ± 2.0 | 444.2 ± 3.8 |
| Ornidazole | 56.5 ± 3.3 | 146.4 ± 9.7 | 253.0 ± 6.9 | 444.6 ± 3.7 |
| Tinidazole | 54.8 ± 5.0 | 145.6 ± 2.2 | 252.6 ± 1.8 | 442.1 ± 3.1 |
| Satranidazole | 48.9 ± 2.7 | 147.7 ± 5.6 | 250.0 ± 5.9 | 451.1 ± 5.1 |

that levels in the above range can be estimated with good accuracy and precision. The C.V. for a concentration of 50 ng/ml ranged from 2.7% for satranidazole to 9.4% for metronidazole.

*Application*

Procedure I has been used to measure the blood levels of metronidazole following a single oral dose of 200 mg to a healthy volunteer. A $C_{max}$ of 4.61 μg/ml was recorded at 1 h and a level of 0.15 μg/ml was detected at 48 h (Fig. 5). Under the conditions of assay, the known metabolite of metronidazole

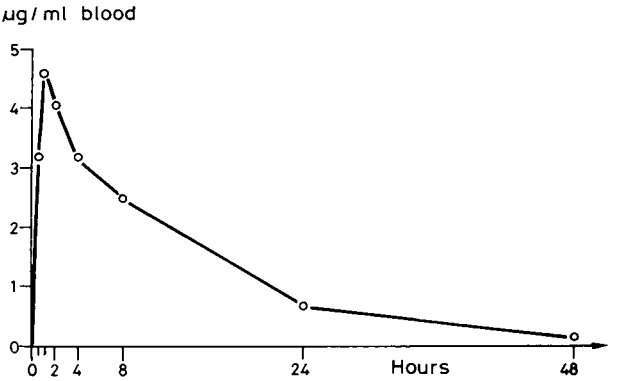



Fig. 5. Blood concentrations of metronidazole after an oral dose of 200 mg of drug to a human volunteer.

[viz. 1-(2-hydroxyethyl)-2-hydroxymethyl-5-nitroimidazole] which would be amenable to derivatization, did not interfere with the analysis of the parent compound (Fig. 3D). Similarly, in the analysis of satranidazole, from the blood of a volunteer receiving a 200-mg dose of the drug, no interference from metabolites was noticed (Fig. 4E).

## DISCUSSION

In procedure I the presence of traces of moisture prior to derivatization led to erratic results, probably due to hydrolysis of the BSTFA reagent or the TMS ester. Care was taken to carefully transfer solvent extracts free from traces of lower aqueous layer, evaporating the extracts not to complete dryness but until a residual film was obtained. BSTFA reagent was then added. Similarly, prior to reconstitution in cyclohexane in procedure I or toluene in procedure II, complete evaporation to dryness under nitrogen was avoided as this resulted in losses due to adsorption on the glass surface.

While derivatization of metronidazole, secnidazole and ornidazole is necessary to decrease column adsorption and increase volatility and sensitivity, for tinidazole and satranidazole — analysed without derivatization — higher temperatures were needed for volatilization. However, the presence of the sulphur atom would lend itself to the enhanced electron-capture sensitivity of these two compounds.

The only other electron-capture gas chromatographic method reported is for a single 5-nitroimidazole, namely ornidazole [13]. The method has an elaborate extraction procedure followed by a TLC clean-up step which involves, first, separation of the external standard from the internal standard and, later, elution of the combined spots of the two standards. Further, the derivatization is effected by the use of two reagents. The methods described in this paper overcome such problems in having a conventional internal standard and a simple extraction procedure free of an additional clean-up step. Moreover, using a single column and stationary phase the quantitative analysis of five different 5-nitroimidazoles from blood, following therapeutic doses, is possible.

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# DOSAGE PAR CHROMATOGRAPHIE GAZ—LIQUIDE D'UN NOUVEL ANTITUMORAL, 1,3,3,5,5-PENTAKIS-(AZARIDINO)-$\lambda^6$,2,4,6,3$\lambda^5$,5$\lambda^5$-THIATRIAZADIPHOSPHORINE-1-OXYDE

## APPLICATIONS AUX ÉTUDES PHARMACOCINÉTIQUES

A. ENGER* et G. DELTOUR

*Institut Jean Godinot, Laboratoire de Radioimmunologie, B.P. 171, 51056 Reims Cedex (France)*

P. CONINX, D. JEZEKOVA et A. CATTAN

*Institut Jean Godinot, Service de Médecine Interne, B.P. 171, 51056 Reims Cedex (France)*

et

R. FOURNAISE

*Faculté des Sciences, Moulin de la Housse, 51100 Reims (France)*

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SUMMARY

*Gas—liquid chromatographic determination of a new anticancer agent, 1,3,3,5,5-pentakis-(azaridino)-$\lambda^6$,2,4,6,3$\lambda^5$,5$\lambda^5$-thiatriazadiphosphorine-1-oxide, for pharmacokinetic investigations*

A method for the assay of 1,3,3,5,5-pentakis-(azaridino)-$\lambda^6$,2,4,6,3$\lambda^5$,5$\lambda^5$-thiatriazadiphosphorine-1-oxide (SOAz), a new anticancer drug of which the clinical trials are in progress, is described. This method is based on capillary gas chromatography using a thermionic detector. The lower detection limit was 100 pg per injection and a coefficient of variation smaller than 5% could be obtained when parathion was used as external standard. The method is suitable for biological samples and therefore has been proposed for clinical pharmacokinetic studies as well as for the determination of patterns of SOAz distribution in several organs of the mouse. A preliminary clinical study showed that the serum decay curves of SOAz could be fitted to an open two-compartment model for drug disappearance.

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## INTRODUCTION

Le 1,3,3,5,5-pentakis-(azaridino)-$\lambda^6$,2,4,6,3$\lambda^5$,5$\lambda^5$-thiatriadiphosphorine-1-oxyde (SOAz) est un médicament appartenant à la série des cyclophosphathiazènes et dérivé directement des cyclophosphazènes. Les cyclophosphazènes sont des composés particulièrement stables qui s'accumulent dans l'organisme et induisent une toxicité cumulative. Le SOAz est issu des recherches de Labarre et collaborateurs [1, 2] qui ont essayé de diminuer la toxicité hématologique observée avec les cyclophosphazènes en remplaçant dans le cycle de la molécule un atome de phosphore par un atome de soufre susceptible de provoquer une déstabilisation et de permettre un meilleur métabolisme.

Après les essais encourageants observés chez l'animal, nous avons testé ce médicament simultanément chez la souris et chez l'homme (essai Phase I et II). Parallèlement aux observations cliniques, nous avons procédé à des études biologiques portant sur les taux plasmatiques tissulaires et les voies d'élimination de cette drogue.

Au début de cette étude nous avons trouvé dans la littérature deux méthodes de dosage, l'une par chromatographie gazeuse (GC) [3]; l'autre par chromatographie liquide haute pression nous avait été communiquée à titre personnel [4]. Cette dernière technique présente un gros inconvénient: son manque de sensibilité.

La technique par GC avec détecteur spécifique azote—phosphore semblait plus performante et nous nous sommes efforcés de la mettre en oeuvre. Nous n'avons jamais pu retrouver les résultats avancés par les auteurs, en particulier en ce qui concerne la reproductibilité des résultats et donc la précision de la méthode et la linéarité de la réponse du détecteur vis-à-vis du SOAz.

On peut évoquer plusieurs raisons pour expliquer ce fait: (1) L'absence d'étalon interne ou externe, la quantification de la drogue se faisant d'après la quantité absolue injectée. On sait que la mesure directe de l'aire du pic obtenu par injection d'une quantité supposée connue de substance est soumise à trop de causes de variation pour obtenir une reproductibilité suffisante. (2) La nature même du SOAz qui s'adsorbe fortement sur tous les supports et donne des résultats totalement différents selon l'état de saturation des différents sites d'adsorption. (3) L'origine différente des chromatographes utilisés pourrait expliquer les distorsions observées au niveau de la détection; la présence simultanée de P, S et N dans la molécule peut aussi être invoquée pour expliquer la réponse non-linéaire du détecteur.

Nous avons donc mis au point notre propre méthode utilisant des colonnes capillaires en silice fondue, un standard externe et la détection spécifique azote—phosphore.

## MATÉRIEL ET MÉTHODES

### *Produits chimiques*

Le SOAz est fourni par Otsuka Chemical (Tokushima, Japon), l'éthylparathion provient de Riedel de Haen (Hannover, R.F.A.).

*Conditions chromatographiques*

Le chromatographe est un Packard Modèle 427 équipé d'un détecteur thermoionique Modèle 905 avec toutes les régulations pneumatiques nécessaires à l'utilisation des colonnes capillaires et tous modes d'injection. Le système d'injection donnant les meilleurs résultats est le "split" que nous utilisons vanne de fuite fermée, la totalité de l'échantillon injecté passe donc dans la colonne. Le courant de bille du détecteur est fixé à 260 pA. Le système est équipé d'une colonne capillaire en silice fondue (15 m × 0.32 mm I.D.). Ces colonnes sont à phase greffée de type apolaire que nous préparons au laboratoire selon un protocole qui sera publié ultérieurement.

Les conditions de travail sont les suivantes: température colonne 245°C, température injecteur 270°C, température de détecteur 290°C, gaz vecteur azote, pression en tête de colonne 1 bar.

*Administration de la drogue*

*Chez l'animal.* Nous utilisons des souris femelles F1 C57 B6/DBA2 porteuses de l'adénocarcinome Ca 755. Le SOAz est administré à la dose de 110 mg/kg de carcasse, après dissolution dans le sérum physiologique, à raison de 0.1 ml par souris et par voie intra-péritonéale. La dose administrée est la même pour chaque animal après détermination d'un poids moyen.

*Chez l'homme.* La drogue est administrée par embol intraveineux à la dose de 220 mg/m$^2$ [5].

*Prélèvements*

*Chez l'animal.* Le sang est prélevé au coin de l'oeil à l'aide d'une pipette Pasteur et recueilli sur tube hépariné. Les organes suivants sont prélevés: reins, foie, rate, poumons, tumeur, cerveau, os et moelle, intestin. Les prélèvements sont recueillis dans des tubes Pyrex de 10 ml contenant chacun 5 ml de tampon phosphate salin (PBS) 0.1 *M* par gramme d'organe.

Les prélèvements peuvent être conservés quelques jours à 4°C ou plusieurs semaines congelés à −25°C.

*Chez l'homme.* Le sang est prélevé sur tube hépariné par ponction veineuse au pli du coude aux temps suivants: 0 (avant injection), 5, 10, 15 et 30 min, et 1, 3, 6, 12, 18, 24 et 36 h.

Les tubes sont conservés à 4°C avant préparation pour dosage.

*Préparation de prélèvements*

*Chez l'animal.* Les organes (sauf l'os) sont broyés pendant 5 min à 4°C à l'aide d'un broyeur Ultra-Turrax IKA T 18.

Ce broyat est centrifugé pendant 20 min à 4°C et 20,000 *g*. Le surnageant est récupéré dans sa totalité. L'os est broyé à l'aide d'un pulvérisateur thermovac refroidi à l'azote liquide. La poudre obtenue est agitée pendant 1 h dans du sérum physiologique.

*Chez l'homme.* Un ml de sang total est prélevé et servira à déterminer la concentration en SOAz des éléments figurés du sang. Avant extraction les globules sont lysés par congélations—décongélations successives. Le SOAz non-lié aux protéines sériques est évalué après ultrafiltration sur membrane Amicon PM 10.

La totalité des urines est recueillie entre 1—12 h, 12—24 h et 24—48 h. Elles

sont stockées au réfrigérateur ou congelées si le dosage doit être différé. Les urines ne reçoivent aucun traitement particulier avant extraction.

*Extraction*

Le protocole d'extraction est le même quels que soient les organes ou liquides physiologiques dans lesquels on veut mesurer le taux de SOAz.

Le surnageant est ajusté à pH 10 avec de la soude 1 *M*. Après addition de 5 ml de dichlorométhane les tubes sont agités lentement sur un agitateur rotatif pendant 5 min. Les tubes sont ensuite centrifugés à 3000 *g* pendant 5 min à 4°C. La phase organique contenant le SOAz est récupérée. On renouvelle l'opération avec 2 ml de dichlorométhane.

Les phases organiques sont ensuites évaporées à sec sous léger courant d'azote et à température ambiante. Le résidu est repris par 100 $\mu$l d'acétone et l'on y ajoute 10 $\mu$g d'éthylparathion dans les tubes prélevés avant le temps 1 h, 5 $\mu$g pour les tubes prélevés entre 1 et 6 h et 2.5 $\mu$g dans les autres tubes.

*Déterminations pharmacocinétiques*

L'expression mathématique des courbes de décroissance plasmatique est déterminée en faisant l'hypothèse "a priori" que l'on se trouve en présence d'un phénomène pouvait être représenté par une somme d'exponentielles du type

$$C(t) = A_0\, e^{-At} + B_0\, e^{-Bt}$$

Les coefficients $A_0$, $A$, $B_0$, $B$ sont obtenus par lissage des valeurs expérimentales selon la méthode de Newton-Raphson et en introduisant une pondération des valeurs expérimentales des concentrations en $1/C(t)^2$. Les volumes des compartiments, $V_{d_1}$ et $V_{d_2}$, le volume de distribution total, $V_d$, les coefficients d'échanges, $K_{12}$ et $K_{21}$, le coefficient d'élimination, $K_{el}$, sont déterminés de façon classique pour un système bicompartimental ouvert. Les clairances urinaires sont déterminées d'après l'équation

$$Cl = \frac{\Delta u}{\int_{t_1}^{t_2} C(t)\mathrm{d}t}$$

$\Delta u$ = quantité de drogue éliminée dans les urines entre les temps $t_1$ et $t_2$.

$\int_{t_1}^{t_2} C(t)\mathrm{d}t$ = aire sous la courbe de décroissance plasmatique entre les temps $t_1$ et $t_2$.

## RÉSULTATS

*Étude de la technique*

Dans les conditions d'analyse définies les temps de rétention sont respectivement: éthylparathion, 1.3 min; SOAz, 4.09 min.

*Linéarité du détecteur.* Pour des quantités de SOAz comprises entre 1 et 25 ng nous obtenons la courbe Fig. 1. La réponse du détecteur n'est pas linéaire, contrairement aux observations d'Uchida et al. [3].

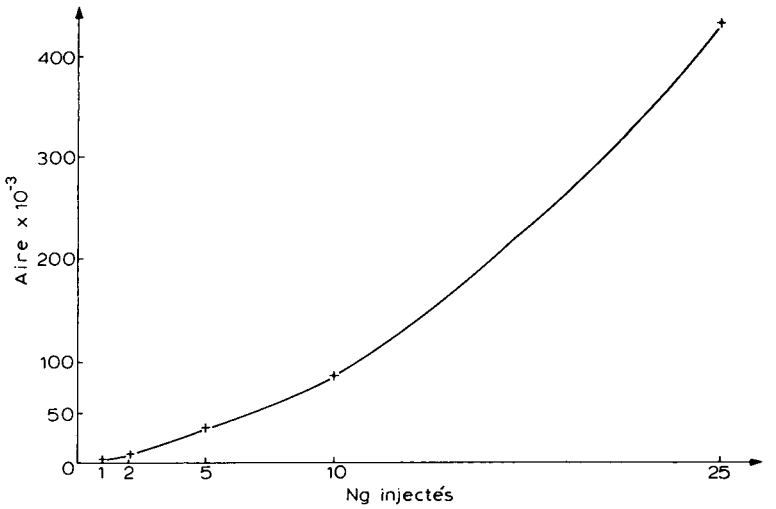


Fig. 1. Réponse du détecteur pour des quantités de SOAz injectées comprises entre 1 et 25 ng ($n$ = 5).

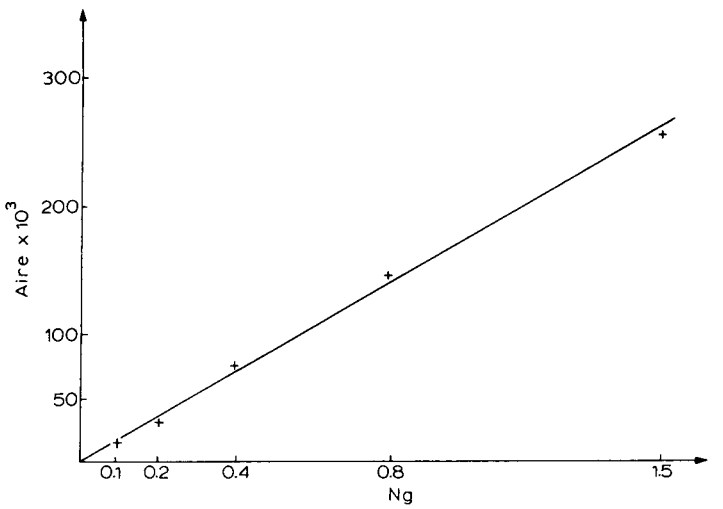


Fig. 2. Réponse du détecteur pour des quantités de parathion comprises entre 0,1 et 1,5 ng ($n$ = 5).

Pour des quantités de parathion comprises entre 0.1 et 1.5 ng nous obtenons le tracé Fig. 2. À la différence du SOAz, la réponse du détecteur est linéaire.

Ces observations nous ont amenés à adopter le protocole de dosage suivant: la quantité de SOAz injectée doit être telle que la surface mesurée de son pic soit comprise entre 18,000 et 23,000. Dans ces conditions l'étalonnage est parfaitement linéaire.

*Limite de détection.* Pour une quantité de SOAz injectée de 97 pg nous obtenons une aire de 368 ce qui correspond à la limite de détection acceptable dans les conditions analytiques standards. Il est possible d'abaisser ce seuil de détection en diminuant l'atténuation de l'électromètre et en augmentant

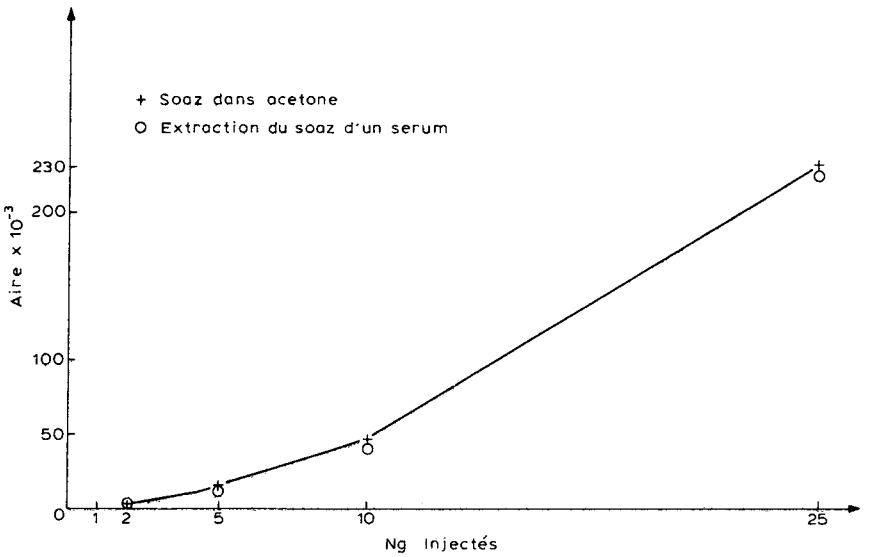


Fig. 3. Comparaison des aires obtenues pour le dosage du SOAz dans une série de sérums surchargés et extraits avec une gamme étalon.

l'intensité du courant de bille du détecteur. Le gain réalisé peut être aisément de 10 mais les conditions de travail deviennent beaucoup plus difficiles.

*Test de récupération.* Des quantités connues et croissantes de SOAz en solution dans l'acétone sont ajoutées à une série de 4 × 1 ml de sérum. Ces sérums sont extraits selon le protocole défini et les résultats sont comparés aux chiffres obtenus par injection de quantités connues de SOAz (Fig. 3). Les pourcentages de récupération varient entre 91 et 108%. Ces chiffres ne sont pas significatifs et correspondent au coefficient de variation de 10% sur la précision des injections. Les pourcentages de récupération ont été mesurés pour les urines et différents organes chez la souris et sont comparables. Nous considérons donc qu'il n'y a pas de coefficient de correction à appliquer pour les extractions de SOAz à partir des différents prélèvements biologiques.

*Reproductibilité intra-essais.* Un pool de sérums humains est aliquoté et surchargés en SOAz à 8, 5, 2.5 et 1 μg/ml. Après extraction selon le protocole défini on ajoute respectivement 10, 5, 2.5 et 1 μg/ml de sérum de parathion. Chaque extrait est dosé dix fois dans les mêmes conditions. Les résultats sont donnés dans le Tableau I.

TABLEAU I

REPRODUCTIBILITÉ INTRA-ESSAI

Dix déterminations par point.

| SOAz (μg/ml) | Moyenne | C.V. (%) |
|---|---|---|
| 8 | 7.77 | 4.46 |
| 5 | 5.02 | 3.7 |
| 2.5 | 2.56 | 3.4 |
| 1 | 0.95 | 4.7 |

*Contrôle inter-essai.* À un pool de sérums humains aliquoté en fractions de 1 ml, on ajoute 5 μg de SOAz dans l'acétone. Après agitation les pools sont congelés et stockés à −25° C.

Pour une série de 11 déterminations la moyenne du sérum de contrôle s'établit à 4.95 μg/ml et le coefficient de variation à 9.7%.

*Étude pharmacocinétique chez l'homme*

La Fig. 4 donne le tracé chromatographique type d'un patient ayant reçu 400 mg de SOAz. Le prélèvement effectué à la quinzième minute contient 10.8 μg de SOAz par ml de sérum. Un prélèvement sanguin effectué avant injection de la drogue permet de s'assurer qu'il n'existe pas dans le plasma du malade de substance pouvant interférer avec le dosage.

Sur la série de 17 patients étudiés et les nombreux pools de sérums utilisés,

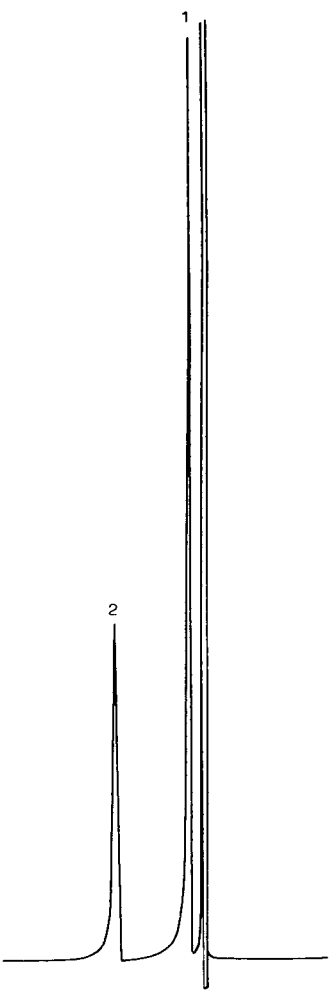


Fig. 4. Tracé chromatographique d'un patient ayant reçu 400 mg de SOAz. Prélèvement à la 15e minute. Pic 1: parathion. Pic 2: SOAz, 10.8 μg/ml.

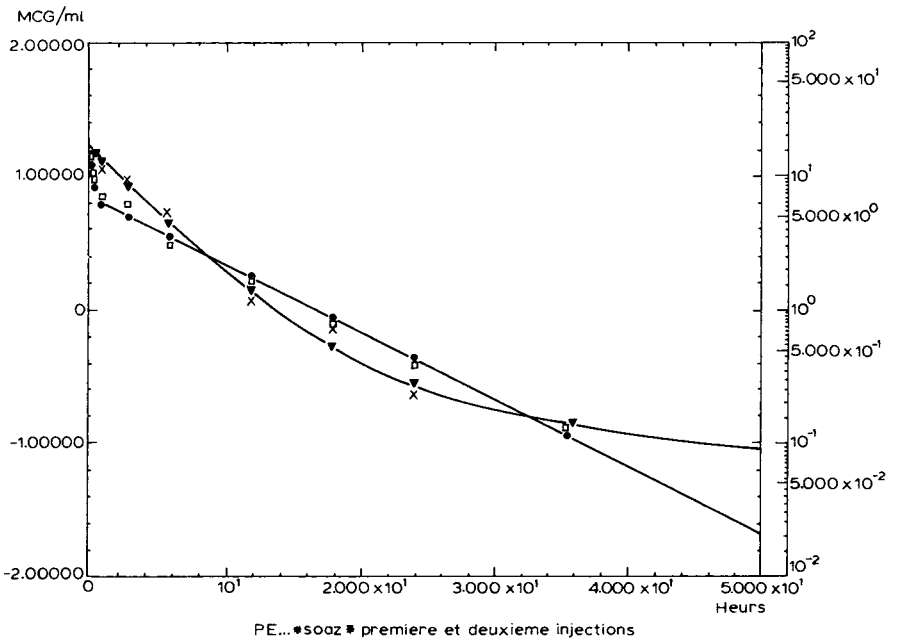


Fig. 5. Courbe de décroissance plasmatique du SOAz chez un patient ayant reçu deux injections à deux mois d'intervalle. 1e injection: 430 mg, voie intraveineuse; (□), points expérimentaux; (•), points théoriques recalculés. 2e injection: 300 mg, voie intraveineuse; (X), points expérimentaux; (▾), points recalculés.

TABLEAU II

VALEURS DES CONCENTRATIONS EN FONCTION DU TEMPS POUR PATIENT P.E.

Le C.V. représente le coefficient de variation observé entre les valeurs expérimentales et les valeurs théoriques calculées d'après l'équation de la courbe. Le signe négatif indique que les valeurs calculées sont inferieures aux valeurs expérimentales.

| Temps (h) | 1e injection* (μg/ml) | C.V.(%) | 2e injection** (μg/ml) | C.V. (%) |
|---|---|---|---|---|
| 0.083 | 16.9 | 0.87 | 15.4 | —2.5 |
| 0.163 | 14.7 | 5.2 | | |
| 0.25 | 10.8 | —8.6 | 16.7 | 8.1 |
| 0.5 | 8.7 | 3.9 | 14.4 | —0.194 |
| 1 | 6.7 | 3.9 | 12 | —7.9 |
| 3 | 5.9 | 16.6 | 8.6 | 2.03 |
| 6 | 3.3 | —5.1 | 5 | 10.5 |
| 12 | 1.6 | —7.9 | 1.16 | —17.6 |
| 18 | 0.8 | —7.5 | 0.76 | 32.4 |
| 24 | 0.4 | —7 | 0.24 | —9.9 |
| 36 | 0.13 | 13.5 | 0.14 | 1.74 |

*430 mg injectés le 20 septembre 1982; poids du patient 86 kg.
**300 mg injectés le 25 novembre 1982; poids du patient 83 kg.

nous n'avons jamais mis en évidence de pic chromatographique interférant avec le SOAz ou le parathion. La Fig. 5 donne les courbes de pharmacocinétique chez un patient ayant reçu lors de la première injection 430 mg de SOAz et 300 mg lors de la seconde.

Le Tableau II donne les valeurs des concentrations en fonction du temps.

## DISCUSSION

La technique de dosage du SOAz décrite ici donne des résultats très reproductibles. Les coefficients de variation pour la reproductibilité intra-essais sont inférieurs à 5% et inférieurs à 10% pour la reproductibilité inter-essais.

La sensibilité permet d'atteindre des concentrations inférieures à 0.1 $\mu$g/ml de sérum en routine et on peut l'abaisser d'un facteur 10 si nécessaire. L'utilisation de colonnes capillaires en silice fondue et à phase greffée autorise l'injection dans la colonne de volumes importants jusqu'à 5 $\mu$l sans dommage pour celle-ci. Après une année d'utilisation la colonne en service présente des caractéristiques toujours acceptables malgré des conditions analytiques particulièrement drastiques. L'utilisation d'un standard externe limite les erreurs dues à l'injection. Ce standard est disponible facilement dans le commerce et dispense de synthétiser un analogue structural du SOAz utilisé comme standard interne par d'autres auteurs [6]. Les techniques d'extractions et de dosages, très simples, s'appliquent à tous les liquides biologiques et

TABLEAU III

PARAMÈTRES PHARMACOCINÉTIQUES DU PATIENT P.E.

1e courbe équation: $13.845\ e^{-4.104t} + 6.971\ e^{-0.1162t}$.
2e courbe équation: $15.707\ e^{-0.2214t} + 0.3719\ e^{-0.02873t}$.

| Paramètre | 1e injection | 2e injection |
|---|---|---|
| $t_1$ ½ min | 10.1 | 187 |
| $t_2$ ½ min | 357.8 | 1447 |
| $V_{d_1}$ l | 20.6 | 18.7 |
| $V_{d_2}$ l | 34.7 | 14.2 |
| $V_{d\ tot}$ l | 58.4 | 124 |
| $K_{12}$ | 0.0406 | $42 \cdot 10^{-5}$ |
| $K_{21}$ | 0.0242 | $55 \cdot 10^{-5}$ |
| $K_{el}$ | 0.00548 | 0.00319 |
| $Cl_{tot}$ ml min$^{-1}$ | 113 | 59.6 |
| Urines | | |
| 0—12 h | 114.5 mg 26.6% | 77.5 mg 26.8% |
| 12—24 h | 26 mg 32.7% | 12 mg 29.8% |
| 24—48 h | 7 mg 34.3%* | 1.3 mg 30.3%* |
| Toxicité (classification EORTC) | | |
| Polynucléaires | II | III |
| Plaquettes | III | III |

*Ces pourcentages représentent la fraction totale de la drogue éliminée dans les urines.

organes et permettent d'aborder la pharmacocinétique plasmatique faite chez l'homme et montrent qu'il existe de très grandes variations entre individus et que l'administration réitérée de ce médicament semble entrainer une profonde modification des paramètres pharmacologiques (voir Tableau III).

L'élimination se fait préférentiellement par voie urinaire sous forme inchangée pour 30 à 60%. Il n'a pas été mis en évidence de forme conjuguée. L'étude de différentes formes de traitements mathématiques des données pharmacocinétiques fait l'objet d'un travail plus approfondi dans notre institut.

REMERCIEMENT

Nous remercions Mme R. Dufour pour sa collaboration technique au cours de nos différents essais.

RÉSUMÉ

Il est décrit une méthode de dosage d'un nouvel antitumoral, le SOAz [1,3,3,5,5-pentakis-(aziridino)-$\lambda^6$,2,4,6,3$\lambda^5$,5$\lambda^5$-thiatriazadiphosphorine-1-oxide] utilisant la chromatographie en phase gazeuse avec colonnes capillaires et détecteur thermoionique. La quantité minimum mesurable est de 100 pg par injection, l'utilisation d'un standard externe permet d'obtenir des coefficients de variation inférieurs à 5%. La méthode est applicable aussi bien aux liquides biologiques qu'aux organes et permet d'étudier la pharmacocinétique chez l'homme et la distribution de la drogue dans les différents tissus chez l'animal. Les études préliminaires chez l'homme montrent que la cinétique est bien décrite par un système bi-compartimental ouvert.

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# AN AUTOMATED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF AMINOGLYCOSIDES IN SERUM USING PRE-COLUMN SAMPLE CLEAN-UP AND DERIVATIZATION

LUDWIG ESSERS

*Institute of Medical Microbiology, University of Essen, D-4300 Essen (F.R.G.)*

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## SUMMARY

An automated high-performance liquid chromatographic method for the determination of the aminoglycosides amikacin, dibekacin, gentamicin, netilmicin, sisomicin and tobramycin is described. The procedure involves sample clean-up by adsorption of the aminoglycosides on a pre-column, subsequent derivatization with *o*-phthalaldehyde and on-line separation of derivatives by column switching. A short cation-exchange column serving concurrently as a guard column in combination with a reversed-phase column was used for separation. Except for the determination of netilmicin an internal standard consisting of an aminoglycoside was used in each assay. The signals of the aminoglycosides determined were linear within the range of 1—16 mg/l serum. The inter-assay imprecision ($n$ = 10) calculated as coefficient of variation was less than 6%. The results were obtained within 20 min after injection of the serum sample. Easy performance and flexibility make the procedure feasible for therapeutic drug monitoring.

## INTRODUCTION

High-performance liquid chromatographic (HPLC) methods, when compared with other assays for the determination of gentamicin levels in serum, reveal excellent performance characteristics concerning linearity, accuracy, recovery and inter-assay precision [1, 2]. Another advantage is versatility, low running costs and a high degree of specificity. Nevertheless, HPLC procedures have not been widely introduced for drug monitoring of aminoglycosides in routine laboratories. A main reason for this is the fact that HPLC techniques for the analysis of aminoglycosides usually require labor-intensive sample preparation including deproteinization and derivatization to a fluorogenic or highly UV-absorbing compound.

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This paper describes an automated method for the assessment of aminoglycoside levels in body fluids using pre-column techniques for sample preparation and on-line separation and detection of phthalaldehyde derivatives.

## EXPERIMENTAL

### *Instrumentation*

The sample preparation device (see Fig. 1) consisted of two pumps (Model 410, Kontron, Zürich, Switzerland) for the delivery of rinsing buffer and derivatization reagent, two motor-driven six-port valves (Latek, Heidelberg, F.R.G.) switched by the integrator, a 40 × 4.6 mm stainless-steel column dry-packed with Bondapak $C_{18}$ Corasil (37—50 μm, Waters, Milford, MA, U.S.A.) and a 250 × 4.6 mm pre-saturator column dry-packed with coarse-grained silica gel (Merck, Darmstadt, F.R.G.).

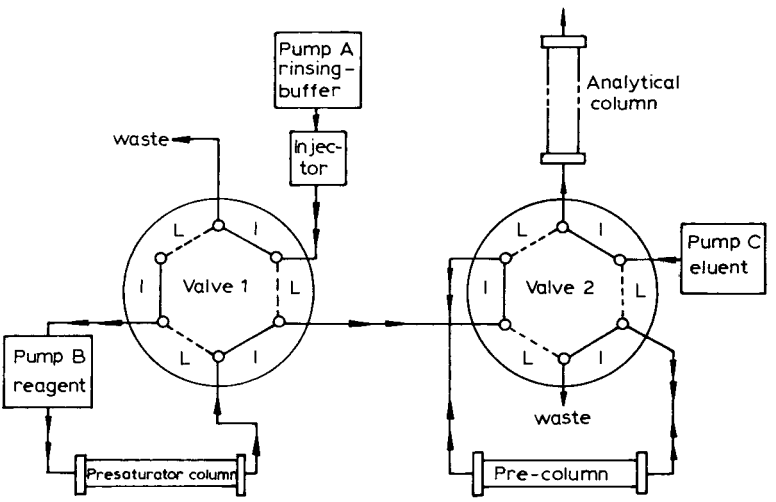


**Fig. 1. Diagram of the pre-column switching system.**

Chromatographic separations were performed with a Hewlett-Packard liquid chromatograph 1081 B equipped with a 7120 Rheodyne sample injector (20-μl loop, Rheodyne, Berkeley, CA, U.S.A.) and a temperature-controlled column compartment. The analytical column system consisted of a 60 × 4.6 mm column filled with Nucleosil SA, 5 μm particle size, adapted to a 125 × 4.6 mm column packed with Nucleosil $C_{18}$ (5 μm). The packing material is available from Macherey & Nagel (Düren, F.R.G.). Pre-packed columns were purchased from Bischoff (Leonberg, F.R.G.).

The temperature of the analytical column was kept at 25°C. The column effluent was monitored by a Perkin-Elmer fluorimeter (Model 2000 (Perkin-Elmer, Norwalk, CT, U.S.A.) equipped with an 18-μl flow cell, a UG 1 excitation filter combined with a 10-mm diaphragm and a KV 418 filter at the emission site. Filters are available from Schott (Mainz, F.R.G.). The detector signal was recorded by an SP 4200 computing integrator (Spectra-Physics, Santa Clara, CA, U.S.A.).

*Materials*

Demineralized water "nanopure" grade was used. Methanol, 2-mercaptoethanol and sodium acetate were p.a. grade from Merck. *o*-Phthalaldehyde purissimum) was purchased from Serva (Heidelberg, F.R.G.). Amikacin sulphate (potency 749 μg/mg) was a gift from Grünenthal (Stolberg, F.R.G.), dibekacin sulphate (704 μg/mg) was from Pfizer (Karlsruhe, F.R.G.), gentamicin sulphate (606 μg/mg) was from Merck, gentamicin components C1 (620 μg/mg), C1A (788 μg/mg) and C2 (641 μg/mg) as well as netilmicin sulphate (581 μg/mg) were gifts from Schering (Bloomfield, NJ, U.S.A.), neamine base was kindly donated by Upjohn (Kalamazoo, MI, U.S.A.), sisomicin sulphate (594 μg/mg) was from Bayer (Leverkusen, F.R.G.) and tobramycin sulphate (928 μg/mg) was from Lilly (Indianapolis, IN, U.S.A.).

Standards were freshly prepared by spiking 950 μl of pooled serum from donors with 50 μl of each of the aminoglycosides to be determined and an internal standard solution (see Table I). Specimens were spiked with a stock solution of the internal standard (usually 10 μl per 200 μl of serum).

TABLE I

CHROMATOGRAPHIC ELUENTS USED FOR THE SEPARATION OF DIFFERENT AMINOGLYCOSIDES WITH THE AUTOMATED METHOD AND APPROPRIATE SUBSTANCES USED AS INTERNAL STANDARD

| Aminoglycoside | Eluent | Internal standard |
|---|---|---|
| Amikacin | 74% Methanol—water<br>0.25 *M* Sodium acetate, pH 7.4 | Neamine |
| Dibekacin<br>Gentamicin<br>Sisomicin | 80% Methanol—water<br>0.1 *M* Sodium acetate, pH 7.4 | Tobramycin |
| Tobramycin | 80% Methanol—water<br>0.1 *M* Sodium acetate, pH 7.4 | Gentamicin C1A |
| Netilmicin | 95% Methanol—water<br>0.2 *M* Sodium acetate, pH 5.0 | Not yet found |

*Chromatographic conditions*

The rinsing buffer for the sample preparation system was a 0.15 *M* sodium acetate buffer pH 7.4. The flow-rate was 1 ml/min.

Derivatizing reagent described by Maitra et al. [3] was pumped at a rate of 0.2 ml/min.

The mobile phase for the analytical column system (flow-rate 1 ml/min) varied depending on the aminoglycoside to be separated. Solvent composition and the appropriate internal standard can be read from Table I.

pH values were adjusted with concentrated nitric acid. Depending on the age of the column and the brand used for each column the amount of methanol and/or sodium acetate required may vary somewhat.

Solutions were passed through a 0.45-μm HVLP filter (Millipore, Bedford, MA, U.S.A.) and deaerated under reduced pressure immediately before use.

*Procedure*

Serum samples were centrifuged in 1.5-ml polypropylene cups (Eppendorf, Hamburg, F.R.G.) at 13,500 *g* (Haemofuge, Heraeus-Christ, Osterode, F.R.G.) for 1 min and spiked with the internal standard. With the injection of 20 $\mu$l of the serum sample the integrator switching the valves was started, whilst the right valve (Fig. 1) was in the "I" (inject) position and the left valve in the "L" (load) position. The sample was rinsed by pump A through the pre-column for 3 min. During this time derivatization reagent was recycled by pump B and the mobile phase used for separation was directly pumped through the analytical column system (pump C). After 3 min valve 1 was switched to the "I" position allowing the *o*-phthalaldehyde reagent to flow through the pre-column for 1 min. Then both valves were positioned to "L" for another 3 min. In this position the aminoglycloside derivative was eluted from the pre-column to the analytical column. By switching valve 2 to the "I" position the cycle was finished. The total time for sample preparation was 4 min; another 16 min were necessary for separation of the aminoglycoside derivatives from the blank.

## RESULTS

As shown in Fig. 2, with the gentamicin components C1, C1A and C2, adsorption of aminoglycosides to reversed-phase material depends on the sodium acetate concentration added to the rinsing buffer of the pre-column. A compromise has been made with a concentration of 0.15 *M* sodium acetate

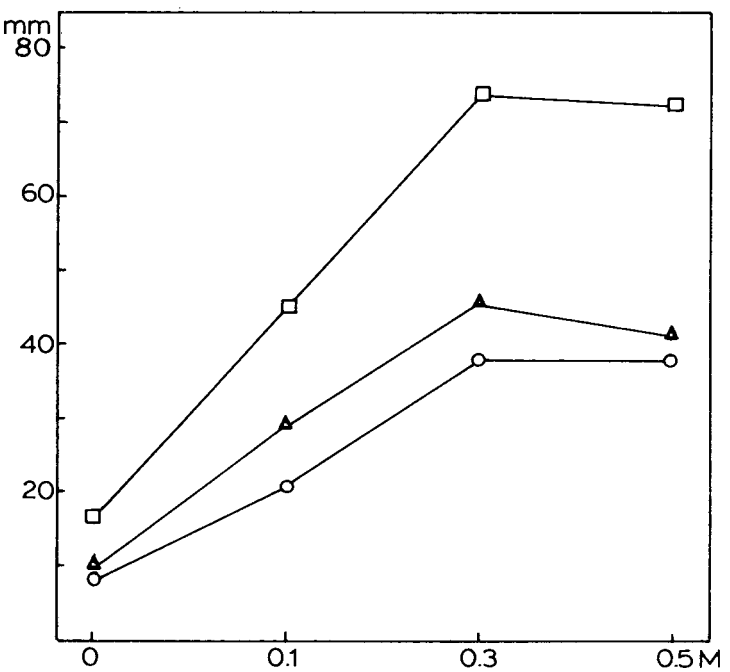


Fig. 2. Adsorption of gentamicin components C1 (□), C1A (▲) and C2 (○) dependent on the sodium acetate concentration of the rinsing buffer. Ordinate: peak height of derivatives eluted. Abscissa: acetate concentration.

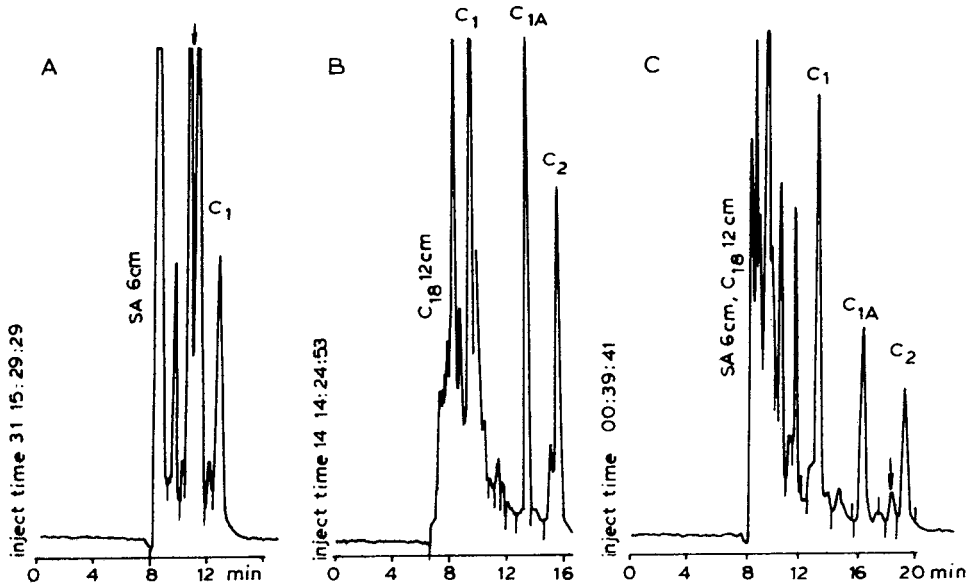


Fig. 3. Separation of gentamicin derivatives from blank peaks: (A) short cation-exchange column (arrow indicates badly resolved C1A and C2 compounds); (B) reversed-phase column; (C) combination of both columns (arrow indicates C2A compound).

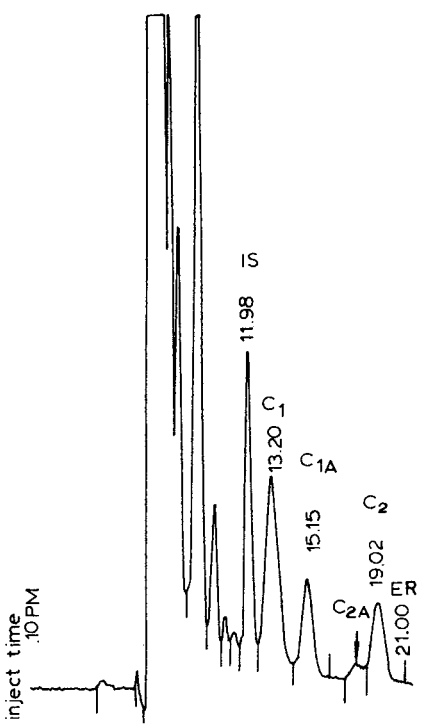


Fig. 4. Chromatogram of a serum sample containing 3.7 mg/l gentamicin spiked with 2.5 mg/ml tobramycin (internal standard, IS).

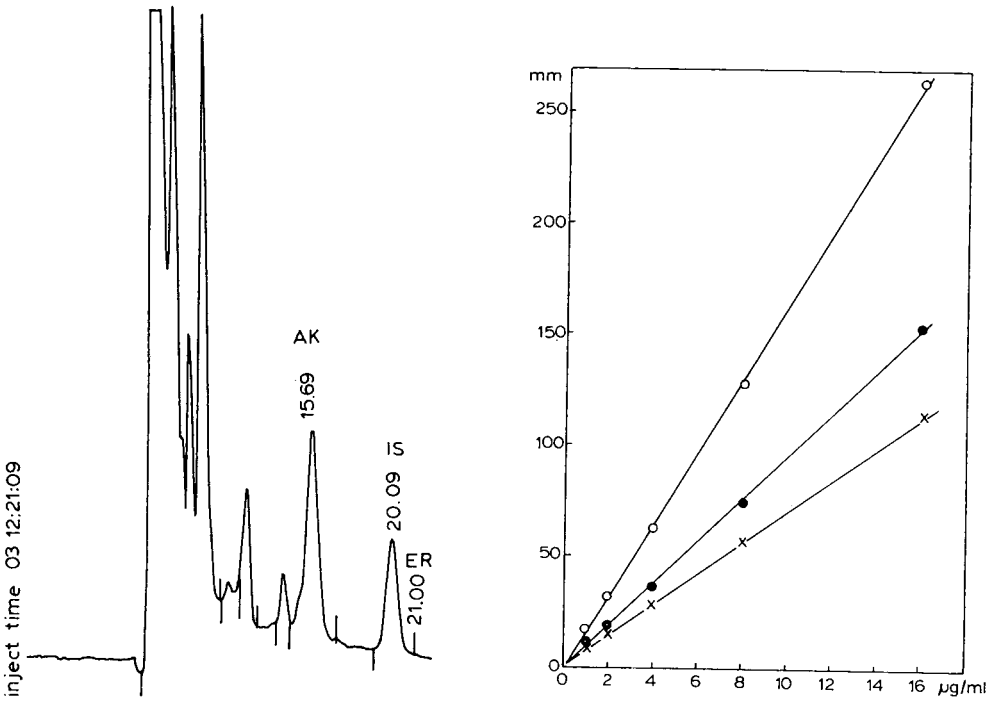


Fig. 5. Chromatogram of a serum sample containing 5 mg/l amikacin (AK) spiked with 2.5 mg/l neamine (internal standard, IS).

Fig. 6. Calibration curve of the automated system determined with the three gentamicin components. (○), C1: $Y = 16.6X - 1.6$, $r = 0.9998$. (●), C1A: $Y = 9.5X - 0.7$, $r = 0.9997$. (×), C2: $Y = 7.2X + 0.3$, $r = 0.9998$.

because amikacin adsorption was reduced at higher concentrations. The pH of the rinsing buffer has been adjusted to pH 7.4 to avoid precipitation of serum components. As indicated by protein determinations, more than 99% of serum proteins are eluted from the pre-column by rinsing for 3 min. Column pressure gradually rises but more than 100 samples can be handled with one pre-column packing.

Gentamicin C1 has a very short retention time compared to the other gentamicin components when eluted from reversed-phase material, resulting in a bad separation from blank peaks (Fig. 3B). The combination of a short cation-exchange column with a reversed-phase column can overcome this problem as the C1 component adsorbs more strongly to a cation-exchanger than gentamicin C1A and C2 (Fig. 3A and C).

Figs. 4 and 5 show typical chromatograms obtained with a serum sample containing gentamicin and amikacin, respectively, spiked with the appropriate internal standard.

Linearity of the analytical system is demonstrated with the calibration curve of gentamicin components (Fig. 6).

Intra-assay precision calculated by the coefficient of variation was determined for gentamicin and amikacin. Values ($n = 10$) were 5.9% at a gentamicin concentration of 4.7 mg/l and 4.9% at an amikacin concentration of 5.8 mg/l.

## DISCUSSION

An automated method for the analysis of drugs by HPLC has been described by Roth et al. [4]. Their procedure cannot, however, be applied to aminoglycosides without modification because these molecules exhibit strong polarity and do not readily adsorb to reversed phases. The problem is solved at low cost by adding sodium acetate to the rinsing buffer which probably acts as an ion-pair reagent.

This is, as far as we know, the first time that pre-column derivatization with on-line separation has been tried for aminoglycoside analysis. Post-column derivatization systems have been described by Anhalt and Brown [5] and Mays et al. [6]. Even though the post-column procedure works reliably, some disadvantages should be mentioned: baseline noise is enhanced by pumping derivatization reagent through the detector; the flow cell may be soiled by reaction products; consumption of reagent is high; and reaction time can only be prolonged by increased dead volume resulting in additional peak broadening and loss of sensitivity. These flaws can be avoided by pre-column derivatization.

One problem of pre-column derivatization is the fact that alkaline *o*-phthaldehyde reagent (pH 10.4) is passed through the pre-column, leading gradually to destruction of the silica skeleton of the stationary phase. This effect can be minimized by passing the reagent through a pre-saturator column filled with coarse-grained silica gel. Efficient separation of four gentamicin components has been performed using ion-pair chromatography with alkylsulphonate [7] or an EDTA—methanol mixture [8] as the solvent.

This paper demonstrates that a high resolution of four gentamicin peaks is possible by the combination of a short cation-exchange column serving concurrently as a guard column with a reversed-phase column.

Variation of retention times caused by aging of the column packing or by different brands can be compensated by small changes of methanol or salt content of the eluent.

The HPLC method described has been integrated into a therapeutic drug monitoring program. Its main advantage is easy performance, reliability, flexibility and reduced running cost. The procedure has been applied with success to the aminoglycosides amikacin, dibekacin, gentamicin, netilmicin, sisomicin and tobramycin. The analysis time of 20 min seems to be acceptable as the number of samples sent to our laboratory is limited.

## ACKNOWLEDGEMENTS

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CHROMBIO. 1916

# SEMI-AUTOMATED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF CYCLOSPORINE IN PLASMA AND BLOOD USING COLUMN SWITCHING

H.T. SMITH* and W.T. ROBINSON

*Drug Metabolism Section, Sandoz, Inc., Route 10, East Hanover, NJ 07936 (U.S.A.)*

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## SUMMARY

A sensitive, semi-automated high-performance liquid chromatographic method utilizing column switching is described for the determination of cyclosporine in plasma and blood. This method involves a short and improved manual protein precipitation of the sample followed by an automated clean-up of the supernatant. After automatic loading of the clean supernatant onto an LC-8 column for initial separation, the segment containing cyclosporine is loaded (automatically) onto an LC-18 column for final separation and quantitation. Cyclosporine is detected by its ultraviolet absorption at 202 nm. The rate of analysis was four samples per h running 24 h per day (ca. 100 samples per day). The method is sensitive enough to measure with confidence cyclosporine concentrations of 8 μg/l in plasma and 20 μg/l in blood with a linear response up to 2500 μg/l using only 0.5 ml of sample. No internal standard is required. The method was applied continuously (24 h per day) to approximately 1000 samples without deterioration in method parameters.

## INTRODUCTION

Cyclosporine (Sandimmune®), a novel immunosuppressive agent [1, 2], is a cyclic undecapeptide [3] currently used in the area of organ transplants. The clinical success of cyclosporine and the utility of measuring blood or plasma concentrations of the drug have been documented by several investigators [4—9]. The purpose of this report is to describe an improved semi-automated high-performance liquid chromatographic (HPLC) method for the determination of cyclosporine in plasma or blood.

Several methods have been reported and are summarized in Table I. The radioimmunoassay (RIA) method [10] was originally published for analysis of plasma but has been applied successfully to both blood and plasma analysis at

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TABLE I

COMPARISON OF PUBLISHED METHODS

| Author | Ref. | Type of analysis | Sample type (vol. in ml) |
|---|---|---|---|
| Donatsch et al. | [10] | RIA | Blood/plasma (0.1) |
| Niederberger et al. | [11] | HPLC-Gradient | Plasma (1) |
| Lawrence and Allwood | [12] | HPLC-Isocratic | Serum (1) |
| Sawchuk and Cartier | [13] | HPLC-Isocratic | Blood/plasma (2) |
| Nussbaumer et al. | [14] | HPLC-Column switching | Blood/plasma (0.5) |
| Leyland-Jones et al. | [15] | HPLC-Isocratic | Plasma (0.6) |
| Kahan et al. | [7] | HPLC-Isocratic | Blood/plasma (1) |
| Yee et al. | [16] | HPLC-Gradient (Isocratic?) | Serum (2) |
| Carruthers et al. | [17] | HPLC-Isocratic | Plasma (1) |
| Smith and Robinson (present publication) | | HPLC-Column switching | Blood/plasma (0.5) |

*Short = protein precipitation and injection; long = extraction and evaporation; very long = additional sample manipulation.
**By nature of the method the average sample preparation plus analysis time is short for a large number of samples.

detection limits better than reported [18]. This method is not totally specific in that cross-reactivity to selected metabolites has been shown [10, 17].

Most of the HPLC methods available have definite disadvantages. In general, sample preparations tend to be labor-intensive and/or chromatography times tend to be long, severely limiting sample through-put. In order to well define the pharmacokinetics of cyclosporine, a method should be able to define plasma or blood concentrations for at least three half-lives [19]. Based on the work of Beveridge et al. [20], detection limits of published HPLC methods seem to present problems and the methods of Niederberger et al. [11] and Nussbaumer et al. [14] have been up to now the most attractive.

In order that we might investigate the pharmacokinetics of cyclosporine in man, it was the objective of the following work to develop and validate an HPLC method with a detection limit of less than 20 μg/l and an improved analysis time over existing methods.

## MATERIALS AND METHODS

### *Reagents*

Chemicals used were cyclosporine (cyclosporin A) (Sandoz, E. Hanover, NJ, U.S.A.); methanol, acetonitrile and hexane (UV; HPLC grade, Fisher Scientific, Fair Lawn, NJ, U.S.A.) and glass-distilled water. Outdated human plasma (N.J. Blood Services, New Brunswick, NJ, U.S.A.) and whole blood (Sera-Tec Biologicals, North Brunswick, NJ, U.S.A.) were used in the preparation of standards.

A stock standard of cyclosporine was prepared by dissolving 12.5 mg of cyclosporine in 100 ml of methanol. Further dilutions of this stock solution

| Sample preparation* | Analysis parameters | | | |
|---|---|---|---|---|
| | Detection limit ($\mu$g/l) | Selectivity | Standard-ization | Chromatography time (min) |
| ** | 1—20 | Metabolite x-reactivity | External | ** |
| Long | 20 | High | Internal | 30 |
| Long | 100 | (?) | External | 5 |
| Very long | 25 | High | Internal | 10 |
| Short | 20 | High | Internal | 30 |
| Long | 100 | High | Internal | 20 |
| Long | 100 | High | Internal | 45 |
| Long | 30—50 (?) | High | Internal | 15 |
| Long | 31 | High | Internal | 30 |
| Short (semi-automated) | 8 | High | External | 15 |

were made with methanol prior to preparing plasma and blood standards containing 20—2500 $\mu$g/l cyclosporine (less than 4% methanol).

*Solutions*

*Mobile phases A and B.* For mobile phase A, 5 l of acetonitrile—water (55:45) and for mobile phase B, 3 l of acetonitrile—water (75:25) are prepared daily. The solutions are filtered/degassed using a vacuum filter system (Millipore, Bedford, MA, U.S.A.) equipped with a 0.45-$\mu$m filter (Nylon 66, Rainin Instrument, Woburn, MA, U.S.A.).

*Precipitation solution.* Prepare 1 l of acetonitrile—water (97.5:2.5).

*Precipitation procedure*

Into 15-ml glass-stoppered test tubes pipet 0.5 ml of each blank, standard or subject plasma (or blood) sample. Add, by use of a Repipet® (Labindustries, Berkeley, CA, U.S.A.), 1.2 ml of the precipitation solution, stopper and mix for 30 sec using a Maxi-Mix® (Thermolyne, Dubuque, IA, U.S.A.). Centrifuge at 1000 *g* for 5 min.

*Automated sample wash and chromatography*

Decant the supernatant from each sample into a 2-ml glass autosampler vial (Cat. No. 3-3123, Supelco, Bellefonte, PA, U.S.A.) which is held in a 5-ml polystyrene sample cup (Technicon Instruments, Tarrytown, NY, U.S.A.), and cover with a 2.5 cm × 2.5 cm piece of aluminum foil. Place the sample cup containing the supernatant into the tray of the Sampler IV® (Technicon Instruments). Start the automated microprocessor-controlled (Altex 420, Altex, Berkeley, CA, U.S.A.) sample wash and chromatography process utilizing

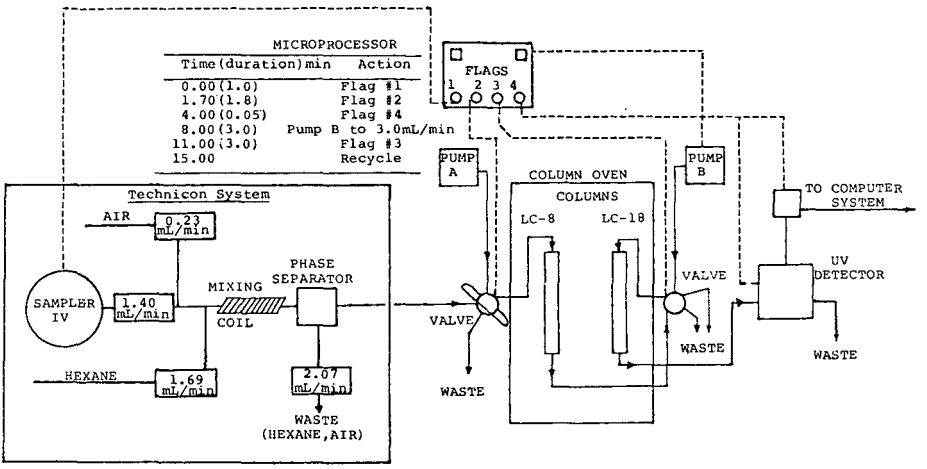


Fig. 1. Schematic of the automated sample preparation and chromatography system. (— — — —) Microprocessor controlled devices.

a Proportioning Pump III® (Technicon Instruments) and the sample wash and chromatography set up shown in Fig. 1. Program the microprocessor (see Fig. 1) to automatically sample, wash (with hexane), separate (the supernatant from the hexane) and load the sample onto the 2.0-ml loop of the pneumatically actuated, remotely controlled injection valve (Model AH-CV6-UHPa-N60 with DVI; Valco Instruments, Houston, TX, U.S.A.). The sample contained in the loop of the injection valve is automatically injected onto an LC-8 column (Supelcosil LC-8, 150 × 46 mm, 5 $\mu$m particle size; Supelco) kept at 75°C in a thermostated column oven (Model LC250; Kratos, Westwood, NJ, U.S.A.). Cyclosporine is eluted with mobile phase A at a flow-rate of 3.0 ml/min using Pump A (Model 110A, Altex). The segment containing cyclosporine is automatically diverted, by means of a second injection valve (Valco Instruments), onto an LC-18 column (Supelcosil LC-18, 150 × 4.6 mm, 5 $\mu$m particle size; Supelco) also at 75°C and cyclosporine is eluted from this column with mobile phase B at a flow-rate of 1.0 ml/min using Pump B (Model 110A, Altex). Cyclosporine is detected by its UV absorption at 202 nm using a variable-wavelength detector (Model LC-75 with autocontrol; Perkin-Elmer, Norwalk, CT, U.S.A.). Peak height measurements, baseline integrations and calculations are performed by a computer system (HP-1000; Hewlett-Packard, Paramus, NJ, U.S.A.) equipped with a computer-automated laboratory system (CALS) software package (Computer Inquiry Systems, Waldwick, NJ, U.S.A.). Concentrations of cyclosporine in plasma and blood are determined by relating their peak height measurements to the standard curve concentration—response measurements run before, during and after analysis of unknown samples.

## RESULTS

### *Chromatograms*

No interfering peaks have been detected in the plasma or blood used for blank standards or from subjects who have been orally dosed with cyclo-

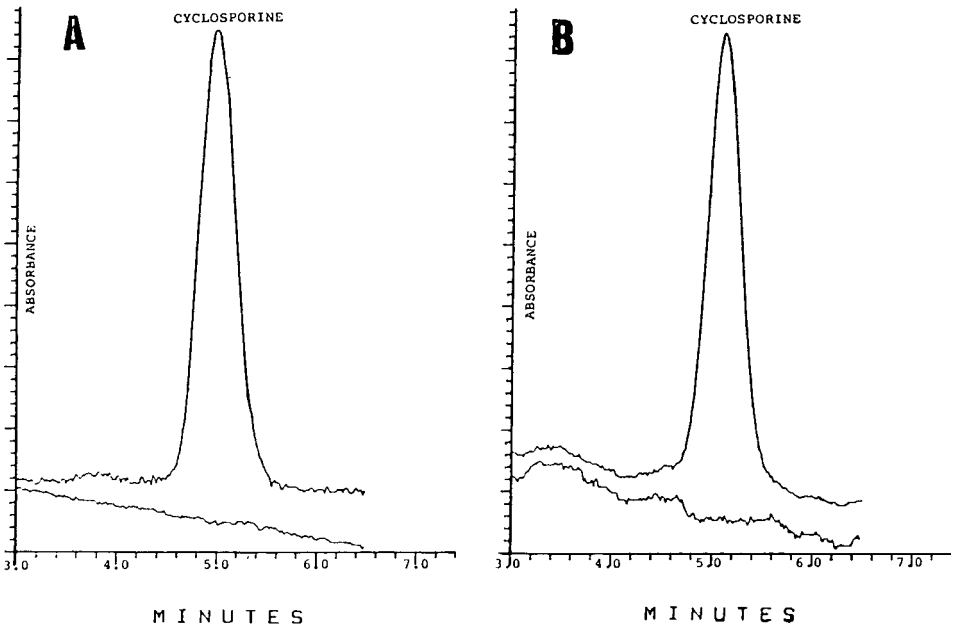


**Fig. 2. Representative chromatograms obtained in the analysis of 0.5 ml of (A) blank plasma (lower trace) and plasma standard containing 200 μg/l cyclosporine and (B) subject plasma samples at zero h (lower trace) and 1.0 h (equivalent to 280 μg/l cyclosporine) after oral administration (1400 mg) of cyclosporine.**

sporine. Fig. 2A shows a chromatogram of a plasma blank and 200 μg/l standard and Fig. 2B shows a zero-h and 1.0-h plasma sample from a normal volunteer who had received a single oral dose (1400 mg) of cyclosporine. The absence of a solvent front is due to a computer user-controlled rejection of the first 3 min of data following sample injection. This eliminates the large offset caused by the large volume of mobile phase A passing through the LC-18 column upon injection of the cyclosporine segment.

*Linearity*

Daily standardization curves ($n$ = 16) for cyclosporine in both plasma and blood resulted in a linear concentration—response relationship. Cyclosporine concentrations of 0, 50, 200, 500, 1000 and 2500 μg/l in plasma and blood were used for standardization and regression analysis of the data resulted in mean slope and $y$-intercept values of 0.024 mV/μg/l and 0.020 mV for plasma and 0.016 mV/μg/l and 0.06 mV for blood, respectively. The corresponding mean correlation coefficients for the plasma and blood standard curves were 0.999 and 0.997, respectively.

*Accuracy, precision and reproducibility*

The accuracy of the method was evaluated by analyzing plasma and blood samples containing known amounts of cyclosporine. Using the $t$-value from a one-tailed Student's $t$-distribution table and the variance of absolute differences between the actual concentrations and the concentrations found (see Table II), the 95% confidence intervals for single determinations of cyclosporine in

TABLE II

VALIDATION OF METHOD APPLIED TO PLASMA AND BLOOD USING SPIKED SAMPLES

| Spiked concentration (μg/l) | Mean ± S.D. (C.V., %) | | | | | Mean absolute difference from true value ± S.D. (μg/l) | 95% Confidence limit percent of true value |
|---|---|---|---|---|---|---|---|
| | Day 1 ($n$ = 4) | Day 2 ($n$ = 4) | Day 3 ($n$ = 4) | Day 4 ($n$ = 4) | Days 1—4 ($n$ = 16) | | |
| Plasma | | | | | | | |
| 30 | 27 ± 3 (11.1) | 31 ± 2 (6.5) | 28 ± 2 (7.1) | 32 ± 3 (9.4) | 29 ± 3 (10.3) | 2.4 ± 2.1 | ± 20.3 |
| 117 | 117 ± 5 (4.3) | 109 ± 1 (0.9) | 108 ± 3 (2.8) | 123 ± 7 (5.7) | 114 ± 8 (7.0) | 7.1 ± 4.0 | ± 12.1 |
| 480 | 517 ± 20 (3.9) | 473 ± 16 (3.4) | 439 ± 7 (1.6) | 455 ± 5 (1.0) | 471 ± 32 (6.8) | 29.0 ± 15.3 | ± 11.6 |
| 1220 | 1172 ± 22 (1.9) | 1220 ± 6 (0.5) | 1152 ± 29 (2.5) | 1201 ± 33 (2.7) | 1186 ± 35 (2.7) | 36.8 ± 31.1 | ± 7.5 |
| 2460 | 2648 ± 41 (1.5) | 2357 ± 67 (2.8) | 2392 ± 23 (1.0) | 2441 ± 30 (1.2) | 2459 ± 123 (5.0) | 94.8 ± 73.5 | ± 9.1 |
| Blood | | | | | | | |
| 25 | 29 ± 4 (13.8) | 29 ± 1 (3.4) | 26 ± 1 (3.8) | 23 ± 2 (8.7) | 26 ± 3 (11.5) | 2.5 ± 2.4 | ± 26.8 |
| 500 | 476 ± 22 (4.6) | 585 ± 59 (10.1) | 465 ± 33 (7.1) | 519 ± 80 (15.4) | 511 ± 68 (13.3) | 52.4 ± 43.3 | ± 25.7 |
| 1000 | 1063 ± 81 (7.6) | 1071 ± 66 (6.2) | 972 ± 73 (7.5) | 1093 ± 96 (8.8) | 1048 ± 86 (8.2) | 73.1 ± 64.5 | ± 18.7 |
| 2400 | 2200 ± 127 (5.8) | 2388 ± 198 (8.3) | 2575 ± 39 (1.5) | 2497 ± 151 (6.0) | 2409 ± 194 (8.1) | 172.7 ± 75.0 | ± 12.7 |

plasma and blood were calculated at all concentrations. The results indicate that any single value would fall within ± 20.3% and 26.8% of its true value for plasma and blood, respectively.

The precision (within-day variability) and reproducibility (day-to-day variability) of the method are also demonstrated by the data in Table II. The coefficients of variation (C.V.) for the within-day variation at any concentration of cyclosporine in plasma ranged from 0.5% to 11.1% while the day-to-day variation for the same set of data ranged from 3.0% to 10.3%. For blood the within-day variation was 1.5% to 15.4% and the day-to-day variation for the same data ranged from 8.1% to 13.3%.

*Sensitivity*

The sensitivity of this method was evaluated by analyzing plasma and blood samples to which cyclosporine had been added in concentrations near the limit of sensitivity. The results from the analysis of these samples are shown in Table III. Although concentrations could be detected to 5 μg/l and 15 μg/l in plasma and blood, respectively, the precision and accuracy deteriorated at these concentrations. Method parameters remained consistent to 8 μg/l in plasma and 20 μg/l in blood. The difference in the limit of sensitivity is due to the lower recovery of cyclosporine from blood samples.

TABLE III

EVALUATION OF DETECTION LIMIT OF CYCLOSPORINE IN PLASMA OR BLOOD

Coefficients of variation (%) in parentheses.

| Cyclosporine concentration (μg/l) | Mean response factor (μg/l/mV) | |
|---|---|---|
| | Plasma | Blood |
| 5 | 61.61 (32.8) | — |
| 8 | 36.16 (15.5) | — |
| 15 | 39.88 (15.9) | 118.28 (16.1) |
| 20 | 34.31 (0.2) | 77.99 (8.6) |
| 50 | 35.69 (0.6) | 63.39 (2.2) |
| 200 | 38.58 (3.8) | 62.96 (6.1) |

*Stability*

The pooled plasma samples initially analyzed as part of the accuracy study were stored at 1°C for 12 weeks. A quadruplicate analysis of these samples after 4 and 12 weeks resulted in the mean values and C.V. values shown in Table IV. No apparent loss in cyclosporine was noted for the 12-week period.

*Application of the method*

Approximately 1000 plasma and blood samples from twelve subjects dosed with cyclosporine have been analyzed by this method. The method was applied on a 24 h per day basis. During this period, no deterioration in method parameters (peak shape, retention time) was noted. Typical blood concentrations after a single 1400-mg dose of cyclosporine are shown in Fig. 3. In most

TABLE IV

ANALYSIS OF PLASMA SAMPLES CONTAINING CYCLOSPORINE AFTER STORAGE AT 1°C FOR 12 WEEKS

| Theoretical concentration (μg/l) | Mean ± C.V. (%) | |
|---|---|---|
| | 4 weeks | 12 weeks |
| 30 | 34 ± 4.2 | 26 ± 6.5 |
| 117 | 104 ± 7.7 | 110 ± 0.5 |
| 480 | 494 ± 12.8 | 497 ± 2.6 |
| 1220 | 1204 ± 3.9 | 1323 ± 0.9 |
| 2460 | 2223 ± 6.8 | 2651 ± 1.7 |

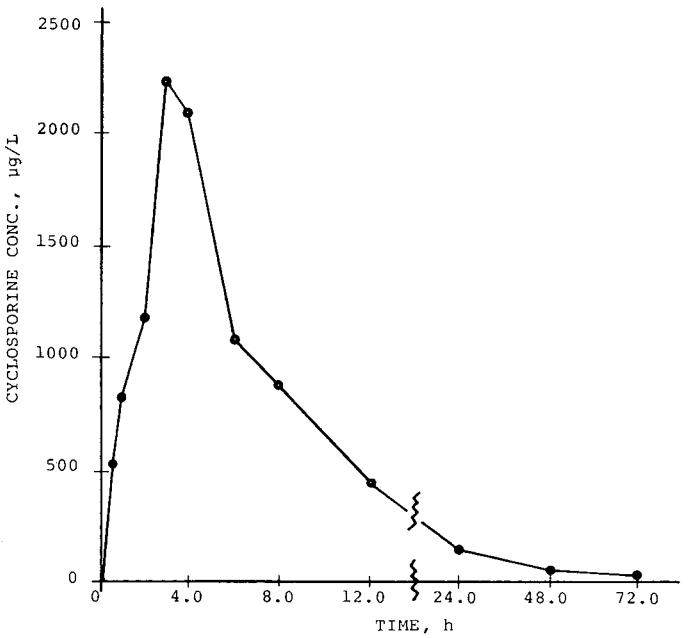


Fig. 3. Concentration of cyclosporine in blood as a function of time from a subject who received a single oral dose of 1400 mg.

cases, for doses down to 350 mg, plasma and blood concentrations above the detection limit of the method could be followed for at least three half-lives.

## DISCUSSION

Although several HPLC methods are reported for the analysis of cyclosporine in plasma and blood, none has approached the level of automation that has been presented here. Following a simple manual protein precipitation of each plasma or blood sample, a fully automated analysis system completes sample preparation, chromatographic separation and data analysis at the rate of four samples per h. Moreover, the method could be applied continuously day and night at a rate of approximately 100 samples per day.

The use of acetonitrile as a protein precipitation medium is more efficient than methanol [21] and therefore, heating of the samples to obtain a clean supernatant as described by Nussbaumer et al. [14] was not necessary.

The use of a hexane wash for removing additional lipophilic material from the sample was introduced by Sawchuk and Cartier [13]. Incorporation of the hexane wash in the automated system provided a sample apparently free of most late eluting components. The remaining material was removed by increasing the mobile-phase flow-rate providing an adequate column clean-up without creating the longer analysis times associated with the step gradient [14] and gradient elution methods [11].

In general, use of an internal standard in liquid chromatography is not necessary. The simple sample preparation in this method allowed us to use external standardization simplifying the method and reducing the chromatography time without loss of linearity, precision or accuracy.

Based on the analysis of samples spiked with drug around the detection limit of the method, concentrations of 8 $\mu$g/l and 20 $\mu$g/l for plasma and blood, respectively, could be measured with confidence. Concentrations below this limit could be detected albeit with a loss in precision and accuracy.

Maintenance of the semi-automated system is limited to routine column care — which is performed daily and consists of replacement of the frit and the packing material at the top of each column — and weekly replacement of pump tubes on the Technicon Proportioning Pump. Total maintenance time is approximately 30 min daily for column care and 5 min weekly for pump tube replacement. The cost of the instrumentation used for this system was approximately $35,000 which does not include the HP-1000 computer system, but does include a gradient HPLC system (with a microprocessor), Technicon Proportioning Pump and Sampler IV which are found in many laboratories.

In conclusion, the method reported here employs (a) an automated sample wash; (b) column switching and (c) computer analysis providing greater sensitivity and greater sample through-put than previously reported. The reliability of the method, the sensitivity of the method and utility of the method have been adequately demonstrated with the routine analysis of approximately 1000 samples.

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# HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF CEFOTETAN EPIMERS IN HUMAN PLASMA AND URINE

F. KEES and H. GROBECKER*

*Department of Pharmacology, University of Regensburg, Universitätstrasse 31, D-8400 Regensburg (F.R.G.)*

and

K.G. NABER

*Urologic Clinic, Elisabeth Hospital, Schulgasse 20, D-8440 Straubing (F.R.G.)*

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## SUMMARY

Cefotetan, a new broad-spectrum 7α-methoxycephalosporin antibiotic, was assayed in plasma and urine by means of reversed-phase high-performance liquid chromatography. Commercially available cefotetan exists in two epimeric forms. The procedure described allows the separation and quantitation of both epimers. For the first time a different pharmacokinetic behaviour ($t_{1/2}$ = 3 h versus 4 h) for each epimer after intravenous injection to healthy volunteers is demonstrated. It is assumed that one epimer is bound to a greater extent to serum proteins and is therefore responsible for the differences observed. As both epimers exhibit similar antibacterial activity, it seems doubtful whether these differences would have clinical significance. Iothalamic acid was determined simultaneously as a marker of kidney function.

## INTRODUCTION

Cefotetan disodium is a new 7α-methoxycephalosporin with a wide spectrum of antibacterial activity and remarkable stability against various β-lactamases. Pharmacokinetic parameters in man have been studied in different races [1—4]. The commercially available product consists of a mixture of two epimers because of the asymmetric carbon atom at the dithietan ring. In this paper the *R* and *S* epimers are called A and B, respectively, because the absolute configuration was not known. In weakly alkaline media the two epimers are

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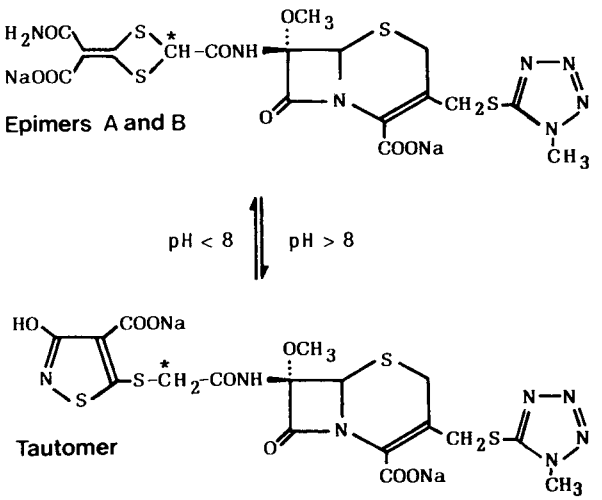


Fig. 1. Chemical structure of cefotetan. The asterisk marks the epimeric centre and the position of this carbon atom in the tautomer.

in equilibrium with a third tautomeric form (Fig. 1). Under physiological conditions the proportion of the tautomer is small, as demonstrated by others [1, 4]. The aim of the present study was to elucidate the pharmacokinetic behaviour of both epimers of cefotetan after intravenous injection of the drug in healthy volunteers.

## MATERIALS AND METHODS

### *Reagents*

Cefotetan was provided by ICI-Pharma (Plankstadt, F.R.G.), Conray 70® (meglumine iothalamate) and iothalamic acid {3-(acetylamino)-2,4,6-triiodo-5-[(methylamino)carbonyl]-benzoic acid} by Byk-Gulden (Konstanz, F.R.G.); acetonitrile and dichloromethane (ChromAR) were obtained from Promochem (Wesel, F.R.G.), the primary and secondary sodium phosphates (analytical grade) from Merck (Darmstadt, F.R.G.), and tetrabutylammonium bromide (purum) from Fluka (Neu-Ulm, F.R.G.). Water was purified with a Milli-Q water purification system (Millipore, Neu-Isenburg, F.R.G.).

The stock solutions of cefotetan and iothalamic acid were prepared in 50 m*M* sodium phosphate buffer (pH 6.2) to yield final concentrations of 1.0 mg/ml and 10.0 mg/ml, and stored in aliquots at −20° C. The stock solutions were then diluted with drug-free plasma to provide assay standards over a concentration range of 1.25—100 μg/ml for cefotetan and 12.5—1000 μg/ml for iothalamic acid. For urine samples the standard solutions were prepared in 50 m*M* phosphate buffer (pH 6.2).

### *Analysis of plasma and urine samples*

A 200-μl volume of plasma was buffered with 200 μl of 0.1 *M* sodium dihydrogen phosphate and mixed for 5 sec with 400 μl of acetonitrile on a Reax 1 shaker (Heidolph, Kelheim, F.R.G.). After 15 min at 4° C the mixture was

centrifuged (1 min, 10,500 *g*) and the clear supernatant transferred to a 12 × 75 mm disposable polypropylene tube. Then 2.0 ml of dichloromethane were added, the tube was capped and mixed (5 min) on a Reax 2 Mixer (Heidolph). After centrifugation (10 min, 4800 *g*) an aliquot (5—50 μl) of the upper aqueous phase was injected onto the column.

Urine samples were centrifuged (10 min, 4800 *g*) and diluted 1:10 with 50 m*M* sodium dihydrogen phosphate (resulting pH 5.8—5.9).

All biological samples were stored at −70°C until required for analysis.

*Chromatography*

The chromatographic system consisted of a pump M 6000A, a fixed-wavelength detector M 440 allowing simultaneous measurement at 254 and 280 nm, an automatic injector WISP 710 B, a data module M 730 and a system controller M 720 (all from Waters Assoc., Königstein/Ts., F.R.G.). A Hibar® stainless-steel column (125 × 4 mm I.D.), prepacked with 5-μm LiChrosorb RP-18 silica (Merck), was used for separation. The flow-rate was maintained at 1.0 ml/min, the resulting back-pressure was 110—115 bar. The eluent was monitored at 254 and 280 nm, integrating the peak area of the signals at 280 nm. For plasma samples the mobile phase was prepared by combining 925 ml of water, 75 ml of acetonitrile, 5.50 g of sodium dihydrogen phosphate monohydrate, 1.80 g of disodium hydrogen phosphate dihydrate and 20 mg of tetrabutylammonium bromide. For urine samples the contents of acetonitrile and tetrabutylammonium bromide were changed to 45—50 ml/l and 22.5 mg/l, respectively. The apparent pH of the buffered solutions was about 6.4.

## RESULTS

*Sample clean-up*

The major disadvantage of high-performance liquid chromatography (HPLC) in comparison with the microbiological assay is the need to remove proteins from the samples before injection onto the analytical column. In order to maintain the precision, a minimal number of clean-up steps (e.g. pipetting or extracting) is desirable. Deproteinization is usually performed by adding organic solvents or acids. Ultrafiltration seems to be a very fast and simple technique and was used for the determination of cefmenoxime in plasma; but addition of sodium dodecylsulphate (SDS), a highly protein-bound displacing agent, is necessary to recover the protein-bound fraction of the drug [5]. By adding 0.5% SDS we obtained nearly quantitative recoveries of both epimers of cefotetan from plasma using the sample clean-up procedure described under Materials and methods. However, the ion-pairing properties of SDS in reversed-phase HPLC caused chromatographic aberrations with peak splitting. Deproteinization by addition of perchloric acid resulted in low recoveries because of apparent co-precipitation of cefotetan. After sample clean-up by extraction of the cephalosporin and iothalamic acid into chloroform—pentanol as described in ref. 6, we obtained 90% recovery for both epimers of cefotetan and 70% recovery for iothalamic acid. Because of its simplicity for routine use, we preferred the procedure described under Materials and methods which is a

modification of other methods known from the literature [7—9]. Acetonitrile acts as an efficient protein precipitation agent. In the following extraction step with dichloromethane, neutral lipophilic substances as well as the acetonitrile used for protein precipitation, are removed. The cephalosporin remains in the undiluted aqueous layer. Using this procedure we achieved quantitative recoveries from biological fluids for the following acid compounds: cefadroxil, cefotaxime, desacetylcefotaxime, cefotiam, cefmenoxime, cefsulodin, ceftazidime and iothalamic acid. For cefotetan the recovery was 85% for epimer A and 98% for epimer B. On the other hand, the recovery for a neutral compound, namely desacetylcefotaxime lactone, was only 10%.

*Chromatography*

In Fig. 2 the chromatograms of an aqueous standard and plasma samples after simultaneous intravenous injection of 1 g of cefotetan and 20 ml of Conray 70 (containing 13.2 g of iothalamic acid) are depicted. The best compromise between run-time and separation of the isomers of cefotetan from each other and from endogenous compounds was obtained by adding small quantities (0.1 m*M*) of tetrabutylammonium salt to the buffered (pH 6.3—6.5) mixture of water and acetonitrile. For urine samples it was necessary to lower

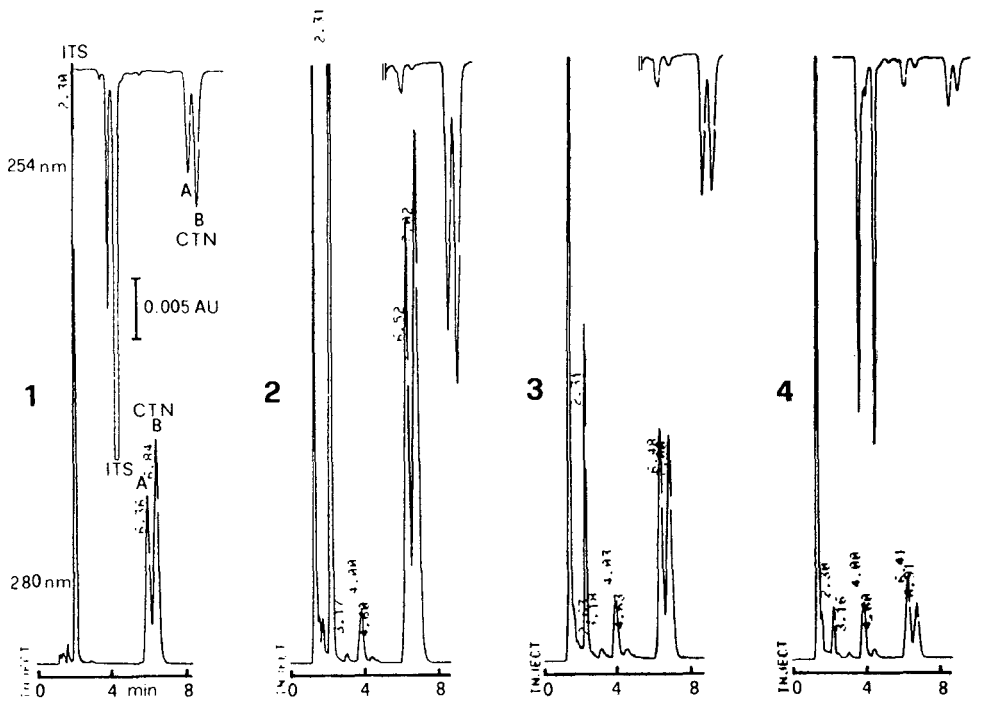


Fig. 2. Chromatograms of (1) an aqueous standard solution of cefotetan (CTN, commercially available mixture of epimers A and B) and iothalamic acid (ITS) (amount injected: ITS, 5 μg; CTN, 500 ng) and of plasma samples from a healthy volunteer 10 min (2), 2 h (3) and 6 h (4) after simultaneous intravenous injection of 1 g of cefotetan and 20 ml of Conray 70 (corresponding to 13.2 g of iothalamic acid). Concentrations (μg/ml): (2) ITS, 849; CTN-A, 56.4; CTN-B, 72.8. (3) ITS, 204; CTN-A, 29.5; CTN-B, 29.9. (4) ITS, 27.8; CTN-A, 10.2; CTN-B, 7.3.

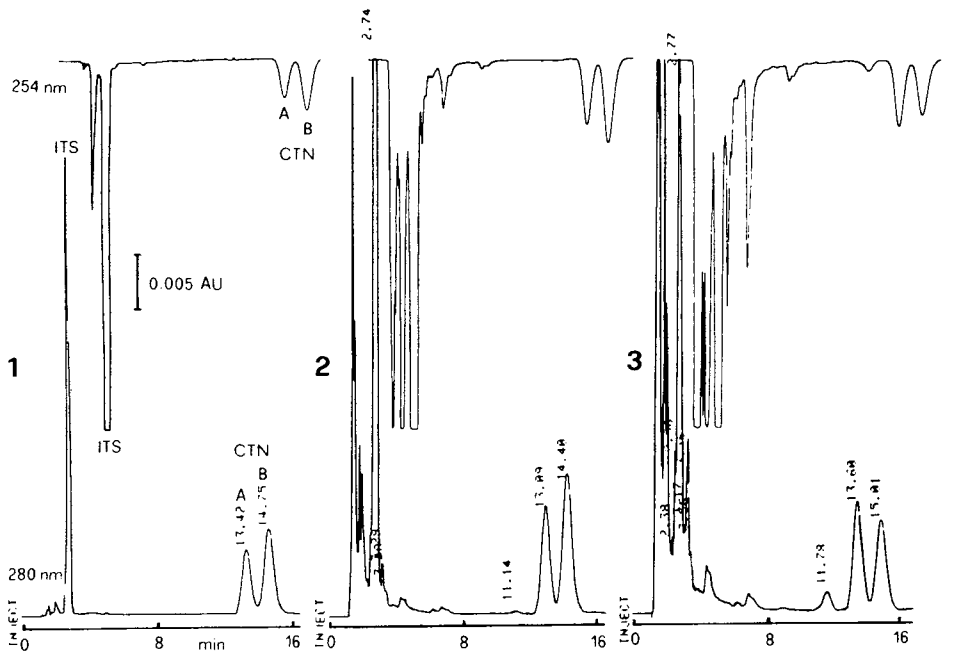


Fig. 3. Chromatograms of (1) an aqueous standard solution of cefotetan (CTN) and iothalamic acid (ITS) (amount injected: ITS, 5 μg; CTN, 500 ng) and of urine samples from a healthy volunteer 0—2 h (2) and 4—6 h (3) after simultaneous intravenous injection of 1 g of cefotetan and 20 ml of Conray 70 (corresponding to 13.2 g of iothalamic acid). Concentrations (μg/ml): (2) ITS, 9870; CTN-A, 325; CTN-B, 502. (3) ITS, 2370; CTN-A, 376; CTN-B, 342.

the acetonitrile content and to change the concentration of the ion-pairing agent in order to maintain the separation from interfering compounds. As a consequence the overall run-time increased to 15 min compared to 8 min for plasma samples (Fig. 3).

*Recovery and sensitivity*

The recoveries of iothalamic acid and cefotetan were checked in the concentration range 12.5—1000 μg/ml and 1.25—100 μg/ml, respectively. Two sets of spiked plasma samples were prepared and each analysed two times on different days. The peak areas found were compared with those of standard aqueous solutions. The results are shown in Table I. The mean recoveries were 100.3 ± 2.4% for iothalamic acid, 84.6 ± 2.2% for isomer A of cefotetan and 97.6 ± 2.4% for isomer B. The recoveries from aqueous samples were 95.1 ± 2.0%, 93.7 ± 3.2% and 93.6 ± 3.7%, respectively. Obviously, the recovery of isomer A from plasma was substantially lower than the recoveries of the other two compounds. The recoveries of the individual spiked plasma samples fluctuated between 74 and 118%. For quantitation of the concentrations found in the unknown plasma samples, the mean value was used. The regression lines obtained in all cases had correlation coefficients better than 0.9996.

The lowest amount detectable on column was 1—2 ng for each epimer of cefotetan but ten-fold higher for iothalamic acid, as the absorption coefficient

TABLE I

RECOVERY OF IOTHALAMIC ACID AND CEFOTETAN (EPIMERS A AND B) FROM HUMAN PLASMA

| Amount added (μg/ml) ITS/CTN | Recovery* (%) | | |
|---|---|---|---|
| | Iothalamic acid (ITS) | Cefotetan (CTN) | |
| | | Epimer A | Epimer B |
| 1000/100 | 100.7 ± 4.9 | 86.4 ± 1.1 | 97.4 ± 3.3 |
| 500/50 | 102.8 ± 3.8 | 87.0 ± 2.6 | 98.3 ± 1.6 |
| 250/25 | 101.7 ± 3.3 | 84.7 ± 2.6 | 97.0 ± 0.8 |
| 100/10 | 100.1 ± 3.9 | 85.5 ± 7.3 | 96.7 ± 4.3 |
| 50/5 | 102.2 ± 7.9 | 83.6 ± 5.1 | 102.5 ± 10.6 |
| 25/2.5 | 98.8 ± 3.8 | 80.4 ± 6.6 | 94.7 ± 1.5 |
| 12.5 /1.25 | 95.9 ± 9.7 | 84.4 ± 11.3 | 96.6 ± 12.4 |
| Mean ± S.D. | 100.3 ± 2.4 | 84.6 ± 2.2 | 97.6 ± 2.4 |

*Mean ± S.D.; $n = 4$.

is low at 280 nm ($\lambda_{max}$ = 240 nm; see also Figs. 2 and 3). For an injection volume of 50 μl this would correspond to a limit of detection of 20—40 ng/ml and 200—400 ng/ml, respectively. This analytical limit of detection could not be reached in biological fluids. In all plasma samples interfering compounds corresponding to up to 1 μg/ml of iothalamic acid were present, whereas in a few samples only an interfering peak corresponding to not more than 0.2 μg/ml of epimer B of cefotetan occurred. The blanks observed in urine were about ten times higher. Since up to 12 h after administration the concentrations of iothalamic acid were much higher than the blank values, no attempt was made to eliminate the unusually high blank value.

*Sample stability*

Especially if automatic injection systems are used for overnight runs, samples must be stable at room temperature . β-Lactam antibiotics are labile in weak basic and strong acid media. The pH of plasma samples kept at room temperature rises to 9 [10]. Therefore plasma samples containing cephalosporins should be buffered to a neutral or weak acid pH [5]. Following the preparation procedure described in this report, no degradation of cefotetan or iothalamic acid was measurable within 12 h. Moreover, at the resulting pH of 5.8—6.0 the ratio of the two epimers of cefotetan was constant too, whereas in unbuffered plasma samples (final pH 8.2—8.3 after sample clean-up) the amount of A increased and that of B decreased in samples with prevalent isomer B. Iothalamic acid was also stable in unbuffered samples.

*Application*

The analytical method described above was used to determine simultaneously both epimers of cefotetan and iothalamic acid in plasma and urine after intravenous injection of 1 g of cefotetan and 20 ml of Conray 70 (containing 13.2 g of iothalamic acid) for pharmacokinetic studies. Iothalamic acid is nearly

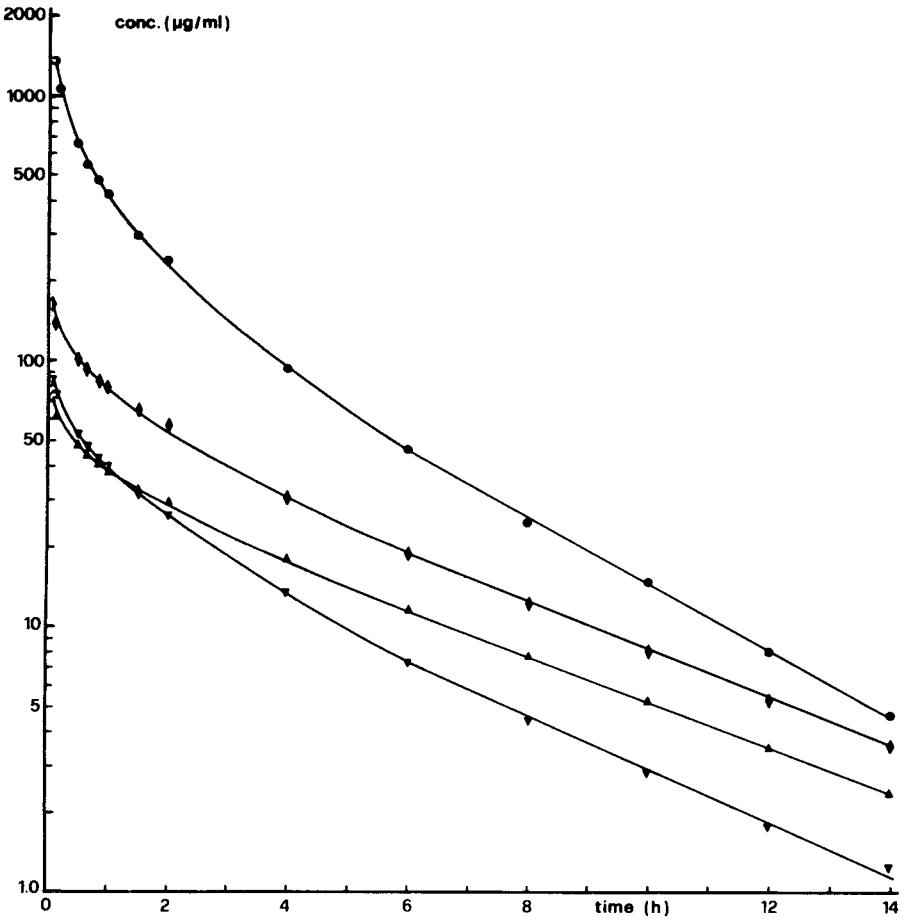


Fig. 4. Mean plasma concentrations of cefotetan (CTN, epimers A and B) and iothalamic acid (ITS) after simultaneous intravenous injection of 1 g of cefotetan and 20 ml of Conray 70 (corresponding to 13.2 g of iothalamic acid) to 12 healthy volunteers. (•), ITS; (♦), CTN (A + B); (▲), CTN-A; (▼), CTN-B.

exclusively eliminated through the kidney by glomerular filtration and served in this study as marker for the function of the kidney [11]. A detailed report will be published elsewhere [12]. In Fig. 4 the mean plasma concentrations of cefotetan obtained from twelve healthy volunteers are shown. As can be seen, the time—concentration curve of isomer A is flatter. The terminal half-life was about 3 h for isomer B and 4 h for isomer A, respectively. These data are in agreement with average half-lives of cefotetan found by others [1—4].

Immediately after cessation of the injection the ratio A/B was 40:60 and the reversed after 12 h. The observed isomerisation in proteinaceous solutions was confirmed in vitro by incubation of cefotetan in phosphate buffer and 7% bovine serum albumin (BSA) at pH 7.4. As can be seen in Fig. 5, within 18 h the ratio shifted nearly to 50:50 in buffer, whereas in BSA solution the resulting ratio (70:30) was similar to that in plasma of volunteers.

DISCUSSION

Beside the microbiological assay, HPLC has been established as a standard method to determine cephalosporins in biological matrices. Because of its precision, high linear range and possibility to detect and assay active metabolites quantitatively, as in the case of the 3-acetoxycephalosporins [7, 13], HPLC is the preferred method for pharmacokinetic studies. In addition, after simultaneous application of different antibiotics, separation and quantitation of the various compounds can be achieved.

In the present paper a different pharmacokinetic behaviour of each epimer of cefotetan has been demonstrated. The reason for this effect is probably a higher ratio of protein binding of epimer A, which can be assumed from the observed lower recovery after deproteinisation with acetonitrile, and the isomerisation in BSA solution leading to another ratio of the epimers than in buffer. On the other hand, lower stability of epimer B could contribute to this effect too, because in BSA solution degradation of cefotetan is enhanced with respect to the buffer (Fig. 5). Similar results were obtained from work with moxalactam. Commercially available moxalactam preparations are also mixtures of two epimers with slightly different protein-binding ratios, biological half-lives and antimicrobial activities [14—19]. As both epimers of cefotetan have a similar antibacterial activity [20], probably no clinical consequences will result from the finding of different serum half-lives for the isomers. Whether accumulation of isomer A will occur in plasma or other body fluids after multiple doses of cefotetan seems questionable, since both epimers are presumably in equilibrium with each other. The analytical method described in the present paper could clarify this problem during further clinical trials.

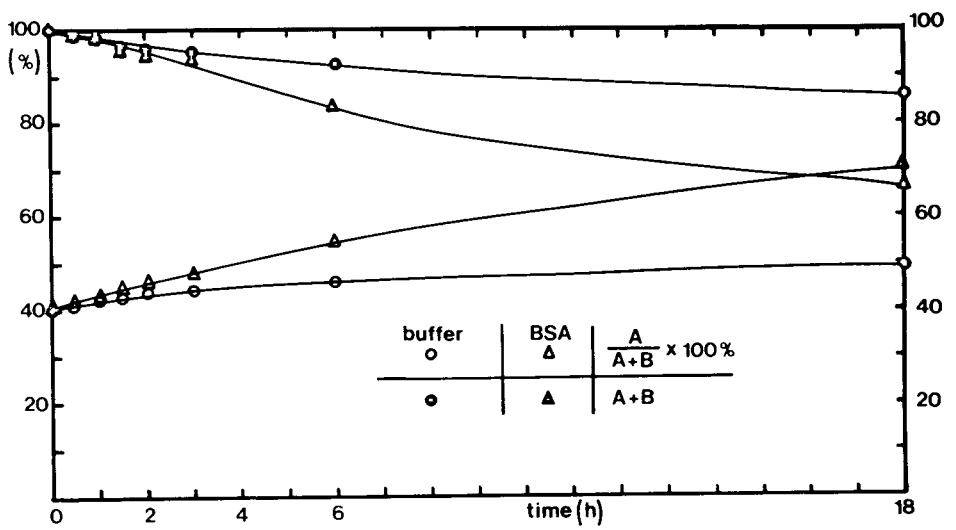


Fig. 5. Degradation of cefotetan (mixture of epimers A and B) at 37°C, pH = 7.4, in 0.1 *M* sodium phosphate buffer (○, ●) and 7% bovine serum albumin (BSA) solution (△, ▲). Increase in fraction of epimer A.

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# DETERMINATION OF SISOMICIN, NETILMICIN, ASTROMICIN AND MICRONOMICIN IN SERUM BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

TAKESHI KAWAMOTO*

*Analytical Applications Department, Shimadzu Corporation, 1-63-1, Shibasaki, Chofu (Japan)*

IKUO MASHIMO and SHIGEKI YAMAUCHI

*Department of Clinical Chemistry, Research Division, Special Reference Laboratory Co., Ltd., 2-4-1, NS Building, Nishishinjuku, Shinjuku Ward, Tokyo (Japan)*

and

MAKOTO WATANABE

*Department of Pharmacy, Tokyo Kosei-Nenkin Hospital, 23 Tsukudo-cho, Shinjuku Ward, Tokyo (Japan)*

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## SUMMARY

A high-performance liquid chromatographic method for monitoring serum levels of the aminoglycoside antibiotics sisomicin, netilmicin, astromicin, and micronomicin, using an amino acid analysis system is described. The procedure involves sample preparation with a CM-Sephadex column, and quantitation using internal standards chosen from each of the other aminoglycosides.

The aminoglycosides were separated by reversed-phase ion-pair chromatography on Zorbax $C_8$ and ODS columns, and detected by the post-column derivatization technique.

The calibration curves of serum concentration for sisomicin, netilmicin, astromicin, and micronomicin were linear over the ranges 0.32—22.8 μg/ml, 0.17—11.6 μg/ml, 0.1—6.3 μg/ml, and 1.0—30 μg/ml, respectively. The coefficients of variation were 2.5, 2.8, 3.1 and 1.9%, at the serum concentrations of 1.3, 1.45, 1.58 and 2.5 μg/ml, respectively ($n$ = 6).

Determination by the internal standard method using another aminoglycoside gives accurate and reproducible results. This method is applicable also to other aminoglycoside antibiotics.

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## INTRODUCTION

Aminoglycoside antibiotics are used for the treatment of serious infections caused by gram-negative bacilli. Because of their ototoxicity and nephrotoxicity, careful monitoring of blood levels is required in order to obtain rational therapy.

Various methods have been developed for the determination of aminoglycoside antibiotics in serum, including microbiological assay, radioenzymic assay, homogeneous enzyme immunoassay, and high-performance liquid chromatography (HPLC).

Determination using microbiological methods is time-consuming and their quantitative variation, which is inherent to microbes, makes its application to drug monitoring difficult. Recently, many authors have reported the determination of aminoglycosides in serum using HPLC because of its accuracy and precision in quantitation [1—5]. Since aminoglycosides have several primary amino groups, they are easily derivatized, and these same authors have described ultraviolet detection of 1-fluoro-2,4-dinitrobenzene derivatives (pre-label) [1, 2] and fluorescence detection of *o*-phthalaldehyde derivatives (post-label) [3—5].

We have already reported the determination of tobramycin in serum by HPLC adopting *o*-phthalaldehyde post-label derivatization [6], this report describes the application of this method to the analysis of sisomicin, netilmicin, astromicin, and micronomicin in serum. Since there is so far no kit commercially available in Japan for the enzymic immunoassay of these antibiotics, this method is valuable from a therapeutic viewpoint.

## EXPERIMENTAL

### *Apparatus*

The Shimadzu liquid chromatographic amino acid analysis system consisted of an LC-3A pump equipped with a column oven CTO-2A and a sample injector SIL-1A, a reagent pump PRR-2A, and a fluorescence detector FLD-1

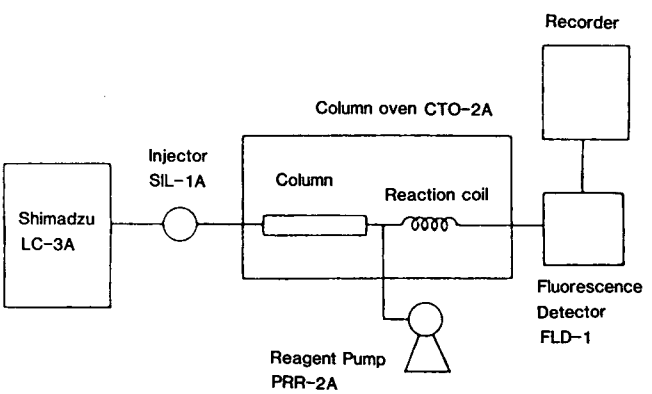


Fig. 1. Flow diagram of the analytical system. Flow-rates: LC-3A: 0.8 ml/min (Zorbax $C_8$), 1.5 ml/min (Zorbax ODS); PRR-2A (reagent pump): 0.5 ml/min. Column oven temperature CTO-2A: 55°C.

with a fluorescence lamp (300—450 nm, maximum 360 nm) and an emission filter EM-4. Fig. 1 shows the flow diagram of the chromatographic system.

The columns used were Zorbax $C_8$ (5 $\mu$m, 15 cm × 4.6 mm I.D.) for the analysis of sisomicin (SISO), netilmicin (NTL), and astromicin (ASTM), and Zorbax ODS (5 $\mu$m, 15 cm × 4.6 mm I.D.) for micronomicin (MCR). The reaction coil, made of stainless steel (70 cm × 0.5 mm I.D.), was stored in the column oven and maintained at 55° C to promote the reaction.

*Reagents*

Sisomicin sulphate and netilmicin sulphate were obtained from Essex Nippon K.K. (Osaka, Japan); astromicin and micronomicin were obtained from Kyowa Hakko Kogyo (Tokyo, Japan). Concentrated aqueous stock solutions of these aminoglycoside antibiotics were stored refrigerated at 4° C and were diluted before use. All antibiotics concentrations were calculated by their potency. *o*-Phthalaldehyde was obtained from Merck (Darmstadt, F.R.G.); 30% solution of Brij-35 was from Technochemical (Tokyo, Japan). Sodium *p*-toluenesulphonate and 2-mercaptoethanol were from Wako (Osaka, Japan). All other chemicals were of analytical grade.

*Mobile phase and reaction reagent*

The mobile phase contained 25 m*M* sodium *p*-toluenesulphonate as a counter-ion for reversed-phase ion-pair chromatography and 20 m*M* sodium dihydrogen phosphate dihydrate; sodium perchlorate anhydrous was added to the mobile phase to adjust the retention time of each aminoglycoside. The pH of the mobile phase was adjusted to about 2.0 with perchloric acid.

The stock reagent was prepared by dissolving 9.4 g of boric acid and 4.8 g of sodium hydroxide in 400 ml of distilled water. Just before use, the reaction reagent was prepared by the addition of 6 ml of an ethanolic solution of 400 mg of *o*-phthalaldehyde, 1 ml of 2-mercaptoethanol, and 2 ml of a 30% solution of Brij-35. The reaction reagent was then diluted to 500 ml, and the pH was adjusted to about 10.5 with sodium hydroxide.

*Sample preparation*

Amino acids in serum interfere with the determination of aminoglycosides because *o*-phthalaldehyde reacts with primary amines and gives fluorescent products. So it is necessary to remove amino acids as well as proteins prior to injection. We adopted and modified the preparation procedure of Anhalt [3].

Fig. 2 shows the procedure to separate aminoglycosides from interfering compounds in serum by a short column with a bed volume of 1 ml of CM-Sephadex (C-25). This column was washed with the initial buffer (0.4 *M* sodium acetate) and the serum sample was applied to the column. The internal standard chosen from other aminoglycosides was dissolved in the initial buffer, and 100 $\mu$l of the solution were added to the column. The column was washed with 2 ml of initial buffer twice in succession. The eluting buffer was changed to 10 m*M* sodium hydroxide in the initial buffer (alkaline buffer), and 600 $\mu$l of this buffer were added to the column. After the column had been drained, 400 $\mu$l of alkaline buffer were added and the eluate collected was injected into the HPLC system.

Sample 400 μl in CM-Sephadex column
↓ ←100μl IS. in initial buffer *
Initial buffer 2ml×2(rinse)
↓ ←600μl Alkaline buffer(rinse) **
↓ ←400μl Alkaline buffer(eluation)
Eluate
↓
Injection to HPLC

* 0.4 M Sodium acetate.
** 10 mM Sodium hydroxide in initial buffer.

Fig. 2. Sample preparation with a CM-Sephadex column.

## RESULTS AND DISCUSSION

Fig. 3 shows chromatograms of sisomicin-spiked serum and the corresponding blank serum. Tobramycin was used as the internal standard at a concentration of 9.4 μg/ml. Chromatogram A is the blank serum containing the internal standard, and B is the sisomicin spiked at a concentration of 22.8 μg/ml. The relation between the peak height ratio (SISO/TOB) and the sisomicin concentration in the serum was linear over the range 0.32—22.8 μg/ml. Linear regression analysis of this calibration yielded the equation $Y = 0.034X + 2.23 \cdot 10^{-3}$ ($r = 0.9997$).

The coefficient of variation (C.V.) of the peak height ratio was 2.5%, which was determined by analyzing six individually prepared serum samples containing 1.3 μg/ml sisomicin and 9.4 μg/ml tobramycin (Table I).

Fig. 4 shows chromatograms of netilmicin-spiked serum and its blank. Astromicin was used as the internal standard at a concentration of 6.3 μg/ml. Chromatogram A is the blank and B is the netilmicin-spiked serum (11.6 μg/ml). The calibration curve was linear over the range 0.17—11.6 μg/ml. Linear regression analysis of this calibration curve resulted in the equation $Y = 0.101X - 8.5 \cdot 10^{-3}$ ($r = 0.9998$). The C.V. for netilmicin was 2.8% at a concentration of 1.5 μg/ml ($n = 6$) (Table I).

In order to analyze astromicin, netilmicin was used as the internal standard

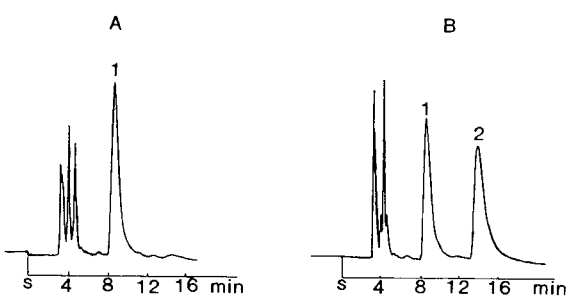


Fig. 3. Chromatograms of serum spiked with sisomicin and blank serum. Peaks: 1 = tobramycin (IS), 2 = sisomicin. (A) Blank serum: tobramycin (IS) 9.4 μg/ml. (B) Spiked serum: sisomicin 22.8 μg/ml. Mobile phase: 25 m*M* sodium *p*-toluenesulphonate, 20 m*M* sodium dihydrogen phosphate dihydrate, 0.3 *M* sodium perchlorate (anhydrous), pH 2.0 with perchloric acid. Flow-rate 0.8 ml/min; sensitivity ×64; injected volume 10 μl.

TABLE I

COEFFICIENTS OF VARIATION FOR THE DETERMINATION OF AMINOGLYCOSIDES IN SERUM

| | C.V. (%) | Serum concentration ($\mu$g/ml) |
|---|---|---|
| Sisomicin | 2.5 | 1.3 |
| Netilmicin | 2.8 | 1.45 |
| Astromicin | 3.1 | 1.58 |
| Micronomicin | 1.9 | 2.5 |

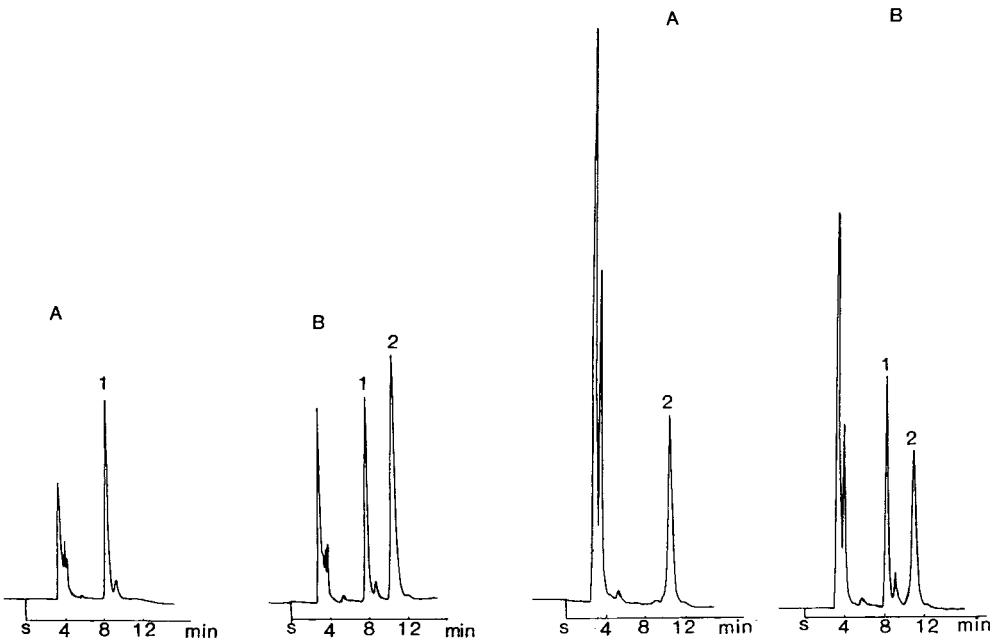


Fig. 4. Chromatograms of serum spiked with netilmicin and blank serum. Peaks: 1 = astromicin (IS), 2 = netilmicin. (A) Blank serum: astromicin (IS) 6.3 $\mu$g/ml. (B) Spiked serum: netilmicin 11.6 $\mu$g/ml. Mobile phase: 0.6 *M* sodium perchlorate (anhydrous); other conditions are the same as in Fig. 3. Injected volume 20 $\mu$l. These analytical conditions were used for the experiments with netilmicin and astromicin.

Fig. 5. Chromatograms of serum spiked with astromicin and blank serum. Peaks: 1 = astromicin, 2 = netilmicin (IS). (A) Blank serum: netilmicin (IS) 5.8 $\mu$g/ml. (B) Spiked serum: astromicin 6.3 $\mu$g/ml.

at a serum concentration of 5.8 $\mu$g/ml. Typical chromatograms of serum containing astromicin and blank serum are shown in Fig. 5. Chromatogram A is the blank serum and B is the astromicin-spiked serum (6.3 $\mu$g/ml). The calibration curve is linear from 0.1 to 6.3 $\mu$g/ml. The equation obtained from linear regression analysis is $Y = 0.244X - 7.75 \cdot 10^{-3}$ ($r = 0.9997$). The C.V. for astromicin was 3.1% at a concentration of 1.6 $\mu$g/ml ($n = 6$) (Table I).

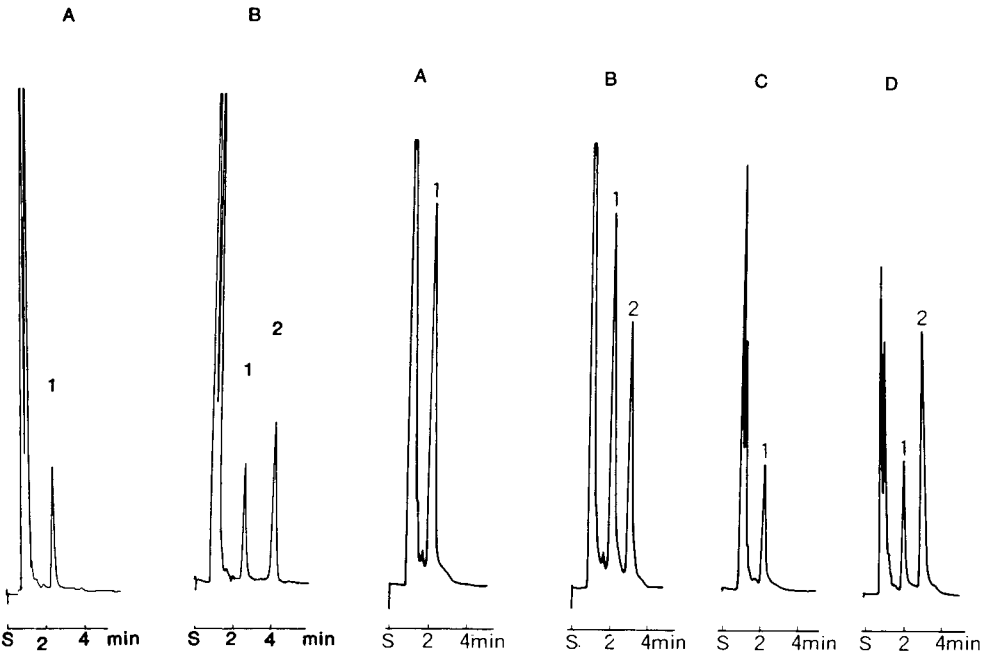


Fig. 6. Chromatograms of serum spiked with micronomicin and blank serum. Peaks: 1 = sisomicin (IS), 2 = micronomicin. (A) Blank serum: sisomicin (IS) 40 μg/ml. (B) Spiked serum: micronomicin 7.5 μg/ml. Mobile phase: 1.0 *M* sodium perchlorate (anhydrous); other conditions are the same as in Fig. 3; injected volume 50 μl.

Fig. 7. Chromatograms of serum and urine after the administration of micronomicin to a patient. Peaks: 1 = sisomicin (IS), 2 = micronomicin. (A) Blank urine: sisomicin (IS) 40 μg/ml. (B) Sample urine. (C) Blank serum: sisomicin (IS) 40 μg/ml. (D) Sample serum. Injected volumes: 150 μl (A, B) and 50 μl (C, D). Other analytical conditions are the same as in Fig. 6.

Experiments concerning sisomicin to astromicin were carried out using the Zorbax $C_8$ column; analytical conditions are described in the legends to the relevant figures.

The column was changed to Zorbax ODS for the analysis of micronomicin. Sisomicin was chosen as the internal standard from the other aminoglycosides, and it was spiked in serum at a concentration of 40 μg/ml. Fig. 6 shows the chromatograms of micronomicin-spiked serum (B, 7.5 μg/ml) and the blank (A). Fig. 7 shows chromatograms of serum and urine after the administration of micronomicin to a patient. Chromatograms A and C are blank urine and serum, B and D are sample urine and serum, respectively. The linearity of the calibration curve was from 1.0 to 30 μg/ml. The C.V. was 1.9% at a concentration of 2.5 μg/ml ($n$ = 6) (Table I). Linear regression analysis of this curve resulted in the equation $Y = 0.082X - 11.7 \cdot 10^{-3}$ ($r$ = 0.9998).

Linearity of the calibration curves which cover the clinically observable ranges of each aminoglycoside, and the low C.V. values indicate that the determination of aminoglycosides in serum by this method is accurate and reproducible. By using internal standards chosen from other aminoglycosides, manipulation errors during sample preparation, or injection errors, are avoided

and accurate determination of aminoglycoside antibiotics in serum is attained even when many samples are treated at the same time.

Sample preparation was examined by various methods such as organic solvent deproteinization, the ion-exchange resin method, and short-column extraction with CM-Sephadex (C-25). Short-column extraction with CM-Sephadex based on Anhalt's method [3] resulted in the highest recovery.

In Anhalt's method, 0.2 *M* sodium sulphate was used as the initial buffer, but its application to the analysis of netilmicin resulted in two split peaks in our experiments. This phenomenon was avoided by changing the initial buffer to 0.4 *M* sodium acetate, but the exact mechanism is unknown (see Fig. 2).

During the experiments for micronomicin, 400 $\mu$l of alkaline buffer were added to elute aminoglycoside from the short column. Another 400 $\mu$l of alkaline buffer were then added to the short column and the 800 $\mu$l of eluate collected were used as sample. This method gave a recovery rate (almost 100%) higher than that obtained by washing the short column once to elute (around 90% recovery: data not shown). But we carried out experiments following the procedure in Fig. 2, in order to avoid sample dilution and complicated procedures. Deproteinization and extraction were performed simultaneously in a short column, so the procedure was easy and fast. Besides that, because of the separation selectivity of reversed-phase ion-pair chromatography, other aminoglycoside antibiotics hardly interfere with the determination.Total analysis time including sample preparation and chromatographic separation was less than 30 min.

In conclusion, the advantages of this method are speed, accuracy, and good reproducibility. Therefore, this method is applicable for therapeutic drug monitoring.

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# DETERMINATION OF BESTATIN IN SERUM BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

JUNICHI ISHIDA, MASATOSHI YAMAGUCHI*, MASAAKI KAI and YOSUKE OHKURA*

*Faculty of Pharmaceutical Sciences, Kyushu University 62, Maidashi, Higashi-ku, Fukuoka 812 (Japan)*

and

MASARU NAKAMURA

*Faculty of Pharmaceutical Sciences, Fukuoka University, Nanakuma, Jonan-ku, Fukuoka 814-01 (Japan)*

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## SUMMARY

A simple method for the determination of bestatin and its major metabolite in man, *p*-hydroxybestatin, in human serum was investigated; the method employs high-performance liquid chromatography with fluorescence detection. Bestatin and *p*-hydroxybestatin are oxidized to phenylacetaldehyde and *p*-hydroxyphenylacetaldehyde, respectively, with periodate, which are then converted into fluorescent compounds with 4,5-dimethoxy-1,2-diaminobenzene. The compounds are separated by reversed-phase chromatography on LiChrosorb RP-18. The detection limits of bestatin and *p*-hydroxybestatin are 0.2 and 0.4 μg/ml serum, respectively. This method permits the precise determination of bestatin in serum (20 μl) from patients administered bestatin. *p*-Hydroxybestatin in serum can not be measured by this method because of its low concentration (less than the detection limit).

## INTRODUCTION

Bestatin, (2*S*, 3*R*)-3-amino-2-hydroxy-4-phenylbutanoyl-S-leucine, inhibits aminopeptidase B and leucine aminopeptidase, and enhances delayed-type

*Present address: Faculty of Pharmaceutical Sciences, Fukuoka University, Nanakuma, Jonan-ku, Fukuoka 814-01, Japan.

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hypersensitivity [1, 2]. Bestatin has been studied as a possible therapeutic drug for cancer, resistant infection and muscular dystrophy.

For the determination of bestatin in serum, only a gas chromatographic—mass spectrometric (GC—MS) method has been reported [3]. This method also permits the determination of *p*-hydroxybestatin, which is a major metabolite in man but occurs at low concentration in serum. For the biomedical investigattions of bestatin and for drug monitoring during experimental therapy, a readily available method of determination was required.

We have developed a simple method for the determination of bestatin and *p*-hydroxybestatin in minute amounts of human serum by high-performance liquid chromatography (HPLC) with fluoresence detection. This method is based on the determination of phenylacetaldehyde and *p*-hydroxyphenylacetaldehyde formed from bestatin and *p*-hydroxybestatin, respectively, by oxidation with periodate. The aldehydes are converted into fluorescent compounds by reaction with 4,5-dimethoxy-1,2-diaminobenzene (DDB), a fluorescent derivatization reagent for aromatic and arylaliphatic aldehydes [4, 5]. The fluorescent products are separated by reversed-phase HPLC on LiChrosorb RP-18. Drug-free serum spiked with bestatin and *p*-hydroxybestatin was used as a model sample to establish suitable conditions for a general analytical method. Sera from patients administered bestatin were subjected to analysis by the method.

## EXPERIMENTAL

### *Materials and reagents*

All chemicals were of reagent grade, unless otherwise noted. Deionized and distilled water was used. Bestatin and *p*-hydroxybestatin are the products of Nihon Kayaku (Tokyo, Japan). DDB monohydrochloride was prepared as described previously [4].

The DDB solution (1.3 m*M*) was prepared by dissolving 26.5 mg of DDB monohydrochloride in 100 ml of 0.3 *M* hydrochloric acid and should be used within 3 h. Normal sera were obtained from healthy volunteers (male, 22—52 years of age) in this laboratory in the usual manner. Sera from patients with muscular dystrophy (male, 4—10 years of age) were supplied from National Nishibeppu Hospital (Beppu, Oita, Japan).

### *Apparatus*

A Toyo Soda 803 D high-performance liquid chromatograph equipped with a Rheodyne 7125 syringe-loading sample injector valve (100-$\mu$l loop) and a Shimadzu RF 530 fluorescence spectromonitor fitted with a 12-$\mu$l flow-cell operating at an emission wavelength of 390 nm and an excitation wavelength of 320 nm was used. The column was LiChrosorb RP-18 (particle size, 5 $\mu$m; 150 × 4 mm I.D.; Japan Merck, Tokyo, Japan). This column can be used for more than 1000 injections (with only a small decrease in the theoretical plate number) when washed with aqueous acetonitrile (1:1, v/v) at a flow-rate of 0.8 ml/min for about 30 min after every day of analyses. Uncorrected fluorescence excitation and emission spectra were measured with an Hitachi MPF-4 spectrofluorimeter in quartz cells (optical path-length, 10 × 10 mm);

spectral band-widths of 5 nm were used in both the excitation and emission monochromators.

*Procedure*

Sample serum (20 μl) was mixed with 20 μl of water and 200 μl of 4.2 m*M* acetic acid. The mixture was heated for about 5 min in a boiling water-bath and centrifuged at approximately 1000 *g* for 5 min. To 100 μl of the supernatant were added 50 μl of 1.5 *M* ammonium hydroxide and 25 μl of 3 m*M* sodium periodate, and the mixture was allowed to stand at room temperature (approximately 25°C) for 20 min. The excess periodate was decomposed by the addition of 25 μl of 12 m*M* sodium sulphite. To the mixture, 200 μl of DDB solution were added and the mixture was warmed at 37°C for 50 min; then, 50 μl of 1.0 *M* sodium hydroxide were added. Drug-free serum (20 μl) was treated in the same way as for the sample serum. A 100-μl volume of the final mixture was injected into the chromatograph.

The mobile phase was a mixture of 0.1 *M* Tris · hydrochloric acid buffer (pH 8.7) and acetonitrile (3:1, v/v). The flow-rate was 0.8 ml/min (approximately 75 kg/cm$^2$). The column temperature was ambient (approximately 25°C). The net peak heights due to bestatin and *p*-hydroxybestatin in the sample serum were calculated. The amounts of bestatin and *p*-hydroxybestatin were calibrated by means of the standard addition method: 20 μl of water added to the serum in the procedure were replaced by 20 μl of a standard mixture of bestatin and *p*-hydroxybestatin (0.7 or 3 μg/ml each).

## RESULTS AND DISCUSSION

*HPLC conditions*

Fig. 1 shows the chromatograms obtained with a standard mixture of bestatin and *p*-hydroxybestatin and with water for reagent blank. The chromatogram (Fig. 1a) was identical to that obtained when a mixture of phenylacetaldehyde and *p*-hydroxyphenylacetaldehyde was treated directly with DDB as in the procedure. The retention times for the DDB derivatives of bestatin and *p*-hydroxybestatin are 4.5 and 10.5 min, respectively. The eluates from peaks 1 and 2 in Fig. 1a have fluorescence excitation spectra with maxima at 325 and 327 nm, respectively, and emission spectra with maxima at 391 and 382 nm, respectively.

The concentration of acetonitrile in the mobile phase affects the separation of the peaks. At a concentration greater than 45%, the peaks for bestatin and *p*-hydroxybestatin partially overlap that of the blank, while a concentration of 20% or less causes delay in the elution with broadening of the peaks for bestatin and *p*-hydroxybestatin. A concentration of acetonitrile less than 30% provides a satisfactory separation of the peaks for DDB and *p*-hydroxybestatin. Greater concentration of acetonitrile gives higher peaks for bestatin and *p*-hydroxybestatin in a range 20—40%. Therefore, 25% acetonitrile in the mobile phase was used for the procedure recommended. When methanol was used in place of acetonitrile in the mobile phase, the half-widths of the peaks doubled. Tris · hydrochloric acid buffer in the mobile phase does not affect the retention times of any of the peaks at concentrations of 0.05—1.0 *M*;

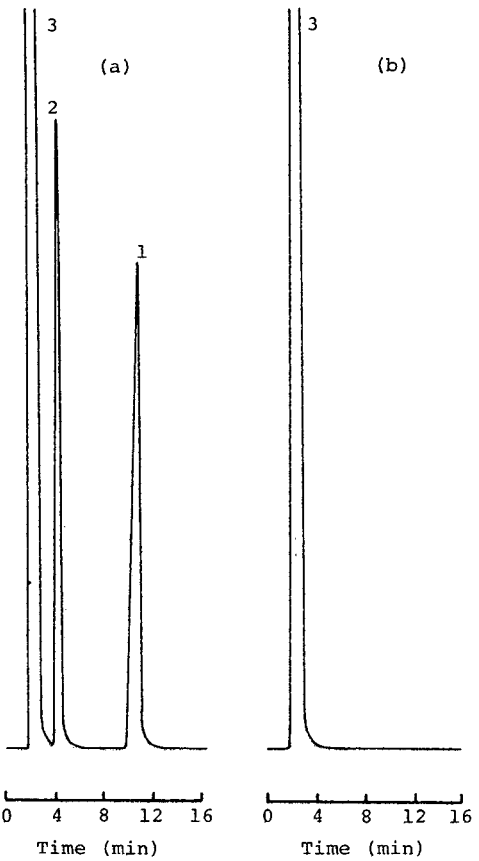


Fig. 1. Chromatograms of (a) DDB derivatives of bestatin and *p*-hydroxybestatin, and (b) reagent blank. Aliquots (20 μl) of a standard mixture of bestatin and *p*-hydroxybestatin (2 μg/ml each), and of water for blank, were treated according to the procedure. Peaks: 1, bestatin; 2, *p*-hydroxybestatin; 3, DDB.

0.1 *M* was selected for convenience. The pH of 0.1 *M* Tris · hydrochloric acid buffer only slightly influences the retention times of the peaks for bestatin and *p*-hydroxybestatin in the range 7.0—9.0, but has a considerable effect on the peak heights. Both peaks increase in height with increasing pH in the range 7.0—9.0. Since the column packing LiChrosorb RP-18 can be used in the limited range of pH less than about 9.0, a 0.1 *M* Tris · hydrochloric acid buffer of pH 8.7 was employed in the recommended procedure.

*Oxidation and derivatization*

Bestatin and *p*-hydroxybestatin are oxidized effectively to phenylacetaldehyde and *p*-hydroxyphenylacetaldehyde, respectively, in ammonium hydroxide solution with sodium periodate solution at a concentration of 1.5 m*M* or greater for bestatin and at 2 m*M* or greater for *p*-hydroxybestatin; 3 m*M* was used for the simultaneous oxidation of the compounds. Ammonium hydroxide solution in a concentration range 0.8—3.0 *M* gives maximum and constant heights of peaks for both compounds; 1.5 *M* was selected as optimum.

The formation of the aldehydes is dependent on the temperature and time of the oxidation procedure. At room temperature (22—27° C) the peak heights reach their maxima after standing for 18 min and remain constant for 25 min or longer, while at 0° C the heights do not reach their maxima even after standing for 40 min. Thus, the oxidation was carried out at room temperature for 20 min. Excessive periodate interferes with the derivatization of the aldehydes with DDB, and should therefore be decomposed with sodium sulphite. Sodium sulphite gives maximum and constant peak heights in a concentration range of 10—14 m*M*; a 12 m*M* solution was employed in the procedure. The recoveries of phenylacetaldehyde and *p*-hydroxyphenylacetaldehyde from bestatin and *p*-hydroxybestatin respectively (2 μg/ml each) under the conditions of the oxidation procedure were 99.3 ± 0.8% (mean ± S.D., $n = 6$).

The resulting aldehydes should be derivatized in dilute hydrochloric acid with DDB. Thus, DDB was dissolved in the acid for convenience. The acid concentration in the range 0.2—0.4 *M* gives maximum and constant peak heights; a 0.3 *M* solution was employed as optimum. The DDB solution gives the most intense peaks at a concentration greater than about 1.0 m*M* in the case of both bestatin and *p*-hydroxybestatin; 1.3 m*M* was used as a sufficient concentration. The peak heights reach maximum and constant values after warming at 37—50° C for more than 30 min in the case of bestatin and for more than 40 min in the case of *p*-hydroxybestatin, and a higher temperature allows the peaks to develop more rapidly, but heating at 100° C for more than 10 min causes a decrease in peak heights. Therefore, warming at 37° C for 50 min was selected as optimum. The resulting mixture is made slightly alkaline by the addition of 1.0 *M* sodium hydroxide to stabilize the DDB derivatives. The final mixture is stable for more than 2 h at room temperature. The limits of detection for bestatin and *p*-hydroxybestatin in a standard mixture were 90 and 68 pg per injection volume of 100 μl, respectively, at a signal-to-noise ratio of 2.

*Determination of bestatin and p-hydroxybestatin in serum*

Serum should be deproteinized, otherwise the column packing for HPLC is considerably damaged. The deproteinization can be done by adding 4.0—4.5 m*M* acetic acid (final concentration 3.3—3.8 m*M*, final pH approximately 5) to water-diluted serum and heating at 100° C for more than 3 min; 4.2 m*M* acetic acid and 5 min heating were employed in the procedure. When serum was deproteinized with perchloric acid (final concentration 1.0 *M*) in the usual manner, a large peak arose very close to the peak for bestatin and interfered with the determination of bestatin. The use of trichloroacetic acid (final concentration 0.5 *M*) caused low recoveries of bestatin and *p*-hydroxybestatin (approximately 50 and 40%, respectively).

Fig. 2 shows typical chromatograms obtained with drug-free serum from a healthy man and the serum spiked with bestatin and *p*-hydroxybestatin. Peaks for bestatin and *p*-hydroxybestatin (Fig. 2a, peaks 1 and 2) are successfully separated under the conditions of HPLC from other peaks due originally to the sample serum. The eluates from peaks 1 and 2 in Fig. 2a have the same fluorescence excitation and emission spectra as those of the eluates from peaks 1 and 2 in Fig. 1a, respectively.

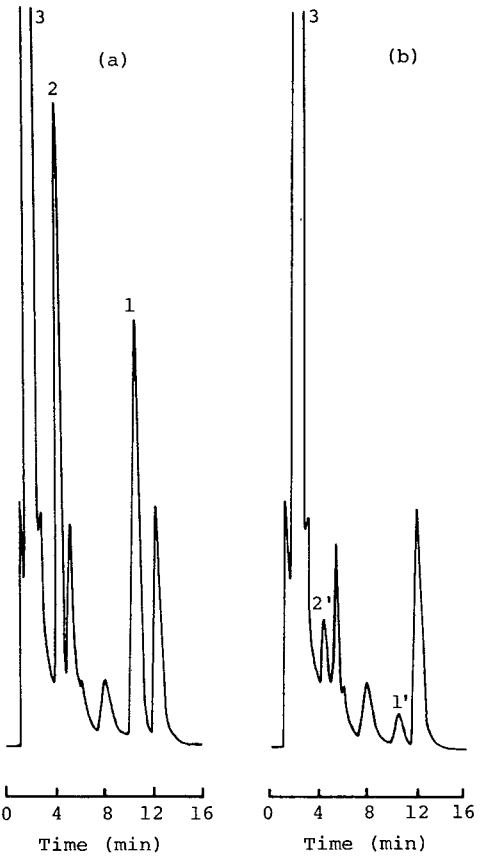


Fig. 2. Chromatograms obtained with (a) drug-free serum spiked with bestatin and *p*-hydroxybestatin (2 μg/ml each) and (b) the drug-free serum, according to the procedure. Peaks: 1, bestatin; 2, *p*-hydroxybestatin; 3, DDB. For peaks 1′ and 2′, see text.

Small peaks in the chromatogram of the drug-free serum (peaks 1′ and 2′ in Fig. 2b) have exactly the same retention times as those of peaks 1 and 2 in Fig. 2a, respectively, and increased in height in proportion to increasing amounts of serum (10—100 μl). These peaks were also observed even when serum was treated without the oxidation procedure, and could not be separated from the peaks for bestatin and *p*-hydroxybestatin by any changes in HPLC conditions. Moreover, peaks 2′ and 1′ were observed even when another reversed-phase column, μBondapak phenyl, was used in place of the LiChrosorb RP-18 column. In this case, the peak height ratios of peak 1′ to peak 1 and peak 2′ to peak 2 were almost same as those ratios in Fig. 2, respectively, although the resolution of the derivatives from bestatin and *p*-hydroxybestatin on μBondapak phenyl was slightly worse than that on LiChrosorb RP-18. The fluorescence excitation and emission spectra of the eluate from peak 1′ are identical in shape and maxima with those of the eluate from peak 1, while the eluate from peak 2′ has rather complex spectra with excitation maxima at 300, 327 and 340 nm, one of which (the maximum at 327 nm) is identical with that for peak 2, and emission maxima at 366 and 382 nm, the latter being identical with the maximum for peak 2.

The above observations indicate that the component of peak 1′ in Fig. 2b should be the DDB derivative of phenylacetaldehyde which may be present in serum as an intermediate metabolite of phenylalanine [6]. Peak 2′ in Fig. 2b is ascribable to some substances occurring in serum, which may include *p*-hydroxyphenylacetaldehyde as a metabolite of tyrosine [6—8]. In addition, the identification of peak 2′, for which the HPLC fraction was collected from ten analyses and successively lyophilized, was carried out by electron-impact mass spectrometry. However, the mass spectrum obtained did not show a peak of the molecular ion (*m/z* 284) corresponding to the fluorescent product from *p*-hydroxyphenylacetaldehyde because of the small amount of the product and decomposition of the product during the collection of peak 2′ during HPLC.

In any case, the heights of peaks 1′ and 2′ should be subtracted from those in the serum sample in calculating peak heights in the procedure. If drug-free serum corresponding to the serum sample is not available, a pooled serum can be used, because almost constant heights of peaks 1′ and 2′, corresponding to approximately 0.13 μg/ml bestatin and approximately 0.28 μg/ml *p*-hydroxybestatin, respectively, were observed with individual drug-free sera from healthy subjects and patients with muscular dystrophy (coefficients of variation for the heights of peaks 1′ and 2′ were 4.8 and 5.0%, respectively; $n$ = 30 each). Other unidentified peaks in the chromatograms in Fig. 2 were not studied.

Linear relationships were observed between the peak heights and the amounts of bestatin and *p*-hydroxybestatin added in the range 0.2—30 μg/ml to drug-free serum. The recoveries of bestatin and *p*-hydroxybestatin added to drug-free serum at concentrations of 3.0 μg/ml each, were 78.2 ± 1.2% and 78.0 ± 1.4% (mean ± S.D., $n$ = 5 each), respectively.

The limits of detection for bestatin and *p*-hydroxybestatin were 0.2 and 0.4 μg/ml serum, respectively. The limit was defined as the amount giving 1.5 times the height of the peak in drug-free serum for the reason that such amounts of these compounds can be measured fairly precisely, as described below.

The precision was established with respect to repeatability using drug-free sera with added bestatin and *p*-hydroxybestatin. The coefficients of variation were 4.3, 3.0 and 1.8% for 0.2, 0.5 and 3.0 μg/ml bestatin, and 2.7 and 2.0% for 0.4 and 3.0 μg/ml *p*-hydroxybestatin, respectively ($n$ = 10 in each case).

Fig. 3 shows examples of the time—concentration curves of the sera of patients administered bestatin orally in single doses. The chromatograms of sera from the subjects were the same in their pattern as those of drug-free sera with added bestatin and *p*-hydroxybestatin. However, the metabolite, *p*-hydroxybestatin, could not be measured precisely because its concentrations in sera were estimated to be less than 100 ng/ml over 24 h after the administration of bestatin. Miyazaki [9] and Koyama et al. [3] reported that most (about 70%) of the bestatin administered orally was excreted as the unchanged form in urine within 24 h, and that a maximum value of *p*-hydroxybestatin concentration in the serum, reached at 2—3 h after the administration of bestatin, was about 1% of that of the maximum serum concentration of bestatin. The serum concentration of bestatin was at a maximum 30 min after oral administration and then decreased nearly at a first-order rate as shown in

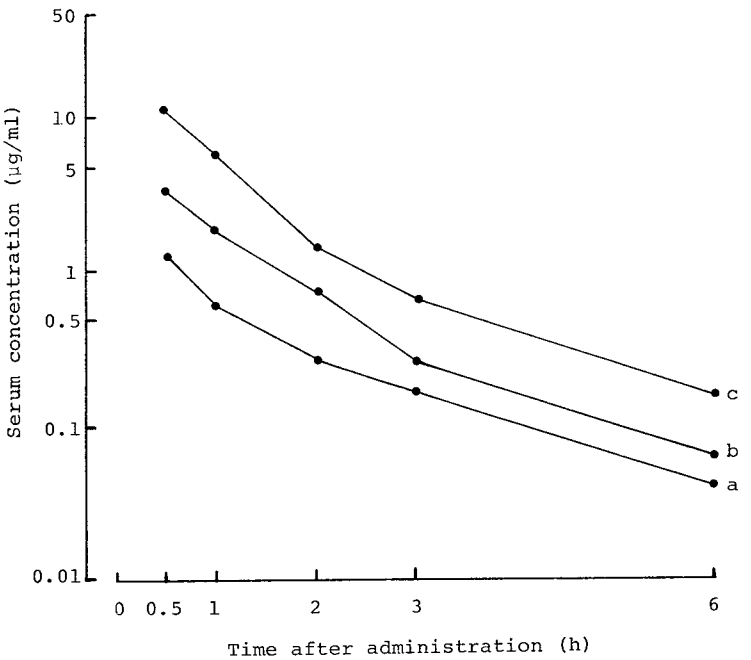


Fig. 3. Serum concentration of bestatin after oral administration of bestatin to patients (male) with muscular dystrophy. Doses of bestatin, body weights and ages: (a) 12 mg, 17 kg, 5 years; (b) 25 mg, 17 kg, 5 years; (c) 60 mg, 16 kg, 9 years.

TABLE I

COMPARISON OF SERUM CONCENTRATIONS OF BESTATIN OBTAINED BY THE PRESENT HPLC METHOD AND THE GC—MS METHOD [3]

| | Serum concentration (μg/ml) | |
|---|---|---|
| | HPLC | GC—MS |
| | 7.13 | 7.97 |
| | 4.83 | 4.41 |
| | 4.80 | 4.40 |
| | 0.70 | 0.87 |
| | 0.32 | 0.37 |
| | 0.31 | 0.30 |
| Mean | 3.02 | 3.05 |
| ± S.D. | 2.95 | 3.09 |

Fig. 3. The biological half-life was 0.7—1.0 h. The pattern of the curves was almost identical with that obtained by the GC—MS method [3].

Comparison with the GC—MS method was made using sera of patients administered bestatin (Table I). The values of bestatin obtained by the present HPLC method are in close agreement with those obtained by the GC—MS method.

This study provides the first HPLC method for the determination of

bestatin. The method is precise and has adequate sensitivity to measure bestatin in 20 $\mu$l of serum, though the sensitivity is not enough to measure low concentrations of *p*-hydroxybestatin in serum. The method is readily performed and may therefore be applied for routine use in biomedical studies of bestatin.

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# DETERMINATION OF TRIMETHOPRIM METABOLITES INCLUDING CONJUGATES IN URINE USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH COMBINED ULTRAVIOLET AND ELECTROCHEMICAL DETECTION

LARS NORDHOLM* and LARS DALGAARD

*Royal Danish School of Pharmacy, Department of Chemistry BC, Universitetsparken 2, DK-2100 Copenhagen Ø (Denmark)*

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## SUMMARY

A high-performance liquid chromatographic method for the determination of trimethoprim metabolites in pig urine was developed. The metabolites — glucuronic acid and sulphuric acid conjugates of phenolic metabolites formed by demethylation of trimethoprim — were quantitated after treatment of urine with β-glucuronidase (*Escherichia coli*). The sulphuric acid conjugate was not susceptible to enzymatic hydrolysis and was therefore assayed as the conjugate by use of ion-pair chromatography on the reversed-phase column. In order to find suitable conditions for enzymatic hydrolysis of the glucuronides, the conjugates were obtained by gel chromatography of urine from a [$^{14}$C]trimethoprim-treated pig.

## INTRODUCTION

Conjugated drug metabolites are often determined indirectly after enzymatic hydrolysis or after cleavage by acid because of difficulties in obtaining standards of the native conjugates. Furthermore, the deconjugated metabolites formed by hydrolysis may be more convenient for a chromatographic analysis, because these products as a rule are less hydrophilic and thus more easy to retain on, for instance, a reversed-phase high-performance liquid-chromatographic (HPLC) column. Hydrolysis of the conjugates may give rise to erroneous results if the hydrolysis is not completed or if some of the conjugates are not substrates of the chosen enzyme.

Trimethoprim [2,4-diamino-5-(3,4,5-trimethoxybenzyl)pyrimidine] (TMP), a synthetic antibacterial, has been reported to be metabolized to six different compounds [1—3] (Fig. 1) in various species. $M_1$ and $M_4$ are partly conjugated

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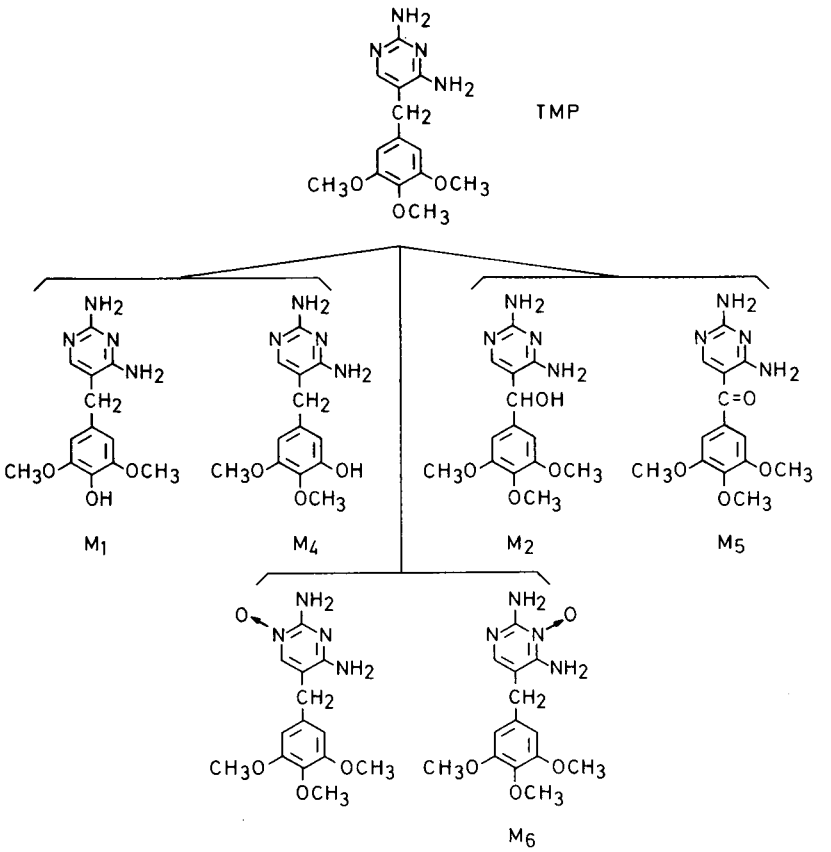


Fig. 1. Structures of trimethoprim and metabolites formed by bio-oxidation.

with glucuronic acid or sulphuric acid. Nielsen and Dalgaard [4] have isolated an $M_4$-sulphate from pig and goat urine. These authors have determined the structure and reported a unique resistance to various arylsulphatases.

Several analytical methods for the quantitation of TMP in body fluids have been used, including microbiological assay [5], spectrofluorimetry [6, 7], autoradiography [1], differential pulse polarography [8, 9], thin-layer chromatography with densitometry [10—12], gas—liquid chromatography [13], and HPLC [14—25]. Only a few methods include quantitation of TMP metabolites [1,8,10,11], and in all cases the determinations were done on phase I and/or deconjugated metabolites. Methods concerning the quantitation of the sulphuric acid conjugate have not previously been published.

The aim of this investigation was to develop an HPLC method which allowed the simultaneous determination of bio-oxidation products (phase I metabolites) and conjugated metabolites of TMP in urine.

In order to optimize the conditions necessary for complete hydrolysis of conjugates, fractions containing $M_1$- and $M_4$-glucuronides were isolated from urine from a pig after intravenous administration of [$^{14}$C]TMP. These conjugates were in turn added to blind pig urine for recovery studies.

## MATERIALS AND METHODS

### *Chemicals*

TMP was a gift from Syntex, Grindsted, Denmark. $M_1$ and $M_4$ were synthesized according to an earlier described procedure [26]. [$^{14}$C]TMP and urine from a pig which had been given 400 mg of [$^{14}$C]TMP (0.5 $\mu$Ci/mg) intravenously were donated by the Department of Pharmacology and Toxicology, Royal Veterinary and Agricultural University, Copenhagen, Denmark. Glucuronides of $M_1$ and $M_4$ and the sulphate conjugate of $M_4$ were isolated in fractions as described below. Standardization of $M_4$-sulphate was done by liquid scintillation counting of a chromatographically (HPLC) pure fraction obtained by the procedure described below. A standard of $M_4$-sulphate was furthermore kindly donated by the Department of Pharmacology and Toxicology, Royal Veterinary and Agricultural University. $\beta$-Glucuronidase (*Escherichia coli*) was obtained in preweighed vials of 1000 sigma units from Sigma (St. Louis, MO, U.S.A.) and diluted with 1 *M* potassium dihydrogen phosphate, pH 6.8. Arylsulphatase (crude solution from *Helix pomatia*) was also obtained from Sigma. All other chemicals were of analytical grade.

### *High-performance liquid chromatography*

HPLC was performed with (I) a Waters Model 6000 solvent delivery system equipped with a Rheodyne injection valve (100 $\mu$l loop), and a Waters Model 440 absorbance detector (254 nm), and (II) Micromeritics equipment consisting of a Model 750 solvent delivery system equipped with a Model 725 autoinjector, and a Model 786 variable-wavelength detector operated at 254 nm. Both systems were equipped with an electrochemical detector consisting of a Model 656 detector cell and a Model 641 VA detector operated in the oxidative mode (Metrohm, Switzerland). Working electrode: glassy carbon. Reference electrode: silver/silver chloride. Auxiliary electrode: glassy carbon. The detectors were connected in series with the electrochemical detector downstream from the ultraviolet (UV) detector. An Omniscribe dual-channel recorder (Houston Instruments, Houston, TX, U.S.A.) was employed. A stainless-steel HPLC column (Knauer, 250 × 4.6 mm I.D.) packed with LiChrosorb RP-18 (5 $\mu$m) particles (Merck, Darmstadt, F.R.G.) was used. The mobile phase consisted of 85 parts of 0.1 *M* potassium dihydrogen phosphate adjusted to pH 7.5 with potassium hydroxide and 15 parts of acetonitrile. Tetrabutylammonium hydrogensulphate (TBA) was added to the mobile phase giving a concentration of $0.7 \cdot 10^{-3}$ *M*. The procedure was carried out at a flow-rate of 1.0 ml/min (250 bars).

### *Isolation of conjugated TMP metabolites*

Gel chromatographic separation was mainly done according to the procedure of Nielsen and Dalgaard [4]. Radioactivities of the urine fractions were determined by mixing 0.5 ml of urine and 3 ml of Ria-Luma (Lumac Systems, Basel, Switzerland) and counting to 1% standard deviation on a Tricarb scintillation counter (Packard Instruments).

The urine sample was evaporated in vacuo at 40°C to a volume of 30 ml. The fraction was centrifuged for 1—2 min at ca. 1200 *g* and the supernatant

applied on a Sephadex G-25 column (100 × 2.6 cm I.D.) (Pharmacia Fine Chemicals, Uppsala, Sweden), eluted with water by use of a Bifok, FIA-08, peristaltic pump. Fractions of 25 ml were collected and 0.5 ml from each fraction was taken for liquid scintillation counting. The column was further eluted with 0.1 *M* ammonium acetate followed by 1 *M* ammonium acetate. Radioactive fractions (almost exclusively from the water eluates) were analysed by HPLC system I using detector settings at (+) 700 mV vs. Ag/AgCl and 254 nm and peaks were collected for liquid scintillation counting. Radioactivity eluted from Sepahdex G-25 was grouped into two parts, the last eluted group containing TMP and phase I metabolites (almost exclusively TMP). The other was applied to a Sephadex G-10 column (33 × 2.6 cm I.D.) and eluted with water. Fractions of 10 ml were collected for liquid scintillation counting giving two groups of fractions containing radioactivity. Radioactive fractions were again analysed by the HPLC system and UV-absorbing compounds were collected for liquid scintillation counting. The last eluted group of fractions from Sephadex G-10 consisted solely of $M_4$-sulphate. The other group of fractions showed on the HPLC UV trace two compounds which by liquid scintillation appeared to be radioactive.

*Hydrolysis of conjugated trimethoprim metabolites*

One millilitre of the first eluted group of fractions from Sephadex G-10 containing radioactivity and 100 μl of β-glucuronidase (*E. coli*, 200 sigma units) were incubated at 38° C. The hydrolysis was followed by HPLC using UV detection at 254 nm and electrochemical detection (ED) at (+) 700 mV vs Ag/AgCl.

One millilitre of the second eluted group from Sephadex G-10 containing radioactivity ($M_4$-sulphate), 100 μl of arylsulphatase (*Helix pomatia*, 400 sigma units), 500 μl of 0.2 *M* potassium dihydrogen phosphate, pH 5, and 100 μl of water, 0.01 *M* barium chloride, or 0.1 *M* barium chloride were incubated at 38° C. The progress of hydrolysis was monitored by HPLC.

Enzymatic hydrolysis of the glucuronides was investigated in the pH range 5.5—7.8 with a diluted urine sample rich in $M_1$-and $M_4$-glucuronides as substrates. Conditions were as described below.

*Quantitation of metabolites*

Urine was diluted ten times with distilled water and 10 μl of diluted urine were injected into HPLC system II for quantitation of $M_1$, $M_4$, and $M_4$-sulphate. Quantitation of $M_1$-glucuronide and $M_4$-glucuronide was done by incubating 1 ml of diluted urine and 100 μl of glucuronidase (*E. coli*, 100 sigma units) at 38° C overnight. The hydrolysate was injected into the HPLC system and the amounts of $M_1$-glucuronide and $M_4$-glucuronide were calculated by subtraction of the $M_1$ or $M_4$ concentration, respectively, before and after enzymatic hydrolysis corrected for differences in dilutions. Concentrations of the metabolites were determined by comparing peak heights of aqueous standards with peak heights of the urine samples. Recovery studies were done by adding standards of $M_1$- and $M_4$-glucuronides obtained from the gel chromatographic separation to urine and subjecting these samples to the described assay.

## RESULTS AND DISCUSSION

Introduction of an ion-pairing agent into the chromatographic system resulted in a decrease of the capacity factor for TMP, $M_1$ and $M_4$. When a new column was employed the TBA concentration had to be adjusted with respect to both the $M_4$-sulphate and TMP, $M_1$ and $M_4$ (a well known problem for reversed-phase columns; see, for example, ref. 27). The relationship between the ion-pairing agent and the capacity factors for TMP, $M_1$ and $M_4$ are outlined in Fig. 2. This behaviour of non-ionic components in reveresed-phase ion-pair HPLC has recently been described thermodynamically by Stranahan and Deming [28] who ascribed the phenomenon to an alteration in the interfacial tension between the mobile phase and an adsorbed phase consisting of a monolayer that is adsorbed on the stationary phase.

Hydrolysis of the radioactive Sephadex G-10 fraction containing $M_4$-sulphate did not take place within 24 h. This was in agreement with earlier findings [4]. A chromatogram of the other radioactive fraction showed two peaks on the UV trace, which disappeared within 4 h of enzymatic hydrolysis giving $M_1$ and $M_4$. The compounds corresponding to the two peaks on the UV trace were not detectable by ED, indicating that a free phenol group or other easily oxidized group was not present. However, a chromatogram of the hydrolysate showed two peaks with the same retention volume and UV/ED ratio as $M_1$ and $M_4$, indicating that the peaks probably were due to $M_1$-glucuronide and $M_4$-glucuronide, respectively (Fig. 3). In the investigations of a suitable pH for hydrolysis of the glucuronides, total hydrolysis appeared to take place within 1 h in the pH interval 5.5—7.0. Above pH 7 the hydrolysis appeared to be complete with 1 h, but the yield of products was lower than for samples treated at pH $\leqslant$ 7.0. So far no explanation can be given for this behaviour. Recoveries of $M_1$ and $M_4$ after hydrolysis of their glucuronides are outlined in Table I.

Investigations of the optimal ED setting were done. The detector response as

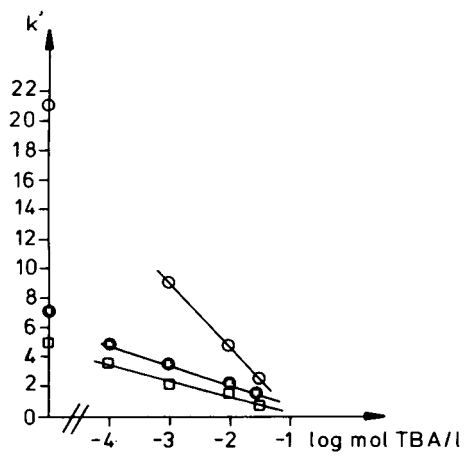


Fig. 2. Reversed-phase ion-pair liquid chromatography of TMP (○); $M_1$ (□); and $M_4$ (●). Column: 25 cm LiChrosorb RP-18 (5 μm). Mobile phase: TBA in acetonitrile—0.1 *M* potassium dihydrogen phosphate adjusted to pH 7.5 with potassium hydroxide (15:85).

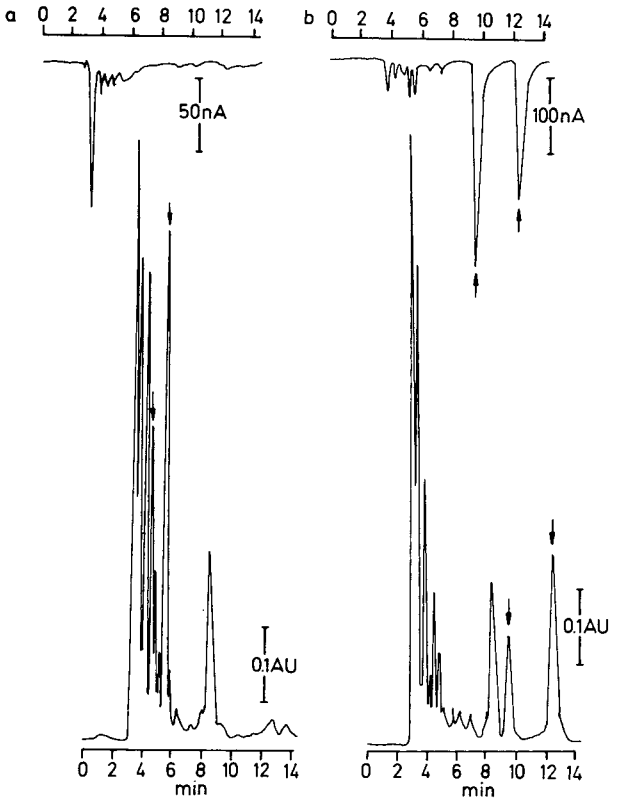


Fig. 3. Chromatograms of a Sephadex G-10 fraction containing $M_1$- and $M_4$-glucuronides before (a) and after (b) hydrolysis with β-glucuronidase (*E. coli*). Upper trace: ED at (+) 700 mV vs. Ag/AgCl. Lower trace: UV detection at 254 nm. Peaks corresponding to radioactive compounds are indicated with arrows.

TABLE I

RECOVERY OF $M_1$ AND $M_4$ AFTER ENZYMATIC HYDROLYSIS OF THEIR GLUCURONIDES IN URINE

Concentrations of $M_1$ and $M_4$ added as glucuronides are determined by liquid scintillation counting. Recovery data represent mean ± S.D. for five determinations of samples at each concentration level obtained by HPLC after enzymatic hydrolysis.

| Concentration of $M_1$ as glucuronide (ppm) | Recovery (%) | Concentration of $M_4$ as glucuronide (ppm) | Recovery (%) |
|---|---|---|---|
| 0.3 | 108 ± 7 | 0.5 | 105 ± 6 |
| 1.2 | 96 ± 4 | 1.9 | 104 ± 2 |
| 6.0 | 98 ± 4 | 9.4 | 104 ± 2 |

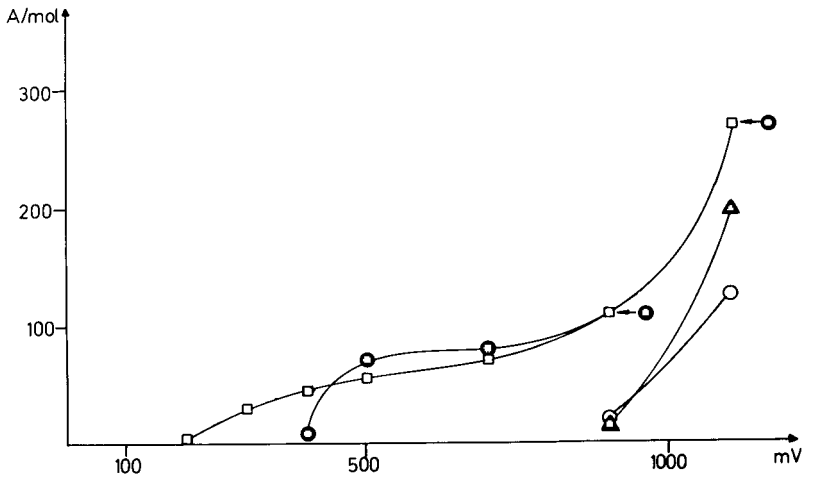


Fig. 4. Applied potential (positive vs. Ag/AgCl) vs. output current. Output current is determined by injecting the same sample of $M_1$ (□); $M_4$ (●); $M_4$-sulphate (▲); and TMP (○) at varying potentials.

a function of applied voltage is shown in Fig. 4. It is seen that in the range of approximately (+) 500—800 mV vs. Ag/AgCl the sensitivity to $M_1$ and $M_4$ is not enhanced. It is therefore most convenient to use (+) 500 mV to obtain maximum selectivity. A minor drawback of selecting (+) 500 mV is that the current yield for $M_4$ is strongly dependent on the applied potential in the range (+) 400—500 mV. In some instances the detector response of $M_4$ appeared to be rather low (compare Figs. 4 and 5). Alteration in the $M_4$ response was only seen when the column and the composition of the mobile phase were changed.

In this work standards were injected for every 5—10 samples in order to check the detector response. Within-day variations of the ED response were usually in the order of 5%.

At (+) 500 mV and 254 nm a linear relationship was found between the concentration of metabolites and their peak heights in the concentration range studied (5—250 ng on-column sample weight). The detection limit in diluted urine defined as twice the baseline noise, was approx. 0.1 ppm using UV detection (254 nm) for $M_1$, $M_4$, and $M_4$-sulphate, and approx. 0.2 ppm for TMP. In the case of ED at (+) 500 mV, the detection limits for $M_1$ and $M_4$ were approx. 0.1 ppm and approx. 0.1—0.2 ppm, respectively, while conjugates and TMP were not detected at (+) 500 mV. The detector response could be drastically improved by using a higher oxidation potential (Fig. 4). This however, caused unacceptable interference from other substances in urine making extraction of the metabolites necessary.

Extraction of $M_1$ and $M_4$ could be performed with ethyl acetate at pH 8 but simultaneous quantitation of $M_1$, $M_4$, and $M_4$-sulphate was not possible by this procedure due to the ionic property of $M_4$-sulphate. In this study ED was therefore mainly included for qualitative detection, but was a valuable supplement to the specificity of the UV detector in cases of interfering peaks on the UV trace. Chromatograms of several blind urine samples from different pigs were obtained. In some cases peaks interfering with $M_1$ and $M_4$ were seen

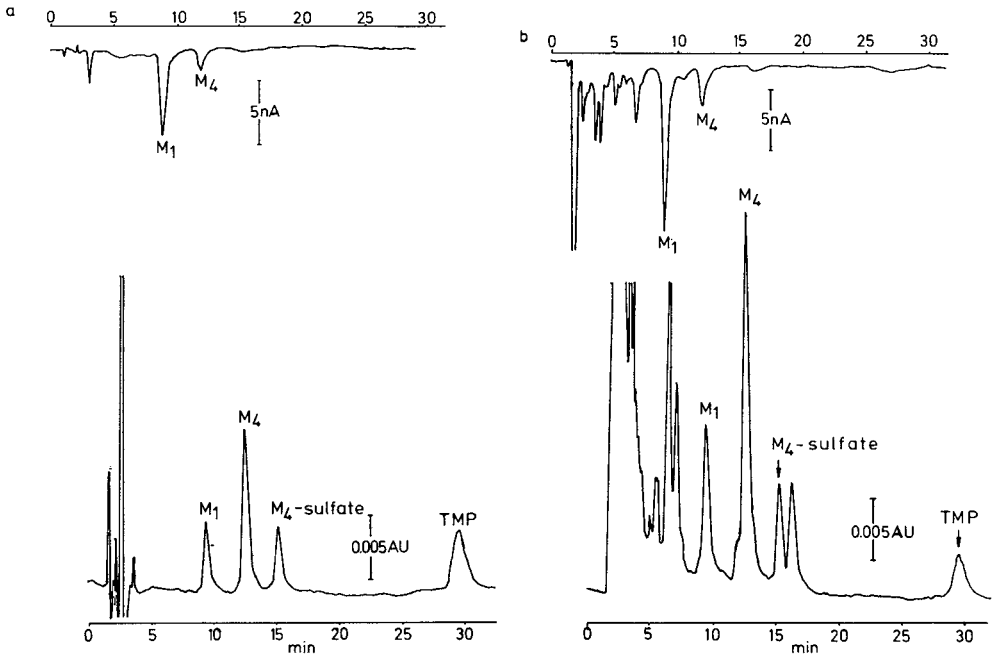


Fig. 5. (a) Standard chromatogram of $M_1$ (44 ng), $M_4$ (99 ng), $M_4$-sulphate (50 ng) and TMP (79 ng). (b) Chromatogram of a hydrolysed urine sample from a 2-month-old pig 2 h after administration of TMP. Upper trace: ED at (+) 500 mV vs. Ag/AgCl. Lower trace: UV detection at 254 nm.

on the UV trace. In such cases ED could always be used for quantitation. A typical chromatogram of urine from a pig after administration of TMP is shown in Fig. 5.

Although this assay includes the possibility of quantitating TMP, this compound would, because of the relatively large retention volume, be better analysed in another system [25]. On gel chromatographic separation only two metabolites were detected ($M_1$ and $M_4$); these metabolites were in turn conjugated to glucuronic acid and sulphuric acid in the case of $M_4$, and to glucuronic acid in the case of $M_1$. These findings were in agreement with earlier published results [4].

A detailed study of the metabolism of TMP in pigs during the first weeks after birth will be reported elsewhere [29].

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CHROMBIO. 1923

# SINGLE-DOSE PHARMACOKINETICS OF PERHEXILINE ADMINISTERED ORALLY TO HUMANS

ALBERT G.B. AMOAH, BARRY J. GOULD and DENNIS V. PARKE*

*Department of Biochemistry, University of Surrey, Guildford, Surrey, GU2 5XH (U.K.)*

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## SUMMARY

A high-performance liquid chromatographic method for the simultaneous determination of perhexiline and its major metabolites, the *cis*- and *trans*-monohydroxyperhexilines $M_1$ and $M_3$, respectively, in human plasma or urine has been developed. Perhexiline and its metabolites are extracted from plasma or urine and derivatized with 1-fluoro-2,4-dinitrobenzene. The extracted dinitrophenyl derivatives of drug and metabolites are separated on a Spherisorb S5 ODS column by gradient elution. The limits of detection for perhexiline and its monohydroxy metabolites were 15 and 3 ng/ml, respectively. The inter-assay coefficients of variation for 100 ng/ml perhexiline, 100 ng/ml $M_1$ and 400 ng/ml $M_3$ were 10.5, 7.6 and 5.6%, respectively ($n$ = 9).

The method has been employed in a limited kinetic study with five healthy adult male volunteers who received 150-mg and 300-mg Pexid tablets at an interval of one week. In four subjects perhexiline exhibited marked first pass effects, with plasma $M_1$ levels higher than unchanged perhexiline; in the urine $M_1$ was the predominant metabolite except in one subject who had higher $M_3$ than $M_1$ in the 300-mg Pexid study. The fifth subject exhibited a defective capacity to hydroxylate perhexiline; $M_1$ and $M_3$ were not detectable in plasma, and the urinary excretion of the monohydroxyperhexilines was relatively less, with $M_3$ present in higher amounts than $M_1$.

## INTRODUCTION

Perhexiline maleate (Pexid) is an effective prophylactic agent in angina pectoris [1]. Analytical methods presently available for measuring perhexiline are either insufficiently sensitive for single-dose kinetic studies, such as the gas—liquid chromatographic methods of Singlas et al. [2] and Cooper and Turnell [3], or do not permit the determination of the major metabolites of perhexiline as in the high-performance liquid chromatographic (HPLC) method of Horowitz et al. [4] which uses fluorescence detection. We now report a sensitive UV spectrophotometric HPLC method for the simultaneous deter-

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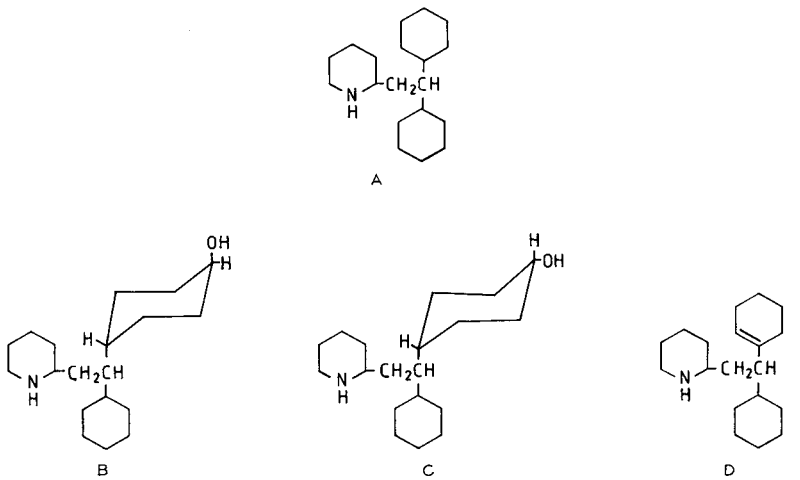


**Fig. 1. Chemical structures of perhexiline (A), its monohydroxylated metabolites $M_1$ (B) and $M_3$ (C), and the HPLC internal standard (D).**

mination of perhexiline and its major metabolites, the *cis*- and *trans*-monohydroxyperhexilines $M_1$ and $M_3$, respectively (Fig. 1). This method is suitable for single-dose kinetic studies with human plasma or urine.

## MATERIALS AND METHODS

### *Reagents*

All aqueous solutions were prepared with deionized water that had been passed through a 0.45-μm MF Millipore filter (Millipore U.K., London, U.K.). Reagent-grade 1-fluoro-2,4-dinitrobenzene, disodium tetraborate and sodium hydroxide (B.D.H. Chemicals, Poole, U.K.), diethyl ether (analytical grade, May and Baker, Dagenham, U.K.) and cyclohexane (analytical grade, B.D.H. Chemicals) were purchased. Methanol (analytical grade, James Burrough, London, U.K.) was passed through a 0.5-μm FH Millipore filter before use. Perhexiline, monohydroxyperhexilines $M_1$ and $M_3$ (1:4 mixture) and internal standard [2-(2′-cyclohexenyl-2-cyclohexylethyl)piperidine hydrochloride] (see Fig. 1) were gifts from Merrell National Labs. (Cincinnati, OH, U.S.A.).

### *Chromatographic system*

A chromatographic system incorporating a Gradient Master and two Constametric I pumps, both from Laboratory Data Control (Riviera Beach, FL, U.S.A.), a Rheodyne syringe loading sample injector (Model 7120, Rheodyne, Berkeley, CA, U.S.A.) with a 100-μl sample loop, and a Spectro Monitor III (Laboratory Data Control) set at 360 nm was used in the analysis. Chromatographic separation of drug and metabolites was achieved with a 250 × 4.6 mm steel column packed with Spherisorb ODS, particle size 5 μm (Phase Separations, Queensferry, U.K.).

### *Chromatographic conditions*

The mobile phase was contained in two reservoirs, A (methanol—water,

85:15) and B (methanol). Elution was performed at 20°C with a flow-rate of 1.0 ml/min. The gradient time and gradient exponent were 20 min and 3, respectively (Service Manual Gradient Monitor Model 1601, Laboratory Data Control). The initial mobile phase composition was 95% A and 5% B with a final composition of 40% A and 60% B. The final mobile-phase composition was maintained for a further 5 min when the run was terminated. The mobile phase was restored to its initial composition and allowed to equilibrate with the column for 10 min before another sample was injected into the chromatograph.

*Sample preparation*

Plasma or urine samples (1.0 ml) containing 250 ng of internal standard, 0.1 ml of 4 *M* sodium hydroxide and 20 ml of diethyl ether were placed into 25-ml screw-capped tubes (Sovirel, Paris, France). Extraction was carried out on a rotary mixer for 30 min and the organic phase removed; the extraction was repeated and the combined organic extracts evaporated to dryness at 40°C. N-Dinitrophenyl derivatives of perhexiline, its metabolites and the internal standard were prepared by a modification of the method of Cox [5] for secondary amines. The dried residue of the ether extract was heated at 80°C for 15 min with 1.5 ml of 26.2 m*M* disodium tetraborate and 0.5 ml of 0.16 *M* 1-fluoro-2,4-dinitrobenzene, and heating continued for a further 1 min after addition of 0.2 ml of 4 *M* sodium hydroxide. The reaction mixture was then cooled and the N-dinitrophenyl derivatives extracted with 20 ml of cyclohexane on a rotary mixer for 30 min. The cyclohexane layer was removed, evaporated to dryness under vacuum at 20°C and the residue redissolved in 100 $\mu$l of methanol—water (85:15) for injection into the chromatograph.

A calibration curve was prepared from blank plasma or urine to which known amounts of perhexiline, and metabolites $M_1$ and $M_3$ had been added. The peak height ratio of drug or metabolite relative to the internal standard was then plotted as a function of drug and metabolite concentration. The drug and metabolite concentrations of samples were determined from the calibration curve.

For recovery studies, known amounts of drug and metabolites were added to normal human blood plasma of which 1.0 ml was extracted with two 20-ml portions of diethyl ether, as previously described.

For precision studies 100 ng/ml perhexiline, 100 ng/ml $M_1$ and 400 ng/ml $M_3$ were added to normal human plasma, which was stored at −20°C until required for analysis.

*Clinical studies*

Following the approval of the ethical committees of the University of Surrey and St. Luke's Hospital, Guildford, five consenting adult male volunteers aged 23—46 years (Table I) were accepted for the clinical study on the basis of a normal medical examination and routine investigations and screening for liver disease. After an overnight fast, each volunteer received 150 mg Pexid orally; fasting was continued for a further 3 h before the subjects were allowed a light lunch. Blood samples were withdrawn over an 8-h period from an intravenous catheter inserted into a forearm vein. A two-hourly urine

TABLE I

HUMAN VOLUNTEERS PARTICIPATING IN THE SINGLE-DOSE PERHEXILINE STUDIES

None of the volunteers was taking other drugs during the study. All volunteers were males.

| Volunteer | Age (years) | Weight (kg) | Low-dose study (mg perhexiline base per kg body weight) | High-dose study (mg perhexiline base per kg body weight) |
|---|---|---|---|---|
| J.D. | 32 | 70 | 2.1 | 4.2 |
| J.B. | 33 | 83 | 1.8 | 3.6 |
| A.D. | 31 | 55 | 2.7 | 5.4 |
| S.G. | 23 | 68 | 2.2 | 4.4 |
| E.E. | 46 | 83 | 1.8 | 3.6 |
| Mean | 33 | 71.8 | 2.1 | 4.2 |

collection was made for the first 8 h after drug ingestion, with a final pooled collection from 8—24 h.

The study was repeated a week later (when perhexiline and metabolites were undetectable in plasma and urine) with 300 mg of Pexid. An additional blood sample was taken at 24 h after drug administration. Plasma and urine samples were analysed by HPLC.

## RESULTS

The recoveries from normal blood plasma were for perhexiline (0.1—10 μg/ml) 95—100%; $M_1$ (0.1—8.0 μg/ml) 90—110%; and $M_3$ (0.4—10 μg/ml) 94—110%. For mixtures of all three (0.5—10 μg/ml of each) the recoveries were 87—118% with a coefficient of variation of 10.5% ($n$ = 9). Chromatograms of blank blood plasma and plasma containing perhexiline, monohydroxyperhexilines $M_1$ and $M_3$, and internal standard are shown in Fig. 2. Plasma samples from patients receiving perhexiline were similar to the spiked plasma (Fig. 2B) and no other peaks were present. The retention times of metabolite $M_3$, $M_1$, the internal standard and perhexiline were 10.75, 12.25, 23.5 and 24.5 min, respectively. The limit of detection for plasma perhexiline was 15 ng/ml and that for monohydroxyperhexilines ($M_1$ and $M_3$) was 3 ng/ml. The calibration curve was linear over the concentration range 15 ng/ml to 10 μg/ml for perhexiline, and over the range 5 ng/ml to 10 μg/ml for the monohydroxyperhexilines. The coefficients of variation of replicate assays performed over a 28-day period for 100 ng/ml perhexiline, 100 ng/ml $M_1$ and 400 ng/ml $M_3$ were 10.5, 7.6 and 5.6%, respectively ($n$ = 9).

### *Clinical studies*

Following the administration of 150 mg of Pexid, perhexiline was undetectable in the plasma of subject J.D. over the 8-h sampling period; this subject had the highest plasma $M_1$ levels (Fig. 3A). Three subjects (A.D., J.B. and S.G.) had significant plasma perhexiline levels with relatively lower $M_1$

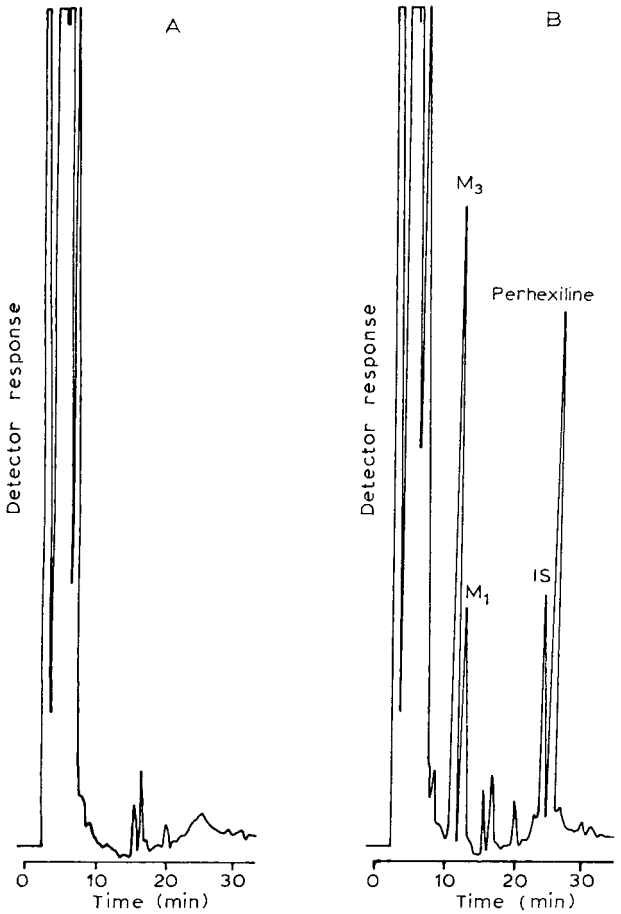


Fig. 2. High-performance liquid chromatograms of ether extracts of (A) human blood plasma and (B) human blood plasma with added perhexiline, monohydroxyperhexilines and internal standard. Perhexiline (500 ng/ml), metabolite $M_1$ (100 ng/ml), metabolite $M_3$ (400 ng/ml) and internal standard (IS) (250 ng/ml) were added to the blood plasma (1.0 ml) before extraction. HPLC conditions were: column, 250 × 4.6 mm Spherisorb ODS; mobile phase, A (methanol—water, 85:15), B (methanol), flow-rate, 1 ml/min; gradient time/exponent, 20 min/3; initial mobile phase, 95% A and 5% B; final mobile phase, 40% A and 60% B.

levels following the 150-mg dose of Pexid. $M_3$ was either undetectable or very low in the plasma of the above four subjects (Fig. 3). The highest plasma perhexiline levels were observed in the fifth volunteer (E.E.); at 8 h the plasma concentration of perhexiline was still rising (Fig. 3E); metabolites $M_1$ and $M_3$ were undetectable in the plasma. At 2 h following drug administration metabolites $M_1$ and $M_3$ were present in the urines of four of the subjects (J.D., J.B., A.D., S.G.) albeit in small amounts (Fig. 4); at 24 h $M_1$ was present in higher amounts than $M_3$. No unchanged perhexiline was detected in any of the urines of the four subjects. In the fifth subject (E.E.) metabolite $M_1$ was not detectable in the urine, $M_3$ was present in relatively small amounts, and significant amounts of unchanged perhexiline were also present (Fig. 4E).

At the higher dose study of Pexid, low concentrations of perhexiline were

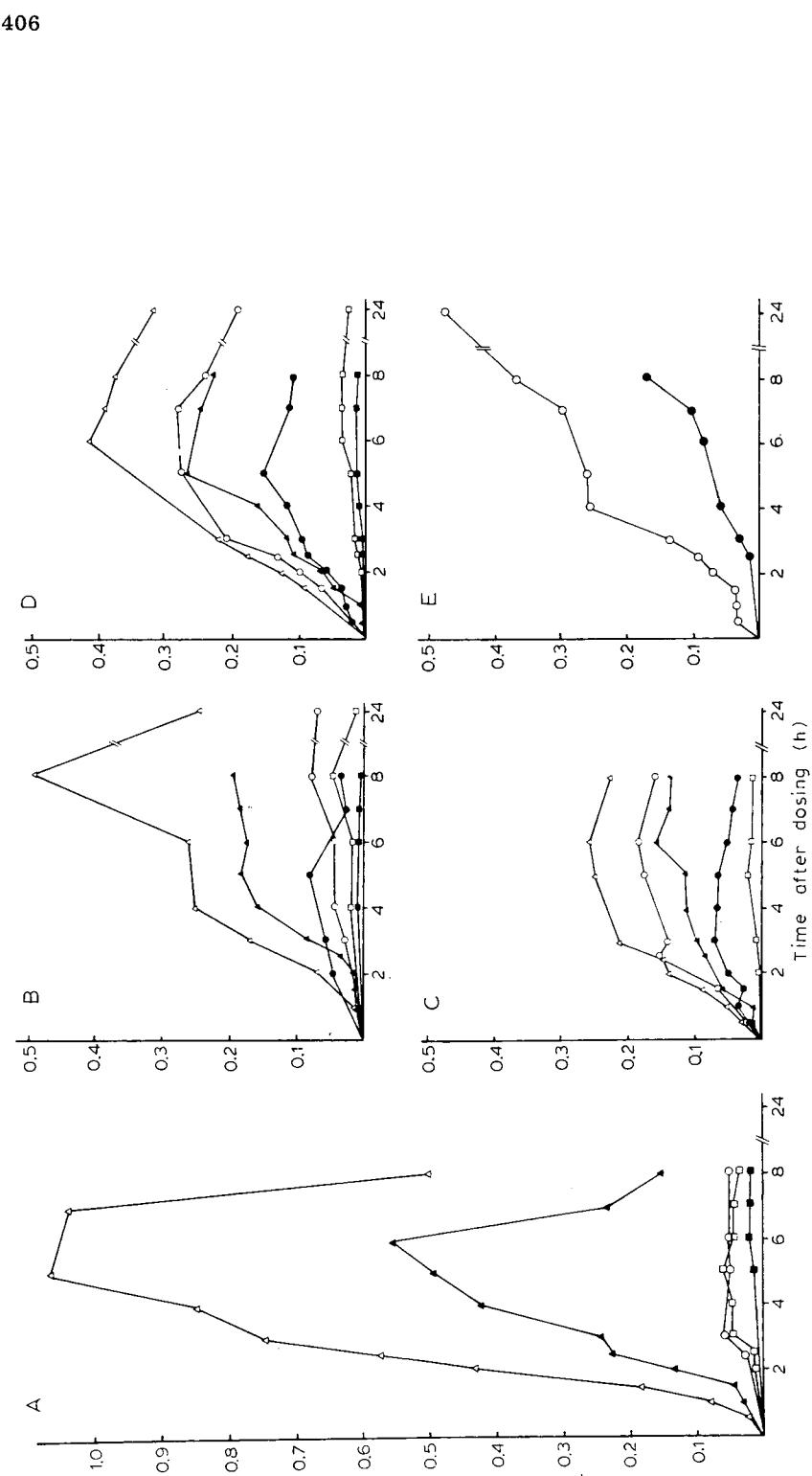


Fig. 3. Plasma concentration—time curve for perhexiline and its monohydroxylated metabolites after oral administration of perhexiline to human volunteers. (A) subject J.D.; (B) subject J.B.; (C) subject A.D.; (D) subject S.G.; and (E) subject E.E. Unchanged perhexiline (•), metabolite $M_1$ (▲), and metabolite $M_3$ (■), are shown after oral dosage with 150 mg of perhexiline; and unchanged perhexiline (○), $M_1$ (△), and $M_3$ (□), after 300 mg of perhexiline.

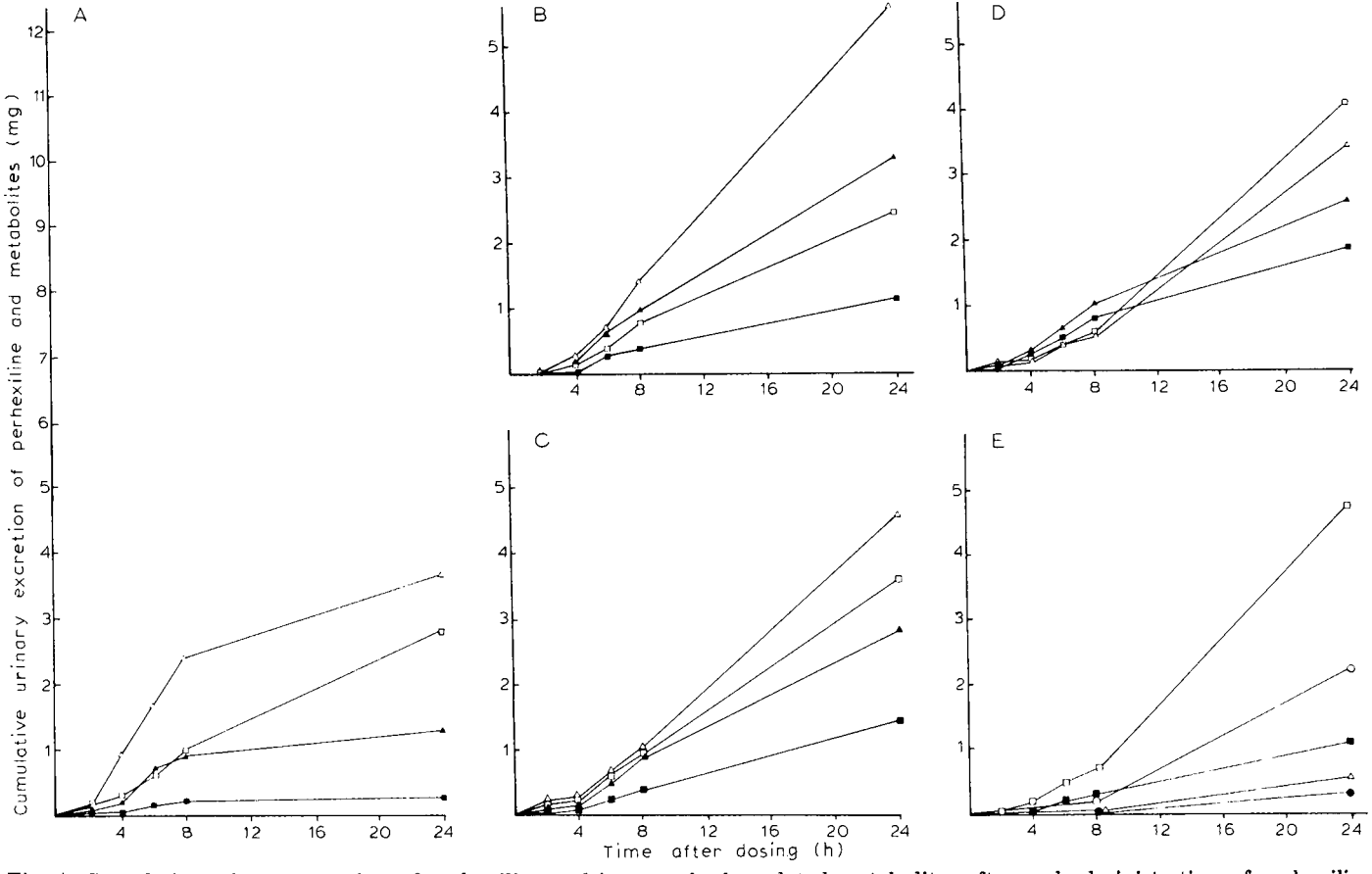


Fig. 4. Cumulative urinary excretion of perhexiline and its monohydroxylated metabolites after oral administration of perhexiline to human volunteers. (A) subject J.D.; (B) subject J.B.; (C) subject A.D.; (D) subject S.G. and (E) subject E.E. Unchanged perhexiline (●), metabolite $M_1$ (▲), and metabolite $M_3$ (■), are shown after oral dosage with 150 mg of perhexiline; and unchanged perhexiline (○), $M_1$ (△), and $M_3$ (□), after 300 mg of perhexiline.

observed in the plasma of subject J.D., with very high levels of metabolite $M_1$ (Fig. 3A). Plasma concentrations of perhexiline and metabolites $M_1$ and $M_3$ were higher at the higher dose of Pexid in subjects J.B., A.D. and S.G. (Fig. 3B—D); at 8 h after dosing, the plasma perhexiline $M_1$ and $M_3$ concentrations were still rising in J.B. (Fig. 3B). Monohydroxyperhexiline metabolites $M_1$ and $M_3$ were again undetectable in the plasma of the fifth subject E.E.; this subject had the highest plasma perhexiline concentration. At 24 h after dosing, metabolite $M_1$ was present in greater amounts than $M_3$ in the urines of three volunteers (Fig. 4). One subject (S.G.) had approximately the same amounts of metabolites $M_3$ and $M_1$ in the urine (Fig. 4D). In the fifth subject (E.E.), $M_3$ was the predominant hydroxylated species present in the urine; metabolite $M_1$ was excreted in the urine, in small amounts, but only at the higher dose; unchanged perhexiline was also present in substantial amounts in the urine of this subject (Fig. 4E).

## DISCUSSION

Despite the proven efficacy of perhexiline maleate in angina pectoris and its reported superiority over some β-adrenergic blocking drugs in the treatment of angina pectoris [6—8] the prescription of perhexiline remains limited because of the frequency and severity of some adverse reactions to the drug.

The mechanism of perhexiline-induced toxicity is still not clear, although a disorder in perhexiline metabolism has been suggested as a possible factor [9]. Singlas et al. [2] have provided evidence in support of this view in that thirteen neuropathic patients showed slower hepatic metabolism of perhexiline, with longer plasma perhexiline half-life and higher mean plasma perhexiline concentration (3.8 μg/ml), than did fourteen patients with no neuropathy (mean plasma perhexiline concentration, 1.0 μg/ml).

The HPLC method now described, with its added advantage of the simultaneous determination of the unchanged drug and two monohydroxy metabolites, has permitted us to carry out a preliminary examination of the single-dose kinetics of perhexiline in human volunteers. In this limited study we observed considerable individuality with respect to the metabolism and clearance of perhexiline which supports the earlier studies using [$^{14}$C]perhexiline [10].

In four subjects (J.D., J.B., A.D., S.G.) perhexiline exhibited marked first-pass effects with plasma metabolite concentrations higher than unchanged perhexiline (Fig. 3). This might explain the observed initial delay between perhexiline administration and the onset of drug effect [11]. The major metabolite in both plasma and urine was $M_1$ in three volunteers. In the fourth subject (S.G.) $M_1$ was also the major metabolite in the plasma, and in the urine at the lower-dose study but at the high-dose study $M_3$ became the major metabolite in the urine at 24 h (Figs. 3 and 4). Perhexiline metabolism could thus be subject to saturable kinetics with respect to metabolite $M_1$. The concentration of free $M_3$ in the urines of all five subjects is greater than might have been expected from the blood concentrations of this metabolite (Figs. 3 and 4).

The fifth subject (E.E.) appeared to have a defective capacity to hydroxylate perhexiline. Metabolites $M_1$ and $M_3$ were not detectable in the blood plasma and

the concentration of unchanged perhexiline in the plasma was much higher than in the four other subjects. Smaller amounts of metabolites were excreted in the urine and there was significant excretion of unchanged perhexiline. The major metabolite in this subject was $M_3$. It is interesting to note that the neuropathic patients of Singlas et al. [2] also formed greater amounts of metabolite $M_3$ than the non-neuropathic patients.

It thus appears that single-dose kinetics of perhexiline may be of value in identifying those subjects at risk of toxic accumulation of perhexiline on conventional dosage regimes. However, further studies are needed to elucidate the complexities of perhexiline metabolism and kinetics, and to fully evaluate the use of single-dose kinetics in predicting potential adverse reactions to perhexiline.

ACKNOWLEDGEMENTS

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# DETERMINATION OF METOPROLOL AND ITS α-HYDROXYLATED METABOLITE IN HUMAN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

JEAN-BAPTISTE LECAILLON*, JACQUES GODBILLON, FRANCOISE ABADIE and GHISLAINE GOSSET

*Ciba-Geigy, Biopharmaceutical Research Center, B.P. 308, 92506 Rueil-Malmaison Cedex (France)*

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## SUMMARY

A high-performance liquid chromatographic method has been developed for the simultaneous determination of metoprolol and its α-hydroxylated metabolite in plasma, Metoprolol, α-hydroxymetoprolol and alprenolol (internal standard) are extracted from plasma at alkaline pH with diethyl ether—dichloromethane (4:1, v/v) and back-extracted with 0.01 *N* sulfuric acid. A 100-μl volume of the acidic extract is injected into the chromatographic system. The compounds are eluted in about 12 min with acetonitrile—acetate buffer (75:25, v/v) on a LiChrosorb RP-8 (5 μm) column. The quantitative determinations are made fluorometrically. Concentrations down to 35 nmol/l (10 ng/ml) of metoprolol base and 30 nmol/l (8 ng/ml) of α-hydroxymetoprolol base in plasma can be determined with good precision and accuracy.

## INTRODUCTION

Metoprolol, a selective β-adrenergic receptor antagonist, is used in the treatment of hypertension and angina pectoris [1]. Its metabolism in humans is well documented [2, 3]. Two of its metabolites (Fig. 1), α-hydroxymetoprolol and O-demethylmetoprolol, display β-blocking activities, but are less potent than the parent drug [2, 4]. O-Demethylmetoprolol has been detected in plasma at very low concentrations, often below 5 nmol/l [3]. This metabolite would not normally contribute to the β-blocking effect of metoprolol [3]. The relative amount of α-hydroxymetoprolol in plasma has been reported to be 0.5—1 times that of metoprolol [1—4]. The object of the present study was to determine both metoprolol and α-hydroxymetoprolol in plasma.

Several methods of determining metoprolol by either gas chromatography

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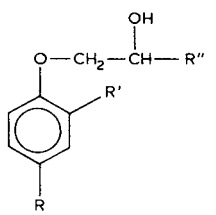
(GC) [5—12] or high-performance liquid chromatography (HPLC) [13—19] have been described. Few of them permit the simultaneous determination of metoprolol and α-hydroxymetoprolol [5, 9, 17]. GC methods [5, 9] need a derivatization step after extraction and evaporation to dryness. One of them [9] is very selective, but it uses a mass spectrometric detector. In the HPLC method [17] the sample is rotated for 1 h for extraction, before evaporating the organic phase to dryness, and the chromatography lasts about 30 min per sample. Its sensitivity is 3 ng of metoprolol and 12 ng of α-hydroxymetoprolol per ml of plasma. The method proposed below is very simple, as it requires no special washing of the glassware and only two extraction steps without evaporation to dryness before chromatography, which lasts 10 min.

## EXPERIMENTAL

### *Chemicals and reagents*

Metoprolol tartrate and the internal standard, alprenolol hydrochloride, were supplied by Ciba-Geigy (Basle, Switzerland). α-Hydroxymetoprolol in the form




| | R | R' | R'' | M.W. |
|---|---|---|---|---|
| Metoprolol | $CH_2—CH_2—OCH_3$ | H | $CH_2—NH—CH(CH_3)_2$ | 267 |
| α — Hydroxymetoprolol | $CH(OH)—CH_2—OCH_3$ | H | $CH_2—NH—CH(CH_3)_2$ | 283 |
| O-Demethylmetoprolol (H 105/22) | $CH_2—CH_2—OH$ | H | $CH_2—NH—CH(CH_3)_2$ | 253 |
| H 117/04 | $CH_2COOH$ | H | $CH_2—NH—CH(CH_3)_2$ | 267 |
| H 104/83 | $CH_2—CH_2—OCH_3$ | H | COOH | 240 |
| Alprenolol | H | $CH_2—CH=CH_2$ | $CH_2—NH—CH(CH_3)_2$ | 249 |

**Fig. 1. Chemical structure of metoprolol, its known metabolites and alprenolol, with their molecular weights (M.W.).**

of its *p*-hydroxybenzoic acid salt was supplied by Hässle (Mölndal, Sweden). The structures of these compounds are given in Fig. 1. All solvents and reagents were of analytical grade (E. Merck, Darmstadt, F.R.G.). Acetonitrile was of spectroscopy quality (Merck 16).

The alkaline solution for extraction was prepared by dissolving 30 g of $K_3PO_4 \cdot 3H_2O$ and 16.8 g of KOH in 100 ml of distilled water.

*Internal standard and reference solutions*

The internal standard solution was prepared in water from alprenolol hydrochloride (concentration: 17.5 μmol/l = 5 μg/ml). Reference solutions of metoprolol and α-hydroxymetoprolol for calibration were prepared from the respective salts dissolved in the aqueous solution of the internal standard. This solution was also used for preparation of validation solutions.

*Sample preparation*

The schematic outline of the sample preparation is given in Fig. 2. Into a 10-ml polypropylene tube are added 1 ml of plasma, 50 μl of the internal standard solution (or reference solution for calibration or validation solution), 1 ml of the alkaline solution and 4 ml of diethyl ether—dichloromethane (4:1, v/v). The tube is shaken on an Infors shaker at 350 rpm for 15 min and centrifuged at 2000 *g* for 10 min. Then 3 ml of the organic phase are transferred

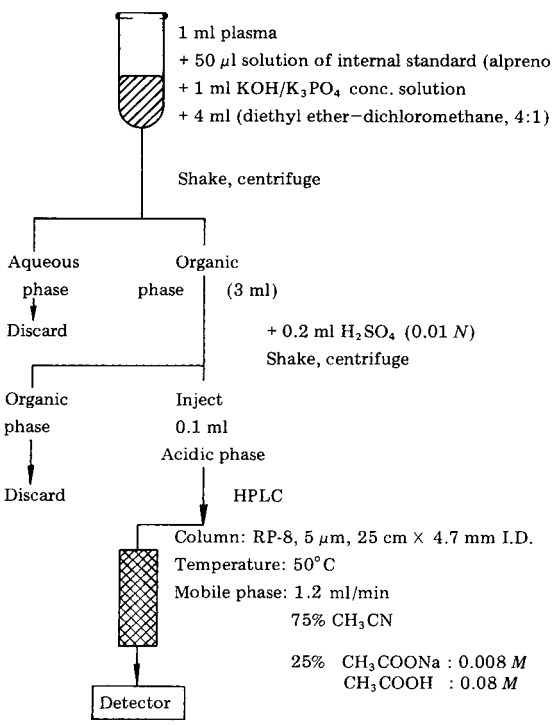



**Fig. 2. Schematic outline of the sample assay.**

into a 10-ml conical glass tube, and 200 $\mu$l of 0.01 $N$ $H_2SO_4$ are added. The tube is shaken for 10 min and centrifuged for 2 min. About 150 $\mu$l of the acidic phase are introduced into a 0.5-ml polyethylene tube of the autosampler and 100 $\mu$l are injected onto the column.

*Chromatography*

The chromatography is performed on a Gilson 302 pumping system equipped with a Gilson 802 manometric system and a Wisp automatic injector from Waters. About 100 4-ml vials marketed by Waters were modified (see Fig. 3) to accommodate small 0.5-ml conical polyethylene tubes (micro-analysis tubes). These tubes are about ten times less expensive than the classical 0.3-ml glass tubes. This modification permits the injection of about 100 $\mu$l from 150 $\mu$l added in the plastic tube. The detector used is a Schoeffel fluorescence detector (Model FS 970) set at wavelengths of 225 nm for excitation and > 320 nm for emission. It is connected to a Spectra-Physics computing integrator (Model 4100).

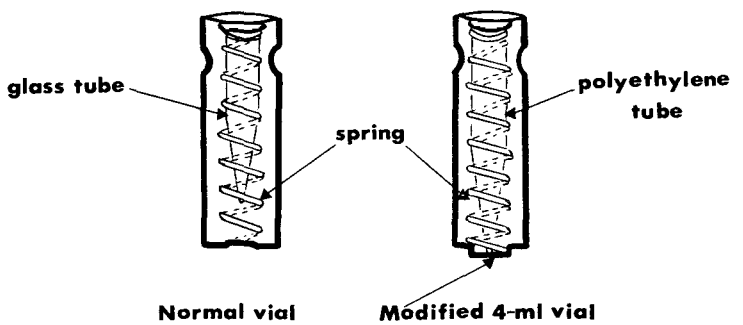



Fig. 3. Modification of a 4-ml WISP autosampler vial to accommodate 0.5-ml polyethylene micro-analysis tubes.

The analytical column (25 cm × 4.7 mm I.D.) is filled with LiChrosorb RP-8, 5 $\mu$m particle size.

The mobile phase, acetonitrile—acetate/acetic acid solution (0.008 $M$ sodium acetate + 0.08 $M$ acetic acid (75:25) is degassed in an ultrasonic bath before use and heated to 40—50°C during use. The column is thermostated at 50°C. The flow-rate is 1.2 ml/min. The retention times of $\alpha$-hydroxymetoprolol, metoprolol and alprenolol are respectively 6.4, 8.2 and 10.1 min (Fig. 4). New LiChrosorb RP-8 batches may give longer retention times with the mobile phase composition described above. In this event, the acetate/acetic acid solution used in the mobile phase should be modified. With the aqueous solution, 0.05 $M$ sodium acetate + 0.5 $M$ acetic acid, the retention times and the separation of the components with new LiChrosorb batches are similar to those normally obtained.

*Calibration*

All metoprolol, $\alpha$-hydroxymetoprolol and alprenolol amounts are expressed in pmol or in nmol of free base. They can be converted to pg or ng, respectively, by multiplying them by the molecular weights (Fig. 1).

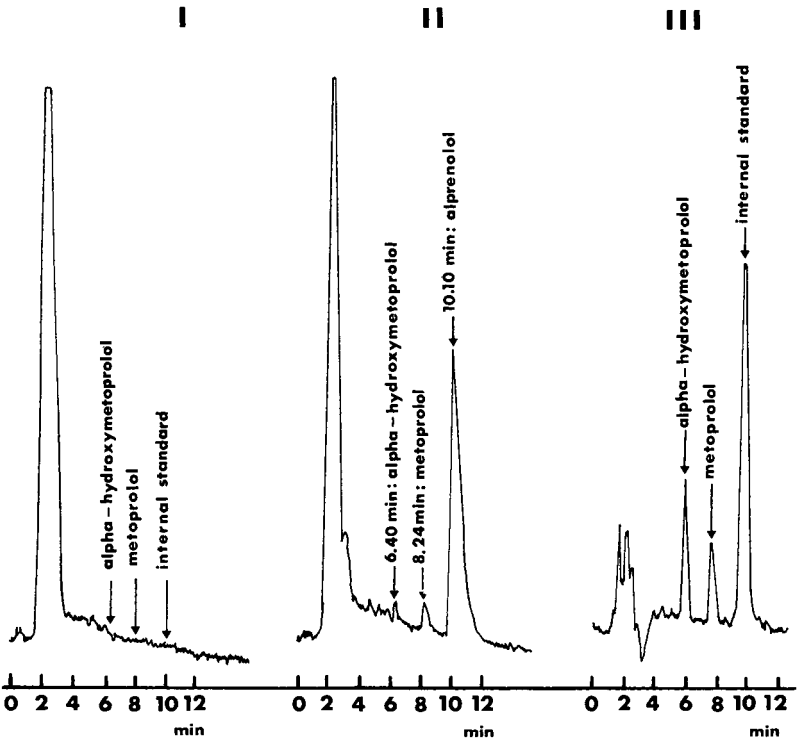



Fig. 4. Chromatograms of extracted plasma: (I) human blank plasma; (II) plasma sample spiked with 40 nmol/l (11 ng/ml) metoprolol base and 28 nmol/l (8 ng/ml) α-hydroxymetoprolol; (III) actual plasma sample.

Calibration samples are prepared by adding 50 μl of calibration solutions containing known amounts of metoprolol (36.5—1462 pmol of free base), α-hydroxymetoprolol (30—1188 pmol) and alprenolol (875 pmol) to 1 ml of plasma according to the procedure described above for the sample preparation. (Calibration solutions were stable for at least one month, when kept at 5°C.)

The calibration curves are established from these calibration samples worked up in duplicate for five concentrations.

The equation of each calibration curve is that of the regression straight line of the log values of peak area ratios (metoprolol or α-hydroxymetoprolol/internal standard) versus the log value of the concentrations.

## RESULTS AND DISCUSSION

### *Precision, accuracy and calibration*

Plasma samples containing metoprolol and α-hydroxymetoprolol at different concentrations were analysed repeatedly. The results in Table I give the precision and accuracy of the method for replicates at concentration levels corresponding to those observed after administration of therapeutic doses of metoprolol. Plasma concentrations down to 35 nmol/l metoprolol (10 ng/ml) and 30 nmol/l α-hydroxymetoprolol (8 ng/ml) can be determined with a coefficient of variation lower than 10%.

TABLE I

PRECISION AND RECOVERY OF THE DETERMINATION OF METOPROLOL AND α-HYDROXYMETOPROLOL IN SPIKED PLASMA SAMPLES

| | Metoprolol* | | | | α-Hydroxymetoprolol** | | | |
|---|---|---|---|---|---|---|---|---|
| Added (nmol/l) | 36.5 | 73 | 292 | 877 | 29.7 | 59 | 238 | 713 |
| Found (nmol/l) | 36.5 | 73.9 | 298 | 861 | 27.2 | 60.9 | 237 | 670 |
| | 38.8 | 71.7 | 285 | 861 | 29.7 | 62.0 | 239 | 689 |
| | 33.8 | 71.3 | 281 | 861 | 27.0 | 63.9 | 223 | 726 |
| | 32.0 | 70.4 | 285 | 892 | 30.0 | 62.0 | 231 | 689 |
| | 36.5 | 73.1 | 293 | 861 | | 61.6 | 223 | 679 |
| | 33.3 | | 297 | 843 | | | 231 | 689 |
| Average | 35.2 | 72.1 | 289.8 | 863 | 28.5 | 62.1 | 231 | 690 |
| Coefficient of variation (%) | 7.2 | 2 | 2.5 | 1.8 | 5.7 | 1.8 | 2.9 | 2.8 |
| Recovery (%) | 96.3 | 98.7 | 99.2 | 98.5 | 95.9 | 105 | 96.9 | 96.9 |
| Overall recovery | 98 | | | | 99 | | | |
| S.D. | 4 | | | | 5 | | | |

*To convert to ng/ml of metoprolol base, multiply the data by 0.267.
**To convert to ng/ml of α-hydroxymetoprolol base, multiply the data by 0.283.

The calibration curves obtained in the determination of more than 1000 clinical samples gave correlation coefficients higher than 0.9990 and slope values within the range 0.93—1.07.

*Selectivity*

Metoprolol and α-hydroxymetoprolol are conveniently separated from plasma components (Fig. 4). The retention times of the other metabolites (Fig. 1) were 2.3, 5.4 and 6.2 min for H 104/83, H 117/04 and H 105/22 (O-demethylmetoprolol), respectively. The last-mentioned metabolite has a retention time very close to that of α-hydroxymetoprolol. However, extremely low concentrations of this metabolite have been recorded in plasma (< 5 nmol/l) [3, 20] after intravenous or oral administration of metoprolol to healthy subjects, or to patients with renal impairment, whether undergoing dialysis or not. The highest level (12 nmol/l) was observed in a uremic patient during steady-state conditions [3]. The other two metabolites are not detected with the sample preparation described.

*Adsorption on glassware and metoprolol stability in extracts*

A significant concentration decrease was observed when alkaline or neutral solutions with low metoprolol concentrations were contained in glass tubes. To avoid adsorption, plastic tubes were used for the first extraction, and the decrease in concentration was no longer observed. The stability of metoprolol in the extracts was studied. No significant decrease in the concentration was found when sample extracts were kept for 15 h at room temperature. This permits the use of an automatic injector.

In conclusion, the described procedure allows the assay of metoprolol and

α-hydroxymetoprolol in plasma with suitable sensitivity and accuracy. The work-up procedure is simpler and faster than that described in the methods previously reported. It is well suited for pharmacokinetic and clinical pharmacology studies.

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CHROMBIO. 1932

# DETERMINATION OF 3,7-DIMETHYL-1-(5-OXOHEXYL)-XANTHINE (PENTOXIFYLLINE) AND ITS 3,7-DIMETHYL-1-(5-HYDROXYHEXYL)-XANTHINE METABOLITE IN THE PLASMA OF PATIENTS WITH MULTIPLE DISEASES USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY*

W. RIECK and D. PLATT*

*Institute of Gerontology, University of Erlangen-Nürnberg and 2nd Internal Medicine, Nürnberg (F.R.G.)*

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## SUMMARY

Pharmacokinetic studies of pentoxifylline in elderly patients with multiple diseases are described. Because of the low plasma levels of pentoxifylline and its hydroxy metabolite a new high-performance liquid chromatographic method was established. Under our chromatographic conditions, piracetam, a nootropic drug, can be easily separated from simultaneously applied pentoxifylline and its metabolite. Piracetam shows no appreciable ultraviolet absorption above 230 nm and cannot be detected at 268 nm.

## INTRODUCTION

The drug Trental® (pentoxifylline) is often used to eliminate or reduce disturbances in the peripheral blood circulation. So far, however, pharmacokinetic parameters of pentoxifylline and its metabolite have been established only for young and healthy persons [1, 2]. Since Trental is also used in the therapy of older patients, a pharmacokinetic study with patients over 60 years old (average age 77.1, S.D. 8.4) with multiple diseases was considered to be necessary. To avoid possible acute side-effects caused by intravenous application of the drug to elderly patients, the drug was applied by infusion (100 mg of Trental during 90 min).

Because of the expected lower plasma levels of pentoxifylline and its hydroxy metabolite, and also because these patients are treated with other

*Dedicated to the 60th birthday of Prof. Dr. E. Buddecke, University of Münster.

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drugs, it was necessary to establish a new high-performance liquid chromatographic (HPLC) method instead of using published procedures [2, 3].

Our new method is particularly flexible in analysing various volumes of plasma (1—4 ml), and it is sensitive and selective.

Preliminary results of the parmacokinetic study based on our method will be given.

## EXPERIMENTAL

### *Reagents*

Pentoxifylline and its hydroxymetabolite were kindly provided by Albert-Roussel Pharma (Wiesbaden, F.R.G.); HPLC-grade water and methanol were supplied by Promochem (Wesel, F.R.G.).

Extrelut® columns from Merck (Darmstadt, F.R.G.) and Sep-Pak® silica cartridges from Waters (Königstein, F.R.G.) were used for sample clean-up.

The stock solution was prepared by dissolving 100 mg of pentoxifylline and 100 mg of metabolite in 1000 ml of water. A standard solution was then made by diluting 300 ml of the stock solution with water to a final volume of 1000 ml.

### *Instrumentation and chromatographic conditions*

The HPLC system consists of two Kontron LC pumps (Model 410), a Kontron mixing chamber, a Rheodyne Model 7125 sampling valve, a Kontron autosampler (Model MSI 660), a Kontron ultraviolet (UV) detector (Model Uvicon 720 LC), a Kontron programmer (Model 200) and a Spectra-Physics integrator (Model SP 4100).

A LiChrosorb RP 18 column (particle size 10 $\mu$m, 25 cm × 4.9 mm I.D.), and an RP 18 guard column (particle size 10 $\mu$m, 3 cm × 4.6 mm I.D.) manufactured by Kontron were used.

The analyses were carried out isocratically using a methanol—water mixture (48:52, v/v) as eluent.

The flow-rate of the mobile phase (saturated with helium) was 2.0 ml/min. Always 20 $\mu$l of sample solution were injected either manually in a 20-$\mu$l loop of the Rheodyne sampling valve or by means of the autosampler. Pentoxifylline and the hydroxy metabolite were detected at a wavelength of 268 nm. The external standard method was chosen for calibration.

UV spectra were run on a Beckman Instruments spectrometer (Model 24).

### *Preparation of standard plasma samples*

Venous blood (containing no pentoxifylline or metabolite) was centrifuged in a heparinized test tube for 10 min at 3000 *g* and the plasma was separated. Then 500, 200, 100, 50, 20, 10 and 5 $\mu$l of the standard solution were added to 1-ml aliquots of the plasma.

### *Method A*

The spiked plasma samples prepared as above were first diluted with water to a volume of 16 ml in a calibrated cylinder, and by rinsing the cylinder twice with 2-ml portions of water were transferred directly onto an Extrelut column.

For 30 min the sample was allowed to soak before elution with 40 ml of methylene chloride was started. The solvent was removed from the eluate on a rotatory evaporator at 30°C maximum water bath temperature, and 300 μl of methanol were added to the residue. After shaking for 1 min, the solution was transferred into an Eppendorf micro test tube and centrifuged (Eppendorf centrifuge, Model 3200) for 2 min at 17,000 *g*; 20 μl of the upper layer were injected into the chromatograph via the autosampler. Thus, reference samples for calibrations of chromatographic peak areas were obtained corresponding to the concentrations: 15, 6, 3, 1.5, 0.6, 0.3 and 0.15 μg/ml.

*Method B*

The spiked plasma samples were chromatographed as described by method A on the Extrelut column. The residue was then dissolved in 3 ml of methylene chloride and transferred to a Sep-Pak cartridge preconditioned with 20 ml of methylenechloride. First, 75 ml of pure methylene chloride were run through the cartridge, then the components were eluted with 20 ml of methylene chloride—methanol mixture (20:1, v/v).

The solvent was removed from this eluate under vacuum and the residue was extracted four times with 500-μl portions of methylene chloride. After transferring 1-ml portions to an Eppendorf micro test tube and evaporation of the solvent by a stream of nitrogen, 300 μl of methanol were added (in some cases only 60 μl). After vortexing for about 1 min and centrifugation for 2 min at 17,000 *g*, 20 μl of the upper layer were injected via the autosampler or manually by the Rheodyne sampling valve.

*Patients' plasma samples*

The plasma was isolated as described for the standard plasma samples. It was stored at −20°C and thawed at room temperature before use. Plasma volumes of 1—3 ml were analysed by method A; 1—4 ml of plasma were analysed if method B was used.

## RESULTS AND DISCUSSION

*Detector study*

In the literature [3, 4] the absorption maximum of pentoxifylline is reported to be at a wavelength of 274 nm. There is, however, a low-wavelength absorption maximum at 210 nm (Fig. 1) with a more than two-fold higher extinction coefficient. For reasons of better sensitivity, this wavelength was chosen alternatively for detection. The proper specifications of the UV system at that low-wavelength range were tested. The better signal-to-noise ratio at 268 nm (absorption maximum in the mobile phase), on the other hand, results in a more accurate determination of peak areas for quantitative measurements.

*Calibration curve*

The calibration curve of pentoxifylline and its metabolite from plasma was linear in the range 0.15–6.0 μg/ml with both method A and method B. Linear regression analysis of these data gave correlation coefficients (*r*) for pentoxifylline and the metabolite consistently greater than 0.999.

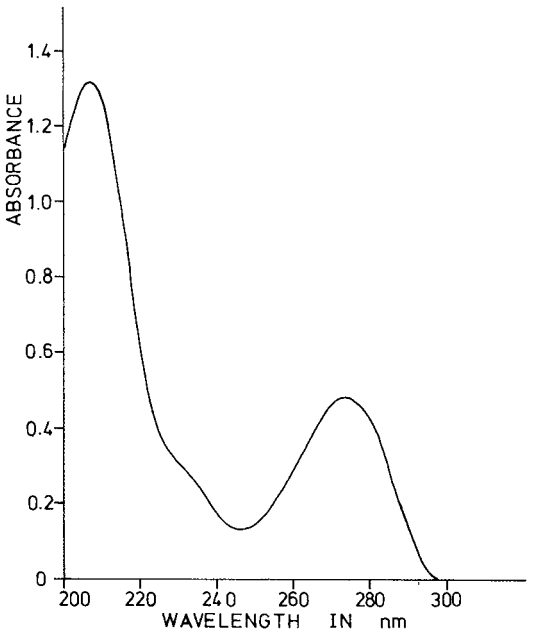



Fig. 1. UV spectrum of pentoxifylline ($c$ = 12.5 μg/ml) in water. Reference: water.

*Recovery rate, precision, accuracy and sensitivity of the assay*

The recovery rate in the concentration range 0.15—15.0 μg/ml using method A is 98.5% (relative S.D. = 7.3%, $n$ = 32) for pentoxifylline and 106.3% (relative S.D. = 11.1%, $n$ = 32) for the metabolite. Using method B, recoveries of 95.7% (relative S.D. = 7.7%, $n$ = 23) for pentoxifylline and 105.4% (relative S.D. = 5.2%, $n$ = 23) for the metabolite are found.

TABLE I

PRECISION AND ACCURACY OF ASSAY METHOD A

| Actual plasma concentration (μg/ml) | | Measured plasma concentration (μg/ml, mean ± S.D.) | | Relative S.D. (%) | | Relative error (%) ($\frac{\text{conc. found}}{\text{conc. actual}} \times 100$) | | No. of determinations |
|---|---|---|---|---|---|---|---|---|
| P* | M** | P | M | P | M | P | M | |
| 6.000 | | 5.989 ± 0.408 | 5.996 ± 0.393 | 6.817 | 6.548 | 99.8 | 99.9 | 11 |
| 4.500 | | 4.664 ± 0.268 | 4.502 ± 0.295 | 5.752 | 6.561 | 103.6 | 100.0 | 4 |
| 2.100 | | 1.997 ± 0.108 | 2.050 ± 0.079 | 5.193 | 3.868 | 95.1 | 97.6 | 4 |
| 0.900 | | 0.896 ± 0.055 | 0.829 ± 0.082 | 6.095 | 9.920 | 99.6 | 92.1 | 5 |
| 0.300 | | 0.285 ± 0.024 | 0.278 ± 0.022 | 8.547 | 7.914 | 95.0 | 92.7 | 4 |

*P = pentoxifylline.
**M = metabolite.

TABLE II

PRECISION AND ACCURACY OF ASSAY B

Analysis end-volume = 300 μl

| Actual plasma concentration (μg/ml) | Measured plasma concentration (μg/ml, mean ± S.D.) | | Relative S.D. (%) | | Relative error (%) ($\frac{\text{conc. found}}{\text{conc. actual}} \times 100$) | | No. of determinations |
|---|---|---|---|---|---|---|---|
| P* M** | P | M | P | M | P | M | |
| 6.000 | 5.869 ± 0.095 | 5.987 ± 0.179 | 1.617 | 2.990 | 97.8 | 99.8 | 4 |
| 4.500 | 4.668 ± 0.125 | 4.696 ± 0.113 | 2.687 | 2.402 | 103.7 | 104.4 | 4 |
| 2.100 | 2.130 ±0.069 | 2.111 ± 0.035 | 3.262 | 1.678 | 101.4 | 100.5 | 4 |
| 0.900 | 0.923 ± 0.043 | 0.851 ± 0.073 | 4.621 | 8.550 | 102.6 | 94.6 | 5 |
| 0.300 | 0.283 ± 0.022 | 0.276 ± 0.025 | 7.614 | 8.897 | 94.3 | 92.0 | 3 |

*P = pentoxifylline.
**M = metabolite.

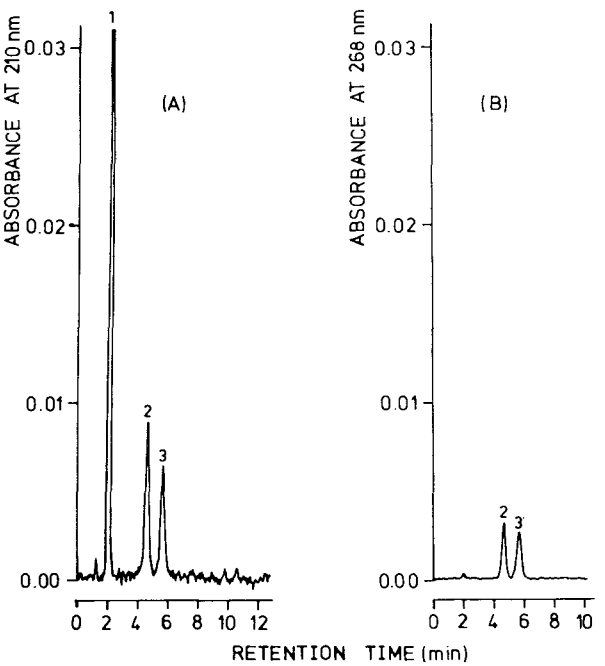
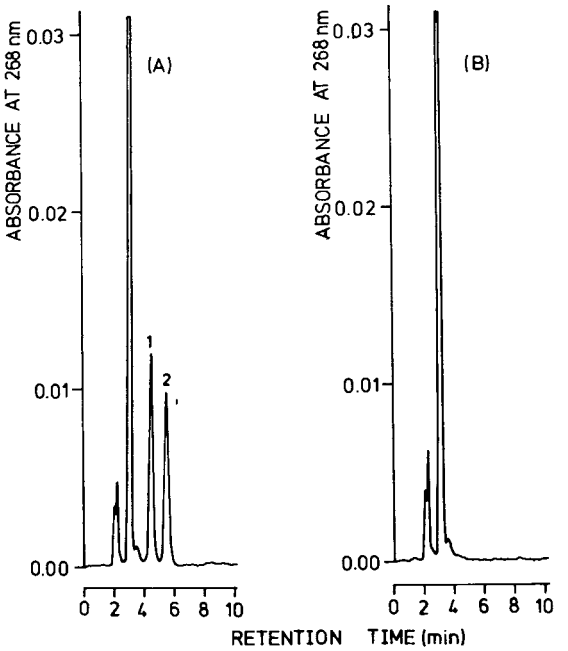



Fig. 2. Chromatograms of a standard solution of piracetam, pentoxifylline and metabolite. (A) absorbance at 210 nm, (B) absorbance at 268 nm. Peaks: 1 = piracetam (2 μg per injection; time 1.9 min), 2 = pentoxifylline (100 ng per injection; time 4.5 min), 3 = metabolite (100 ng per injection. time 5.5 min). Injection volume: 20 μl. For chromatographic conditions see text.



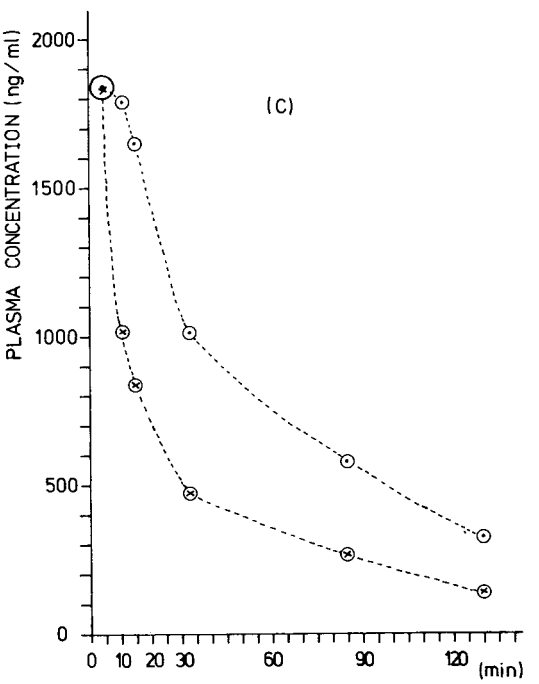




Fig. 3. Chromatograms of a patient's (TR-12) plasma samples: (A) after administration of pentoxifylline; (B) before administration of pentoxifylline. Samples prepared by method A. Peaks: 1 = pentoxifylline (time 4.4 min), 2 = metabolite (time 5.4 min). (C) Profiles of pentoxifylline (×) and metabolite (•) plasma concentrations versus time after an infusion of 100 mg of pentoxifylline (Trental) during 90 min.

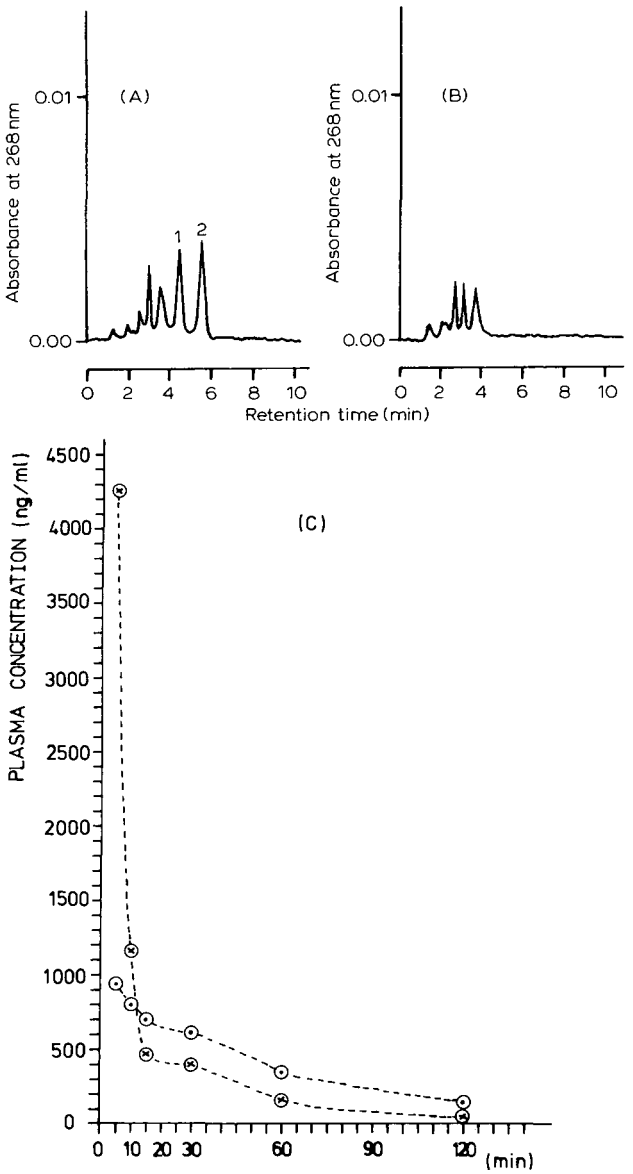



Fig. 4. Chromatograms of a patient's (TR-15) plasma samples: (A) after administration of pentoxifylline; (B) before administration of pentoxifylline. Sample preparation by method B (3 ml of plasma, final analysis volume 300 $\mu$l). Peaks: 1 = pentoxifylline (time 4.4 min), 2 = metabolite (time 5.4 min). (C) Profiles of pentoxifylline (×) and metabolite (•) plasma concentrations versus time after an infusion of 100 mg of pentoxifylline (Trental) during 90 min.

The recovery rates are calculated by comparing the peak areas for pentoxifylline and metabolite extracted from spiked plasma samples to those of an equal amount injected directly into the chromatograph. The efficiency of each method is shown by the precision data (Tables I and II).

Following method B and using 3 ml of spiked plasma, the minimum measurable concentration for pentoxifylline and the metabolite is 10 ng/ml, allowing a signal-to-noise ratio of approximately 3. In comparison, method A (3 ml of spiked plasma) gives a minimum measurable concentration for both components of 50 ng/ml with a signal-to-noise level of approximately 3.

Two different procedures for the analysis of the plasma samples (referred to as methods A and B) are essential because with method A, with an analysis end-volume of less than 300 $\mu$l recognition of the metabolite peak (retention time 5.4 min) is difficult due to interference from an unidentified component (eluted at 5.9 min). Method B eliminates the problem of peak interference in the concentration range 10—50 ng/ml. Method A, on the other hand, is faster and more economic.

*Practical application of the method*

The patients examined in our pharmacokinetic study very often, besides a disturbed peripheral blood circulation, showed defective cerebral circulation. This factor, of course, effects the analytical results because such diseases are normally treated simultaneously with high dosages (3—9 g) of Nootrop® (piracetam). Under our chromatographic conditions, piracetam can be easily separated from pentoxifylline and its metabolite. Furthermore, piracetam shows no significant UV absorption above 230 nm and cannot be detected at 268 nm (Fig. 2).

During the study, there were no signs of interference in the analysis of pentoxifylline and its metabolite from the other prescribed drugs.

In figs. 3 and 4 typical chromatograms for two patients are shown, together with the individual kinetic curves. Table III shows the medications administered to these patients.

TABLE III

MEDICATION ADMINISTERED TO TWO PATIENTS WITH MULTIPLE DISEASES

| Patient | Drugs | Patient | Drugs |
|---|---|---|---|
| TR-15 | Trental | TR-12 | Trental |
| | Nootrop | | Digimerck |
| | Novodigal | | Coffein |
| | Bisolvon | | Kalinor-Br. Tabl. |
| | Sultanol | | |
| | Minipress | | |
| | Sanasthmyl | | |
| | Euphyllin | | |
| | Laevulose | | |
| | Fructose | | |
| | Bactrim | | |
| | Tutofusin K 10 | | |
| | Liquemin | | |

## CONCLUSION

In summary, it has been demonstrated that, by using our HPLC method, pharmacokinetic studies with older patients who are treated especially with high doses of piracetam, and even a low dose of Trental, can be established.

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CHROMBIO. 1937

# ANALYTICAL ISOTACHOPHORESIS UTILIZING COMPUTER SIMULATION

## II. ASSESSMENT OF OPTIMUM SEPARATION CONDITIONS FOR URINARY TRIFLUOROACETIC ACID METABOLIZED FROM ANAESTHETIC HALOTHANE

TAKESHI HIROKAWA*, HIROKATSU TAKEMI and YOSHIYUKI KISO

*Applied Physics and Chemistry, Faculty of Engineering, Hiroshima University, Shitami, Saijo, Higashi-hiroshima 724 (Japan)*

and

REIKO TAKIYAMA, MICHIO MORIO, KOHYU FUJII and HIROSATO KIKUCHI

*Department of Anesthesiology, School of Medicine, Hiroshima University, Kasumi 1-2-3, Minami-ku, Hiroshima 734 (Japan)*

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SUMMARY

Experimentally determined optimum separation conditions for a metabolite of anaesthetic halothane in urine, trifluoroacetic acid, were assessed by means of computer simulation of the isotachophoretic steady-state. The simulation confirmed that urinary acids and trifluoroacetic acid can be separated in the limited pH range of 3.5—3.7 buffered by β-alanine, as far as the pH dependence of effective mobility is utilized. The separated fraction of the trifluoroacetic acid zone was identified by mass spectrometry. The simulated coefficient of the calibration curve agreed well with the observed value.

## INTRODUCTION

Trifluoroacetic acid (TFA) is the main metabolite of halothane (2-bromo-2-chloro-1,1,1-trifluoroethane) which is a volatile anaesthetic widely used in clinical anaesthesia and has traditionally been considered an inert anaesthetic. However, hepatitis following halothane anaesthesia has been reported [1] and

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it was suggested that the toxicity of halothane may be associated with the metabolism of the anaesthetic [2]. Therefore, it is necessary to elucidate the metabolism of this drug. It is generally recognized that halothane is metabolized into TFA, $F^-$, and $Br^-$ in urine together with $CF_2CHCl$ and $CF_3CH_2Cl$ in expiratory gas. $Br^-$ is a natural component of the human body; therefore precise determination of $Br^-$ metabolized from halothane is difficult. On the other hand, TFA is not present in the body and the total urinary excretion of TFA is the largest among the metabolites.

In a previous paper [3] we have shown that the isotachophoretic determination of TFA is more advantageous than previously described techniques such as paper chromatography [4], thin-layer chromatography [5], and gas chromatography [6]. Namely, the latter methods require complicated sample pretreatment, well trained technicians and long periods for analysis.

In the present paper, the experimentally determined optimum separation conditions and coefficient of the calibration curve for quantification of TFA were analysed by means of a computational method based on the simulation of isotachophoretic qualitative [7] and quantitative indices [8] to test the analytical utility of the present method in biochemical fields.

## ASSESSMENT OF OPTIMUM SEPARATION CONDITIONS

To assay TFA by means of isotachophoresis, the optimum separation conditions have been assessed experimentally among the normal urinary acids, lactic acid, phosphoric acid, pyruvic acid, and the objective metabolites (TFA, $F^-$, and $Br^-$) [3].

The optimum electrolyte system found was as follows: leading electrolyte 0.01 *M* hydrochloric acid buffered by β-alanine (pH 3.5—3.8) and terminating electrolyte 0.01 *M* caproic acid. A surfactant Triton X-100 was added to the leading electrolyte (0.2%). The above electrolyte conditions were assessed by computer simulation.

The absolute mobility and the acid dissociation constants used in simulation are summarized in Table I. The $pK_a$ value of TFA has not yet been reported. It was therefore assumed as 0.6 by analogy with trichloroacetic acid ($pK_a$ = 0.64).

TABLE I

PHYSICOCHEMICAL CONSTANTS USED IN SIMULATION (25°C)

$m_0$ = absolute mobility ($cm^2\ V^{-1}\ sec^{-1}$) × $10^5$.
$pK_a$ = thermodynamic acidity constants, assumed values being used for $Cl^-$ and trifluoroacetic acid.

| Cations | $m_0$ | $pK_a$ | Anions | $m_0$ | $pK_a$ |
|---|---|---|---|---|---|
| β-Alanine | 36.7* | 3.552 | $Cl^-$ | 79.08 | —3 |
| ε-Aminocaproic acid | 28.8* | 4.43 | $F^-$ | 57.4 | 3.173 |
| Creatinine | 37.2* | 4.828 | Trifluoroacetic acid | 42.7* | 0.6 |
| Histidine | 29.6* | 6.04 | Pyruvic acid | 42.3 | 2.490 |
| Imidazole | 29.5* | 8.08 | Phosphoric acid | 35.1* | 2.12 |
| Tris(hydroxymethyl)aminomethane | 29.5* | 8.08 | | 61.5* | 7.470* |
| | | | | 71.5 | 12.360 |
| Amediol | 29.5* | 8.78 | Lactic acid | 36.5 | 3.860 |
| Ethanolamine | 44.3 | 9.498 | | | |

*The absolute mobilities were obtained isotachophoretically and the other constants were taken from ref. 9.

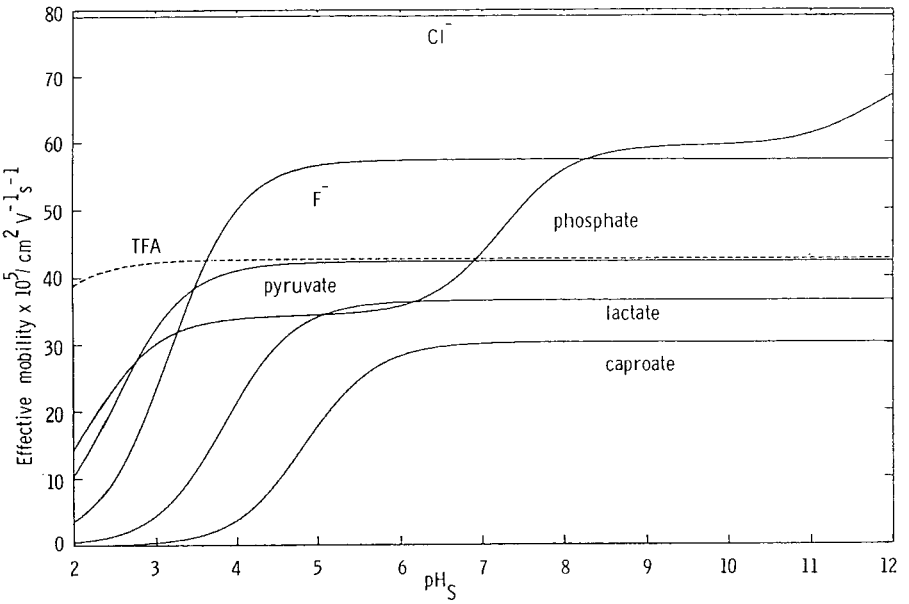


**Fig. 1. The pH dependence of effective mobility of $Cl^-$, $F^-$, trifluoroacetate (TFA; broken curve), pyruvate, phosphate, lactate and caproate ions using absolute mobility and $pK_a$ in Table I. The curves are not for the isotachophoretically steady state.**

Fig. 1 shows the pH dependence of effective mobilities of the treated samples using absolute mobility and thermodynamic $pK_a$ values listed in Table I. Although the curves in Fig. 1 are not for the isotachophoretically steady state, the curves are useful for a rough estimation of the optimum pH range for the separation. As is apparent from Table I and Fig. 1, the absolute mobility of pyruvate and TFA ions differ slightly ($4 \times 10^{-6}$ cm$^2$ V$^{-1}$ sec$^{-1}$); therefore, the separation of these ions may be impossible in the medium and high pH range at which these ions are in the fully charged state. On the other hand, the $pK_a$ value of pyruvate is 2.49 and it differs from the $pK_a$ of TFA by approximately 2; thus these acids may be separated in the low pH range utilizing the pH dependence of effective mobility. However, in the low pH range ($< 4$) the pH versus effective mobility curve of $F^-$ intersects those of TFA and pyruvate, and the optimum pH may be very critical, supporting the reported narrow pH range for separation. For the precise estimation of separability of these ions, a simulation technique is necessary.

As shown in the previous paper, the qualitative index ($R_E$) at isotachophoretically steady state [7] can be simulated at any pH of the leading electrolyte ($pH_L$) and the separability of the samples can be estimated by comparing the simulated indices. The index, $R_E$, is the ratio of the potential gradient of a leading zone to that of a sample zone. The ratio is equal to the inverse relative mobility as follows:

$$R_E = E_S/E_L = h_S/h_L = \overline{m}_L/\overline{m}_S \quad (1)$$

where $E$, $h$ and $\overline{m}$ denote potential gradients, step heights of isotachopherogram and effective mobilities, respectively, and L and S denote leading and

sample zones (ions). The difference of $R_E$ values among samples is a measure of the separability at a certain $pH_L$, since the separability is closely related to the difference in the effective mobility of the objective samples. Practically, when the $R_E$ values of two samples at the steady state differ by about 0.15, the samples may be separated.

The simulation conditions were as follows. The leading ion was 10 m*M* $Cl^-$ and the detection of $Br^-$ was not intended. Namely, the absolute mobility of $Br^-$ ($81.0 \times 10^{-5}$ $cm^2$ $V^{-1}$ $sec^{-1}$) is greater than that of $Cl^-$; therefore $Br^-$ precedes $Cl^-$. For the terminating ion, caproate was used. Then, the pH values of the leading electrolytes ($pH_L$) were varied in the range 2.8—4.2 by β-alanine (Ala), 4.0—5.0 by ε-aminocaproic acid (AMC), 4.4—5.6 by creatine (Cre), 5.4—6.6 by histidine (His), 6.4—7.6 by imidazole (Im), 7.4—8.6 by tris-(hydroxymethyl)aminomethane (Tris), 8.4—9.2 by 2-amino-2-methyl-1,3-propanediol (amediol; Am), and 9.0—9.7 by ethanolamine (EA). In the above $pH_L$ range, $R_E$ values were simulated for lactic acid, phosphoric acid, pyruvic acid, TFA, and $F^-$ at an isotachophoretically steady state. Fig. 2 shows the $pH_L$ dependence on the $R_E$ values. Apparently, in the high $pH_L$ range (> approximately 4) the curves cross each other at several $pH_L$ values, suggesting the difficulty of separation. In the low $pH_L$ range the curve of $F^-$ crosses those of pyruvate and TFA; however, they can be separated in the pH range approximately 3.4—3.6. Thus, the estimated optimum $pH_L$ was in the

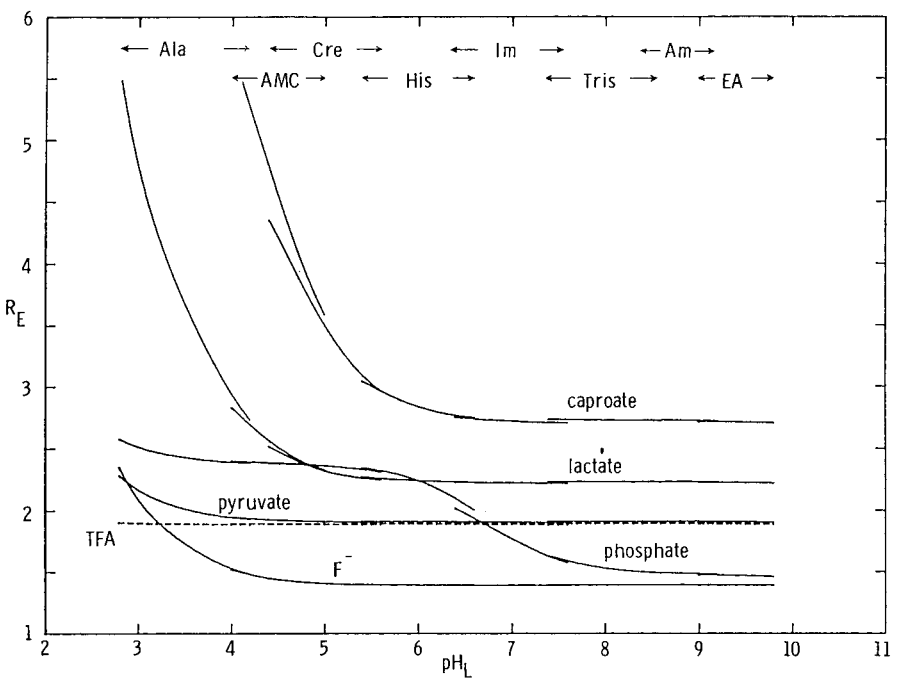


Fig. 2. The pH dependence of simulated $R_E$ values of $F^-$, trifluoroacetic acid (TFA: broken curve), pyruvate, phosphate, lactate and caproate ions at isotachophoretically steady state. The leading electrolyte is 10 m*M* $Cl^-$. The buffers used were β-alanine (Ala; $pH_L$ 2.8—4.2), ε-aminocaproic acid (AMC; 4—5), creatine (Cre; 4.4—5.6), histidine (His; 5.4—6.6), imidazole (Im; 6.4—7.6), tris(hydroxymethyl)aminomethane (Tris; 7.4—8.6), 2-amino-2-methyl-1,3-propanediol (amediol, Am; 8.4—9.2), and ethanolamine (EA; 9.0—9.7).

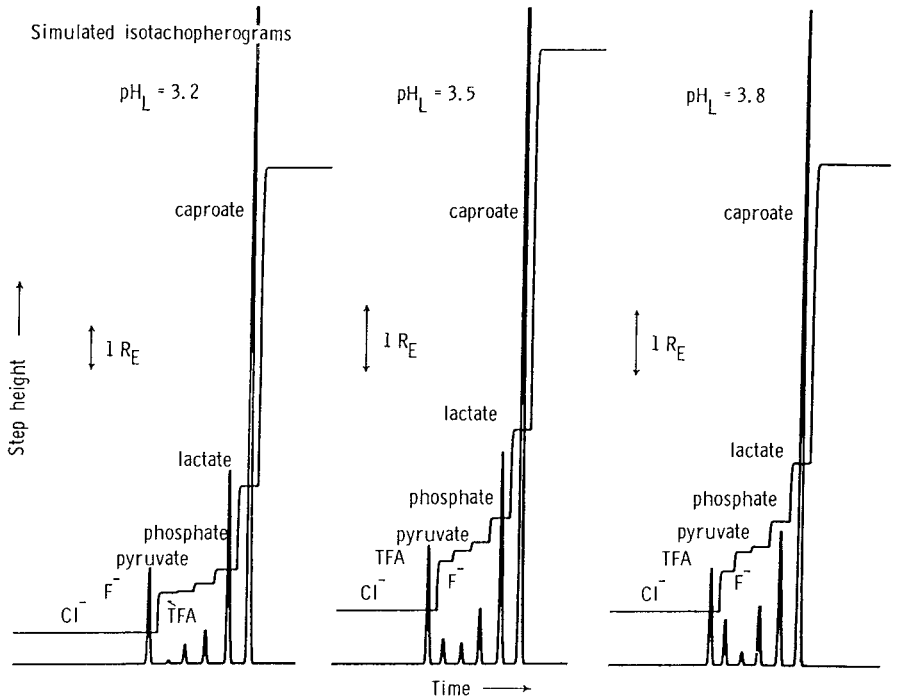


**Fig. 3. The simulated isotachopherograms at pH$_L$ 3.2, 3.5, and 3.8 for equimolar F$^-$, TFA, pyruvate, phosphate, and lactate ions (buffer β-alanine). The leading ion is Cl$^-$ and the terminating ion is caproate.**

very narrow pH$_L$ range and this estimation agreed with the experimentally assessed optimum pH$_L$ range.

Fig. 3 shows the simulated isotachopherograms at pH$_L$ 3.2, 3.5 and 3.8. At pH$_L$ = 3.2, F$^-$ ($R_E$ = 1.92) and TFA (1.89) may form a mixed zone. At pH$_L$ 3.5, F$^-$ ($R_E$ = 1.73), TFA (1.89) and pyruvate (2.02) can be separated, confirming the previous electrolyte conditions [3]. At pH$_L$ 3.8, the separation of TFA (1.89) and pyruvate (1.97) becomes difficult.

## QUANTITATIVE ANALYSIS

A passing time of zone $t$ (sec) of a sample $n$ (nmol) can be expressed as

$$t = 10^{-6}\, n\, \kappa_S / I\, C_S^t\, \bar{m}_S \qquad (2)$$

where $\kappa_S$ is the specific conductivity (S/cm), $I$ the driving current (A), $C_S^t$ the total concentration (M) of the sample in the separated zone, and $\bar{m}_S$ the effective mobility (cm$^2$ V$^{-1}$ sec$^{-1}$) of the sample zone. The passing time can be calculated for a certain amount of sample using the simulated values of $\kappa_S$, $C_S^t$, and $\bar{m}_S$ under an arbitrary driving current $I$. Namely, a proportional coefficient between $n$ (nmol) and $t$ (sec) in the conventionally used calibration curve can be easily obtained by simulation technique. Fig. 4 shows the coefficients of the calibration curve ($a$) of treated samples under a 50 μA driving current in the

$pH_L$ range of 3—10:

$$n = a\,t$$

$$a = C_S^t\,\bar{m}_S \times 50/\kappa_S \qquad (3)$$

Apparently from Fig. 4, the proportional coefficients depend not only on the mobility of the sample but also on that of the buffer ion used. The details of the quantitative aspects in isotachophoresis have been reported in Part I [8].

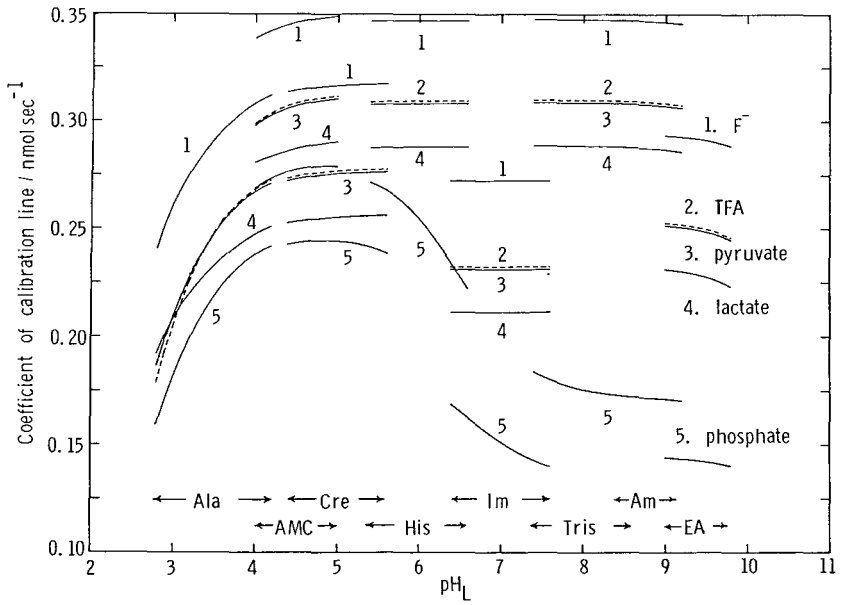


Fig. 4. The simulated coefficient ($a$) of calibration line $n$ (nmol) = $a\,t$ (sec) of $F^-$, trifluoroacetate (TFA; broken curve), pyruvate, phosphate, and lactate ions. For the electrolyte system used, see legend of Fig. 2.

## EXPERIMENTAL

To identify the separated TFA, a mass spectrogram of a prepared target fraction was obtained. For this purpose the optimized electrolyte system was used. The leading electrolyte was 10 m$M$ hydrochloric acid and the $pH_L$ was adjusted by β-alanine at 3.5. The terminating electrolyte was 10 m$M$ caproic acid. The pH measurements were carried out using an Iwaki glass pH meter Model 225, and the isotachopherograms were obtained using a Shimadzu isotachophoretic analyser, IP-2A, equipped with a potential gradient detector designed for preparative use. The temperature of the separation compartment was thermostatted at 25°C. A main separating tube, 6 cm × 0.5 mm I.D., was connected to a preseparating column, 2 cm × 1 mm I.D. The driving current was stabilized at 50 μA during the detection of sample zones. To measure $R_E$ values precisely, an asymmetric potential of the potential gradient detector was corrected using the simulated $R_E$ values of an internal standard, lactic acid. The reagents used for control samples of the urinary acids and the metabolites

were commercial guaranteed grade. An isotachopherogram of urine from a patient anaesthetized by halothane was also obtained without any pretreatment. A mass spectrogram of prepared TFA was obtained using an Hitachi mass spectrometer Model RMS-4. The experimental conditions were: chamber voltage, 80 V; total emission, 80 $\mu$A; target current, 50 $\mu$A; and chamber temperature, 250°C.

## RESULTS AND DISCUSSION

Fig. 5 shows an isotachopherogram of control samples, lactic acid, phosphoric acid, pyruvic acid, fluoride, and TFA at $pH_L$ 3.5. As expected the mixture was separated under the electrolyte conditions. A good agreement was obtained between the simulated isotachopherogram in Fig. 3 and the observed one in Fig. 5, confirming the present simulation. Table II shows the observed and simulated $R_E$ values of these acids, their effective mobility and concentrations in the separated zones. Except for $F^-$ the observed and the simulated $R_E$ values agreed well. Then, these electrolyte conditions were applied to urine samples. Fig. 6 shows the isotachopherogram of urine of a patient who was

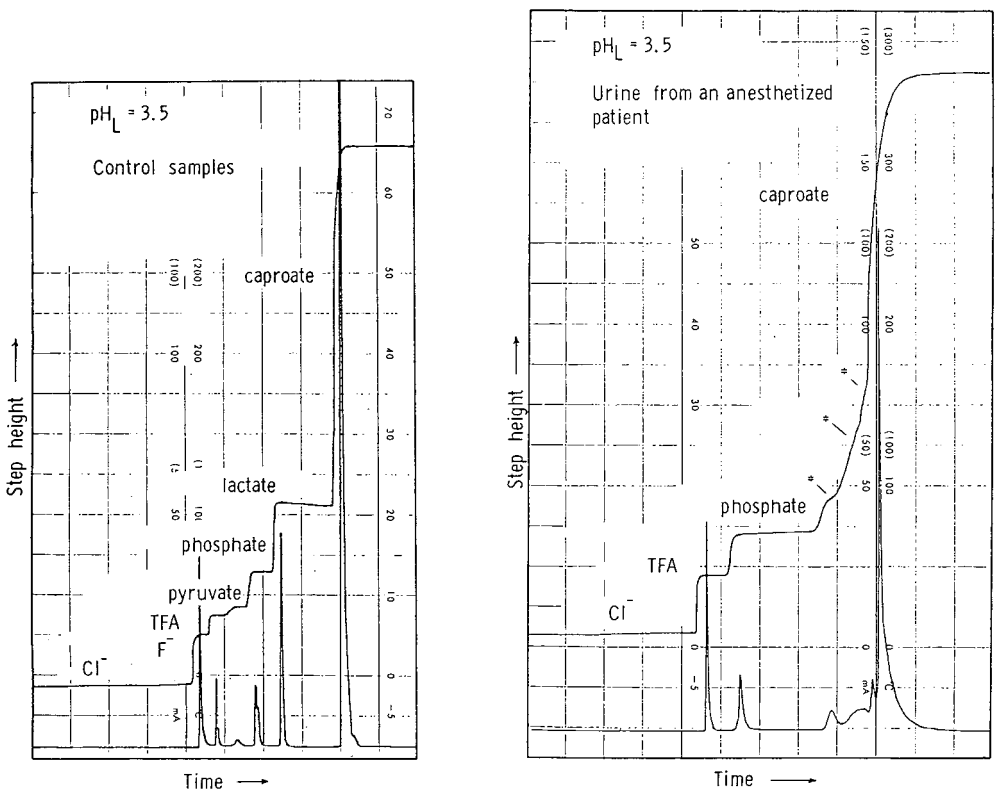


Fig. 5. The observed isotachopherograms of control samples: $F^-$, TFA, pyruvate, phosphate, lactate ions at $pH_L$ 3.5 under 50 $\mu$A driving current.

Fig. 6. The observed isotachopherograms of urine from an anaesthetized patient at $pH_L$ 3.5 under a driving current of 50 $\mu$A. Asterisks show unidentified components.

TABLE II

OBSERVED AND SIMULATED $R_E$ VALUES OF FLUORIDE, TRIFLUOROACETATE, PYRUVATE, PHOSPHATE AND LACTATE, EFFECTIVE MOBILITY AND CONCENTRATION OF ZONE CONSTITUENTS AT $pH_L$ 3.5 (25°C)

Leading electrolyte = 10 m$M$ $Cl^-$, $\beta$-alanine.
$R_E$ = ratio of potential gradients, $E_S/E_L$.
$\bar{m}_S$ = effective mobility ($cm^2\ V^{-1}\ sec^{-1}$) of sample ion × $10^5$.
$pH_S$ = pH of sample zone.
$C_S^t$ = total concentration (m$M$) of sample.
$C_{B,S}^t$ = total concentration (m$M$) of buffer ion.
$\bar{m}_{B,S}$ = effective mobility ($cm^2\ V^{-1}\ sec^{-1}$) of buffer ion × $10^5$.
$\kappa_S$ = specific conductivity (mS/cm) of sample zone.
$I$ = ionic strength × $10^3$.

| Ions | $B_E$ | | $\bar{m}_S$ | $pH_S$ | $C_S^t$ | $C_B^t$ | $\bar{m}_{B,S}$ | $\kappa_S$ | $I$ |
|---|---|---|---|---|---|---|---|---|---|
| | Corr.* | Calc** | | | | | | | |
| Fluoride | 1.62 | 1.73 | 43.00 | 3.729 | 8.887 | 16.35 | −14.19 | 0.656 | 7.07 |
| TFA | 1.81 | 1.89 | 39.44 | 3.642 | 7.539 | 15.52 | −15.82 | 0.601 | 7.53 |
| Pyruvate | 1.98 | 2.02 | 36.99 | 3.683 | 7.531 | 15.45 | −15.05 | 0.564 | 7.11 |
| Phosphate | 2.42 | 2.37 | 31.47 | 3.711 | 6.746 | 14.87 | −14.53 | 0.480 | 6.59 |
| Lactate | 3.67 | (std.) | 20.30 | 3.996 | 7.083 | 14.78 | −9.547 | 0.310 | 4.21 |

*Corrected $R_E$ values; the internal standard was lactate ion.
**Simulated $R_E$ values.

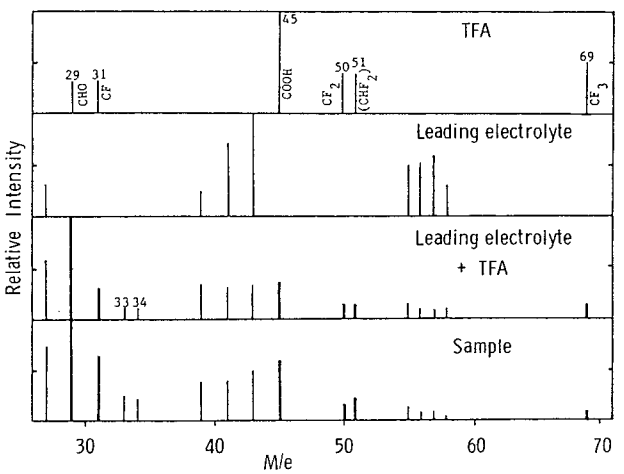


Fig. 7. The observed mass spectra of neat trifluoroacetic acid (TFA), leading electrolyte ($pH_L$ 3.5, $\beta$-alanine buffer), leading electrolyte + TFA, and prepared TFA zone.

anaesthetized by halothane. A zone corresponding to TFA could be found as well as phosphoric acid, lactic acid, and unidentified anions, although $F^-$ and pyruvate could not be detected in this case. This assignment of zones was confirmed by mass spectometry. Fig. 7 shows the mass spectra obtained of neat TFA, leading electrolyte, leading electrolyte + TFA, and the separated zone of

TFA in urine from a patient anaesthetized by halothane. The spectrum of the separated fraction has the following fragment ion peaks, *m/e*: 29 (CHO), 31 (CH), 45 (COOH), 50 ($CF_2$), 51 ($CHF_2$), 69 ($CF_3$), which can be assigned to the fragments of TFA in comparison with those of neat TFA. The molecular ion peak of $m/e = 114$ was not detected. The other peaks can be assigned for the fragments of $\beta$-alanine in leading and TFA zones.

The coefficient of the calibration curve of TFA was obtained under a 50 $\mu$A driving current using the above electrolyte system. The experimentally obtained relation between sample amount $n$ (nmol) and zone-passing time $t$ (sec) was as follows:

$$n = 0.25t \tag{4}$$

The coefficient of correlation of the obtained slope was 0.997 for fifteen replicates, varying $n$ in the range 1.5—15 nmol. The simulated coefficient was 0.247 (nmol/sec) and the agreement was good. The minimum detectable amount of TFA was 1.5 nmol and it took 6 sec for detection.

Thus the isotachophoretic technique was demonstrated to be useful in analysis of urinary TFA without time-consuming pretreatment. This is quite important in clinical analysis. And the use of a computer simulation technique has proved to be useful for the separation and quantification problem in isotachophoresis.

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CHROMBIO. 1914

## Note

# Direct determination of 3-methoxy-4-sulphonyloxyphenylglycol (MHPG sulphate) in urine using gas—liquid chromatography

P.C.N. EICHHOLTZ*, D. BINKHUYZEN and R.E. THIEME

*Department of Psychiatry, Free University, Valerius Clinic, Valeriusplein 9, 1075 BG Amsterdam (The Netherlands)*

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3-Methoxy-4-hydroxyphenylglycol (MHPG) is a metabolite of noradrenaline. MHPG is excreted in human urine in free form and as glucuronide and sulphate conjugates. MHPG sulphate (3-methoxy-4-sulphonyloxyphenylglycol) is supposed to be primarily of cerebral origin [1, 2]. Urinary excretion of MHPG sulphate can be used as an index for central noradrenaline turnover in psychiatric and pharmacologic research.

Three methods for the determination of MHPG sulphate in urine have been described. The enzymatic method of Bond and Howlett [3] has the disadvantage of possible enzyme impurities and the absence of an internal standard. Moreover, it is not a direct method because MHPG sulphate has to be calculated after determination of free and glucuronide-conjugated MHPG. Two different non-enzymatic methods for MHPG sulphate were described by Yeh et al. [4] and Murray et al. [5]. In our hands, however, both methods resulted in low recoveries of 25% and 30%, respectively.

We think there is a need for an improved method for the isolation and determination of urinary MHPG sulphate. In this paper we describe a simple method for the separation of MHPG sulphate on Sephadex with subsequent enzymatic hydrolysis and an assay by gas—liquid chromatography (GLC). Results for 21 healthy volunteers are reported.

## EXPERIMENTAL

### *Chemicals*

All chemicals used in this procedure were pro-analysi. Ethyl acetate and acetonitrile were of Uvasol quality. Extrelut was obtained from E. Merck

0378-4347/84/$03.00 

(Darmstadt, F.R.G.); heptafluorobutyric anhydride was obtained from Pierce (Rockford, IL, U.S.A.); DEAE-Sephadex A-25 was obtained from Pharmacia (Uppsala, Sweden); arylsulphatase (*Helix pomatia*) (EC 3.1.6.1) suspension 5 mg/ml was from Boehringer-Mannheim (Mannheim, F.R.G.); β-glucuronidase from *Escherichia coli* (EC 3.2.21) type VII, No. G-1758, purified soluble powder, was from Sigma (St. Louis, MO, U.S.A.). 3-Methoxy-4-sulphonyl-oxyphenylglycol potassium was a gift from Hoffmann-La Roche (Basle, Switzerland).

*Pre-wash*

Three grams of DEAE-Sephadex A-25 were saturated with distilled water for 24 h, then washed successively with 0.1 *M* hydrochloric acid, 12.5% ammonia, 100% acetic acid, 4 *M* ammonium acetate pH 7.0, with distilled water in between.

The chloride test was negative and the resin resaturated with distilled water. The anion-exchanger DEAE-Sephadex A-25 then has acetate as a counter ion.

*Extraction procedure*

To an aliquot (5 mg of creatinine) of a 24-h urine sample adjusted to pH 3.0 with 100% acetic acid, 20 ml of 1% acetic acid (pH 3.0) were added. This mixture was passed through a polypropylene column (4 cm × 0.7 cm I.D.) containing 200 mg of pre-washed DEAE-Sephadex A-25. Then the column was washed with 10 ml of acetic acid, twice with 10-ml portions of distilled water and the MHPG sulphate eluted with 10 ml of 1 *M* sodium acetate (pH 8.9). Finally, 0.50 ml of saturated barium chloride and 0.05 ml of aryl-sulphatase suspension were added to this eluate and after adjustment to pH 6.2 the volume was diluted to 20.0 ml with distilled water.

Another aliquot of urine was treated likewise after adding 40 μg of MHPG sulphate potassium as the internal standard.

The samples were incubated for 16—18 h at 37.0°C. The MHPG liberated from MHPG sulphate was then extracted with 135 ml of ethyl acetate at pH 7.5 using the Extrelut column. The eluate, dried with sodium sulphate, was evaporated to a small volume (± 1 ml), transferred quantitatively to a vial, and further evaporated to dryness in a vacuum desiccator. The obtained residue was dissolved in a mixture of 200 μl of acetonitrile, 100 μl of ethyl acetate and 100 μl of heptafluorobutyric anhydride (HFBA). The vial was tightly capped, mixed thoroughly and left standing overnight (16 h).

Next day the sample was evaporated under a stream of dry air at room temperature and the vial placed in a vacuum desiccator (1 h). Finally, the residue was dissolved in 100 μl of ethyl acetate and 2 μl were injected into the GLC column.

*Gas—liquid chromatography*

The analysis was carried out with a Packard Model 428 gas chromatograph equipped with a flame ionisation detector, under the following conditions: column, 2 m × 2 mm I.D.; liquid phase 5% OV-17; support, Supelcoport, 80—100 mesh (Supelco, Bellefonte, PA, U.S.A.); column temperature from 140°C to 180°C at 4°C/min; detector temperature 200°C; injection port tem-

perature 200°C; attenuation 32; supporting gas, helium, at a flow-rate of 20 ml/min.

Calculations were done using a Hewlett-Packard Model 3380A integrator.

## RESULTS AND DISCUSSION

The use of DEAE-Sephadex A-25 for isolation of MHPG sulphate was first introduced by Yeh et al. [4], who used 0.15 *M* hydrochloric acid for elution of the sulphate. Replicating their procedure we had a yield of MHPG sulphate of only 25%. This can be explained by the fact that MHPG sulphate was unstable under this acid condition [5].

The method described by Murray et al. [5] includes the use of Amberlite XAD-2 for extraction of all three MHPG variants. They mention a yield of only 30% in this procedure, which we confirm. Sephadex LH-20 was subsequently used for the isolation of MHPG sulphate. This method, however, is very laborious and time-consuming.

The reproducibility of our analytical procedure was tested by determination of MHPG sulphate in ten aliquots of one 24-h urine sample. Determinations were implemented on different occasions and carried out by different laboratory assistants. In the test samples we consistently found a recovery of more than 80%. The mean value obtained was 0.82 mg per g of creatinine with a standard deviation of 0.16. Recovery appears to be "urine-dependent" to a certain extent (age, diet, drugs) and varies between 60 and 95% according to the internal standard.

After adding free MHPG to the sample no free MHPG could be detected in the eluate. The eluate of urine samples was tested for the presence of MHPG glucuronide by incubating with purified β-glucuronidase. No MHPG could be

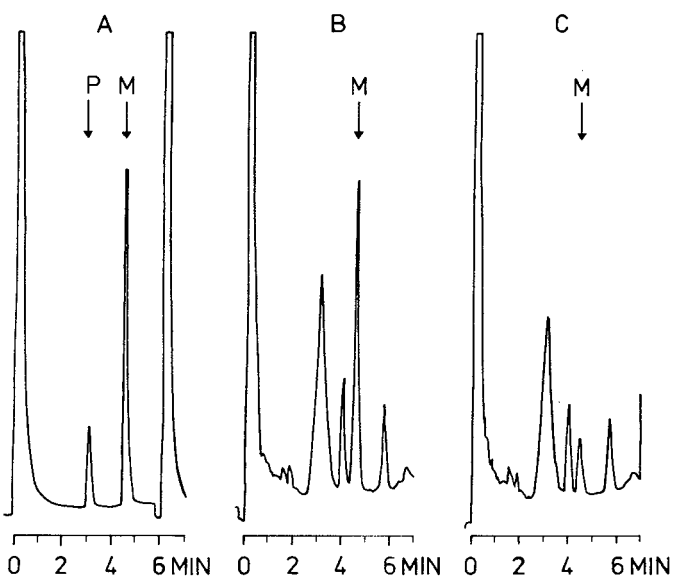


Fig. 1. Chromatograms of (A) reference standard (495 ng), (B) blank urine plus MHPG sulphate standard, serving as a recovery standard (452 ng), and (C) blank urine (77.5 ng) (recovery value 76.4%). P = piperazine (unfluoridated) and M = MHPG (fluoridated).

TABLE I

EXCRETION OF MHPG SULPHATE IN HEALTHY VOLUNTEERS COMPARED WITH VALUES GIVEN BY BOND AND HOWLETT AND MURRAY ET AL.

Values are expressed as mean ± S.D., *n* in parentheses.

| | mg MHPG sulphate per g creatinine* | |
|---|---|---|
| | Males | Females |
| Present paper | 0.60 ± 0.16 (9) | 0.69 ± 0.17 (12) |
| Bond and Howlett [3] | 0.85 ± 0.34 (7) | 0.98 ± 0.37 (6) |
| Murray et al. [5] | 0.67 ± 0.15 (7) | 0.65 ± 0.21 (3) |

*Calculated as free MHPG.

detected in the eluate afterwards. This demonstrates a complete purification of MHPG sulphate by DEAE-Sephadex A-25 in acetate at pH 3.

In the final stage of their analytical procedure Murray et al. [5] claimed "quantitative conversion in a single step" of MHPG sulphate by trifluoroacetic anhydride and ethyl acetate at room temperature to MHPG tris-trifluoroacetic acid. In our replication we found only 25% conversion after 2 h of incubation and 42% after 16 h. When we used HFBA in the same procedure there was almost 60% conversion of MHPG sulphate while fluoridation was complete. Enzymatic hydrolisis of MHPG sulphate appears to be necessary for a high yield. We used an arylsulphatase preparation from Boehringer which did not contain MHPG itself. MHPG sulphate potassium was only poorly soluble in ethylacetate, and we therefore added acetonitrile to increase its solubility and to serve as a catalyst in the fluoridation reaction.

Fig. 1 shows a typical chromatogram. It concerns a depressed male patient without medication. The excretion was 0.61 mg per g of creatinine, and the recovery 76.4%.

In a group of 21 healthy university students using a catecholamine-restricted diet, MHPG sulphate excretion was estimated in 24-h urine samples. In Table I these values are compared to those given by Bond and Howlett [3] and Murray et al. [5] for a normal population. Neither of these latter studies mention dietary conditions. We think ours is an improved method for the estimation of MHPG sulphate in urine.

ACKNOWLEDGEMENTS

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## Note

# Possibilities and limitations in the analysis of amino acid oxazolidinones in the femtomole range by gas chromatography with electron-capture detection

PETR HUŠEK* and VLADIMÍR FELT

*Research Institute of Endocrinology, Národní třída 8, 116 94 Prague 1 (Czechoslovakia)*

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Both of the automated chromatographic techniques — high-performance liquid chromatography (HPLC) and gas chromatography (GC) — are nowadays routine tools in the analysis of amino acids because there is the possibility of separating these compounds several times more quickly than with the classical amino acid analysers. Simultaneously, there is a determined effort to increase the sensitivity of detection by use of selective detectors, such as the fluorometer in HPLC or the electron-capture detector in GC. Fluorescent derivatives for HPLC amino acid analysis are most frequently prepared by treatment of the compounds with *o*-phthaldialdehyde (OPA) [1—3], dansyl chloride [4—6] or, more recently, with 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-F) [7], which in contrast to OPA reacts also with the secondary amino group of proline and hydroxyproline. Analysis at the picomole level is usual, the detection limit being in the range of a few femtomoles.

In GC analysis the necessary pre-column derivatization of amino acids leads in most cases to formation of fluorine-rich compounds, so that electron-capture detection (ECD), generally three orders of magnitude more sensitive than flame-ionization detection (FID), is the obvious choice. However, problems arise in conjunction with temperature-programmed operation as the increase in baseline with temperature programming of packed columns causes deterioration in the analysis of the higher amino acid members, especially when derivatives of lower volatility are used [8]. Under isothermal operation femtomole amounts of N(O,S)-TFA (trifluoroacetyl), or HFB (heptafluorobutyryl) amino acid alkyl esters could be analysed successfully [9—12]. Thus, the use of selective detectors with either of the chromatographic techniques affords comparable results concerning the sensitivity.

As the recently introduced procedure of amino acid treatment by action of

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a perhalogenated acetone [13, 14] can compete well with the commonly used esterification approaches [13], and a higher abundance of chemically introduced halogen atoms predestines the derivatives for ECD, we have studied the chromatographic behaviour of the compounds in mass ranges down to femtomoles. The GC—ECD temperature-programmed analysis of the acylated amino acid oxazolidinones succeeded well with both packed and open-tubular columns; however, variable losses of the compound amino acids were observed in packed columns at lower injected mass. Possibilities for analysis in the subpicomole range together with limitations, caused by the presence of chromatographic support and/or reagent impurities, are discussed.

## EXPERIMENTAL

Five nanomoles down to 100 pmol of each amino acid in an equimolar mixture of twenty protein amino acids, including hydroxyproline, ornithine and S-methylcysteine (not included in the sample for the capillary column), were subjected to derivatization with 1,3-dichlorotetrafluoroacetone (DCTFA) and reactive anhydrides, the heptafluorobutyric anhydride (HFBA) or the pentafluoropropionic anhydride (PFPA), as described previously [13, 14]. Following the procedure the derivatives were dissolved and injected in heptane only, so arginine and the two amides asparagine and glutamine could not be determined [13].

Gas chromatography was carried out with a Hewlett-Packard 5736A gas chromatograph equipped with a linear $^{63}Ni$ electron-capture detector and two flame-ionization detectors. A capillary inlet system 18740B with splitless injection mode was used for analysis with a 25 m × 0.31 mm capillary column made from fused silica and containing OV-1 cross-linked methylsilicone gum. Like previously [14] the column was run in a temperature range of 60 to 230°C (16°C/min) under a hydrogen flow-rate of 4.6 ml/min (100 cm/sec) and make-up nitrogen for ECD of 20 ml/min. A dual set of glass-packed columns (2 mm I.D.), a 2-m column with 3% OV-17 on Chromosorb W HP and a 0.5-m column with 1.5% SE-30 on Chromosorb G HP (or Chromosorb 750), operating at 8°C/min linear increase in the temperature ranges 80—230°C and 150—200°C under nitrogen flow-rates of 20 ml/min and 30 ml/min, respectively, was used [13] for the comparison studies. The injector and electron-capture detector temperatures were 200°C and 250°C for both types of column used.

## RESULTS AND DISCUSSION

Already in our previous studies [13, 14] on the GC behaviour of the amino acid oxazolidinones it was concluded that these perhalogenated compounds are more prone to adsorption to the column fillings than the acylated amino acid alkyl esters. Especially the derivatives of histidine, cystine and tryptophan could not be eluted from packed columns with common fillings based on diatomaceous supports of the Chromosorb W type. As even the Chromosorb 750 support, being considered the best deactivated one, did not allow the elution of the three troublesome derivatives completely [14], the exclusion of

any support at all would be the best way how to succeed with quantification.

Using selective and halogen-sensitive ECD it was possible to evaluate the possible adsorption losses of the amino acid oxazolidinones near or below the picomole level. Success with GC—ECD temperature-programmed analysis of N(O,S)-HFB amino acid isobutyl esters in the picomole range using support-coated open-tubular (SCOT) columns was reported recently [15, 16]. The relative molar responses showed that some derivatives were detectable in much smaller amounts than others. Corkill et al. [17] studied responses of strong electrophores in the GC—ECD system and found that the most sensitive compounds were derivatized iodothyronines (the N,O-perfluoroacylated

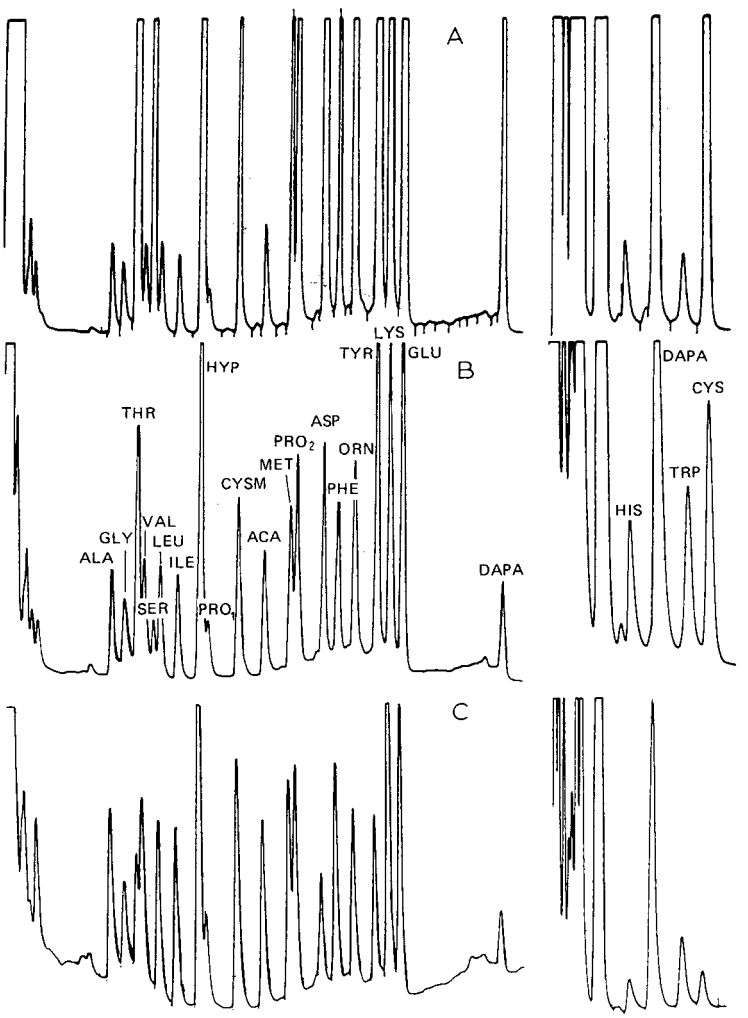


Fig. 1. GC—ECD analysis of (N,O)-HFB amino acid oxazolidinones in a dual set of packed columns [13] after derivatization of an equimolar mixture containing 5 nmol of each amino acid. The derivatives were submitted to analysis after dilution with heptane to the following final amount of each derivative per injected sample: (A) 100 pmol (attenuation × 512); (B) 10 pmol (× 64); (C) 1 pmol (× 4). The analytical conditions in all three cases were the same so that a constant elution order was maintained.

methyl esters) which were essentially twenty times more sensitive than lindane.

Fig. 1 shows GC—ECD-programmed analysis of N(O)-HFB amino acid oxazolidinones in packed columns in a range of 100 to 1 pmol per injected compound. In accordance with our previous study [13] it can be accepted that no or minimal adsorption losses of amino acid oxazolidinones occur in the packed columns down to 100 pmol per amino acid injected. This means that the responses in Fig. 1A represent real differences in ECD caused by the presence of an unequal number of electron-capturing groups in the molecule. Among the "low sensitive" electron-capturing derivatives we find the aliphatic amino acids histidine and tryptophan, i.e. amino acids containing the bis(chlorodifluoromethyl)oxazolidinone ring only. A higher response is given by the sulphur-containing amino acids (S-metylcysteine and methionine) and also phenylalanine because of the additional effect of the aromatic ring. "Highly sensitive" are the compound amino acids in which the side-chain reactive groups are converted to HFB-acylated forms, and also the diamino-dicarboxylic acids with two oxazolidinone rings in the molecule. Also the high response for the second derivative of proline ($Pro_2$, eluted after methionine in Fig. 1.) confirms our assumption that the HFB group participates in its formation. The presence of the additional electrophore in the molecule of the compound amino acids causes unexpectedly high enhancement of the ECD response, which is on average one order higher than that of the "low sensitive" oxazolidinones of the simple amino acids. Very similar results were obtained with the N(O,S)-HFB amino acid isobutyl esters [16], where the relative molar response (RMR) difference between the least sensitive valine (RMR 0.90) and the most sensitive tyrosine (RMR 32.7) was about 36-fold. Two to five times higher responses were shown by the sulphur-containing amino acids in comparison with the aliphatic ones, again in close agreement with our findings.

The picture, however, alters when the injected amount is lowered by one or two orders down to 1 pmol per amino acid derivative (Fig. 1B and C). Adsorption losses in the column fillings occur with the compound amino acids, where the presence of additional fluorine atoms in the HFB moiety makes the derivatives more prone to adsorption than those with an oxazolidinone ring only. The most drastic decline in RMR was observed with the hydroxyl-containing amino acids (except hydroxyproline, the large peak of which in Fig. 1C does not represent the pure compound but is a co-elution with an unknown impurity), especially serine, the adsorption of which is complete in the picomole range (disappearance of Ser from Fig. 1C). Also the response of cystine declines rapidly with the mass reduction. As the oxazolidinones of the simple amino acids do not seem to be adsorbed with the mass lowering, the relative responses of the protein members are close to equality in the picomole range. From Fig. 1C it is also apparent that the N,N-diHFB oxazolidinone of lysine is less prone to adsorption than any other compound amino acid and its analysis down to 10 fmol was possible with the packed column used.

Approximately ten times higher sensitivity can be achieved using a capillary column (Fig. 2). As the fused-silica column with bonded OV-1 methylsilicone does not separate the HFBA-treated oxazolidinones completely, the PFPA-treated oxazolidinones were used instead [14]. The lower amount of fluorine atoms in the PFP moiety results in a partial lowering of the ECD responses for

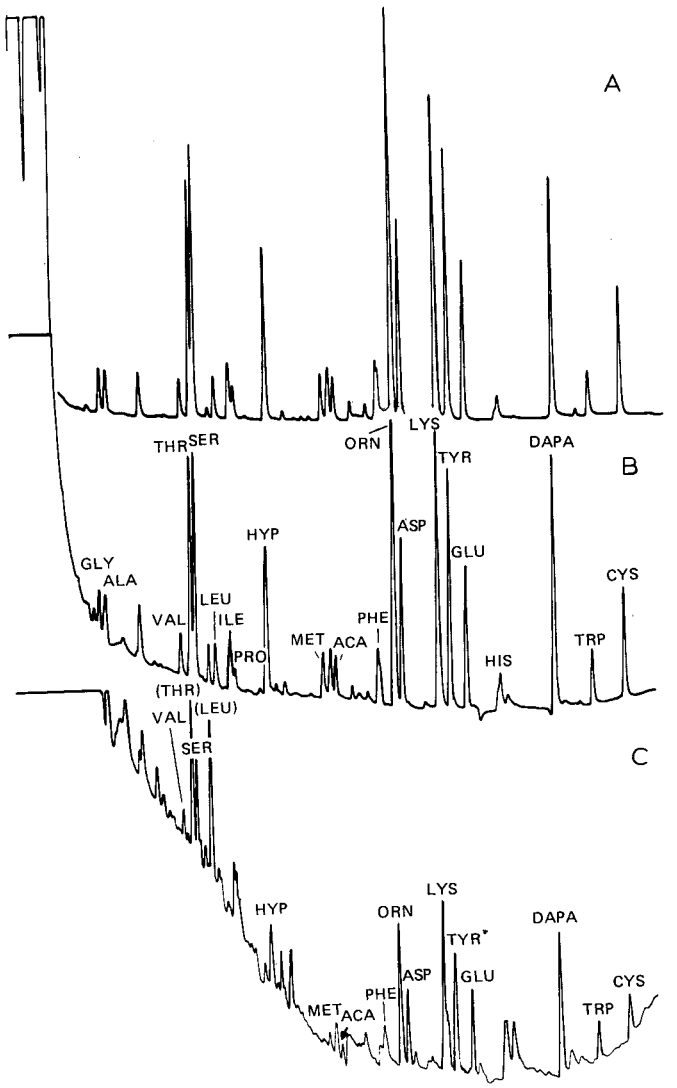


Fig. 2. GC—ECD analysis of (N,O)-PFP amino acid oxazolodinones in a fused-silica capillary column with OV-1 bonded phase [14]. The same initial amount as in Fig. 1 was derivatized and the sample was diluted to the following final amounts injected: (A) 1 pmol (attenuation × 32); (B) 0.1 pmol (× 4); (C) 0.01 pmol (× 1). Amino acids in brackets are co-eluted with an unknown impurity having identical retention time.

the compound amino acids. From Fig. 2A it can be seen that the highest response is given by the dibasic amino acids (Orn and Lys), being approximately ten times higher than that of the aliphatic amino acids. However, the most important finding is that the responses of the protein members are independent of the mass injected. From Fig. 2A—C it follows visually that the RMR values are comparable for all concentration ranges down to 10 fmol and that they were essentially the same in the range of 1 to 0.01 pmol of injected mass. This means, therefore, that employment of open-tubular columns without a chro-

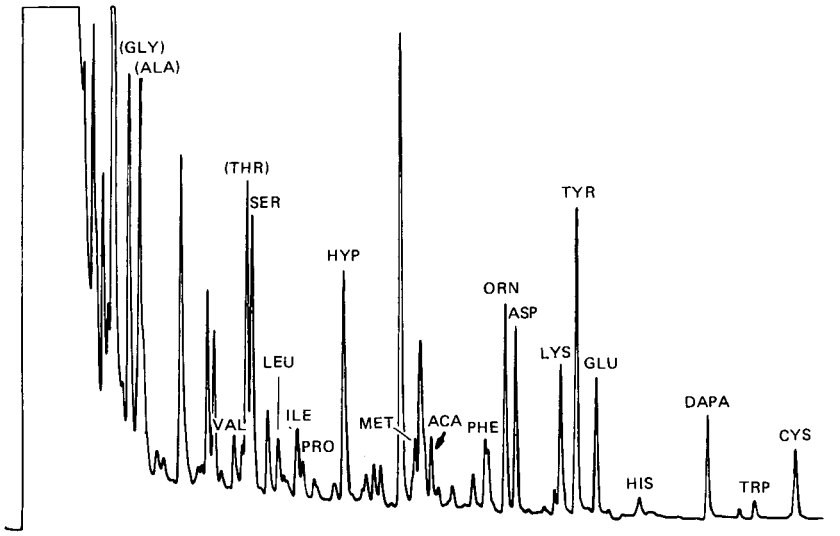


Fig. 3. GC—ECD analysis of a sample containing 100 pmol of each amino acid initially. After derivatization, 1 pmol of each amino acid derivative was injected into the capillary column as in Fig. 2. The brackets around some amino acids follow the same meaning as in Fig. 2.

matographic support is the only means of obviating losses of the perfluoroacylated amino acid oxazolidinones at concentration ranges below 100 pmol per injected amino acid derivative. Next to this, it is worth noting that the presence of hydrogen, being the carrier gas for the capillary column, did not seem to influence the process of electron-capture in the detector provided that nitrogen as the make-up gas was added. Both the sensitivity and the linearity did not change significantly as far as we noticed.

At higher instrument sensitivities, i.e. lower mass injections, another problem arises with the presence of impurities, as can be seen from Figs. 2C and 3. Even when the samples with the lowest derivative concentrations are prepared by dilution of the initial concentrated sample (nanomole range) by heptane of nanograde purity, extraneous peaks appeared on the chromatogram (Fig. 2C) and the quantification of the first protein members was impossible. Impurities with identical retention times to those of some amino acid derivatives present a serious problem when samples with a low amino acid content are derivatized (Fig. 3). We found later that many of the impurities originated from the analytical grade hexane (Fluka, Buchs, Switzerland) used for extraction [13] and that its repeated distillation brought a great improvement, even though some extraneous peaks survived. The results show that the GC—ECD analysis of perfluoroacylated amino acid oxazolidinones near and below the picomole range is well possible; however, one has to face impurities of various origins when the initial derivatized amount is low.

## APPLICATION

The capillary column with OV-1 cross-linked phase connected to an electron-capture detector (attenuation × 512) or even a flame-ionization detector

(attenuation × 4) enabled us to estimate the protein amino acids in 5 μl of human serum routinely. The amino acids were isolated by means of cation-exchange resin (Dowex 50W-X2, $H^+$, 100—200 mesh), which was placed with a plug of synthetic wool directly in the lower part of a polypropylene conical tip (used for the push-button pipettes and cut off below at O.D. of about 2 mm) thus forming a bed of 6—8 mm of wet resin. The 5-μl serum sample was diluted ten times with 25% aqueous acetic acid containing 0.5 nmol of the internal standards (α-aminocaprylic acid, diaminopimelic acid) [13], dropped on the resin bed and left to penetrate it. The bed was then washed with two times 30 μl of water and the amino acids were eluted subsequently with 150 μl of 2 *M* aqueous ammonia. The amino acids in dry residue were then converted to the N(O)-PFP oxazolidinones and after extraction and evaporation of the extraction solvent the amino acid derivatives were dissolved in 40 μl of heptane and subjected to GC analysis. Minimal presence of disturbing peaks was observed.

## CONCLUSIONS

The GC—ECD temperature-programmed analysis of PFP- or HFB-acylated amino acid oxazolidinones brought results comparable to those obtained with N(O,S)-HFB amino acid isobutyl esters [15, 16]. Amino acids with the perfluoroacylated side-chain reactive groups afford responses of about one order higher than the others; however, they tend to be adsorbed in the column filling more readily than those with the oxazolidinone ring only. Significant adsorption losses appear in packed columns at levels below 100 pmol per amino acid injected. Elimination of chromatographic support in the capillary columns resulted in undisturbed analysis down to femtomole amounts without apparent losses of the troublesome amino acid derivatives; however, problems with impurities appear to be serious at that level. Hydrogen can be used as carrier gas for the capillary without deterioration in the performance of the electron-capture detector if nitrogen as the make-up gas is added in excess.

The capillary column was used for estimation of the protein amino acids in 5 μl of human serum either by employment of ECD or FID routinely.

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## Note

# Determination of creatine in body fluids and muscle

EMILIO MUSSINI, LAURA COLOMBO, GLORIA DE PONTE and FRANCA MARCUCCI*

*Istituto di Ricerche Farmacologiche "Mario Negri", Via Eritrea 62, 20157 Milan (Italy)*

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The non-enzymatic methods generally employed for determining creatine in biological samples lack specificity, because of interference from several compounds normally present. The widely used Folin method [1] for determination of creatinine and creatine in serum and urine is based on the Jaffé reaction after conversion of creatine into creatinine [2, 3]. Unfortunately, though, it is subject to gross errors when applied to urine which contains a large amount of creatine [4, 5]. Adult human males usually excrete very little creatine in the urine. However, during fasting and in certain muscle diseases blood creatine rises above the levels that can be reabsorbed by the kidneys, and creatinuria results [6]. During studies of muscle diseases, evaluation of the methods available for measuring creatinine and creatine in biological samples led us to the conclusion that they can be measured respectively the Jaffé picrate [2] and the α-naphthol—diacetyl reaction [7] with satisfactory results if preliminary ion-exchange chromatography is carried out. In this laboratory we have recently developed a satisfactory method for determination of creatinine in plasma and urine [8].

This paper now describes a method for creatine determination in body fluids. It includes prior removal of interfering substances from biological samples, using a strong cation-echange resin, followed by separation of creatinine from creatine by a weak cation-exchanger. The ease and speed of analysis make this method very attractive for routine clinical determination of creatine and creatinine in muscle pathologies.

## MATERIALS

### *Chemicals*

Creatine monohydrate, creatinine and α-naphthol were purchased from

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Merck (Darmstadt, F.R.G.). Guanidinoacetic acid was obtained from Fluka (Buchs, Switzerland) arginine from Mann Research Labs. (New York, NY, U.S.A.) and picric acid aqueous solution (1.2%) from BDH Chemicals (Poole, U.K.).

All chemicals used in the procedure and those screened in the interference study were reagent grade. Creatine standard solutions in the range 4.5—450 μmol/l, were prepared with distilled water from a stock solution of 0.1 g/l.

Ion-exchange resins Duolite C 225 (52—100 mesh, $H^+$) and Bio-Rex 70 (100—200 mesh, $H^+$) were obtained from BDH and from Bio-Rad Labs. (Richmond, CA, U.S.A.), respectively.

*Reagents for creatine determination by the α-naphthol—diacetyl reaction*

An alkali stock solution is prepared by dissolving 60 g of sodium hydroxide and 160 g of anhydrous sodium carbonate in 1 l of water and stored in a warm place.

A 1.6% (w/v) solution of α-naphthol in the alkali stock solution is prepared immediately before use and filtered. It should not be darker than a pale straw-yellow and can be used for at least 2 h after preparation.

A 1% (v/v) stock solution of diacetyl in water is kept at 4°C and diluted 1:20 with water immediately before use.

## PROCEDURE

*Isolation and separation of creatine and creatinine from biological materials.*

*Urine.* A 1 ml volume of urine, adjusted to pH 5—6 with hydrochloric acid, was applied to a column (15 × 0.5 cm I.D.) packed with Duolite C-225 (52—100 mesh, $H^+$). The column was washed with 15 ml of water and eluted with 15 ml of 2 *M* ammonium hydroxide. The first 3 ml were discarded, and 6 ml of the next 12 ml were passed through a column (10 × 1 cm I.D.) packed with Bio-Rex 70 (100—200 mesh, $H^+$); 30 ml of 0.1 *M* acetic acid were used for elution. The first 5 ml were discarded; the next 10 ml, which contained creatine, and the last 15 ml, containing creatinine, were collected in 1-ml fractions. Each fraction was used for assay of creatine and creatinine by the α-naphthol—diacetyl reaction and the Jaffé picrate reaction, respectively. Creatine and creatinine, used as standards, 0.885 mmol/l, each dissolved in 2 *M* ammonium hydroxide, were applied to the Bio-Rex 70 column.

*Plasma and muscle.* Plasma (1 ml) was deproteinized with 0.6 *M* perchloric acid (1:5, v/v) and rat muscle homogenate (0.2 ml) in 1.15% potassium chloride (1:8, w/v) with 0.6 *M* perchloric acid (1:1, v/v). After standing 10 min in ice, samples were centrifuged at 3000 *g* for 10 min. Portions of the supernatants were then applied to the first chromatographic column and processed as described for urine.

*Recovery*

The recovery of creatine added to control human plasma, to pooled patient's plasma, or urine (diluted twenty times) was determined by diluting portions of each of these specimens (1) with an equal volume of a standard solution of creatine (45 mmol/l) and (2) with an equal volume of water. Creatine was then

isolated from the samples by the two ion-exchange procedures, and measured by the α-naphthol—diacetyl reaction, as follows. A portion of each eluate (7 ml) was added to 2 ml of α-naphthol solution and 1 ml of diacetyl solution; the procedure was calibrated against similarly treated creatine standards with absorbance measured at 525 nm against a column blank. Recoveries were calculated from the results.

*Determination of other interfering components in eluates*

The check of purity of eluates from the ion-exchange columns, control plasma, urine (diluted twenty times) and pooled control rat muscles were processed as above. Final eluates were desalted by passing through ion-exchange resin Duolite C-225 (200—400 mesh) as previously described [8]. After washing with distilled water (50 ml), the absorbed creatine was eluted with 50 ml of 2 *M* ammonia. Eluates were dried under vacuum and spotted on precoated silica gel 60 F 254 thin-layer aluminium sheets (20 × 20 cm, Merck). The developing solvent was a mixture amyl alcohol—pyridine—water (21:42:37). The detection reagent was prepared as follows: (A) 1% α-naphthol in 8% sodium hydroxide (dissolved in 80% ethyl alcohol), (B) 0.1% diacetyl dissolved in 90% ethyl alcohol. Immediately before use 1 volume of A was mixed with 1 volume of B. Plates were placed for 3 min at 100° C.

## RESULTS AND DISCUSSION

As shown in Fig. 1, creatine adsorbed on the Bio-Rex 70 column from plasma, urine, muscle or aqueous solutions of creatine was eluted (99 ± 1%) with 6 ml of 0.1 *M* acetic acid. The following 8 ml of eluate (from fractions 19 to 26) containing creatinine can be analysed by the Jaffé reaction, with minor modifications [9].

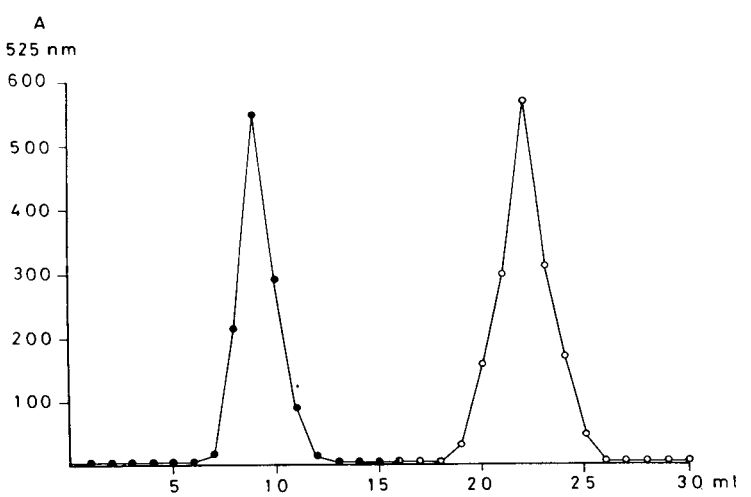


Fig. 1. Elution pattern of creatine (•) and creatinine (○) (aqueous solutions, 100 μg each) absorbed on the Bio-Rex 70 column; analyses are as described under Materials and Procedure. Fractions of 1 ml.

*Recovery studies*

Recoveries of creatine added at two different amounts (2.5 mg/l and 5.0 mg/l) to seven different control plasma, pooled patient's plasma and urine (diluted twenty times) and pooled control rat muscle, isolated by the two-step cation-exchange procedure and assayed by the α-naphthol—diacetyl reaction, were, respectively, 95 ± 2%, 98 ± 2% and 94 ± 3%. These results indicate that the isolation procedure was quantitative.

*Precision and accuracy*

Reproducibility of the method was checked by assaying pooled plasma and urine samples twenty times. Mean values of 7.20 mg/l and 23.52 mg/l for pooled plasma were found by this method with standard deviations (S.D.) of ± 0.05 mg/l and ± 0.05 mg/l and coefficients of variation (C.V.) of ± 0.69% and ± 0.21%. For urine, mean values were 46.10 mg/l and 411.0 mg/l with S.D. ± 0.09 mg and ± 2.0 mg/l and C.V. ± 0.19% and ± 0.50%, respectively.

These pooled samples, stored frozen at −20° C, were determined in duplicate over a one-month period, with the following results: 7.25 mg/l and 23.00 mg/l for mean values of pooled plasma; S.D. = ± 0.04 mg/l and ± 0.05 mg/l; C.V. = ± 0.55% and ± 0.22%. For urine pools, the mean values were 45.10 mg/l and 415.12 mg/l; S.D. = 0.05 mg/l and ± 1.92 mg/l; C.V. = ± 0.11% and ± 0.46%.

Reproducibility and accuracy were checked employing pooled rat skeletal muscles. Mean values of 22.29 μmol/g were found; S.D. = ± 0.30 μmol/g; and C.V. = ± 1.34%. Duplicate determinations of these pooled muscles, kept frozen at −20° C as described for plasma and urine, led to the following results: 23.09 μmol/g and 20.53 μmol/g for mean values of pooled muscles; S.D. = ± 0.29 μmol/g and ± 0.25 μmol/g; C.V. = ± 1.26% and ± 1.22%.

*Calibration*

Standard curves for the procedure, prepared from the aqueous standards, are linear up to 50 mg/l (plasma) and 800 mg/l (urine) and pass through the origin. Calibration was done daily to ensure maximum precision.

*Application*

The method has been applied for routine clinical analysis of urine of patients with Duchenne muscular dystrophy and plasma of uraemic patients. Creatine concentrations in urine and plasma of these patients are reported in Table I, together with normal values. In order to clarify the relationship between creatine and creatinine in healthy and diseased subjects Table I also reports results of a previous study on creatinine determination [8] and creatine levels in pooled rat skeletal muscle.

*Interferences*

Not many compounds are likely to interfere in this procedure, because of the double passage through two different cation-exchange resins. Anions, proteins and neutral molecules wash through the cation-exchange resin Duolite C-225. Thin-layer chromatography of desalted eluates from control human plasma and urine, indicates that they contain only creatine. The endogenous and exogenous materials we tested (Table II) did not interfere in creatine determination.

TABLE I

CREATININE AND CREATINE IN PLASMA AND URINE OF NORMAL HUMAN CONTROLS AND PATIENTS

| Clinical diagnosis | Creatinine | | Creatine | |
|---|---|---|---|---|
| | Plasma (mmol/l ± S.E.) | Urine (mmol/l ± S.E.) | Plasma (mmol/l ± S.E.) | Urine (mmol/l ± S.E.) |
| Duchenne muscular dystrophy ($n$ = 50) | n.d.* | 3.30 ± 0.2 | n.d. | 3.56 ± 0.2 |
| Uraemia ($n$ = 50) | 0.74 ± 0.02 | n.d. | 0.10 ± 0.03 | n.d. |
| Normal controls ($n$ = 50) | 0.09 ± 0.001 | 6.50 ± 0.5 | 0.04 ± 0.001 | 0.24 ± 0.006 |
| | Creatinine ($\mu$mol/g) | | | |
| Pooled control rat skeletal muscle | 22.29 ± 1.2 | | | |

*n.d. = not detectable.

TABLE II

COMPOUNDS TESTED FOR INTERFERENCE WITH CREATINE ASSAY BY THE $\alpha$-NAPHTHOL—DIACETYL REACTION

| Compound | Test concentration (mg/l) |
|---|---|
| Creatinine | 50 |
| Arginine | 60 |
| Guanidine | 60 |
| Methionine | 50 |
| Pyruvic acid | 400 |
| Urea | 600 |
| Glutathione | 80 |
| 2,3-Diphosphoglyceric acid | 6000 |
| Guanidinacetic acid | 50 |
| Uric acid | 70 |

Creatine is the end-product of the metabolism of glycine and arginine. It is eliminated via the kidneys by glomerular filtration, but is normally reabsorbed in renal tubules, resulting in very little creatine in the urine. Quantitative determination of creatine in plasma and urine is important in the diagnosis of muscular diseases. High serum creatine values have been reported in patients with amyotrophic lateral sclerosis, progressive dystrophia musculorum and dermatomyositis [10]. Urine creatine levels are reported by the same authors to be high in glomerulonephritis. Studies are in progress on the correlation between creatine and creatinine in urine and muscle in muscle-wasting diseases.

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## Note

# Analytical peptide mapping by ion-exchange high-performance liquid chromatography: application to haemoglobin variants

TAKASHI IMAMURA*, JUN SUGIHARA, EISUKE YOKOTA, MASAAKI KAGIMOTO, YASUSHI NAITO and TOSHIYUKI YANASE

*First Department of Internal Medicine, Faculty of Medicine, Kyushu University, Fukuoka 812 (Japan)*

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High-performance liquid chromatography (HPLC) has become increasingly important in the study of amino acid sequences in proteins [1, 2]. A number of advantages of HPLC include convenience and efficiency, high sensitivity, and ready application to both analytical and preparative purposes. Although peptide separations have been commonly made by HPLC with a reversed-phase column, the use of an ion-exchange column provides a new dimension. This report describes our experiences with separation of tryptic peptides of haemoglobins (Hb) by the use of an ion-exchange column and phosphate—acetonitrile gradient elution. The results indicate that this procedure is a useful technique for routine peptide mapping.

## MATERIALS AND METHODS

Human haemoglobin variants investigated were those detected in this laboratory [3]. The methods for purification of haemoglobins, preparation of globins, separation and aminoethylation of the $\alpha$- and $\beta$-globin chains were described previously [4, 5]. Tryptic peptides were made from aminoethylated globins in ammonium bicarbonate buffer at pH 8.0 and 37°C for 4 h. A JASCO (Japan Spectroscopic Co., Tokyo, Japan) Model TWINCLE high-pressure liquid chromatograph equipped with a Model GP-30 gradient elution system, a VL-611 variable-loop sample injector, and a UVIDEC 100-III variable-wavelength detector was employed. All separations were performed either on a TSK

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(Toyo Soda Manufacturing, Tokyo, Japan) IEX-510 SP SIL cation-exchange column (5 μm particle size, 0.4 × 30 cm), or on a JASCO FINEPAK SIL $C_{18}$ reversed-phase column (10 μm particle size, 0.46 × 25 cm). Elution of peptides was achieved by the use of a linear gradient of phosphate buffers and acetonitrile. Two solvents were used for ion-exchange HPLC: developer 1 was 49 m*M* potassium dihydrogen phosphate and 5.4 m*M* orthophosphoric acid, pH 2.86; developer 2 was 40% acetonitrile in 50 m*M* potassium dihydrogen phosphate and 50 m*M* disodium hydrogen phosphate, pH 6.8, at a flow-rate of 0.8 ml/min. Developer 1 at pH 2.86 was used to elute the peptides from the reversed-phase column, with a gradient of acetonitrile at a flow-rate of 1.0 ml/min [2]. The elution was monitored at 220 nm. Most of our experiments were with 0.5—1 mg of the lyophilized samples on either of the two columns. No more than 0.1 mg was necessary for a pilot run. All column fractions from ion-exchange HPLC were lyophilized, redissolved in 100—150 μl of developer 1, and rechromatographed on the reversed-phase column. A 0.1% solution of trifluoroacetic acid was also used instead of phosphate buffer [6] when desalting of the final peptide was desired. The presence of phosphate salts in the sample interfered neither with analyses of the amino acid compositions, nor with rechromatographing the peptides by reversed-phase HPLC.

## RESULTS

Fig. 1 (A and B) illustrates the separations of tryptic peptides from Hb A by ion-exchange HPLC. The developer sequence was initially a 12-ml isocratic elution with developer 1, and a 102-ml linear gradient from developer 1 to 2, the final level of which was continued isocratically. The peaks are very sharp and symmetrical for the most part in the overloaded chromatograms. Thus, each peptide emerged in a maximum volume of 0.5—1 ml, except for the αT6 peptide, which was eluted in a volume of 2—4 ml because of the substantial trailing pattern. The broader peak was also characteristic of the altered αT6 peptide from Hb Kokura in which a glycine residue is substituted for an aspartic acid residue at position 47. Peptides αT12-13, αT12, and αT13 did not derive from the soluble portion of the α-chain. Fig. 1 shows that these core peptides could have been isolated by ion-exchange HPLC. This knowledge was used to devise methods that may hopefully lead to the isolation of the core peptides. Indeed, these materials from the insoluble portion of the α-chain are barely soluble in the solvents at acidic pH, the majority of which precipitates and is then separated either by centrifugation or by filtration. The difficulty in dissolving these peptides upon application may be overcome by the use of 0.05% nonylamine—33% β-mercaptoethanol as solubilizer in a 0.1% phosphoric acid solution containing 5% methanol. The materials, once dissolved, did not form precipitates during chromatographic runs under the conditions used. Other attempts to isolate the core in pure form by reversed-phase HPLC were unsuccessful. Since problems were encountered with the solubility of the core, special emphasis has not been placed on the determination of the yield of core peptides. In all instances, nearly quantitative elution may be anticipated from chromatography on the cation-exchange column used for the initial study.

Fig. 2 provides a comparison of the positions of tryptic peptides from Hb A

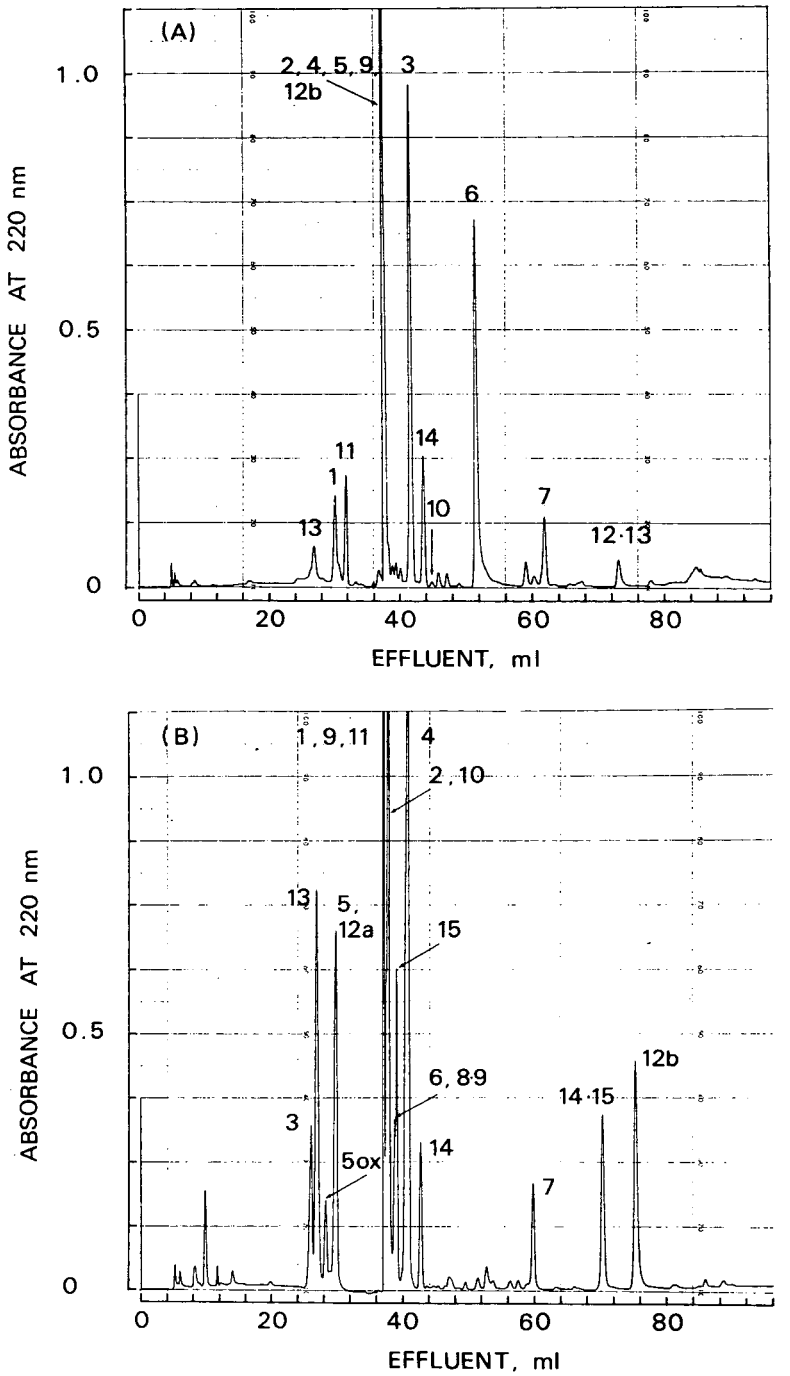


Fig. 1. Separation of tryptic peptides from (A) α-globin, and (B) β-globin chains of Hb A on a 0.4 × 30 cm TSK IEX-510 SP SIL cation-exchange column. See text for developer sequence.

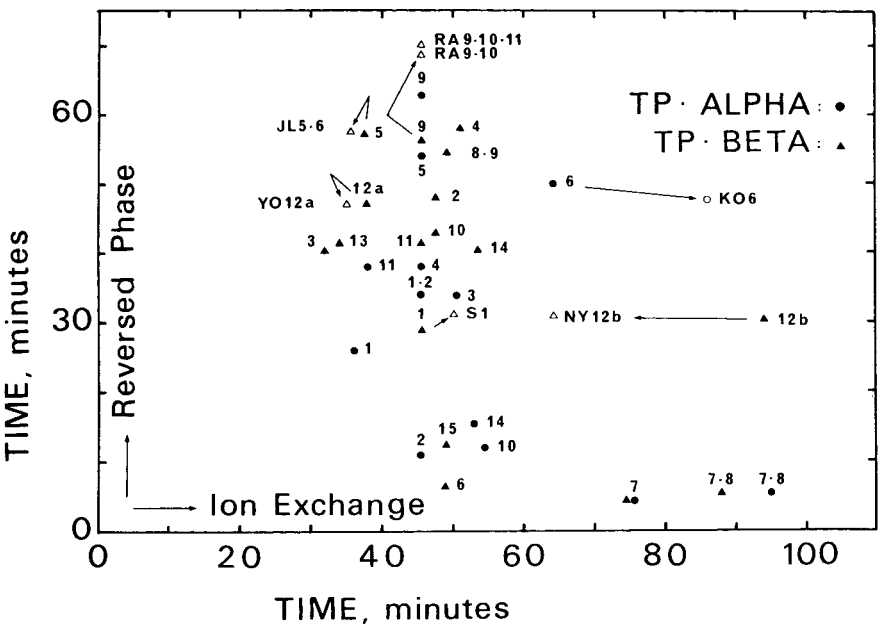


Fig. 2. Comparison of elution volumes of tryptic peptides of Hb A and the variants in two HPLC systems. The compositions of solvents are given in the text. Tryptic peptides (TP) are numbered in the sequence in which they occur in the polypeptide chains. Open symbols denote the positions of altered peptides from haemoglobin variants: RA = Hb Rahere ($\beta$82 lysine → threonine); JL = Hb J Lome ($\beta$59 lysine → asparagine); YO = Hb Yoshizuka ($\beta$108 asparagine → aspartic acid); KO = Hb Kokura ($\alpha$47 aspartic acid → glycine); S = Hb S ($\beta$6 glutamic acid → valine); and NY = Hb New York ($\beta$113 valine → glutamic acid). Structural identifications are cited in refs. 3 and 7.

in the two HPLC systems. The open symbols and arrows which connect the positions of an altered peptide from haemoglobin variant with the normal counterpart show how different the movement can be. It is apparent that some electrophoretic variants could be identified more precisely on these two-dimensional maps than on the one-dimensional chromatograms.

## DISCUSSION

In applying ion-exchange chromatography to the study of haemoglobin variants, we have tried to extend the unique possibilities of peptide separation by HPLC as much as does the successive use of electrophoresis and chromatography for the peptide mapping on paper support media. As shown, ion-exchange HPLC could identify peptides without much change in the molecular weight, but with alteration in charge. Although the hydrophilic matrices covered with hydroxyl groups have some properties of molecular sieving, the separation of peptides appears to be mainly due to the effect of net charge of the peptide. This was apparent when most of the neutral peptides, including some with +1 net charge, emerged in a single fraction. When this system produces a mixture, rechromatographing by reversed-phase HPLC usually effects the needed separation, since these two systems have entirely

different elution patterns. The substitution of an aspartic acid for an asparagine residue, or of glutamic acid for a glutamine residue, may be identified on ion-exchange HPLC. These substitutions may not cause much change in behaviour on reversed-phase HPLC [2]. Therefore, it is advantageous for the study of electrophoretic variants to use ion-exchange HPLC in the first dimension. If this system failed to show any abnormality, rechromatographing some of the fractions by reversed-phase HPLC could usually detect an alteration in the peptide compositions.

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## Note

# Separation of the cross-linking amino acids of elastin on thin-layer plates

STEPHEN KELLER*, ANJAN K. GHOSH, AJIT K. GHOSH, GERARD M. TURINO and INES MANDL

*Columbia University College of Physicians and Surgeons, New York, NY 10032 (U.S.A.)*

(First received June 6th, 1983; revised manuscript received July 29th, 1983)

There has long been a need for a simple and rapid method of separating the cross-linking amino acids of elastin. Such a procedure would greatly assist both structural and metabolic studies of this important protein, which is present in most types of connective tissue. Until recently the only methods available for separating the cross-linking amino acids in elastin hydrolyzates have been modified amino acid analyzer programs [1—3] and one-dimensional thin-layer chromatography (TLC) [4]. The original paper chromatographic procedure [5] as well as more recent electrophoresis methods [6, 7] do not separate desmosine from isodesmosine. Even though the analyzer procedures [1—3] separate the four cross-links, desmosine (DES), isodesmosine (IDE), merodesmosine (MD) and lysinonorleucine (LNL), from each other, these methods are complex and expensive. The previously reported TLC procedure [4] separates all four cross-links from one another but is not able to separate the cross-links from certain other amino acids, such as lysine, arginine and proline. To resolve this problem we have now devised a two-dimensional TLC procedure which completely separates the four cross-linking amino acids from each other and from all other amino acids present in elastin hydrolyzates.

## EXPERIMENTAL

The *n*-propranol, *n*-butanol, concentrated ammonia (29%), glacial acetic acid and acetone were reagent grade obtained from Fisher Scientific (Springfield, NJ, U.S.A.). The E. Merck silica gel G powder and the 20 × 40 cm blank glass plates were obtained from Brinkmann Instruments (Westbury, NY, U.S.A.); the precoated 20 × 40 cm E. Merck silica gel G plates (250 $\mu$m layer) were obtained from Analtech (Newark, DE, U.S.A.). The desmosine and iso-

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desmosine standards were obtained from Elastin Products (Pacific, MO, U.S.A.); the merodesmosine and lysinonorleucine were kindly provided by Dr. Barry Starcher then at Washington University (St. Louis, MO, U.S.A.). The other amino acids used as standards were obtained from Nutritional Biochemicals (Plainview, NY, U.S.A.). The ninhydrin was obtained from Pierce (Rockford, IL, U.S.A.) and the glass atomizer for spraying the TLC plates as well as the 5-$\mu$l microcapillary pipettes were obtained from Fisher Scientific.

The cross-link standards were put in aqueous solutions at concentrations of 2 mg/ml and stored at −20° C. The remaining amino acid standards were made up to concentrations of 4 $\mu M$. Bovine ligamentum nuchae elastin (250 mg) was hydrolyzed in five sealed ampoules with a total volume of 15 ml of 6 $M$ hydrochloric acid at 100° C for 18 h. After evaporation to dryness on a steam bath, the hydrolyzates were made up to a total volume of 5 ml with water and stored at −20° C. Thin-layer plates were prepared by suspending 30 g of silica gel G in 67 ml water and spreading a 250-$\mu$m layer onto 20 × 40 cm glass plates. The solvent for the first (20 cm) dimension was $n$-butanol—acetic acid—water (4:1:1). The second dimension solvent was $n$-propanol—water—ammonia (8:11:1). The amino acid standards, as well as the elastin hydrolyzate were all spotted on the silica gel plates in 3-$\mu$l aliquots, with a total of four applications per spot, using cool forced air drying between applications. Chromatography in the first dimension in the short direction of the plates was carried out in a polyethylene tank, 37 × 47 × 30 cm, height, width, depth. Solvent (100 ml) was placed into a parafilm-lined trough, and the glass plate was carefully inserted into it. Chromatography in the second dimension was carried out in the long direction, using a cylindrical glass jar 46 cm high, 25 cm diameter. When the run was completed, plates were dried at room temperature for 24 h, and then sprayed with a solution of 1 g of ninhydrin in 100 ml of acetone.

## RESULTS AND DISCUSSION

When a one-dimensional separation of standard amino acids in the first system, $n$-butanol—acetic acid—water, is carried out the elastin cross-links all stay at, or near the sites of application, while all other amino acids placed on the plate migrate much farther. It is likely that the strong binding to the stationary phase is a result of the higher charge density of the cross-links which along with their relatively high p$K$ values renders them extremely hydrophilic. To enable these substances to migrate and separate in the second dimension, a high pH provided by ammonia was applied. The replacement of $n$-butanol by $n$-propanol which is miscible with water in all proportions allowed the formulation of a solvent system containing more water than is usual for amino acid separation. In this system the cross-links moved away from the origin [4]. After one-dimensional separation of the amino acids in the $n$-propanol—water—ammonia system, along the long axis of the plates, the four cross-links were seen to have very different $R_F$ values. However, in the one-dimensional system lysine has the same $R_F$ value as LNL, proline has an $R_F$ value similar to IDE and arginine has an $R_F$ value similar to that of MD. The two-dimensional separation of a standard amino acid mixture containing the four cross-links, along with lysine (LYS), arginine (ARG), proline (PRO), glycine, alanine,

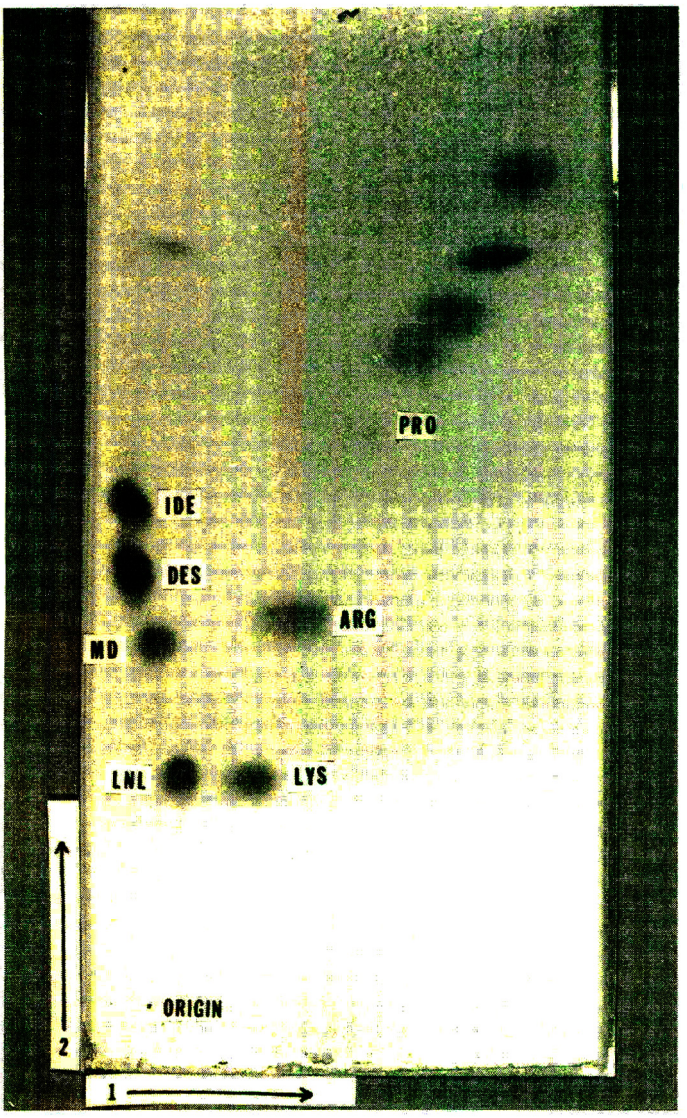


Fig. 1. Separation of standard amino acids on a silica gel plate by the two-dimensional method using *n*-butanol—acetic acid—water (4:1:1) in the first dimension and *n*-propanol—ammonia—water (8:1:11) in the second.

valine and leucine by a combination of the two systems is shown in Fig. 1. The four cross-links are seen to be completely separated along the left border of the plate, and well removed from all the other amino acids contained in the mixture. Gly, Ala, Val and Leu are the four spots located above and to the right of Pro. The same separation procedure was used to resolve the components of a hydrochloric acid hydrolyzate of bovine ligamentum nuchae elastin. Fig. 2 shows that the cross-links are again situated on the left border of the plate, completely separated from each other and from all other amino acids in the hydrolyzate. The amount of merodesmosine in elastin is much lower than that of the other three cross-linking amino acids which accounts for the fact that it is not visible on the chromatogram.

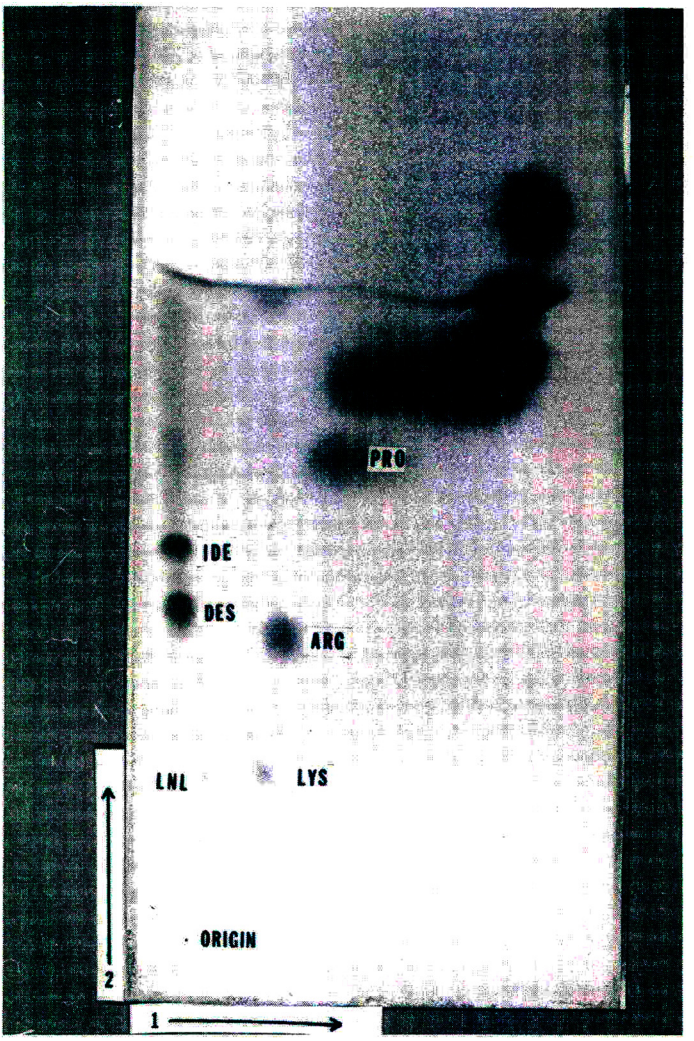


Fig. 2. Separation of the amino acids in an acid hydrolyzate of elastin by the two-dimensional method used in Fig. 1.

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## Note

# Method for the determination of oltipraz, a new antischistosomal agent, in blood

H.M. ALI*

*Department of Pharmacology, Faculty of Pharmacy, University of Khartoum, P.O. Box 1996, Khartoum (Sudan)*

J.L. BENNETT*

*Department of Pharmacology and Toxicology, Michigan State University, B420 Life Sciences Building, East Lansing, MI 48824 (U.S.A.)*

S.M. SULAIMAN

*Bilharzia Research Unit, National Health Laboratories, Ministry of Health, Khartoum (Sudan)*

and

J. GAILLOT

*Department of Biodynamics, Institute de Biopharmacie Rhone-Poulenc, 182/184, Avenue Aristide Briand, 92160 Antony (France)*

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Oltipraz, methyl-4-(pyrazinyl-2)-5-dithiole-1,2-thione, is a highly lipid soluble compound which has significant antischistosomal activity when given orally to patients infected with *S. mansoni* or *S. heamatobium* [1—3]. The recommended dose for patients with *S. mansoni* is 20—25 mg/kg while patients with *S. haematobium* infections receive 25—30 mg/kg.

Assay methods based on thin-layer chromatography [1], gas—liquid chromatography (GLC) [1] and high-performance liquid chromatography (HPLC) [3] were previously used for the determination of oltipraz in biological fluids, in animals and in humans. Prior to chromatographic analysis, oltipraz was

*Address for reprint requests.

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extracted from the biological fluids by multiple extraction procedures using relatively large volumes of organic solvents, e.g. diethyl ether [3]. After performing the above procedures we felt that the multiple extraction procedure was too expensive, i.e. in countries like Sudan, the cost of solvents and glassware is very high. For this reason we attempted to employ a single-extraction technique, prior to chromatographic analysis, using small volumes of chloroform for analyzing oltipraz in urine, serum and plasma. Similar methods involving the single-extraction technique have been previously reported [4—7]. In this paper we describe an assay method for the analysis of oltipraz based on a single-extraction step followed by GLC analysis.

## EXPERIMENTAL

### *Compounds and materials*

Oltipraz (RP-35972) and its butyl derivative (RP-35919), Fig. 1, were kindly supplied by Rhone-Poulenc Sante (Paris, France). Thiodiglycol (lot 25c-0026-2) was purchased from Sigma (St. Louis, MO, U.S.A.).

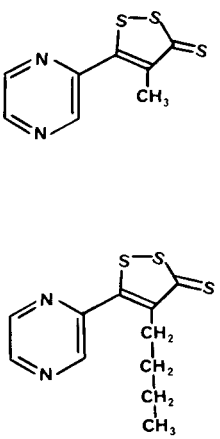

Fig. 1. Structure of oltipraz (upper) and the internal standard RP-35919 (lower).

### *Gas—liquid chromatography*

A Perkin-Elmer F33 gas chromatograph with a flame ionization detector and linked to a Hitachi Perkin-Elmer 1.0-mV Model 56 recorder was used. A glass column (1.0 m × 5.0 mm O.D. and 3.0 mm I.D.) packed with Chromosorb Q (100—120 mesh) and coated with 3% OV-17 (Perkin-Elmer) was used under the following operating conditions: nitrogen, 100 kN/m$^2$; hydrogen, 100 kN/m$^2$; air, 180 kN/m$^2$; oven temperature, 235°C; and injection port temperature, 250°C. The column was conditioned at 260°C for 24 h before use. The retention times of oltipraz and its butyl derivative were 8.5 and 13.5 min, respectively.

### *Determination of oltipraz in biological fluids*

To 1.0 ml of urine, serum, plasma or aqueous solution containing oltipraz in a glass-stoppered conical centrifuge tube were added oltipraz butyl derivative

(RP-35919, 50 μm/ml, 1.0 ml) as the internal standard, sodium bicarbonate (saturated solution, 1.0 ml), water (1.0 ml), thiodiglycol (0.1 ml) and chloroform (120 μl). Aqueous solutions of oltipraz and its butyl derivative were prepared in an absolute alcohol—distilled water mixture (1:4). The tube was shaken thoroughly on a Whirlmixer (Vortex Genie) for 1.0 min and then centrifuged at 3800 *g* for 2.0—3.0 h until a clear chloroform layer was obtained. A 10-μl syringe was inserged through the aqueous phase into the chloroform layer and 5—8 μl of the organic phase were carefully withdrawn and injected onto the GLC column.

The peak height ratio (PHR: oltipraz/oltipraz butyl derivative) was calculated and the concentration of oltipraz in the biological fluid sample was determined by reference to the appropriate calibration curve. Calibration curves in water, urine, serum or plasma were separately prepared by repeating the above procedure. The calibration curve(s) data were statistically analyzed using the regression analysis method.

*Recovery of compounds from biological fluids*

The recovery of oltipraz from various volumes (1, 5 or 10.0 ml) of water, urine, serum and plasma was tested by spiking these media with 10 μg of the drug (in 1.0 ml of water) and extracted as described above. A reference sample (non-extracted) was prepared in chloroform (10 μg/ml) and was considered as the 100% recovery level. The recovery of oltipraz butyl derivative was examined similarly using 50 μg of this compound. A 10-μl aliquot of the chloroform extract was withdrawn and injected onto the GLC column and the corresponding peaks were measured. Duplicate injections were made in each case and the recovery of the compounds was tested on six separate replicates for each media. The peak height obtained for each of the reference samples (non-extracted chloroform solutions) was considered equivalent to 10 and 50 μg of oltipraz and its butyl derivative, respectively. The amount extracted for each compound from water, urine, serum and plasma was then determined by comparison to the reference samples. The mean values were obtained and expressed as percentages of the original amount of compound added.

The data obtained for oltipraz recovery from the six replicate runs in each media were further used to test the repeatability of the assay method.

## RESULTS AND DISCUSSION

Using the GLC conditions described in the experimental section we were able to obtain sharp, symmetrical and separated peaks for oltipraz and its butyl derivative (Fig. 2). Extracts of various volumes (1, 5, or 10.0 ml) of blank serum and plasma of humans and/or animals receiving oltipraz, gave no interfering peaks, when chromatographed. Since the butyl derivative of oltipraz is almost identical to oltipraz in its physicochemical properties (Fig. 1), we selected the butyl derivative as an internal standard for the assay of oltipraz. We observed that the pH in the aqueous phase for optimal extraction of oltipraz into chloroform ranged between 5.0 and 7.0. More importantly, we were able to perform this extraction in a small volume of chloroform. In previous methods, for GLC and HPLC, 20.0 ml of diethyl ether were used per

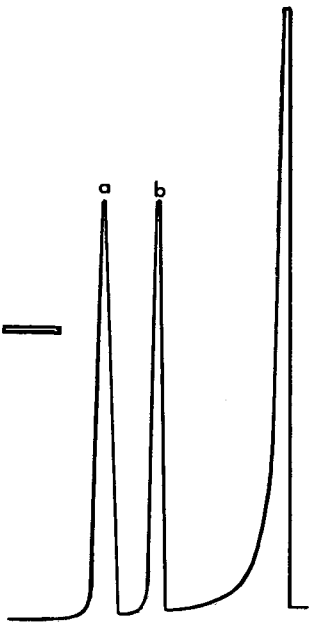


Fig. 2. A representative gas–liquid chromatogram of (a) oltipraz and (b) oltipraz butyl derivative (RP-35919). The retention times (min) of a and b were 8.5 and 13.5, respectively. Time scale equals 5 min. (Blank serum and plasma gave no interfering peaks.)

assay [1, 3]. (This reduction in solvent use becomes extremely important in countries like Sudan where the cost of solvents is 5 to 10 times higher than in the U.K. or U.S.A.) We made no attempt to identify other suitable organic solvents for this method. We did study the time required to extract oltipraz from various aqueous phases (from 20 to 0.5 min) and found that 1 min was optimal.

In our trials with humans we have measured the concentration of oltipraz in blood. Peak plasma concentration in 5.0-ml samples from humans after a 25 mg/kg dose is 1.07 $\mu$g per ml of blood. Recent work by the manufacturers on oltipraz in primates are unlikely to warrant use of urine as a reliable source of the drug for pharmacokinetic studies.

The average amounts ($X$ + S.E.) of oltipraz extracted from a given aqueous media (1, 5 or 10.0 ml) spiked with 10 $\mu$g/ml drug, using our single-extraction GLC method, were almost identical. This was true with all four extraction media tested: water (9.32 + 0.18 $\mu$g/ml), urine (9.29 + 0.17 $\mu$g/ml), serum (6.20 + 0.08 $\mu$g/ml) and plasma (6.08 + 0.15 $\mu$g/ml). We have also tested the reproducibility on a given sample of human sera spiked with 10 $\mu$g/ml oltipraz (the sample was assayed daily over a period of five days) and found the mean value and standard error to be almost identical to that reported above for serum.

When oltipraz blood levels were determined, following oral administration of the drug to humans or monkeys, using this assay method, the lower limit of detection was approximately 150 ng. The lower limit of quantification using serum and plasma ranged between 1.0 and 2.0 $\mu$g/ml. At present we are using

this assay in analyzing the effects of food on the bioavailability of oltipraz in humans. Dietary components and/or endogenous compounds have not interfered with the chromatographic analysis of oltipraz in human and animal trials.

The extractability of oltipraz and/or is butyl derivative from the various media was different. This implies that the calibration curves are to be prepared using the same media in question and that extrapolation from one media to another should not be attempted. These findings were further confirmed when the absolute recoveries of oltipraz and its butyl derivative were compared when extracted from water, urine, serum and plasma samples. The correlation coefficient values obtained for the four different aqueous media ranged between 0.9989 to 0.9999 and all the curves passed through the origin, indicating a linear relationship between the concentration of oltipraz (2.0—16.0 $\mu$g/ml) and the peak height ratio.

In the light of above findings, the single-extraction GLC assay method used for the determination of oltipraz from aqueous and biological fluids has good reproducibility, sensitivity and concentration: peak height ratio linearity. Furthermore, the single-extraction GLC assay method, compared to the previously described ones for oltipraz [1, 3], has some advantages: (1) reduction in chances of losing drug since transfer has been avoided; (2) reduction in usage of glassware; and (3) a reduction in the amount of organic solvent.

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## Note

# Determination of sodium azodisalicylate, salazosulphapyridine and their metabolites in serum, urine and faeces by high-performance liquid chromatography

R.A. VAN HOGEZAND*

*Department of Medicine, Division of Gastroenterology, St. Radboud Hospital, P.O. Box 9101, 6500 HB Nijmegen (The Netherlands)*

H.C.J.G. VAN BALEN

*Department of Organic Chemistry, University of Nijmegen, Toernooiveld 2, 6525 ED Nijmegen (The Netherlands)*

A. VAN SCHAIK, A. TANGERMAN and P.A.M. VAN HEES

*Department of Medicine, Division of Gastroenterology, St. Radboud Hospital, P.O. Box 9101, 6500 HB Nijmegen (The Netherlands)*

B. ZWANENBURG

*Department of Organic Chemistry, University of Nijmegen, Toernooiveld, 2, 6525 ED Nijmegen (The Netherlands)*

and

J.H.M. VAN TONGEREN

*Department of Medicine, Division of Gastroenterology, St. Radboud Hospital, P.O. Box 9101, 6500 HB Nijmegen (The Netherlands)*

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Salazosulphapyridine (SASP) is extensively used in the treatment of inflammatory bowel disease. The mode of action of SASP is unknown. After oral ingestion the larger part of SASP reaches the colon intact where it is split

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by colonic bacteria at the azo bond into sulphapyridine (SP) and 5-amino-salicylic acid (5-ASA). Virtually all SP is absorbed and subsequently metabolized and excreted in the urine. Some of the 5-ASA is absorbed and excreted in the urine in the acetylated form. The larger part of 5-ASA is excreted in the faeces [1—3].

Recent studies have demonstrated that 5-ASA is the active moiety of SASP [4, 5]. SP functions as a carrier to transport 5-ASA to the distal part of the bowel. Most side-effects of SASP are attributable to SP [6]. Therefore, the treatment of inflammatory bowel disease has been focused on the use of 5-ASA [7, 8]. A promising compound in which 5-ASA is linked to another 5-ASA molecule is sodium azodisalicylate (di-5-ASA) [9, 10]. In pharmacokinetic studies of this new compound a reliable method for the determination of di-5-ASA is necessary. No satisfactory analytical method for measuring di-5-ASA and 5-ASA is available. This paper presents a suitable method for the determination of di-5-ASA, 5-ASA, acetyl-5-ASA (ac-5-ASA) and also for SASP in serum, urine and faeces.

## EXPERIMENTAL

### *Reagents and material*

5-Amino-2-hydroxybenzoic acid (5-ASA) and 4-amino-2-hydroxybenzoic acid (PAS) were purchased from Merck (Darmstadt, F.R.G.). 5-ASA was purified prior to use. Salazosulphapyridine, sulphapyridine and acetylsulphapyridine were supplied by Pharmacia (Uppsala, Sweden).

Acetyl-5-ASA (ac-5-ASA) and di-5-ASA were synthesized by the Department of Organic Chemistry of the University of Nijmegen. Propionyl-PAS and propionyl-5-ASA were synthesized by the reaction of PAS and 5-ASA with propionic anhydride and purified by recrystallization from water. All other reagents were of analytical grade (Merck).

For the clean-up of serum samples, Baker chemical extraction columns (3 ml) filled with quaternary amines (No. 7091) were used. They could be regenerated about ten times.

### *Apparatus*

High-performance liquid chromatographic (HPLC) analyses were performed on a Spectra Physics instrument (Model SP 740) equipped with a Chrompack 3 mm × 150 mm analytical column packed with LiChrosorb RP-18 (5 $\mu$m) coupled to a Chrompack guard-column (12 mm × 75 mm).

Detection was performed with a Schoeffel fluorescence monitor Model FS 970, with excitation at 305 nm and a cut-off filter at 396 nm. For injection of the samples an automatic sampler was used.

### *Chromatographic conditions*

The mobile phase consisted of deionized water adjusted to pH 3.3 (for SASP pH 2.85) with 0.01 *M* citric acid and 20% methanol. A flow-rate of 1.4 ml/min was established. The run-time was 16 min (for SASP 25 min).

*Standards*

Standards of 5-ASA, ac-5-ASA, di-5-ASA and SASP were prepared in serum (range 0.1—20 μmol/l), urine (range 20—1000 μmol/l) and faeces (range 20—1000 μmol/l).

*Sample preparation*

*Urine.* To 1 ml of urine were added propionyl-PAS as internal standard (0.1 ml of a solution of 10 mmol/l; for SASP 0.5 ml) and 20 μl of propionic anhydride. After standing for a few minutes at room temperature, 9 ml of methanol were added (for SASP 8.5 ml of methanol). After Vortex mixing, the mixture was left for at least 30 min and centrifuged. To 0.5 ml of the supernatant 4.5 ml of distilled water were added and 20 μl of this mixture were injected into the HPLC apparatus, assaying ac-5-ASA and free 5-ASA, the latter as propionyl-5-ASA.

For the determination of di-5-ASA or SASP an extra step was inserted, viz. cleavage of the azo bond. Before addition of propionic anhydridie 300 μl of freshly prepared 10% sodium dithionite ($Na_2S_2O_4$) were added to 1 ml of urine. After warming the mixture at 30°C for 5 min, 20 μl of propionic anhydride were added. The mixture was then treated as described above.

*Faeces.* Faeces were sampled directly into about 300 ml of mercury chloride (0.2%) and mixed thoroughly to prevent cleaving of the azo bond by colonic bacterial enzymes, then stored at —20°C. With 1 g of this suspension the same procedure as mentioned above for urine was followed.

*Serum.* To 0.5 ml of serum were added propionyl-PAS as internal standard (0.1 ml of a solution of 500 μmol/l) and 20 μl of propionic anhydride. After standing for a few minutes at room temperature 4 ml of a phosphate buffer (0.01 *M*, pH 8) were then added and after centrifugation transferred to a quaternary amine extraction column (pre-equilibrated with 10 ml of phosphate buffer, 0.01 *M*, pH 8). The column was washed with 3 ml of the same buffer. The compounds were extracted with 2 ml of phosphate buffer (0.2 *M*, pH 5.7) into a test tube; 20 μl of this mixture were then injected into the HPLC apparatus.

For the determination of di-5-ASA or SASP the azo bond was cleaved as described above for urine and faeces. Before addition of propionic anhydride, 200 μl of freshly prepared 10% sodium dithionite were added to 0.5 ml of serum. After warming the mixture at 30°C for 5 min, 20 μl of propionic anhydride were added. The same procedure as described above was then followed.

## RESULTS

The chromatograms of the samples, prepared as described in the preceding section, showed three well separated peaks. Some typical chromatograms are shown in Fig. 1. The retention time ($t_R$) for ac-5-ASA was 4.5 min. Free 5-ASA was propionylated to give propionyl-5-ASA with a $t_R$ of 6.5 min. The internal standard (propionyl-PAS) had a $t_R$ of 12.5 min. Under these conditions the acetylated sulphapyridine, sulphapyridine and propionylsulpha-

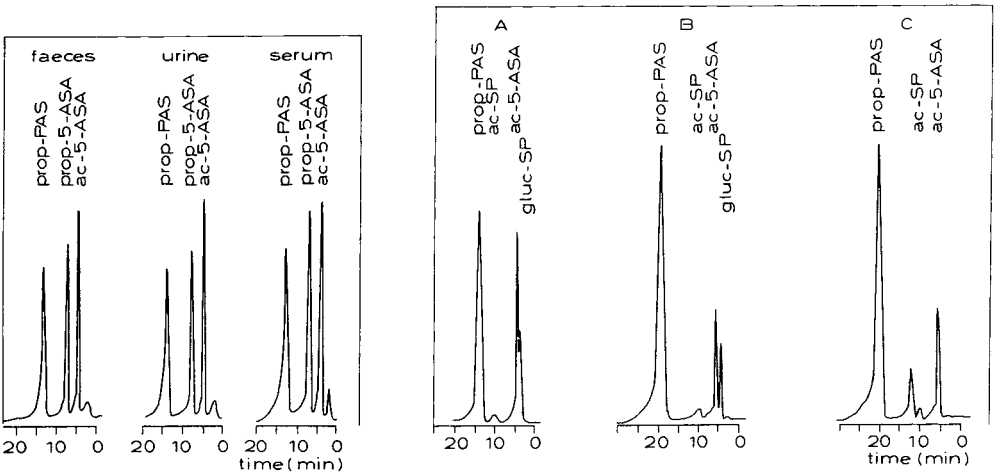


Fig. 1. Typical chromatograms of faeces, urine and serum samples. Concentrations measured: 5-ASA: faeces 502 μmol/l, urine 480 μmol/l and serum 20.7 μmol/l. Ac-5-ASA: faeces 464 μmol/l, urine 499 μmol/l and serum 19.6 μmol/l.

Fig. 2. Typical chromatograms of a urine sample of a patient who ingested a single dose of 2.3 g of salazosulphapyridine; (A) with mobile phase pH 3.3, (B) with mobile phase pH 2.85, and (C) with mobile phase pH 3.3, after deglucuronidation of gluc-SP. Ac-5-ASA concentrations measured: 587 μmol/l (B) and 561 μmol/l (C).

pyridine could hardly be detected and there was no interference with the peaks we wished to measure.

The only interfering metabolite peak we found was glucuronyl-SP (gluc-SP) resulting from SASP (Fig. 2A). This peak interfered with that of ac-5-ASA and could be separated when the pH of the mobile phase was reduced to 2.85 (Fig. 2B). The run-time was then 25 min, resulting in a low internal standard peak, which could be raised by adding more propionyl-PAS (0.5 ml). An alternative way (Fig. 2C) was deglucuronidation of gluc-SP by which 20 μl of β-glucuronidase (Boehringer Mannheim, 100,000 Fishman units /ml) were added to the sample and incubated for 3 h, before adding propionic anhydride or before cleavage with sodium dithionite. By the latter method the gluc-SP peak disappeared, but another peak of unknown origin appeared. In the chromatograms no interfering peaks were observed arising from other compounds in urine, serum and faeces.

Calibration graphs were constructed by plotting the peak height ratios (ac-5-ASA/internal standard and prop-5-ASA/internal standard) against the concentrations of ac-5-ASA and prop-5-ASA (Fig. 3). Straight lines were obtained. The detection limit of ac-5-ASA and prop-5-ASA amounted to 0.1 μmol/l.

Table I demonstrates the intra-assay variation for different concentrations of ac-5-ASA, 5-ASA, di-5-ASA and SASP in urine, faeces and serum. The coefficients of variance are quite low.

The prepared samples, worked up in vials, ready to be injected into the HPLC apparatus, could be stored for at least 14 days at −20°C. There was no decrease in the content of the samples, nor did additional peaks appear.

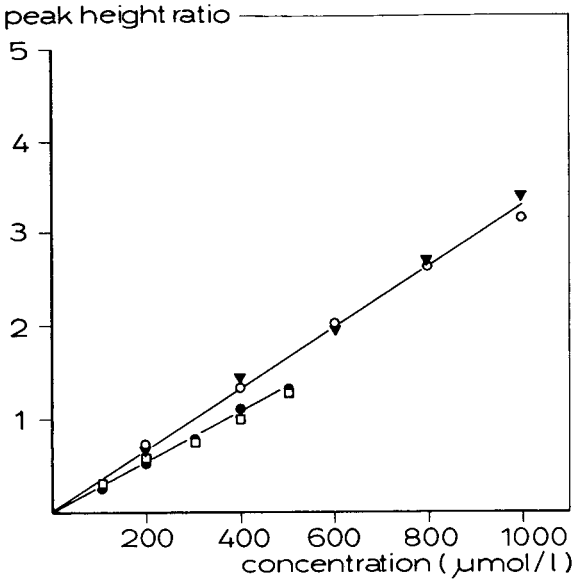


Fig. 3. Peak height ratios (ac-5-ASA/internal standard and prop-5-ASA/internal standard) against the concentrations of ac-5-ASA and prop-5-ASA. (▾), ac-5-ASA in urine; (○), ac-5-ASA in faeces; (●), prop-5-ASA in urine; (□), prop-5-ASA in faeces.

TABLE I

INTRA-ASSAY VARIATION FOR DIFFERENT CONCENTRATIONS OF ac-5-ASA, 5-ASA, di-5-ASA AND SASP IN URINE, FAECES AND SERUM

| | Concentration (μmol/l) | Mean amount detected ($n$ = 6) (μmol/l) | S.D. | Coefficient of variation |
|---|---|---|---|---|
| Urine | | | | |
| ac-5-ASA | 1000 | 1010.3 | 22.9 | 2.3 |
| ac-5-ASA | 100 | 99.1 | 3.9 | 4.0 |
| 5-ASA | 500 | 484.9 | 5.0 | 1.0 |
| di-5-ASA | 100 | 101.2 | 1.8 | 1.8 |
| SASP | 100 | 99.6 | 3.6 | 3.6 |
| Faeces | | | | |
| ac-5-ASA | 1000 | 1017.4 | 16.3 | 1.6 |
| ac-5-ASA | 100 | 104.2 | 1.9 | 1.8 |
| 5-ASA | 500 | 480.1 | 3.0 | 0.6 |
| di-5-ASA | 100 | 93.5 | 7.6 | 8.1 |
| SASP | 100 | 97.1 | 3.7 | 3.8 |
| Serum | | | | |
| ac-5-ASA | 20 | 21.9 | 1.0 | 4.4 |
| ac-5-ASA | 10 | 9.8 | 0.3 | 3.5 |
| 5-ASA | 20 | 20.6 | 0.7 | 3.3 |
| di-5-ASA | 20 | 21.5 | 1.8 | 8.6 |
| SASP | 20 | 20.1 | 0.6 | 3.0 |

## DISCUSSION

In 1973 a colorimetric detection of SASP and its metabolites in serum, urine and faeces, and bile was described [11]. Currently, for the determination of these compounds the focus is on the use of an HPLC assay. Several investigators demonstrated good detection in serum and urine [12—17]. Sulphapyridine and ac-5-ASA could be analysed satisfactorily as well. With the method described in this article we demonstrate the determination of di-5-ASA, SASP, ac-5-ASA and 5-ASA in serum, urine and faeces. SASP and di-5-ASA have been measured indirectly by cleaving the azo bond with sodium dithionite into a molecule of 5-ASA and SP and two molecules of 5-ASA, respectively. In the earlier colorimetric assay of SASP [11] titanium trichloride was used for this cleavage. However, it has been suggested that Ti ions can form complexes between the carboxy and hydroxy functions of the aminosalicylic molecule and the reversed-phase material (unpublished observations). This problem of complex formations with Ti ions can be circumvented by using sodium dithionite for the azo bond cleavage.

In view of the pharmacokinetic and toxicological experiments with di-5-ASA we focused our attention on the metabolites ac-5-ASA and 5-ASA. By propionylating 5-ASA with propionic anhydride propionyl-5-ASA could be determined independently from ac-5-ASA. This method has an obvious advantage over the alternative procedure in which the amount of free 5-ASA is determined as the difference of total ac-5-ASA (obtained after acetylation of samples containing free 5-ASA and ac-5-ASA) and ac-5-ASA present prior to acetylation. Considerable errors will be introduced in this differential method because of the rather large ratios of ac-5-ASA and free 5-ASA.

The present method does not suffer from peaks that interfere with the detection. Characteristic peaks of serum, urine and faeces samples were found to appear in the front of the chromatograms. The use of fluorescence detection is to a large extent responsible for the high degree of selectivity.

In conclusion, the method described here can be used for monitoring SASP, di-5-ASA, ac-5-ASA and 5-ASA in serum, urine and faeces and therefore will be helpful in pharmacokinetic, toxicological and clinical studies with di-5-ASA.

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## Note

# Simultaneous determination of dipyrone metabolites in plasma by high-performance liquid chromatography

ESTER ZYLBER KATZ and LIORA GRANIT

*Clinical Pharmacology Unit, Department of Medicine A, Hadassah University Hospital, Kiryat Hadassah, P.O. Box 12000, Jerusalem (Israel)*

DENNIS E. DRAYER

*Division of Clinical Pharmacology, Cornell University, Medical College, New York, NY 10021 (U.S.A.)*

and

MICHA LEVY*

*Clinical Pharmacology Unit, Department of Medicine A, Hadassah University Hospital, Kiryat Hadassah, P.O. Box 12000, Jerusalem (Israel)*

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Dipyrone {sodium [N-(1,5-dimethyl-3-oxo-2-phenylpyrazolin-4-yl)-N-methylamino]methanesulphonate monohydrate} is an effective analgesic, antipyretic and anti-inflammatory drug widely used for nearly 60 years. During the last decade several attempts were made to study dipyrone metabolism; however, many aspects of the drug pharmacokinetics have not been fully elucidated. Initial studies revealed that after oral administration of [$^{14}$C]dipyrone, absorption was rapid and distribution uniform. The half-life of elimination of the radioactivity from the plasma was 7 h and 90% of the dose was recovered in the urine [1]. Further studies, using thin-layer chromatography, have shown that following oral and intramuscular administration in man, dipyrone as such is undetectable in the plasma since absorption is preceded by hydrolysis to 4-methylaminoantipyrine (MAA), which is further metabolized to 4-aminoantipyrine (AA) [2]. Another MAA metabolite, 4-formylamino-

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antipyrine (FAA), was identified by Volz and Kellner [3]. It is formed by the oxidation of the N-methyl side-chain of MAA [4]. AA is further metabolized to 4-acetylaminoantipyrine (AAA), which is the major metabolite appearing in the urine [3] (Fig. 1). All these metabolites (MAA, AA, AAA and FAA) are also metabolites of aminopyrine [4].

Additional methods to study pyrazolone derivatives have employed spectrophotometric [5], gas chromatographic [6] and mass spectrometric [7] determinations.

High-performance liquid chromatography (HPLC) was first applied for the determination of antipyrine in biological fluids [8] and more recently for the quantitation of aminopyrine and MAA, AA and AAA after it had been added to urine [9].

We hereby report an HPLC method for the simultaneous determination of four dipyrone metabolites (MAA, AA, AAA, FAA) in human plasma, without using derivatization steps. Employing this method, a preliminary study on single-dose kinetics of dipyrone metabolites in a healthy volunteer is presented.

## MATERIALS AND METHODS

### *Chemicals*

All of the chemicals used were of analytical grade. The solvents were LiChrosolv grade from Merck (Darmstadt, F.R.G.). The pure metabolites MAA, AA, FAA, AAA and the internal standard (4-propylaminoantipyrine, PAA) were provided by Hoechst (Frankfurt/M., F.R.G.).

### *Apparatus*

The HPLC system consisted of a Spectra Physics Model 3500 liquid chromatograph with an M6K injector. A JASCO Model UVIDEC-100-III spectrophotometric detector (cell volume, 5 $\mu$l; path length, 10 mm) was operated at 257 nm. A stainless-steel column (300 mm × 3.9 mm I.D.) packed with 10-$\mu$m $\mu$Bondapak $C_{18}$ (part No. 27324, Waters Assoc., Milford, MA, U.S.A.) was used. The mobile phase was 8% methanol in 0.01 *M* sodium acetate, adjusted to pH 3.0 with concentrated hydrochloric acid. The column was maintained at room temperature and the flow-rate was 1.6 ml/min. The recorder was operated at 10 cm/h.

### *Standard solutions*

Stock solutions of 500 $\mu$g/ml MAA, AA, FAA and AAA were prepared in methanol. A 50 $\mu$g/ml PAA internal standard solution was also prepared in methanol. Normal plasma was spiked with known amounts of the metabolites over the range 0.625—5.0 $\mu$g/ml and with 2.5 $\mu$g of PAA. For the range of 5—20 $\mu$g/ml, 10 $\mu$g of PAA were added as internal standard.

### *Extraction procedure*

To 1.0 ml of plasma sample or standard in a 15-ml glass conical tube, 50 or 200 $\mu$l of internal standard solution (PAA, 50 $\mu$g/ml), 100 $\mu$l of 1 *M* sodium hydroxide and 5 ml of chloroform were added. The contents of the tubes were mixed for 1 min on a Vortex-type mixer and then centrifuged for 10 min at

850 *g*. The organic phase was transferred to a tapered centrifuge tube and evaporated to dryness in a 40°C water bath under a stream of air. The aqueous phase was re-extracted with another 5 ml of chloroform following the same procedure. To concentrate the residues at the bottom of the conical tubes, 0.5 ml of chloroform was added, vortexed and again evaporated. The residue was redissolved in 50 $\mu$l of methanol and 5—10 $\mu$l aliquots were injected into the HPLC system.

## RESULTS AND DISCUSSION

The structure of dipyrone and the four major metabolites which can be identified in plasma are shown in Fig. 1. A representative chromatogram obtained when a plasma sample containing 10 $\mu$g/ml MAA, AA, AAA, FAA and 10 $\mu$g PAA as internal standard was extracted by the method described, is shown in Fig. 2. Complete separation of the four metabolites was obtained, with the following retention times: MAA 14, AA 17, FAA 21, AAA 25, and PAA 41 min. When drug-free plasma was extracted in the same manner, no

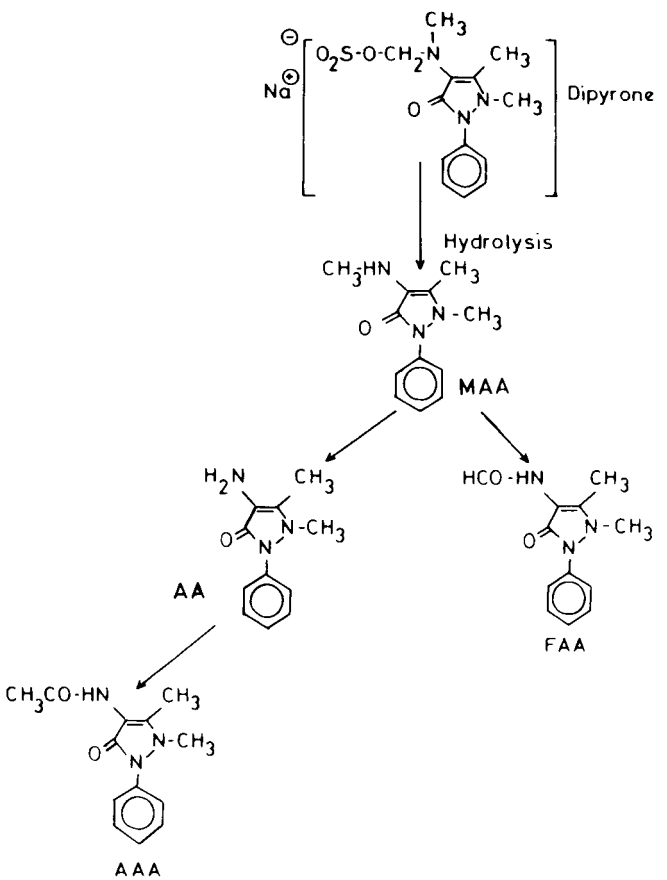


Fig. 1. Structure of dipyrone and its metabolites in man. MAA = 4-methylaminoantipyrine; AA = 4-aminoantipyrine; AAA = 4-acetylaminoantipyrine; FAA = 4-formylaminoantipyrine.

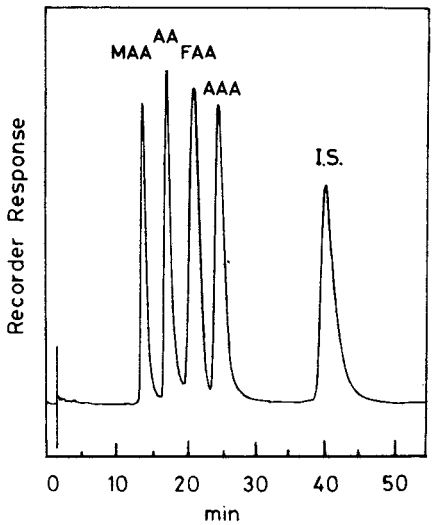


Fig. 2. A representative HPLC chromatogram of human plasma containing 10 μg/ml MAA, AA, FAA, AAA and 10 μg PAA as internal standard (I.S.).

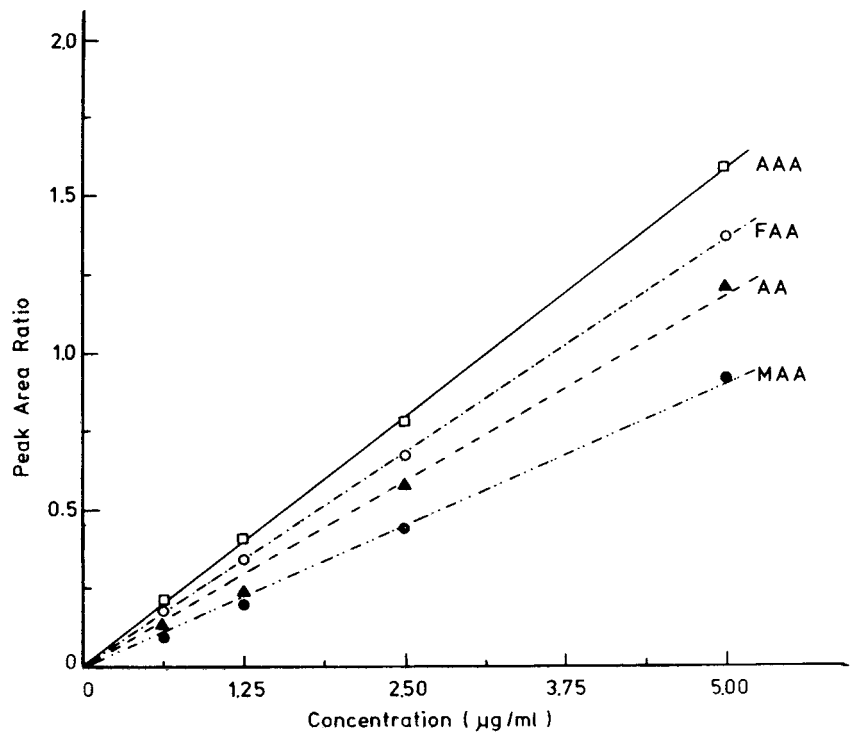


Fig. 3. Calibration curves for dipyrone metabolites with 2.5 μg PAA as internal standard.

interfering peaks from endogenous plasma constituents were observed. Plasma samples obtained after drinking coffee showed a peak with a retention time of 36 min which was identified as caffeine; this did not interfere with the assay.

The complete separation of the four metabolites was possible only when an appropriate mobile phase was found; 8% methanol in 0.01 *M* sodium acetate, adjusted to pH 3.0, resulted in the optimum resolution. From our previous experience, when the pH is raised to 5.0, FAA and AAA appeared as one peak eluting first from the column, followed by PAA, AA and MAA. The conditions employed in previously reported methods [8, 9] did not allow the simultaneous separation of the four metabolites.

Results of the calibration curves for each metabolite are given in Figs. 3 and 4. We observed that the linearity is better over the range 0.625—5.0 μg/ml if 2.5 μg of internal standard (PAA) were used. Correlation coefficients were 0.996, 0.998, 0.999 and 0.999 for MAA, AA, FAA and AAA, respectively. For higher concentrations of the metabolites (5.0—20.0 μg/ml), 10 μg of PAA were used and the correlation coefficients obtained were 0.999, 0.998, 0.999 and 0.999, respectively.

The precision of the method was determined by repeated analyses of plasma specimens containing known concentrations of the metabolite. As shown in Table I, within-day precision varied between 1.5% and 3.6% and day-to-day precision between 2.4% and 6.7%.

We measured the analytical recovery from plasma of the four metabolites. To obtain a good extraction reproducibility the double-extraction method was selected mainly to improve the recovery of MAA and AA. The recovery was calculated from a plot of the absolute method against the extractive method,

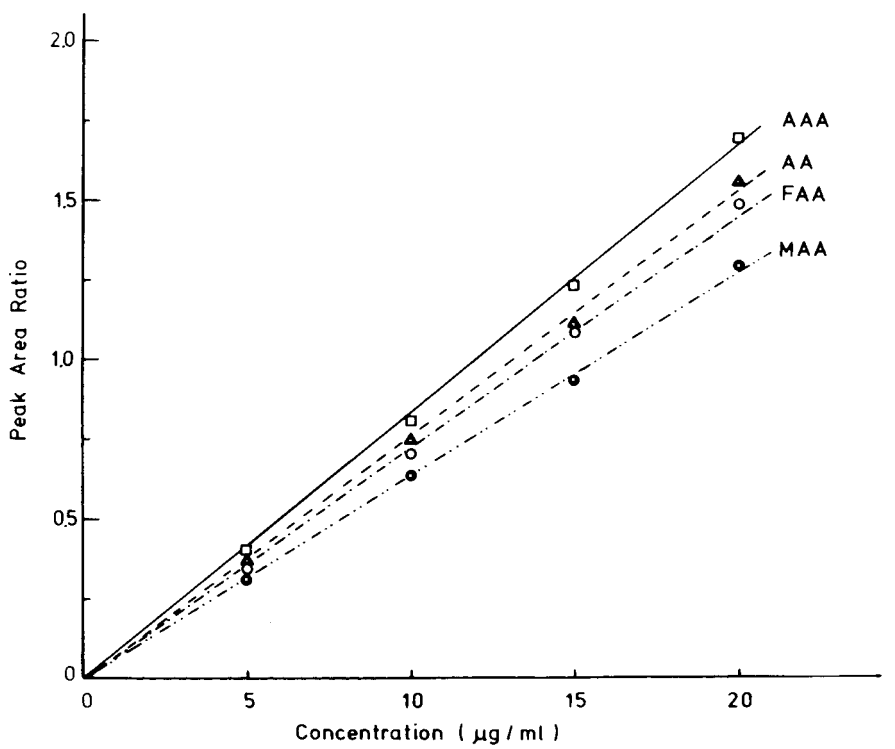


Fig. 4. Calibration curves for dipyrone metabolites, with 10 μg PAA as internal standard.

TABLE I

PRECISION OF THE DETERMINATION OF DIPYRONE METABOLITES APPLIED TO SPIKED HUMAN PLASMA SAMPLES

$n = 6$.

| Metabolite | Within-day precision range | | Day-to-day precision range | |
|---|---|---|---|---|
| | μg/ml (± S.D.) | C.V. (%) | μg/ml (± S.D.) | C.V. (%) |
| MAA | 9.99 ± 0.33 | 3.3 | 9.99 ± 0.66 | 6.7 |
| | 5.00 ± 0.18 | 3.6 | 4.99 ± 0.31 | 6.2 |
| AA | 9.99 ± 0.15 | 1.5 | 10.00 ± 0.43 | 4.3 |
| | 5.00 ± 0.10 | 2.0 | 5.00 ± 0.12 | 2.4 |
| FAA | 9.99 ± 0.16 | 1.6 | 10.00 ± 0.51 | 5.1 |
| | 5.00 ± 0.16 | 3.1 | 4.99 ± 0.14 | 2.8 |
| AAA | 10.0 ± 0.25 | 2.5 | 10.00 ± 0.50 | 5.0 |
| | 4.99 ± 0.13 | 2.5 | 5.01 ± 0.15 | 3.2 |

exactly the same amount of internal standard being added after the extraction. The recovery was essentially complete for the FAA and AAA metabolites (96.0—100%) and ranged from 70.6% to 87.8% for MAA and AA. With a plasma sample volume of 1.0 ml, concentrations as low as 0.1 μg/ml for the four metabolites can be detected.

## HUMAN EXPERIMENT

A healthy male volunteer (age 29 years, weight 100 kg) gave written consent to participate in the study. He was given 1.0 g of dipyrone [two Novalgin (Hoechst) tablets] orally with 200 ml of water following an overnight fast. Venous blood samples (10 ml) were drawn at 0, 1, 2, 4, 6, 8, 10, 14, 24, 36, and 48 h after ingestion; the plasma was separated and analysed as described. Chromatograms of the samples drawn at 4, 10, and 24 h are shown in Fig. 5. Results of the analysis were plotted semilogarithmically as concentration—time curves (Fig. 6). The major metabolite detected in the first 6 h following administration was MAA. Peak values were measured at 2 h. Corresponding with the decrease in MAA levels, AA and FAA concentration increased between 4 and 6 h after dipyrone administration. AAA appeared later than the other metabolites. These observations served as preliminary data for the planning of further pharmacokinetic studies.

A quantitative determination of dipyrone metabolites in plasma is needed for studies on the pharmacokinetics of the drug. The HPLC method described is easy to perform giving a good separation between all metabolites. This could be achieved without any derivatization steps. The separation of FAA and AAA depends, according to our previous experience, on the characteristics of the mobile phase.

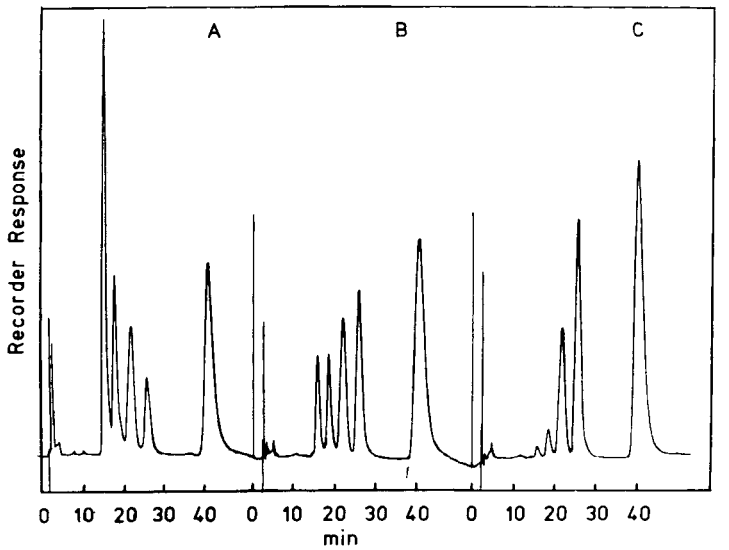


Fig. 5. Chromatograms of plasma samples drawn at 4 (A), 10 (B) and 24 (C) h following the oral administration of 1 g of dipyrone to a healthy volunteer. The metabolite concentrations measured were: (A) MAA 6.0, AA 2.0, FAA 1.3 and AAA 0.4 μg/ml; (B) MAA 1.3, AA 1.0, FAA 1.2 and AAA 1.6 μg/ml; (C) MAA 0.12, AA 0.24, FAA 0.9 and AAA 1.8 μg/ml. A constant 2.5 μg internal standard (PAA) was included in each sample. For retention times see text.

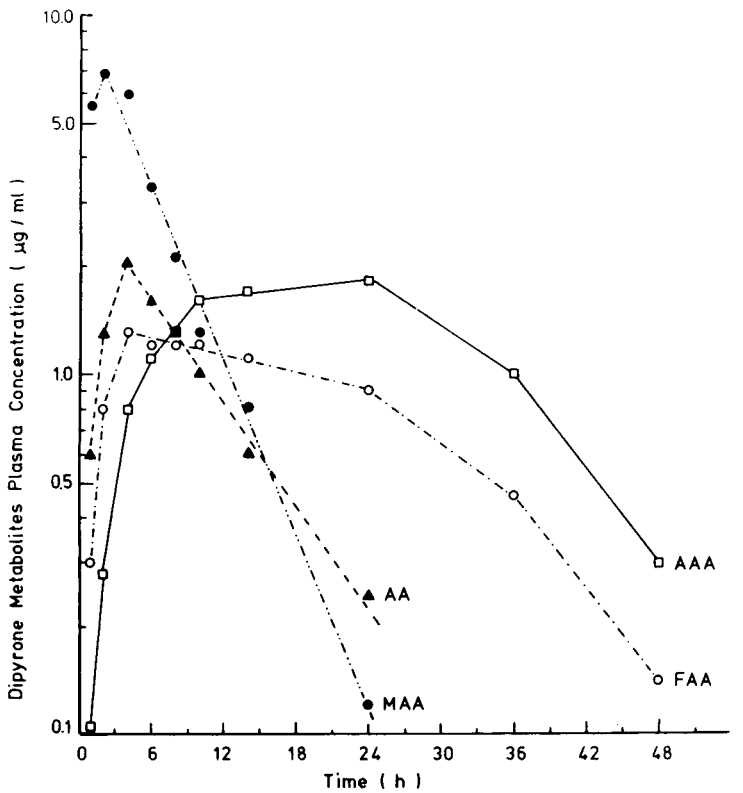


Fig. 6. Plasma concentration—time curves of dipyrone metabolites following the oral administration of 1.0 g of dipyrone to a healthy volunteer.

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## Note

# Reversed-phase high-performance liquid chromatographic determination of salbutamol in rabbit plasma

NAHOKO KUROSAWA, SEIJI MORISHIMA, EIJI OWADA and KEIJI ITO*

*Hokkaido Institute of Pharmaceutical Sciences, 7-1 Katsuraoka-cho, Otaru, Hokkaido 047-02 (Japan)*

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Although salbutamol, $\alpha^1$-[(*tert.*-butylamino)methyl]-4-hydroxy-*m*-xylene-$\alpha,\alpha'$-diol, is widely used as a bronchodilator drug, the pharmacokinetic studies of this drug are limited because of the difficulty of determining the blood levels. Evans et al. [1] reported the disposition of salbutamol in various animals using radioactive salbutamol. Martin et al. [2] and Leferink et al. [3] developed a gas chromatographic—mass spectrometric method to determine salbutamol concentrations in human plasma. However, these methods are very complicated and technically limited. Recently, a high-performance liquid chromatographic (HPLC) method using two columns (ion-exchange column and reversed-phase column) connected to an electrochemical detector was reported by Oosterhuis and Van Boxtel [4]. In spite of the high sensitivity and selectivity, the method seems to have some drawbacks of short lives of columns and detector.

In this report we describe a new HPLC method to study the relations between administration routes and bioavailability in rabbits. This method includes the clean-up procedure by Sep-Pak cartridge and fluorometric determination after separation on a reversed-phase column. Salbutamol concentrations in rabbit plasma could be determined without any interference after oral, rectal and intravenous administration.

## EXPERIMENTAL

### *Materials and reagents*

Salbutamol sulphate was obtained from Chemische Fabrik Schweizerhall (Basel, Switzerland) and its free base, which was used as a standard substance,

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was prepared from salbutamol sulphate by the method of Plavšić [5]. Ethenzamide as an internal standard was purchased from Shizuoka Caffeine (Shizuoka, Japan). Sodium-1-heptane sulphonate (specially prepared for ion-pair reagent) was purchased from Nakarai Chemical (Kyoto, Japan). Methanol (HPLC grade, Cica-Merck) was purchased from Kanto Chemical (Tokyo, Japan). Diethyl ether (extra pure reagent, Kanto Chemical) was used after further purification by distillation. The water used was double-distilled in an all-glass still after passage through an ion-exchange column. The other reagents employed were of analytical grade. The Sep-Pak $C_{18}$ cartridge was purchased from Water Assoc. (Milford, MA, U.S.A.).

*Apparatus and conditions*

A Shimadzu (Kyoto, Japan) Model LC-3A high-performance liquid chromatograph equipped with a fluorescence spectromonitor (Shimadzu Model RF-530) was employed. The analytical column and the guard column were Zorbax $C_8$ (25 cm × 4.6 mm, DuPont Instruments) and LiChrosorb RP-8 (particle size 10 μm, RP-GU, Brown Labs.), respectively, and were warmed at 60°C. The mobile phase was a mixture methanol—5.8 m*M* phosphate buffer (pH 6.0) (31:69), containing 5 m*M* sodium heptane sulphonate as an ion-pair reagent. The flow-rate was 1.0 ml/min, and detection wavelengths were 273 nm for excitation and 310 nm for emission.

*Experiment in rabbits*

White rabbits (3.5—4.0 kg) with stomach-emptying rate controlled by the method of Maeda et al. [6] received oral, rectal or intravenous administration of salbutamol (as salbutamol sulphate) in the form of an aqueous solution (4 mg per 0.4 ml per body volume). Heparinized blood samples were taken from the marginal vein over a period of 9 h, then the separated plasma samples were stored at −20°C until analysis.

*Sample preparation*

The Sep-Pak cartridge, connected to a 5-ml syringe as eluent reservoir, was previously washed with 20 ml of methanol—diethyl ether (1:1) and 20 ml of water. Two millilitres of 2.5 m*M* sodium heptanesulphonate, 0.5 ml of plasma sample and 200 μl of ethenzamide aqueous solution (2 μg/ml) were poured into the cartridge, and were passed through with 2 ml of water twice. After centrifuging at 2300 *g* for 10 min to exclude the aqueous solution, the cartridge was twice eluted with 7 ml of methanol—diethyl ether (1:1) and the eluate was evaporated under vacuum at 20°C. The residue was redissolved in 200 μl of the mobile phase, then a 60-μl aliquot of the solution was injected into the chromatograph.

## RESULTS AND DISCUSSION

The separation of salbutamol from biological components using solvent extraction is relatively difficult because of its hydrophilic character. In our preliminary experiments, salbutamol was poorly extracted with diethyl ether and some other organic solvents. Salbutamol extraction using the Sep-Pak cartridge [4] was more effective; however, the extraction ratio from the aque-

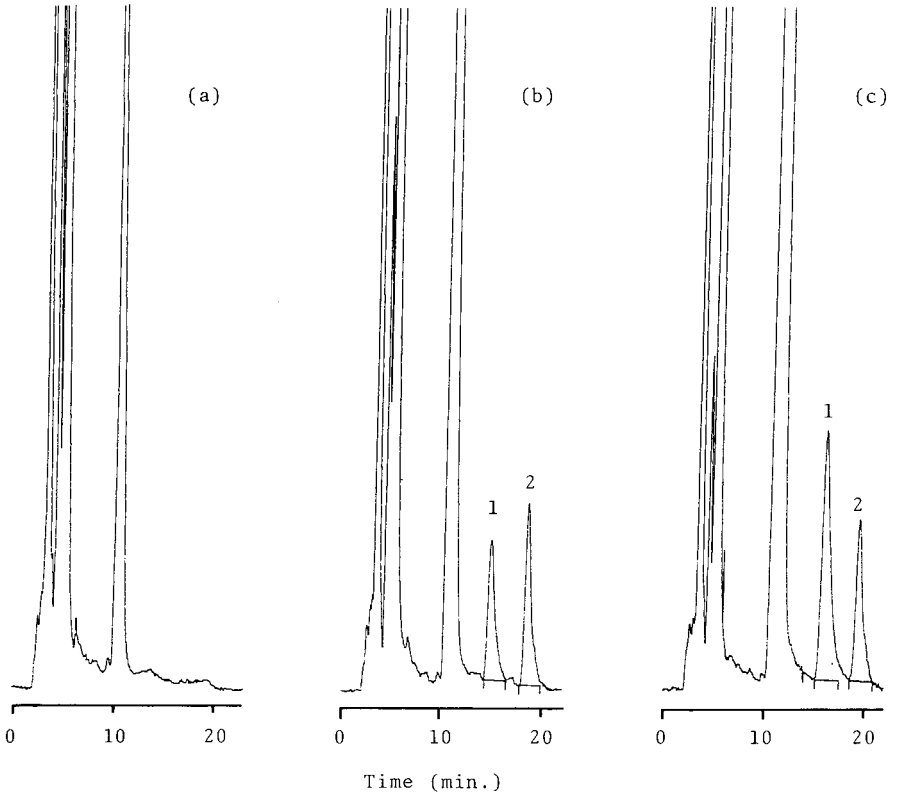


Fig. 1. Chromatograms of (a) rabbit plasma blank, (b) plasma spiked with salbutamol (50 ng) and internal standard, and (c) plasma obtained 1 h after intravenous administration of salbutamol. 1 = salbutamol, 2 = ethenzamide (internal standard).

ous solution was not sufficient: 50.5% ± 16.23 [mean ± C.V. (%), $n$ = 3]. To modify the extraction ratio, we used an ion-pair reagent for the Sep-Pak extraction. In this method, the average extraction ratio was sufficiently high and had less variation: 89.9% ± 2.79 [mean ± C.V. (%), $n$ = 3].

Typical chromatograms of rabbit plasma samples are shown in Fig. 1. The retention times of salbutamol and ethenzamide were approximately 15 and 19 min, respectively. There was no chromatographic interference by plasma constituents.

The calibration curve obtained from the peak height ratios and the amounts of salbutamol extracted from the aqueous standard solution was linear over the range 10—300 ng/ml and almost passed through the origin ($Y = 0.0083X + 0.0174$; $r = 0.999$).

The recovery of the drug from the rabbit plasma was satisfactory as shown in Table I. The minimum detection limit estimated from the signal-to-noise ratio was about 4 ng/ml plasma.

Fig. 2 shows the plasma concentration curves of salbutamol after oral, rectal and intravenous administration of the drug to rabbits. The maximum plasma concentrations after oral and rectal administration were about 30 ng/ml. As these levels were very low compared with that after intravenous administration,

TABLE I

RECOVERY OF SALBUTAMOL FROM RABBIT PLASMA

| Amount added (ng/ml) | Recovery (%) | |
|---|---|---|
| | Mean ± S.D.* | C.V. (%) |
| 10 | 107.6 ± 8.60 | 7.99 |
| 100 | 100.1 ± 2.76 | 2.77 |
| 300 | 104.9 ± 2.75 | 2.62 |

*$n = 5$.

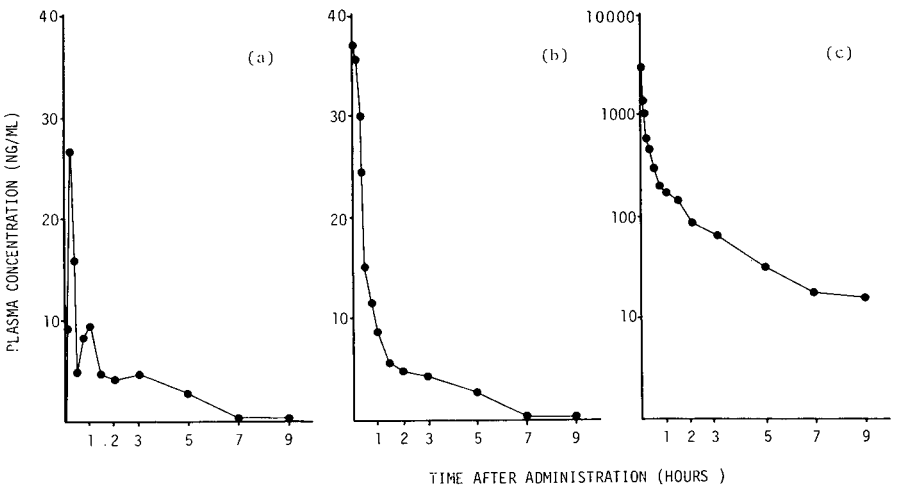


Fig. 2. Plasma concentrations of salbutamol after (a) oral, (b) rectal and (c) intravenous administration of 4 mg of salbutamol to rabbit.

it was suggested that the high metabolic first-pass effect might be in the oral and rectal routes.

In conclusion, the relatively simple method for plasma salbutamol determination described here has enough sensitivity and reproducibility for a salbutamol bioavailability study in rabbits, and could be extended to the determination of the salbutamol conjugates [7] in plasma.

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Note

# Estimation of nadolol levels in plasma using high-performance liquid chromatography with recirculating eluent flow

C.D. KINNEY

*Department of Therapeutics and Pharmacology, The Queen's University of Belfast, The Whitla Medical Building, 97, Lisburn Road, Belfast, BT9 7BL (U.K.)*

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In order to measure plasma levels of nadolol, a long-acting beta-adrenoceptor antagonist drug [1], a reliable and uncomplicated assay procedure is required. Other assays have been described but these have their own problems and disadvantages. The method of Ivashkiv [2] requires several millilitres of plasma and uses a time-consuming acid back-extraction and derivatization procedure, followed by fluorometric quantitation. The assay of Patel et al. [3] does not present an extraction procedure from plasma. Others, Schäfer-Korting and Mutschler [4], Surmann [5] and Funke et al. [6] employ instrumentation which is unavailable to many analysts (fluorodensitometry, high-performance liquid chromatography (HPLC) with electrochemical detection and gas chromatography—mass spectrometry, respectively).

However, most laboratories using HPLC will have the facility of UV detection, and the advantages of simple extraction and recirculated eluent flow make the assay presented a useful tool in the analysis of plasma samples for nadolol. Table I shows the results of some recent work carried out in this department with nadolol.

## MATERIALS AND METHODS

### *Reagents*

Nadolol was supplied by Squibb (Middlesex, U.K.) and acebutolol, internal standard, by May and Baker (Dagenham, U.K.). The extraction solvent was diethyl ether anaesthetic grade and the methanol used was Pronalysis 'Ar', both obtained from May and Baker. Octane sulphonic acid was from Aldrich (Gillingham, U.K.). The water used was double-glass-distilled.

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TABLE I

PLASMA NADOLOL LEVELS OF PATIENT D.O. FOLLOWING ONCE-DAILY ADMINISTRATION OF A 80-mg DOSE

| | Time (h) | Nadolol level (ng/ml) |
|---|---|---|
| Day 1 | 0 | 0 |
| | 2 | 79 |
| | 3 | 70 |
| | 5 | 30 |
| | 8 | 19 |
| Day 2 | 0 | 9 |
| | 3 | 47 |
| Day 3 | 0 | 16 |
| | 3 | 30 |
| Day 5 | 0 | 5 |
| | 3 | 67 |
| Day 6 | 0 | 12 |
| | 2 | 33 |
| | 3 | 52 |
| | 5 | 48 |
| | 8 | 40 |

The following precautions are taken: (1) ensure that all glassware tubes etc. are scrupulously clean, by soaking in methanol overnight; (2) each new bottle of diethyl ether is checked for purity be evaporating a 5-ml aliquot to dryness, and chromatographing a reconstituted fraction.

*Chromatography*

A Shimadzu LC4A liquid chromatograph with a Shimadzu SPD 2AS variable-wavelength UV detector was used with a wavelength of 220 nm and a range of 0.01 a.u.f.s. The recorder was a Perkin-Elmer Model 056. The column used was a 10-cm Hypersil, 5-$\mu$m particle size, ODS column. The eluent was methanol—water (57:43) containing 0.1% octane sulphonic acid, flow-rate 1.0 ml/min. The injector used was either a manual, Rheodyne Model 7125 or an Automatic-Shimadzu Model SIL 2AS, the injection volume used was 10 $\mu$l.

*Calculation*

In order to calculate the amount of nadolol in the test samples, a calibration curve of peak height ratio (nadolol/acebutolol) versus concentration of known

TABLE II

PERCENT EXTRACTION AT CONTROL CONCENTRATIONS OF NADOLOL

| Added (ng/ml) | Percent extraction |
|---|---|
| 20 | 94 |
| 50 | 88 |
| 100 | 106 |
| 200 | 97 |
| Mean $\overline{X}$ | 96 |

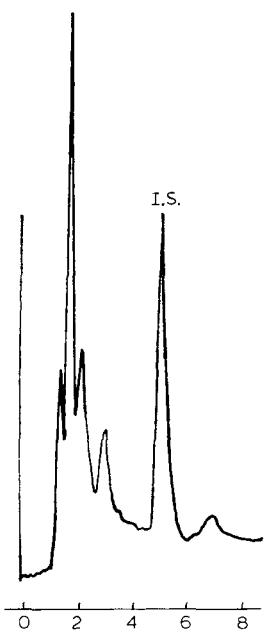
control was drawn. The graph was linear in the range 0—400 ng/ml ($r$ = 0.998) and passed through the origin. From this graph the concentration of the test samples can be derived. Percent extraction at control concentrations can be seen in Table II.

*Extraction*

To 1.0 ml of control or test plasma, were added 100 $\mu$l of acebutolol, the internal standard. To this was added 1.0 ml of a 10 $M$ sodium hydroxide solution, followed by 4.0 ml of diethyl ether, the extraction solvent. The extraction tube was then capped and very briefly vortexed, prior to mixing on a rotary mixer for 15 min. After mixing, the sample was then centrifuged for 5 min at 2000 $g$ and 3.2 ml of supernatant were removed to conical-bottom glass centrifuge tubes and allowed to evaporate to dryness in a water bath at 40°C. The sample was then reconstituted (see Discussion) in 100 $\mu$l of methanol—water (57:43) and 10 $\mu$l were injected into the liquid chromatograph.

*Standards*

The nadolol stock solution was 1 mg/ml in a methanol—water (50:50) solution. From this stock solution was prepared a 20 $\mu$g/ml working standard, which was added to 1 ml of control plasma to give concentrations of 0, 20, 50, 100, 200 ng/ml nadolol (i.e. 0, 1, 2.5, 5, 10 $\mu$l of working standard). These control samples are treated in a similar manner to those of test patient samples.



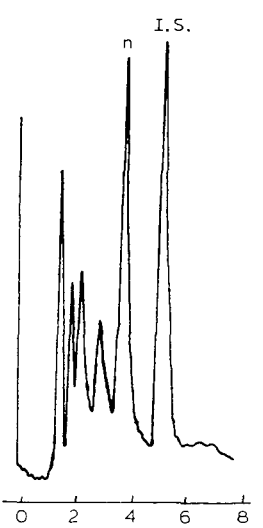




Fig. 1. Chromatogram of a blank patient sample containing internal standard (I.S.), retention time 5.1 min.

Fig. 2. Chromatogram of a patient sample containing 182 ng/ml nadolol (n), retention time 3.8 min, and internal standard (I.S.).

The acebutolol stock solution (internal standard) was 1 mg/ml in a methanol—water (50:50) solution.

From this was prepared a 2.0 μg/ml working standard. A 100-μl aliquot of this solution was added to all tubes (patient and control samples) to give an approximate concentration of 200 ng/ml in plasma.

Typical chromatograms from patient plasma are seen in Figs. 1 and 2.

## RESULTS

In order to ascertain the precision and reproducibility of the assay, three sets of six tubes each containing 1.0 ml of plasma were prepared. In the first set, the concentration of spiked nadolol was 190 ng/ml; second set 95 ng/ml and third 35 ng/ml. The results obtained on analysis of each concentration were as follows: at 190 ng/ml level: coefficient of variation (C.V.) 4.3% (mean = 190.8 ng/ml, S.D. = 8.2 ng/ml); at 95 ng/ml level: C.V. 4.2% (mean = 93.2 ng/ml, S.D. = 3.9 ng/ml); at 35 ng/ml level: C.V. 6.8% (mean = 32.8 ng/ml, S.D. = 2.2 ng/ml).

## DISCUSSION

*Extraction procedure*

*Percentage extraction versus pH (15 min mixing and 5 min centrifugation); see Table III.* From the results shown in Table III the best extraction was obtained using 1.0 ml of 10 *M* sodium hydroxide. Therefore, in order to prevent tube gelling during extraction, the initial vortex must be very brief and also the laboratory temperature* should not be allowed to rise unduly otherwise gelling becomes a problem. Furthermore, any increased mixing time will cause gelling. Also, reduction of mixing time to 5—10 min resulted in a recovery of only 64—83% as opposed to 96% for 15 min mixing time.

*Extraction cleanliness.* (a) In many patient samples a large interfering peak was present (Fig. 3) which appeared just after the nadolol peak. In general this peak was not a problem, but should it be desirable to reduce this peak size, it can be achieved by employing a neutral pH wash, i.e. after adding the internal standard solution to the test or control plasma, 2 ml of diethyl ether were added, the tube was vortexed for 10 sec and centrifuged for 1 min and then the supernatant layer aspirated off to waste. The normal extraction may then proceed by adding the sodium hydroxide solution and diethyl ether etc. Figs. 3 and 4 show one patient sample without and with a neutral pH wash, respectively. With this step, little or no nadolol is lost to waste, and there is also no detriment to the accuracy of the assay.

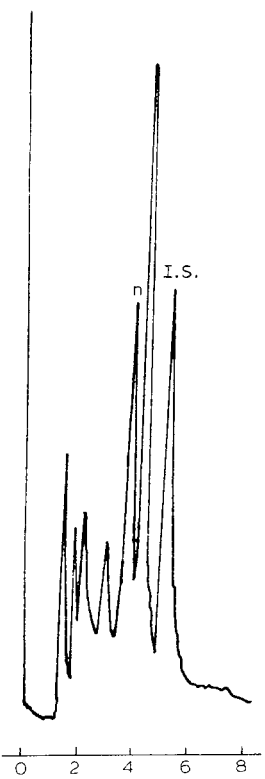
(b) One further point with regard to this peak, is that it would appear from analysing patient plasma samples, that there is a tendency for this peak to increase in magnitude and frequency of occurrence with increased sample storage time. With this point observed, the analysis of samples should be carried out as soon as possible after patient sampling. However, this factor has not

*If the laboratory temperature is very high (> 23°C) chilling of tubes in a freezer (—18°C) prior to centrifugation will alleviate problems with gelling.

TABLE III

PERCENTAGE EXTRACTION VERSUS pH (15 min MIXING AND 5 min CENTRIFUGATION)

| pH | Volume of 10 *M* sodium hydroxide added ($\mu$l) | Percent extraction |
|---|---|---|
| 1.0 | — | Dirty extract |
| 4.0 | — | Dirty extract |
| 7.0 | — | 8 |
| 9.0 | — | 21 |
| 11 | — | 38 |
| 14 | 100 | 53 |
| 14 | 500 | 68 |
| 14 | 700 | 78 |
| 14 | 800 | 94 |
| 14 | 1000 | 96 |



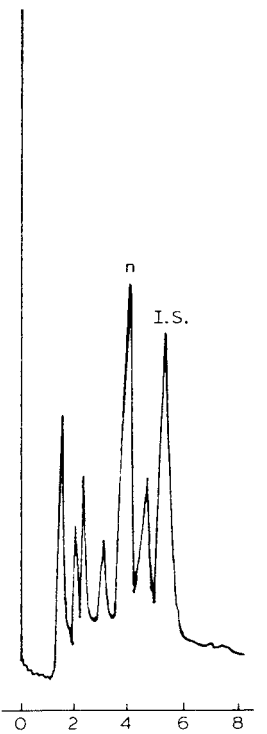




Fig. 3. Chromatogram of a patient sample containing nadolol (n) and internal standard (I.S.). A large peak appears at 4.6 min (without neutral pH diethyl ether wash).

Fig. 4. Chromatogram of a patient sample (as Fig. 3) after neutral pH diethyl ether wash.

been fully investigated as yet, and, as with all plasma sample analyses, it is good policy to analyse as soon as possible.

*Recirculating eluent*

The facilty of recirculated eluent is simply obtained by allowing the effluent from the detector to pass back to the eluent reservoir by means of a piece of tubing. The eluent reservoir should be fairly tightly sealed, as is usual, to prevent any evaporation of constituents which would effect the retention times of the sample components. In general, we used one new batch of 300 ml of eluent for every 100 samples which were chromatographed. In practice this meant making up this volume of eluent every three days and allowing the pump to operate continuously over this period. With this system the baseline drift was never greater than 10% full scale deflection per day and the baseline can be corrected to recorder zero once daily. If the eluent was used for greater than this period of time or number of samples, there was a tendency for the baseline noise to increase which would effect the signal-to-noise ratio of the chromatogram. However, larger volumes of eluent may allow a greater number of samples and longer eluent batch-life to be used. However, the batch of 300 ml suited our particular application needs quite adequately. As already stated the advantages of this system are many, especially with respect to labour saving where it is not necessary to make up a new batch of eluent every day and then wait for the system to equilibrate. This recirculating system has also been used very successfully for other drug analysis procedures within this department. Furthermore, this can be a considerable economy measure, as the amount of materials used is greatly reduced.

*Reconstitution*

Samples should not be reconstituted in eluent as nadolol is not stable in methanol—water containing octane sulphonic acid. Indeed all trace of nadolol had disappeared from the extracts within five days when reconstituted in eluent, whereas no change was seen even after eight days when reconstituted in methanol—water alone.

*Column life*

Although we did not use a guard column, in order to preserve the efficiency of the column, it would possibly be beneficial to employ one if greater number of samples were to be analysed.

## CONCLUSION

From this we may conclude that the assay presented provides a useful method for the analysis of nadolol in patient plasma samples and allows the turnover of many samples per day in a simple and uncomplicated procedure.

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## Note

# Quantitation of 1,3-bis(2-chloroethyl)-1-nitrosourea in plasma using high-performance liquid chromatography

R.L. YEAGER*,*

*Clinical Pharmacokinetics Research Laboratory, Department of Pharmacy, The Clinical Center, N.I.H., Bethesda, MD 20205 (U.S.A.)*

E.H. OLDFIELD

*Surgical Neurology Branch, NINCDS, N.I.H., Bethesda, MD 20205 (U.S.A.)*

and

D.C. CHATTERJI

*Clinical Pharmacokinetics Research Laboratory, Department of Pharmacy, The Clinical Center, N.I.H., Bethesda, MD 20205 (U.S.A.)*

(Received August 11th, 1983)

1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU) is one of the more commonly used nitrosoureas [1—3], and has been shown to be effective in the treatment of certain brain tumors [4]. There is, however, a paucity of information about its pharmacokinetics and disposition in man, attributable partly to the lack of specific and sensitive assays for BCNU in biological samples. In order to study the pharmacokinetics of BCNU after intracarotid administration in patients, a simple and specific BCNU assay sensitive enough to quantitate the drug well into its terminal elimination phase was needed.

A spectrophotometric assay for BCNU was reported [5], but this method lacked sensitivity and specificity. Gas chromatography—mass spectrometry [6] and chemical ionization mass spectrometry [7, 8] assays reported for BCNU have the required sensitivity and specificity, but require sophisticated

*Present address: Merck, Sharp and Dohme Research Laboratories, Merck & Co., P.O. Box 2000, Rahway, NJ 07065, U.S.A.

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instrumentation, sample derivatization and/or labelled materials. A high-performance liquid chromatographic (HPLC) method reported by Krull et al. [9] called for a large sample volume, lacked adequate sensitivity and used phenytoin, a commonly co-administered drug to brain tumor patients as internal standard.

We present a simple, sensitive and selective assay for BCNU in plasma using reversed-phase HPLC. The assay is being used to study the pharmacokinetics of BCNU in humans and monkeys following intracarotid administration.

## MATERIALS AND METHODS

### *Reagents*

BCNU was obtained from Drug Development Branch, NCI, (Bethesda, MD, U.S.A.). Propyl paraben (PP) used as internal standard was purchased from Sigma (St. Louis, MO, U.S.A.). Glass-distilled and certified HPLC-grade acetonitrile was obtained from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Acetic acid (Fisher Scientific, Fairlawn, NJ, U.S.A.), diethyl ether (Mallinckrodt, Paris, KY, U.S.A.), and ethyl alcohol (USPHS, SSC, Perry Point, MD, U.S.A.) were all reagent grade. HPLC solvents were filtered using a 0.45-$\mu$m filter (Millipore, Bedford, MA, U.S.A.) and degassed by applying vacuum.

### *Apparatus*

The chromatographic system consisted of a Spectra-Physics (Santa Clara, CA, U.S.A.) Model 3500B solvent pumping system, equipped with a Schoeffel (Kratos Instruments, Westwood, NJ, U.S.A.) Spectroflow 773 variable-wavelength ultraviolet detector set at 230 nm. The analytical column was an Ultrasphere ODS reversed-phase column, 25 cm × 4.6 mm I.D., 5 $\mu$m particle size, (Altex, Berkeley, CA, U.S.A.), and was preceded by a 7 cm × 2 mm guard column packed with Co:Pell ODS, 30—38 $\mu$m particle size, (Whatman, Clifton, NJ, U.S.A.). The mobile phase was 35% (v/v) acetonitrile and 0.1% glacial acetic acid in water. The flow-rate was 1.2 ml/min and the backpressure was 28 MPa.

### *Procedure*

Blank control plasma was acidified by adding 4% (v/v) glacial acetic acid to a final pH of approximately 4. Blood samples were cooled on an ice-bath as they were drawn, they were centrifuged for 5 min at 2000 *g* while still cool. Plasma was removed and acidified as above, mixed and stored on ice until frozen.

Plasma standards were made fresh in acidified plasma and samples were thawed. To 0.5 ml plasma, treated as above, were added 1.68 $\mu$g of PP [in 5% (v/v) ethyl alcohol in water] as internal standard. The mixture was vortexed for 5 sec and then 4.0 ml of 1.25% (v/v) absolute ethyl alcohol in diethyl ether were added. The samples were mixed on an automatic shaker (Eberbach, Ann Arbor, MI, U.S.A.) for 15 min and centrifuged at 2000 *g* for 10 min. The ethyl alcohol—diethyl ether layer (top) was transferred to clean vials and evaporated under a gentle stream of air at a temperature of 35°C to a dry residue. The samples were then stored in the referigerator until assayed. This

residue was reconstituted with HPLC mobile phase, vortexed for 10 sec and injected into the HPLC system.

BCNU was quantitated by comparison of peak height ratios of drug to internal standard by means of a calibration curve. Calibration curves were analyzed by a weighted least-squares linear regression (weight = $1/c^2$) [10]. Weighted regression analysis was utilized due to the wide range (two orders of magnitude) of sample concentrations. Unweighted regressions were found to be biased toward the higher concentrations with large errors associated with the lower concentrations. The recoveries were determined by comparing absolute peak heights of BCNU and PP with those of aqueous standards injected into the chromatograph each day.

The stability of BCNU in frozen plasma (pH adjusted to 4) was determined by freezing 0.5 ml of plasma standards II, V and VII (0.256, 2.07 and 34.7 μg/ml, respectively). With each curve done over the following month, one of each standard was thawed and assayed. Similarly, the stability of BCNU in refrigerated evaporated plasma extract was measured at the same three concentrations. Plasma standards were extracted as above and refrigerated. With each curve run over the next month, one of each standard was reconstituted and assayed.

## RESULTS AND DISCUSSION

Ethyl alcohol added to diethyl ether increased the recovery of BCNU. However, above a certain concentration interferences also increased. The best solvent for extraction was 1.25% ethyl alcohol in diethyl ether.

Fig. 1 shows representative chromatograms from plasma extracts. Fig. 1A is a chromatogram of blank control plasma. Fig. 1B is of a spiked plasma standard, 0.256 μg/ml with internal standard (PP). Fig. 1C is a patient plasma sample drawn 5 min into a 45-min, 6 mg/kg BCNU infusion. The concentration of this peripheral vein sample was found to be 0.380 μg/ml.

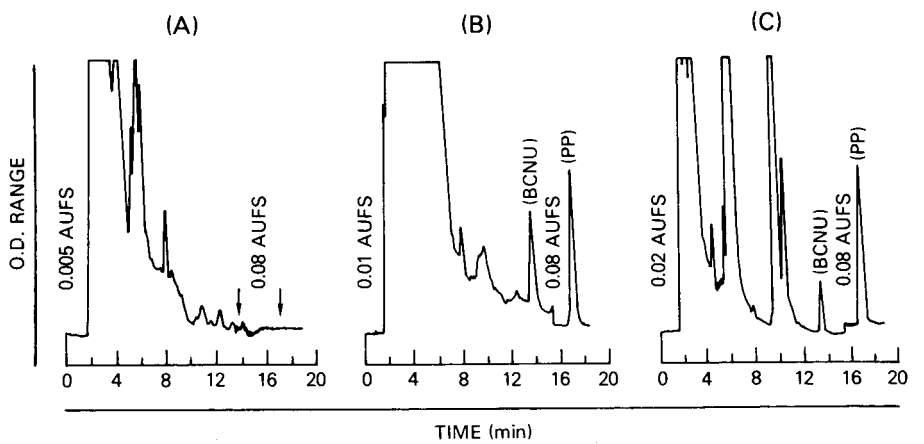


Fig. 1. Representative chromatograms. (A) Plasma blank; (B) spiked plasma standard containing 0.256 μg/ml BCNU and internal standard, PP; (C) representative patient sample following BCNU infusion (assayed and found to contain 0.380 μg/ml BCNU) with internal standard.

Preliminary studies indicated that the half-life of BCNU degradation in plasma at 37°C and pH 7.4 was about 0.25 h [7]. Since nitrosoureas are known to be more stable around pH 4 [11], the pH of the same plasma was adjusted to 4. The half-life increased to 5.3 h at 37°C and pH 4. For this reason, and due to the lack of interferences from adjustment of pH, acetic acid was used to adjust all samples prior to assay to reduce degradation.

The in vitro stability of BCNU at pH 4 in frozen plasma and in refrigerated evaporated plasma extracts was reasonably good. However, the frozen plasma sample chromatograms had more noise. Also after being frozen at pH 4 for more than two or three days the thawed plasma samples were gelled rather than completely liquid. Although these cosmetic changes did not interfere with the assay, sample storage as the refrigerated extract for more than two days is preferred. Samples stored in this manner showed no appreciable loss of BCNU for over a month, but analysis as soon as possible is recommended. Patient samples without pH adjustment were frozen for five days and assayed. BCNU was approximately 70% intact compared to the same samples adjusted to pH 4.

TABLE I

INTER-DAY VARIABILITY

| Standard | I | II | III | IV | V | VI | VII | VIII |
|---|---|---|---|---|---|---|---|---|
| Concentration (μg/ml) | 0.101 | 0.256 | 0.592 | 1.01 | 2.07 | 10.5 | 34.7 | 75.2 |
| Observed | | | | | | | | |
| concentration (μg/ml) | 0.110 | 0.244 | 0.448 | 1.22 | 2.05 | 12.0 | 43.2 | 70.1 |
| | 0.0970 | 0.286 | 0.631 | 0.928 | 1.82 | 12.3 | 37.4 | 71.4 |
| | 0.128 | 0.189 | 0.724 | 1.05 | 1.77 | 13.7 | 42.7 | 59.1 |
| | 0.0992 | 0.266 | 0.633 | 0.923 | 2.32 | 11.3 | 36.3 | 65.1 |
| | 0.0991 | 0.272 | 0.556 | 1.05 | 2.12 | 10.5 | 36.6 | 71.1 |
| Mean | 0.107 | 0.251 | 0.598 | 1.03 | 2.02 | 12.0 | 39.2 | 67.4 |
| S.D. | 0.0130 | 0.0380 | 0.103 | 0.121 | 0.225 | 1.19 | 3.42 | 5.27 |
| C.V. (%) | 12.2 | 15.1 | 17.2 | 11.7 | 11.2 | 9.99 | 8.70 | 7.83 |

Mean C.V. = 11.7%.

TABLE II

INTRA-DAY VARIABILITY

| Standard | II | V | VII |
|---|---|---|---|
| Concentration (μg/ml) | 0.256 | 2.07 | 34.7 |
| Peak height ratios | 0.0430 | 0.371 | 4.45 |
| | 0.0440 | 0.344 | 3.81 |
| | 0.0402 | 0.388 | 4.83 |
| | 0.0428 | 0.347 | 4.54 |
| | 0.0473 | 0.359 | 3.98 |
| Mean | 0.0435 | 0.362 | 4.32 |
| S.D. | 0.00256 | 0.0181 | 0.419 |
| C.V. (%) | 5.90 | 5.01 | 9.70 |

Mean C.V. = 6.87%.

Table I shows the inter-day variability of the assay over a two-week period ($n$ = 5) as demonstrated by observed concentrations. Observed concentrations were calculated by entering individual peak height ratios into the equation of the line for the entire standard curve. The coefficients of variation (C.V.) were consistent over the whole range of the assay and their mean was 11.7%.

Table II reports the intra-day variability of the assay at three concentrations (0.256, 2.07 and 34.7 μg/ml) as demonstrated by a comparison of peak height ratios ($n$ = 5); again the coefficients of variation were consistent over the whole range of the assay, and the mean found to be 6.87%.

The slope of the calibration curve was 0.203 ± 0.020 $(\mu g/ml)^{-1}$ (mean ± S.D.). The mean squared correlation coefficient ($r^2$) was 0.97 and the mean standard error for the slope was less than 10%.

The mean recoveries of BCNU ranged from 47.6 to 73.4% (58.9 ± 9.74%, mean ± S.D.). The retention times were 13.8 min and 16.9 min for BCNU and PP, respectively. The mean signal-to-noise ratio (S/N) for STD I (0.101 μg/ml) was 11.0. Using a S/N ratio limit of 3.0 the assay sensitivity is at least 0.050 μg/ml.

The assay presented here has been shown to be selective and sufficiently sensitive for use in pharmacokinetic analyses. Fig. 2 is a representative concentration versus time profile from a patient receiving BCNU, 220 mg/m$^2$ over 45 min. The reliability and reproducibility are demonstrated by low coefficients of variation for both intra-day and inter-day variability.

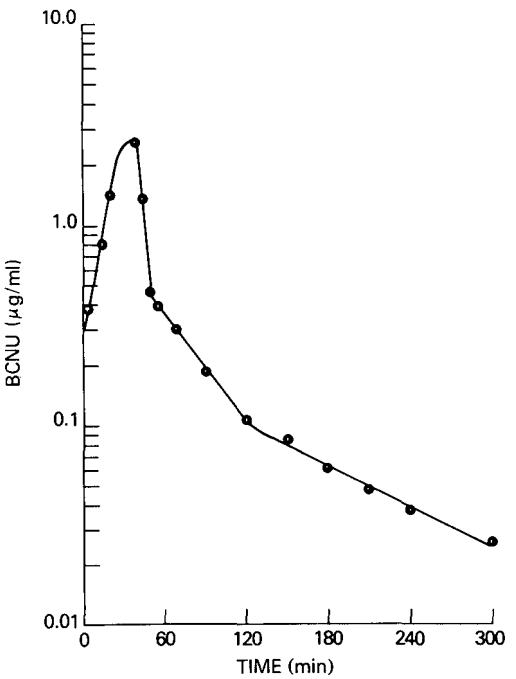


Fig. 2. Representative patient BCNU concentration versus time profile following 387-mg infusion over 45 min. Samples at 180 min and following were assayed by doubling sample volumes.

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Note

# Quantification of antimalarial drugs.

## I. Simultaneous measurement of sulphadoxine, $N_4$-acetylsulphadoxine and pyrimethamine in human plasma

MICHAEL EDSTEIN

*Army Malaria Research Unit, Milpo, Ingleburn, Sydney, N.S.W. 2174 (Australia)*

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Increasing resistance of malaria parasites to 4-aminoquinoline drugs is a major world health problem. Fansidar® (Hoffman-La Roche, Switzerland) is frequently used for the prophylaxis and treatment of malaria in areas where chloroquine-resistant strains of *Plasmodium falciparum* are prevalent [1]. Each tablet of Fansidar contains 500 mg sulphadoxine ($N^1$-5,6-dimethoxy-4-pyrimidinyl-sulphanilamide) (SULPH) and 25 mg pyrimethamine (2,4-diamino-5-*p*-chlorophenyl-6-ethylpyrimidine) (PYR). SULPH and PYR act synergistically to block enzymes in plasmodial pyrimidine synthesis.

Analytical methods for the determination of SULPH include spectrophotometry [2] and high-performance liquid chromatography (HPLC) [3, 4] and for PYR spectrophotometry [5], thin-layer chromatography (TLC) [6—8], gas chromatography (GC) [9, 10] and HPLC [11, 12]. Weidekamm et al. [13] developed a microbiological method for quantifying SULPH and PYR which required the separation of the compounds before analysis. The spectrophotometric methods are not specific, and analyses using the spectrophotometric, TLC, GC and microbiological methods are time-consuming. Recently, Bonini et al. [14] reported a GC method for the determination of SULPH and PYR in blood and urine. In this method the compounds were extracted from biological fluids under acidic and alkaline conditions. A method for the simultaneous quantification of SULPH and PYR would greatly simplify the estimation of Fansidar concentrations in biological fluids.

This paper describes a simple, selective and sensitive HPLC method for simultaneously quantifying SULPH and PYR, and a major metabolite of

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SULPH, $N_4$-acetylsulphadoxine (NASULPH) in human plasma. Reversed-phase ion-pair chromatography was used to separate the compounds and the eluent was monitored for UV absorbance.

## MATERIALS AND METHODS

### *Chemicals and standards*

HPLC grade acetonitrile, methanol and 1-pentanesulphonic acid (PIC B-5) were used (Waters Assoc., Australia). All other reagents were of analytical reagent grade and were used without further purification.

SULPH (Imperial Chemicals Industries, Australia), NASULPH (Hoffmann-La Roche) and PYR (Wellcome, Australia) were donated by the respective companies. A stock standard solution was prepared containing 5.0 mg of SULPH, 0.5 mg of NASULPH and 0.5 mg of PYR per ml of methanol. Intermediate and working standard solutions were prepared by diluting the stock standard solution with methanol. Quinine, as the dihydrochloride salt was used as the internal standard. Solutions were stored at 4°C in amber glass bottles.

A 12 m*M* phosphate buffer solution, pH 3.40, was prepared by adding 0.1 ml of acetic acid to 9.9 ml of phosphate buffer. This solution was used to produce an acidic condition for extraction of the compounds.

### *Instrumentation and chromatographic conditions*

A Pye Unicam LC-XPD pump was used with a Model 440 UV absorbance detector (Waters Assoc.) operated at 254 nm with a sensitivity setting of 0.005 a.u.f.s. A Model 710B sample programmer W.I.S.P. (Waters Assoc.) was used for sample injection and peak areas were measured by a Pye Unicam DP88 integrator. The column was a 30 cm × 3.9 mm I.D., particle size 10 μm, μBondapak $C_{18}$ (Waters Assoc.).

The mobile phase consisting of methanol—acetonitrile—water (25:15:60) containing 0.005 *M* 1-pentane sulphonic acid (pH 3.40) was pumped at a flow-rate of 1.5 ml/min (backpressure of approximately 115 bar) at ambient temperature. The mobile phase was filtered (FHUP 04700, Millipore) prior to use and was purged with helium (50 ml/min) during analysis.

### *Procedure*

To a plasma sample (0.5 ml) in a 15-ml glass culture tube (PTFE-lined screw cap) were added 25 μl of quinine solution (125 ng base per 25 μl), 100 μl of phosphate buffer, pH 3.40, 0.5 ml distilled water and ethylene dichloride (6 ml). The tube was shaken for 20 min on a Dymax shaker (100—120 strokes per min) then centrifuged at 1000 *g* for 10 min to separate the phases. After discarding the aqueous phase, the organic phase was transferred to a clean glass tube and evaporated to dryness at 60°C using a gentle stream of air. The residue was dissolved in 100 μl of the mobile phase and 40 μl of this solution was injected.

To minimise adsorption of the compounds onto glass surfaces, glassware used in extraction was silanised using 0.2% Aquasil (Pierce, Rockford, IL, U.S.A.).

*Calibration*

Calibration curves were prepared by analysing 0.5-ml plasma samples spiked with known amounts of the compounds. The ranges of standards were 0.23—45.45 μg per 0.5 ml for SULPH and 0.023—2.273 μg per 0.5 ml for both NASULPH and PYR, which encompasses the range of plasma concentrations reported by Weidekamm et al. [13] following a single oral dose of Fansidar. Peak area ratios of SULPH/quinine and NASULPH/quinine and the peak height ratio for PYR/quinine were used for calibration. Peak height measurements were found to be more reproducible for PYR quantification than peak area measurements. Calibration standards were run on each day of analysis.

*Determination of precision and recovery*

Within-day and day-to-day reproducibility of the method were determined by repeated assay of several concentrations of each compound. Analytical recovery was determined by comparing peak areas of each compound extracted from spiked plasma with areas obtained by direct injection of the compound.

*Stability of SULPH, NASULPH and PYR*

The stabilities of the compounds were determined by storing plasma standards and working standard solutions for six months at −15°C and 4°C, respectively. Concentrations were determined periodically using the described HPLC method.

## RESULTS

The separation of SULPH, NASULPH, internal standard and PYR extracted from plasma is shown in Fig. 1a. Retention times for SULPH, NASULPH, quinine and PYR were 3.8, 4.7, 7.4 and 9.7 min, respectively. No interfering peaks were present in drug-free plasma extract at the retention times corresponding to NASULPH, quinine and PYR (Fig. 1b). An endogenous component in plasma appearing at a retention time close to that of SULPH is considered negligible when compared to the high therapeutic concentrations of SULPH (i.e. 98.4 μg/ml estimated steady-state concentration) found in plasma [13]. A chromatogram from a plasma sample extract obtained from a volunteer following a single oral dose of Fansidar is shown in Fig. 1c.

Calibration curves for the three compounds showed good linearity with correlation coefficients of 0.995 or better. The limit of quantification was 50 ng/ml for SULPH, 3 ng/ml for NASULPH and 5 ng/ml for PYR. The within-day coefficient of variation averaged 4.6% for SULPH, 5.2% for NASULPH and 2.8% for PYR and the day-to-day coefficient of variation averaged 7.4% for SULPH, 8.0% for NASULPH and 4.7% for PYR (Table I). Extraction recoveries were on average 79, 75 and 86% for SULPH, NASULPH and PYR, respectively (Table II).

No significant degradation was detected for any of the compounds during storage in plasma at −15°C and in methanol at 4°C, for over six months. Interference in the assay was not detected with the following antimalarial drugs: chloroquine, mefloquine, primaquine and proguanil. Maloprim®, because it contains PYR does interfere with the analysis.

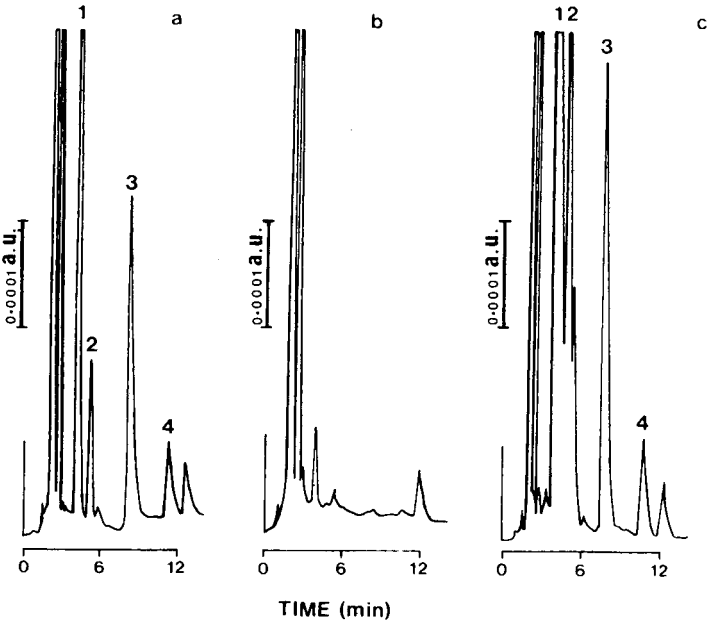


Fig. 1. Chromatograms of (a) extracted spiked plasma sample containing SULPH, 0.45 μg per 0.5 ml (1); NASULPH, 0.045 μg per 0.5 ml (2); internal standard (quinine), 125 ng base (3); and PYR, 0.045 μg per 0.5 ml (4); (b) extracted drug free plasma; and (c) extracted plasma sample obtained 168 h after Fansidar administration to a healthy volunteer (concentrations found in this sample were: SULPH, 19.8 μg per 0.5 ml (1); NASULPH, 0.678 μg per 0.5 ml (2); quinine, 125 ng base (3); and PYR, 0.039 μg per 0.5 ml (4).

TABLE I

PRECISION OF THE HPLC METHOD FOR SULPH, NASULPH AND PYR IN PLASMA (SPIKED SAMPLES)

The number of observations per compound per concentration = 5 in all cases.

| Compound | Concentration (μg per 0.5 ml) | Coefficient of variation (%) | |
|---|---|---|---|
| | | Within-day | Day-to-day |
| SULPH | 0.45 | 6.5 | 13.7 |
| | 1.82 | 4.1 | 6.7 |
| | 4.13 | 4.7 | 4.8 |
| | 22.73 | 2.9 | 4.2 |
| Mean ± S.D. | | 4.6 ± 1.5 | 7.4 ± 4.4 |
| NASULPH | 0.045 | 7.7 | 10.9 |
| | 0.182 | 6.8 | 8.3 |
| | 0.413 | 3.1 | 7.3 |
| | 2.273 | 3.0 | 5.5 |
| Mean ± S.D. | | 5.2 ± 2.5 | 8.0 ± 2.3 |
| PYR | 0.045 | 3.2 | 4.4 |
| | 0.182 | 3.9 | 6.1 |
| | 0.413 | 1.9 | 4.6 |
| | 2.273 | 2.2 | 3.7 |
| Mean ± S.D. | | 2.8 ± 0.9 | 4.7 ± 1.0 |

TABLE II

RECOVERY OF THE HPLC METHOD FOR SULPH, NASULPH AND PYR IN PLASMA

The number of observations per compound per concentration = 5 in all cases.

| SULPH | | NASULPH | | PYR | |
|---|---|---|---|---|---|
| Concentration (μg per 0.5 ml) | Recovery (%, ±S.D.) | Concentration (μg per 0.5 ml) | Recovery (%, ±S.D.) | Concentration (μg per 0.5 ml) | Recovery (%, ±S.D.) |
| 0.45 | 58 ± 6.8 | 0.045 | 71 ± 2.0 | 0.045 | 86 ± 6.8 |
| 1.82 | 80 ± 4.5 | 0.182 | 72 ± 3.7 | 0.182 | 87 ± 7.3 |
| 4.13 | 76 ± 2.4 | 0.413 | 84 ± 1.8 | 0.413 | 83 ± 1.5 |
| 22.73 | 95 ± 1.5 | 2.273 | 76 ± 1.3 | 2.273 | 86 ± 1.3 |
| 45.45 | 84 ± 2.4 | 4.545 | 72 ± 3.1 | 4.545 | 86 ± 4.1 |
| Mean ± S.D. | 78.6 ± 13.5 | | 75.0 ± 5.4 | | 85.6 ± 1.5 |

## DISCUSSION

The ease of the sample preparation using a single-extraction step with ethylene dichloride, the small sample volume required, the low limit of detection of the compounds and the short retention times all contribute to make the present HPLC method suitable for routine analysis of Fansidar. The limit of quantification of the compounds was found to be substantially lower than the expected trough concentrations following recommended prophylactic dosages [13, 15]. The speed of the method was such that 40 samples could be analysed by one operator within 10 h. Recently, the extraction and centrifugation times have been halved without loss of efficiency.

The main advantages of the present HPLC method over the microbiological method of Weidekamm et al. [13] are that, firstly there is no need to separate the compounds before analysis and secondly, NASULPH can also be quantified. A drawback of the GC method of Bonini et al. [14] is the requirement of two extraction steps and the collection of fractions. Previous dedicated HPLC methods for SULPH [3, 4] and PYR [11, 12] are selective and sensitive, but they do not simultaneously quantify the compounds.

Quinine was found to be a good internal standard as it showed reproducible extraction, suitable retention and was well resolved from other peaks. Because quinine is often administered with Fansidar in the treatment of *P. falciparum* malaria [1], other alternative internal standards were investigated. Primaquine, an 8-aminoquinoline antimalarial drug, was extracted using the described conditions and had a retention time of 13.5 min.

Recently, Fansidar resistance has been reported [16, 17]. The monitoring of drug concentrations is required in the studies of Fansidar efficacy because lack of compliance to recommended dosages is a common cause of supposed failure of malaria prophylaxis regimens. The HPLC method described is used in our laboratory both for routine clinical analyses and for pharmacokinetic studies.

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## Note

# Fluorimetric determination of maprotiline in urine and plasma after thin-layer chromatographic separation*

MONIKA PRINOTH and ERNST MUTSCHLER*

*Pharmakologisches Institut für Naturwissenschaftler der Johann Wolfgang Goethe-Universität, Theodor-Stern-Kai 7, 6000 Frankfurt/Main (F.R.G.)*

(First received June 14th, 1983; revised manuscript received September 16th, 1983)

Maprotiline {Ludiomil®, Ciba-Geigy; N-methyl-[3-(9,10-ethanoanthracene-9(10H)-yl)-propyl] aminhydrochloride } is a widely used antidepressive agent.

Several methods for the detection of maprotiline in plasma and urine have already been developed. Riess [1] determined maprotiline by a double-radioisotope derivative technique. Since not every laboratory is equipped with the corresponding instrumentation, a gas—liquid chromatographic method for estimating maprotiline was suggested by Geiger et al. [2]. This procedure involves a re-extraction step and the formation of a derivative, both of which are rather time-consuming. Further gas chromatographic methods for the determination of maprotiline have been introduced by Gupta et al. [3], Sioufi and Richard [4], Charette et al. [5], and Kärkkäinen and Seppälä [6].

For compliance studies it seemed desirable to our group to find a simple and rapid procedure for the quantitative measurement of maprotiline.

The method we developed consists of four steps: (1) extraction from an alkaline sample with *n*-heptane—isopropanol (99:1); (2) derivatization with NBD chloride (7-chloro-4-nitrobenzofurazan); (3) separation of maprotiline from urine or plasma constituents by thin-layer chromatography (TLC); and (4) measuring the fluorescence of the product.

## EXPERIMENTAL

### *Chemicals and materials*

Maprotiline hydrochloride was supplied by Ciba-Geigy (Basle, Switzerland).

*Part of the dissertation of M. Prinoth (Frankfurt/Main).

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Ethanolic solutions of maprotiline, containing 10 mg per 100 ml and 1 mg per 100 ml, were kept in stock.

NBD chloride as well as solvents (analytical grade) and TLC materials were obtained from E. Merck (Darmstadt, F.R.G.). NBD chloride was used as a solution containing 0.1 g in 100 ml of methylisobutyl ketone for extraction.

The TLC plates (20 × 20 cm) used were pre-coated with silica gel 60 (layer thickness 0.25 mm) without a fluorescence indicator and with a concentrating zone (2.5 × 20 cm). The plates were used without any activation prior to use.

*Equipment*

A chromatogram spectrophotometer KM 3 from Carl Zeiss with a Linseis recorder was used.

*Procedure for assay of urine and plasma samples*

*Extraction.* Screw-capped centrifuge glass tubes (not more than 1.2 cm in diameter) are first moistened with 1 ml of a 1 *M* sodium hydroxide solution. Then 1 ml of urine or plasma is added. The contents are then extracted for 30 min on a mechanical shaker with 6 ml of *n*-heptane—isopropanol (99:1). After centrifugation 5 ml of the organic layer are aspirated, transferred to another centrifuge tube and completely evaporated. The evaporation is carried out in a vacuum centrifuge at 30° C (Speed Vac Concentrator, Bachofer Laboratoriumswerke).

*Derivatization procedure.* A 0.1 *M* sodium bicarbonate solution (0.2 ml) and 0.2 ml of a 0.1% NBD chloride solution are added to the extraction residue in the centrifuge tubes. The tube is screwed up tightly and shaken on a vortex mixer for 10 sec. The reaction mixture is kept at 80° C for 3 min. After cooling to room temperature 0.1 g of sodium chloride is added. The contents are then centrifuged for 5 min. At this point it is critical that the tubes are handled carefully so that the two layers do not become mixed again.

*Thin-layer chromatography.* A 50-µl· volume of the upper organic layer is carefully aspirated (from the surface) with a 100-µl Hamilton syringe and applied to a TLC plate in an 8-mm strip with a Linomat III (Camag, Switzerland). Urine or plasma standards of maprotiline of four different concentrations (50, 100, 200 and 500 ng/ml) are also spotted so that a calibration line is included on each plate. The plate is developed (at room temperature) in an unlined glass tank (Desaga) containing 100 ml of chloroform—ethyl acetate (99:1). After developing for 15 cm, the plate is air dried and scanned with a spectrophotometer. On the plate maprotiline is identified by applying its fluorescent derivative without prior extraction ($R_F$ 0.61).

*Densitometric evaluation of the chromatogram.* The spectrophotometer was operated in the fluorescence mode. The light source was a mercury lamp St 41, the 434-nm line being selected for excitation. Emission was filtered with a monochromatic filter having its maximum transmission at 546 nm. The slit chosen was 1 × 6 mm. The peak areas are then calculated. Results are quantified by computing the slope $m$ and the $y$-intercept $b$ of the linear regression curve. This curve links the peak areas to the amount of maprotiline. The unknown $X$ values, representing the amounts of maprotiline, are calculated by means of the linear equation: $Y = mX + b$ ($Y$ = value of the peak areas).

## RESULTS

Recovery studies were performed by analysing spiked urine and plasma samples and comparing the peak areas with those of non-extracted standards. Mean recovery values of 99.8% for urine and 96% for plasma were obtained (mean of three determinations).

The linear behaviour was tested between 0 and 1000 ng/ml with samples containing 0, 5, 10, 20, 50, 100, 200, 300, 500, 700 and 1000 ng/ml maprotiline. For urine, the linear regression curve shows linearity between 20 and 1000 ng/ml with a correlation coefficient of 0.995. The lower limit of detection is 10 ng/ml. For plasma, linearity was tested between 0 and 700 ng/ml with samples containing 0, 5, 10, 20, 50, 100, 200, 300, 500 and 700 ng/ml with a correlation coefficient of 0.999. The lower limit of detection is 20 ng/ml.

Reproducibility experiments were carried out by investigating five samples per concentration and three different concentrations of maprotiline in urine and plasma. The results obtained are summarized in Table I.

TABLE I

SUMMARY OF REPRODUCIBILITY EXPERIMENTS

| Sample | Theoretical amount of maprotiline (ng/ml) | | |
|---|---|---|---|
| | 100 | 200 | 300 |
| Urine | 98 | 204 | 271 |
| | 94 | 206 | 270 |
| | 101 | 198 | 295 |
| | 90 | 204 | 295 |
| | 92 | 201 | 293 |
| Mean | 95.0 | 202.6 | 284.8 |
| C.V. (%) | 4.7 | 1.5 | 4.6 |
| Plasma | 96 | 218 | 313 |
| | 97 | 203 | 306 |
| | 106 | 199 | 310 |
| | 97 | 202 | 296 |
| | 96 | 216 | 292 |
| Mean | 98.4 | 207.6 | 303.4 |
| C.V. (%) | 4.3 | 4.2 | 3.0 |

*Drug interferences*

There are no interferences from normal constituents of urine and plasma with maprotiline (see Fig. 1). No interference is observed by the major metabolite desmethylmaprotiline or by oxaprotiline.

Drugs which can not be determined simultaneously with maprotiline are, for example, desipramine and nortriptyline.

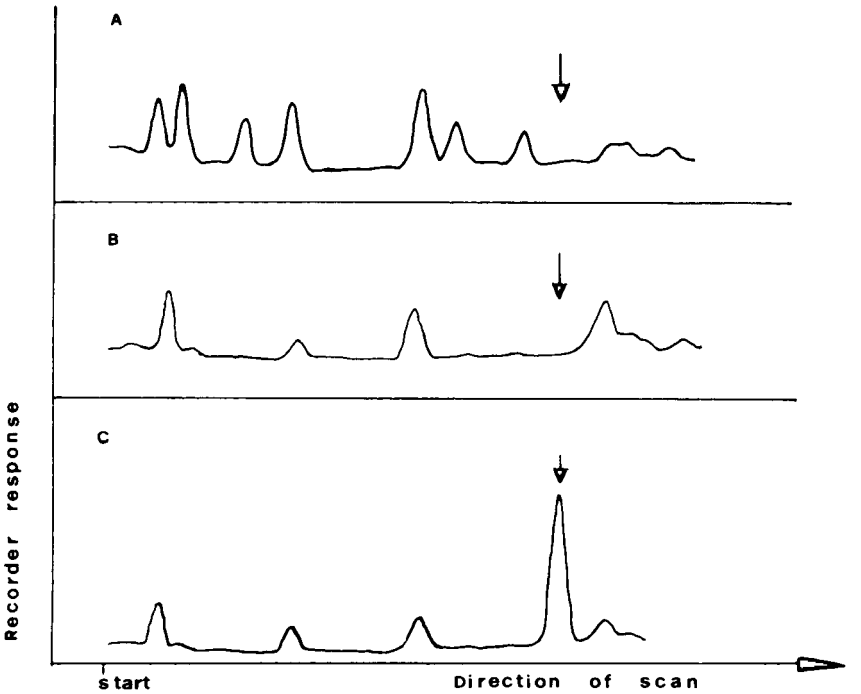


Fig. 1. Representative chromatograms of (A) blank urine, (B) blank plasma, and (C) spiked plasma (50 ng/ml).

The applicability of the method was tested by analysing seven urine samples from patients receiving maprotiline in a daily dose of three times 10—25 mg. The urine was collected and stored at −18°C until analysed. The concentrations found ranged from 122 to 913 ng/ml.

## CONCLUSIONS

The procedure described in this paper allows the determination of maprotiline in a simple and rapid way. After the addition of sodium chloride and final centrifugation of the reaction mixture, attention must be paid to the fact that the two layers can easily become intermixed and thus lead to imprecise results.

## ACKNOWLEDGEMENT

The authors gratefully acknowledge supprot of this study from Dr. Robert-Pfleger-Stiftung, Bamberg, F.R.G.

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4 A. Sioufi and A. Richard, J. Chromatogr., 221 (1980) 393.
5 C. Charette, I.J. McGilveray and K.K. Midha J. Chromatogr., 224 (1981) 128.
6 S. Kärkkäinen and E. Sepälä, J. Chromatogr., 221 (1980) 319.

*Journal of Chromatography*, 305 (1984) 512—513
*Biomedical Applications*
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1921

## Letter to the Editor

### Quantitation of gangliosides in the picomolar range

Sir,

We wish to report a methodology for the reproducible quantitation of picomolar amounts of individual ganglioside species. The procedure, utilizing scanning densitometry of high-performance thin-layer chromatography (HPTLC) plates, requires 75 pmol of ganglioside sialic acid for a triplicate determination and 300 pmol of the ganglioside for a densitometric standard curve. By comparison, spectrophotometric assays require approximately 20 nmol of ganglioside sialic acid for quantitation [1, 2].

HPTLC plates (10 × 10 cm) from E. Merck (Darmstadt, F.R.G., 5 μm particle size silica gel 60) are prewashed by migration in chloroform—methanol (2:1) and activated at 140°C for 10 min. Samples to be quantitated and ganglioside standards in chloroform—methanol (2:1) are applied 10 mm from the plate bottom with a microsyringe (Hamilton No. 7005) in 1—5 μl under a constant flow of warm air (30—40°C). Ganglioside solutions are applied in small overlapping spots which coalesce into 3 × 1 mm bands. The mobile phase is chloroform—methanol—water—1% calcium chloride (60:35:7:1). To insure a saturated atmosphere in the development tank, plates are suspended above the mobile phase for 30 min prior to development. The front is allowed to reach 80 mm above the origin (approximately 25 min). Plates are dried at room temperature and then heated at 140°C for 10 min to remove all traces of solvent prior to spraying with freshly prepared resorcinol—hydrochloric acid reagent [1]. After spraying, plates are covered with clean glass covers and heated at 140°C for 10 min to visualize the blue ganglioside bands.

Ganglioside bands are scanned the same day at 580 nm in the transmission mode at 6 mm/min with a Shimadzu (Kyoto, Japan) dual-wavelength TLC scanning densitometer, Model CS-910. The slit length is adjusted to be 10% greater than the longest band after development and the slit width is 0.2 mm. The peak area of each band is determined after subtraction of the background absorbance at 720 nm by a data processor (Shimadzu C-R1B). Neither zig-zag function nor linearizer program is used.

Densitometric standard curves for gangliosides $G_{M4}$, $G_{M1}$, $G_{D1a}$, $G_{D1b}$ and $G_{T1b}$ are highly linear to 50 pmol of ganglioside sialic acid (Fig. 1). As little as 1 pmol of $G_{M1}$ can be reproducibly detected with a signal-to-noise ratio of

0378-4347/84/$03.00 

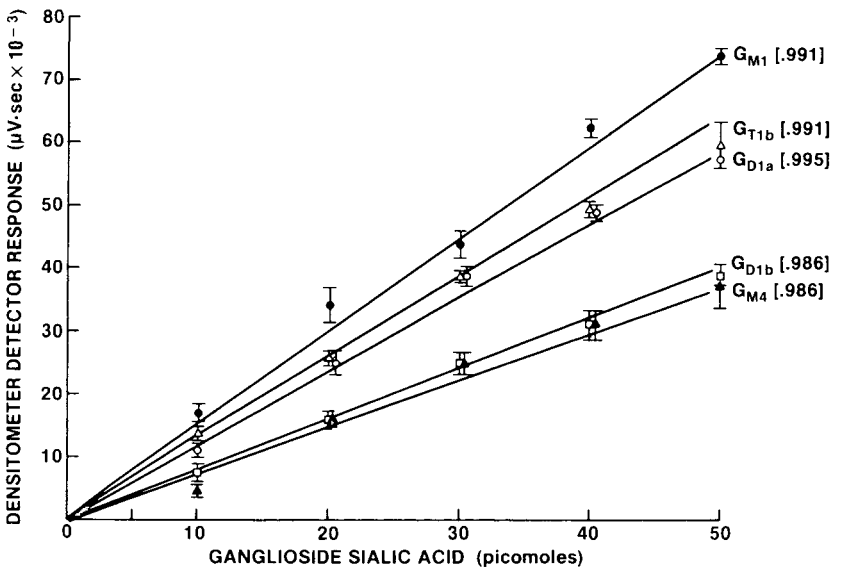


Fig. 1. Ganglioside densitometric standard curves. Densitometric values are the mean ± S.D. of triplicate samples. A separate HPTLC plate was used for each ganglioside species. Correlation coefficients for each standard curve are given in brackets.

5:1. This is 90 times more sensitive than the densitometric procedure of Ando et al. [3]. This enhanced sensitivity allows a more accurate assessment of ganglioside purity. Although sensitivity could be increased further by applying gangliosides in bands less than 3 mm, experimental errors increased significantly due to difficulty in alignment of the densitometer light slit.

To minimize errors due to differences in gel layers, spraying, and ganglioside band geometry [4], gangliosides to be quantitated should be developed on the same HPTLC plate with ganglioside standards used for densitometric standard curves. The variability of this method is 4%.

Picomolar amounts of individual ganglioside species are rapidly quantitated by this method, thus conserving purified gangliosides.

We acknowledge support from the National Multiple Sclerosis Society.

*Uniformed Services University of the Health Sciences, School of Medicine, 4301 Jones Bridge Road, Bethesda, MD 20184 (U.S.A.)*

BRIAN R. MULLIN*
CHARLES M.B. POORE
BONNIE H. RUPP

REFERENCES

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(First received June 27th, 1983; revised manuscript received September 8th, 1983)

*Journal of Chromatography*, 305 (1984) 514
*Biomedical Applications*
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1917

## Book Review

*Iodinated density-gradient media: A practical approach*, edited by D. Rickwood, IRL Press, Oxford, Arlington, VA, 1983, 244 pp., price US $17.00, ISBN 0-904147-51-7.

It is perhaps a little unusual to review in a chromatographic journal a book that is not primarily devoted to chromatographic techniques. However, separation of giant molecules and supramolecular structures that are of fundamental importance in biomedical research is done by density-gradient centrifugation, a fact that is not always recognized by most chromatographers. Moreover, a combination of both approaches, e.g. density-gradient centrifugation with chromatography or electrophoresis, has not yet been exploited, obviously due to the instrumentation problems. On the other hand, such a combination would bring new possibilities to separation science and in the reviewer's eyes it is just a matter of time before these possibilities are exploited. The present volume brings just sufficient information to people in chromatography about other separation techniques and may serve as a source of inspiration.

The book covers separations based on particle size and density and supplies the reader with information about the most common applications to e.g. nucleic acids, nucleoproteins, nuclei, nucleoli, cell membrane fractions, cell organelles, whole cells and viruses, along with an exhaustive bibliography on these topics. It could certainly be recommended to all chromatographers involved in macromolecular separations as supplementary and pleasant reading.

## Related articles published in *Journal of Chromatography*, Vols. 280—283

*Volume 280*



*Volume 281*

*Volume 283*

# Author Index

# Subject Index

Vol. 305, No. 2

JOURNAL OF CHROMATOGRAPHY
BIOMEDICAL APPLICATIONS

# NEWS SECTION

## MEETINGS

### 5th INTERNATIONAL SYMPOSIUM ON MASS SPECTROMETRY IN LIFE SCIENCES, GHENT, BELGIUM, MAY 15–18, 1984

The above symposium is being sponsored by the Faculty of Pharmaceutical Sciences of the State University of Ghent, the National Foundation of Scientific Research (N.F.W.O.–F.N.R.S.) and the Ministry of National Education of Belgium, and the Ministry of Dutch Culture. Contributed papers and posters will cover the following topics: drug metabolism, clinical chemistry, biochemistry, pharmacokinetics, toxicology, ecology and isotope labelling. All papers must be presented in English and no simultaneous translation will be provided. The deadline for receipt of abstracts is February 15, 1984.

Further information may be obtained from: Professor Dr. A. De Leenheer, Laboratoria voor Medische Biochemie en voor Klinische Analyse, Harelbekestraat 72, B-9000 Ghent, Belgium. Tel.: (091) 21 89 51, ext. 324.

### 2nd INTERNATIONAL CONFERENCE ON CHROMATOGRAPHY AND MASS SPECTROMETRY IN BIOMEDICAL SCIENCES, MILAN, ITALY, JUNE 18–20, 1984

The Italian Group for Mass Spectrometry in Biochemistry and Medicine is organizing the above conference. The conference will illustrate and discuss all the latest aspects of chromatography, mass spectrometry (MS) and chromatography–MS, and their areas of application, including biochemistry, medicine, toxicology, drug research, nutrition science and food safety, forensic science, clinical chemistry and pollution.

A major aim of the Conference is to stimulate the exchange of information among scientists working in the above-mentioned fields. Therefore, the conference will consist of lectures by prominent invited speakers, contributed papers and discussions. Facilities will be available for participants to display poster communications. There will also be a book exhibition and displays of manufacturers' literature on chromatography and mass spectrometry and related instrumentation.

For further details, please contact: Dr. Alberto Frigerio, Italian Group for Mass Spectrometry in Biochemistry and Medicine, Via Eustachi 36, I-20129 Milan, Italy; or, Dr. Hubert Milon, P.O. Box 88, CH-1814 La Tour-de-Peilz, Switzerland. Tel.: (24) 42 11 91; Telex: 457302 LINO CH.

INTERNATIONAL SYMPOSIUM ON QUANTITATIVE LUMINESCENCE SPECTROMETRY IN BIOMEDICAL SCIENCES, GHENT, BELGIUM, SEPTEMBER 3–6, 1984

An international symposium on quantitative luminescence spectrometry in biomedical sciences, sponsored by the Faculty of Pharmaceutical Sciences of the State University of Ghent, the National Foundation of Scientific Research (N.F.W.O. – F.N.R.S.), and the Ministry of Education, will be held at the Farmaceutisch Instituut in Ghent.

Contributed papers will cover the following topics: drug and bioanalysis via fluorescence and phosphorescence (LTP, RTP, micellar); fluorescence and chemilumiaescence immunoassays; detection techniques in chromatography (fluorescence, RTPL, etc.); solid surface luminescence methods; chemical derivatization methods; luminescence applications and drug metabolism, clinical chemistry, biochemistry, pharmacokinetics, toxicology, ecology, protein tagging. The conference will be conducted in English, and no simultaneous translation will be provided. Facilities for technical exhibitions will be arranged.

Five plenary lectures will be presented by outstanding specialists in the field of quantitative luminescence spectrometry: R.P. Ekins (fluoroimmunoassays, Institute of Nuclear Medicine, The Middlesex Hospital Medical School, London, U.K.); R.W. Frei (detection techniques, Vrije Universiteit Amsterdam, Amsterdam, The Netherlands); G.G. Guilbault (fluorescence techniques, University of New Orleans, New Orleans, LA, U.S.A.); J.N. Miller (solid surface methods, University of Technology, Loughborough, Leics., U.K.); and J.S. Woodhead (chemiluminescence immunoassays, University of Wales, Cardiff, U.K.).

Before and after the Symposium all correspondence should be sent to: Dr. W. Baeyens, Symposium Chairman, Laboratoria voor Farmaceutische Chemie en voor Ontleding van Geneesmiddelen, Rijksuniversiteit Gent, Harelbekestraat 72, B-9000 Ghent, Belgium.

## CALENDAR OF FORTHCOMING EVENTS

April 8–13, 1984
St. Louis, MO, U.S.A.

**187th National Meeting of the American Chemical Society**
Contact: Meetings Department, American Chemical Society, 1155 Sixteenth Street, NW, Washington, DC 20036, U.S.A.

April 10–13, 1984
Munich, G.F.R.

**9th Conference on Biochemical Analysis (BIOCHEMISCHE ANALYTIK 84) & ANALYTICA 84 Exhibition**
Contact: Secretary General, Dr. Rosmarie Vogel, Abteilung für Klinische Chemie und Klinische Biochemie in der Chirurgischen Klinik Innenstadt der Universität München, Nussbaumstrasse 20, D-8000 München 2, G.F.R. Tel.: (089) 15 30 32; Telex: 5 216 018 bird d. (Further details published in Vol. 276, No. 1.)

April 16–18, 1984
Neuherberg, F.R.G.

**3rd International Workshop on Trace Element Analytical Chemistry in Medicine and Biology**
Contact: Dr. P. Schramel, Gesellschaft für Strahlen- und Umweltforschung, Institut für Angewandte Physik, Physikalisch-Technische Abteilung, Ingolstädter Landstrasse 1, D-8042 Neuherberg, F.R.G.

April 16–19, 1984
Exeter, U.K.

**Royal Society of Chemistry Annual Congress**
Contact: Royal Society of Chemistry, Burlington House, London W1V 0BN, U.K. Tel.: 01-734 9971.

April 16–19, 1984
New York, NY, U.S.A.

**20th International Symposium on Chromatography**
Contact: Professor A. Zlatkis, Chemistry Department, University of Houston, Houston, TX 77004, U.S.A. (Further details published in Vol. 278, No. 2.)

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| April 17–19, 1984<br>Noordwijkerhout,<br>The Netherlands | **Analytical Methods and Problems in Biotechnology – An International Symposium**<br>Contact: W.A. Scheffers, Symposium Analytical Methods and Problems in Biotechnology, Delft University of Technology, Laboratory of Microbiology, Julianalaan 67A, NL-2628 BC Delft, The Netherlands. Tel.: (015)-782411. (Further details published in Vol. 276, No. 2.) |
| April 29–May 4, 1984<br>Rio de Janeiro, Brazil | **12th International Congress of Clinical Chemistry, 7th Latin American Congress of Clinical Biochemistry & 12th Brazilian Congress of Clinical Analysis**<br>Contact: 12th International Congress of Clinical Chemistry, Rua Vicente Licinio 95, Tijuca, 20270 Rio de Janeiro, RJ, Brazil. |
| May 9–11, 1984<br>Dourdan, France | **4th Weurman Flavour Research Symposium**<br>Contact: J. Adda, Laboratoire de Recherches sur les Arômes, 17 rue Sully, 21034 Dijon Cedex, France. |
| May 15–18, 1984<br>Ghent, Belgium | **5th International Symposium on Mass Spectrometry in Life Sciences**<br>Contact: Prof. Dr. A. De Leenheer, Symposium Chairman, Laboratoria voor Medische Biochemie en voor Klinische Analyse, Harelbekestraat 72, B-9000 Ghent, Belgium. Tel.: (091) 21.89.51. |
| May 20–25, 1984<br>New York, NY, U.S.A. | **8th International Symposium on Column Liquid Chromatography**<br>Contact: Professor Cs. Horváth, Mason Laboratory, Yale University, P.O. Box 2159, Yale Station, New Haven, CT 06520, U.S.A. (Further details published in Vol. 272, No. 2.) |
| June 10–14, 1984<br>Toronto, Canada | **International Symposium on Drug Quality Assurance in the Hospital**<br>Contact: Organizing Committee, International Symposium on Drug Quality Assurance in the Hospital, Toronto General Hospital, Public Relations, Bell Wing 1-602, 101 College Street, Toronto, Ontario M5G 1L7, Canada. |
| June 12–14, 1984<br>Szeged, Hungary | **2nd Symposium on the Analysis of Steroids**<br>Contact: Professor S. Görög, Chairman of the Organizing Committee of the 2nd Symposium on the Analysis of Steroids, c/o Hungarian Chemical Society, H-1061 Budapest, Anker köz 1, Hungary. (Further details published in Vol. 278, No. 2.) |
| June 18–20, 1984<br>Milan, Italy | **2nd International Conference on Chromatography and Mass Spectrometry in Biomedical Sciences**<br>Contact: Dr. Alberto Frigerio, Italian Group for Mass Spectrometry in Biochemistry and Medicine, Via Eustachi 36, I-20129 Milan, Italy; or, Dr. Hubert Milon, P.O. Box 88, CH-1814 La Tour-de-Peilz, Switzerland. Tel: (24) 42 11 91; Telex: 457302 lino ch. |
| June 18–21, 1984<br>Ronneby, Sweden | **International Symposium on Liquid Chromatography in the Biomedical Sciences**<br>Contact: Swedish Academy of Pharmaceutical Sciences, P.O. Box 1136, S-11181 Stockholm, Sweden. (Further details published in Vol. 276, No. 2.) |
| June 20–22, 1984<br>St. Andrews, U.K. | **Quality Control of Packaging: Food, Beverages, Pharmaceuticals and Cosmetics**<br>Contact: Royal Society of Chemistry, Burlington House, London W1V 0BN, U.K. Tel.: 01-734 9971. |

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| July 29–Aug. 3, 1984<br>Washington, DC, U.S.A. | **36th National Meeting of the American Association for Clinical Chemistry**<br>Contact: American Association for Clinical Chemistry, 1725 "K" Street, NW, Washington, DC 20006, U.S.A. |
| Aug. 21–24, 1984<br>Colombo, Sri Lanka | **Analytical Chemistry in Development**<br>Contact: Centre for Analytical Research and Development, Department of Chemistry, University of Colombo, Colombo, Sri Lanka; or, Trace Analysis Research Centre, Chemistry Department, Dalhousie University, Halifax, N.S. B3H 4J1, Canada. |
| Aug. 26–31, 1984<br>Philadelphia, PA, U.S.A. | **188th National Meeting of the American Chemical Society**<br>Contact: A.T. Winstead, American Chemical Society, 1155 16th Street, NW, Washington, DC 20036, U.S.A. |
| Aug. 26–Sept. 1, 1984<br>Cracow, Poland | **EUROANALYSIS V – 5th European Conference on Analytical Chemistry**<br>Contact: Professor Zygmunt Kowalski, Secretary-General, Euroanalysis V, Academy of Mining and Metallurgy, Mickiewicza 30, 30-059 Kraków, Poland. (Further details published in Vol. 261, No. 3.) |
| Aug. 27–31, 1984<br>Göttingen, F.R.G. | **Electrophoresis 84. 4th International Meeting of the Electrophoresis Society**<br>Contact: Dr. V. Neuhoff, Max-Planck-Institut für experimentelle Medizin, Hermann-Rein-Strasse 3, D-3400 Göttingen, F.R.G. Tel.: 0551-303248. |
| Sept. 2–6, 1984<br>Hradec Králové,<br>Czechoslovakia | **4th International Symposium on Isotachophoresis – ITP 84**<br>Contact: ITP 84, Dr. Z. Prusik, C.Sc., Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, Flemingovo nám. 2, CS-166 10 Praha 6, Czechoslovakia. (Further details published in Vol. 272, No. 2.) |
| Sept. 3–6, 1984<br>Ghent, Belgium | **International Symposium on Quantitative Luminescence Spectrometry in Biomedical Sciences**<br>Contact: Dr. W. Baeyens, Symposium Chairman, Laboratoria voor Farmaceutische Chemie en voor Ontleding van Geneesmiddelen, Rijksuniversiteit Gent, Harelbekestraat 72, B-9000 Ghent, Belgium. |
| Sept. 10–14, 1984<br>Szeged, Hungary | **Advances in Liquid Chromatography (4th Annual American–Eastern European Symposium on Liquid Chromatography; and, International Symposium on Thin-Layer Chromatography with Special Emphasis on Overpressured Layer Chromatography)**<br>Contact: Dr. Huba Kalász, Department of Pharmacology, Semmelweis University of Medicine, P.O. Box 370, Budapest 1445, Hungary; or, Dr. E. Tyihák, Research Institute for Plant Protection, P.O. Box 102, Budapest 1525, Hungary. (Further details published in Vol. 278, No. 2.) |
| Sept. 23–28, 1984<br>Philadelphia, PA, U.S.A. | **11th Annual Meeting of the Federation of Analytical Chemistry and Spectroscopy Societies**<br>Contact: R.F. Hirsch, Division of Analytical Chemistry, American Chemical Society, 304 Beach Wood, Orange, NJ 07050, U.S.A. |
| Oct. 1–5, 1984<br>Nürnberg, G.F.R. | **15th International Symposium on Chromatography**<br>Contact: Gesellschaft Deutscher Chemiker, Abteilung Fachgruppen, Postfach 90 04 40, Varrentrappstrasse 40–42, D-6000 Frankfurt (Main), G.F.R. (Further details published in Vol. 281.) |

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| Oct. 8–10, 1984<br>Tarrytown, NY, U.S.A. | **3rd International Symposium on Capillary Chromatography**<br>Contact: Dr. A. Zlatkis, Chemistry Department, University of Houston, Houston, TX 77004, U.S.A. |
| Oct. 24–26, 1984<br>Montreux, Switzerland | **3rd Workshop on LC–MS and MS–MS**<br>Contact: Prof. Dr. R.W. Frei, Department of Analytical Chemistry, Free University, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands. (Further details published in Vol. 276, No. 1.) |
| Nov. 11–16, 1985<br>Yalta, U.S.S.R. | **5th Danube Symposium on Chromatography**<br>Contact: Dr. L.N. Kolomiets, The Scientific Council of Chromatography, Academy of Sciences of the U.S.S.R., Institute of Physical Chemistry, Lenin-Prospect 31, Moscow 117312, U.S.S.R. (Further details published in Vol. 281.) |
| Nov. 19–24, 1984<br>Barcelona, Spain | **EXPOQUIMIA 84 – Salón Internacional de la Quimica**<br>Contact: EXPOQUIMIA, Feria de Barcelona, Barcelona 4, Spain. |
| Nov. 22–24, 1984<br>Barcelona, Spain | **14th Annual Symposium on Analytical Chemistry of Pollutants**<br>Contact: 3rd International Congress on Analytical Techniques in Environmental Chemistry/EXPOQUIMIA, Av. Reina Ma. Christina, Palacio No. 1, Barcelona 4, Spain. Tel.: 223.31.01; telex: 50458 FOIMB-E. |
| Nov. 22–24, 1984<br>Barcelona, Spain | **3rd International Congress on Analytical Techniques in Environmental Chemistry**<br>Contact: 3rd International Congress on Analytical Techniques in Environmental Chemistry/EXPOQUIMIA, Av. Reina Ma. Christina, Palacio No. 1, Barcelona 4, Spain. Tel.: 223.31.01; Telex: 50458 FOIMB-E. |
| Feb. 25–March 1, 1985<br>New Orleans, LA, U.S.A. | **36th Pittsburgh Conference and Exposition on Analytical Chemistry and Applied Spectroscopy**<br>Contact: Linda Briggs, Pittsburgh Conference, 437 Donald Road, Dept. J-005, Pittsburgh, PA 15235, U.S.A. |
| April 28–May 3, 1985<br>Miami Beach, FL, U.S.A. | **189th National Meeting of the American Chemical Society**<br>Contact: Meetings Department, American Chemical Society, 1155 Sixteenth Street, NW, Washington, DC 20036, U.S.A. |
| July 1–5, 1985<br>Edinburgh, Scotland, Great Britain | **9th International Symposium on Column Liquid Chromatography**<br>Contact: J.H. Knox, Department of Chemistry, University of Edinburgh, Edinburgh EH9 3JJ, Scotland, Great Britain. |
| Sept. 8–13, 1985<br>Chicago, IL, U.S.A. | **190th National Meeting of the American Chemical Society**<br>Contact: Meetings Department, American Chemical Society, 1155 Sixteenth Street, NW, Washington, DC 20036, U.S.A. |
| Sept. 9–13, 1985<br>Manchester, Great Britain | **30th International Congress of Pure and Applied Chemistry**<br>Contact: The Royal Society of Chemistry, Burlington House, London W1V 0BN, Great Britain. |

## NEW BOOKS

**Prostaglandins and related substances** (*New Comprehensive Biochemistry*, Vol. 5), edited by C. Pace-Asciak and E. Granström, Elsevier, Amsterdam, New York, 1983, XX + 232 pp., price US$ 49.75 (U.S.A. and Canada), Dfl. 129.00 (rest of world), ISBN 0-444-80517-6.

**Extrahepatic drug metabolism and chemical carcinogenesis** (*Proc. Int. Meet., Stockholm, May 17–20, 1983)*, edited by J. Rydström, J. Montelius and M. Bengtsson, Elsevier, Amsterdam, New York, 1983, 648 pp., price US$ 99.75 (U.S.A. and Canada), Dfl. 259.00 (rest of world), ISBN 0-444-80538-9.

**Current and future therapies with insulin** (*Proc. 1st Int. Symp. Treatment of Diabetes Mellitus, Nagoya, September 30–October 1, 1982) (International Congress Series*, No. 607), edited by N. Sakamoto and K.G.M.M. Alberti, Excerpta Medica, Amsterdam, New York, 1983, 432 pp., price US$ 88.50 (U.S.A. and Canada), Dfl. 230.00 (rest of world), ISBN 0-444-90302-X.

**Theoretical drug design methods** (*Pharmacochemistry Library*, Vol. 7), by R. Franke, Elsevier, Amsterdam, Oxford, New York, Tokyo, 1984, *ca.* 432 pp., price US$ 83.00 (U.S.A. and Canada), Dfl. 195.00 (rest of world), ISBN 0-444-99634-6.

**Quantitative analysis of steroids** (*Studies in Analytical Chemistry*, Vol. 5), by S. Görög, Elsevier, Amsterdam, Oxford, New York, Tokyo, 1983, 440 pp., price US$ 95.75 (U.S.A. and Canada), Dfl. 225.00 (rest of world), ISBN 0-444-99698-2.

**Auranofin** (*Proc. SK&F Int. Symp., November 14–16, 1982, Amsterdam; Current Clinical Practice*, Vol. 7), edited by H.A. Capell, D.S. Cole, K.K. Manghani and R.W. Morris, Elsevier, Amsterdam, Oxford, New York, Tokyo, 1983, 510 pp., price US$ 149.00 (U.S.A. and Canada), Dfl. 350.00 (rest of world), ISBN 0-444-90334-8.

**Progress in medicinal chemistry, Vol. 20,** edited by G.P. Ellis and G.B. West, Elsevier, Amsterdam, Oxford, New York, Tokyo, 1983, VIII + 368 pp., price US$ 108.00 (U.S.A. and Canada), Dfl. 254.00 (rest of world), ISBN 0-444-80501-X.

**Clinical experiences with Norcuron®** (*Current Clinical Practice*, Vol. 6), edited by S. Agoston, Excerpta Medica, Amsterdam, New York, 1983, 98 pp., price US$ 25.50 (U.S.A. and Canada), Dfl. 60.00 (rest of world), ISBN 0-444-90331-3.

**Chromatography of antibiotics – second, completely revised edition** (*Journal of Chromatography Library*, Vol. 26), by G.H. Wagman and M.J. Weinstein, Elsevier, Amsterdam, Oxford, New York, Tokyo, 1984, *ca.* 530 pp., US$ 125.50 (U.S.A. and Canada), Dfl. 295.00 (rest of world), ISBN 0-444-42007-X.

**Elsevier's Dictionary of chemistry including terms from biochemistry – English, French, Spanish, Italian and German,** by A.F. Dorian, Elsevier, Amsterdam, Oxford, New York, Tokyo, 1983, VI + 686 pp., price US$ 125.50 (U.S.A. and Canada), Dfl. 295.00 (rest of world), ISBN 0-444-42230-7.

## PUBLICATION SCHEDULE FOR 1984

*Journal of Chromatography* (incorporating *Chromatographic Reviews*) and *Journal of Chromatography, Biomedical Applications*

| MONTH | D 1983 | J | F | M | |
|---|---|---|---|---|---|
| Journal of Chromatography | 282 | 283<br>284/1 | 284/2<br>285/1 | 285/2<br>285/3<br>286<br>287/1 | The publication schedule for further issues will be published later |
| Chromatographic Reviews | | 300/1 | | | |
| Bibliography Section | | 304/1 | 304/2 | | |
| Biomedical Applications | | 305/1 | 305/2 | 306 | |

## INFORMATION FOR AUTHORS

(Detailed *Instructions to Authors* were published in Vol. 264, No. 3, pp. 491-494. A free reprint can be obtained by application to the publisher.)

**Types of Contributions.** The following types of papers are published in the *Journal of Chromatography* and the section on *Biomedical Applications:* Regular research papers (Full-length papers), Short communications and Notes. Short communications are preliminary announcements of important new developments and will, whenever possible, be published with maximum speed. Notes are usually descriptions of short investigations and reflect the same quality of research as Full-length papers, but should preferably not exceed four printed pages. For review articles, see page 2 of cover under Submission of Papers.

**Submission.** Every paper must be accompanied by a letter from the senior author, stating that he is submitting the paper for publication in the *Journal of Chromatography.* Please do not send a letter signed by the director of the institute or the professor unless he is one of the authors.

**Manuscripts.** Manuscripts should be typed in double spacing on consecutively numbered pages of uniform size. The manuscript should be preceded by a sheet of manuscript paper carrying the title of the paper and the name and full postal address of the person to whom the proofs are to be sent. Authors of papers in French or German are requested to supply an English translation of the title of the paper. As a rule, papers should be divided into sections, headed by a caption (*e.g.,* Summary, Introduction, Experimental, Results, Discussion, etc.). All illustrations, photographs, tables, etc., should be on separate sheets.

**Introduction.** Every paper must have a concise introduction mentioning what has been done before on the topic described, and stating clearly what is new in the paper now submitted.

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# Modern Liquid Chromatography of Macromolecules

by **B.G. BELENKII** and **L.Z. VILENCHIK,** Institute of Macromolecular Compounds, Academy of Sciences of the USSR, Leningrad, USSR

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Researchers, technologists and students in the fields of physics, chemistry, biology and medicine will all find this book of interest. It considers the chromatography of high-molecular-weight compounds and its main theoretical and methodological features.

Recommendations are made for preparing high-performance chromatographic systems and for selecting the optimum conditions for their operation. Particular attention is paid to the problems of interpreting chromatographic data in order to obtain various molecular-weight and structural characteristics of the macromolecules investigated: AMW, MWD, indices of polymer branching, the compositional homogeneity of copolymers, the functionality of oligomers, etc. The authors also provide examples of various combinations of chromatographic and other methods that can be used to analyse complex polymer systems.

Written mainly for the reader who has some practical experience in chromatography, the book also contains much useful information for the beginner.



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