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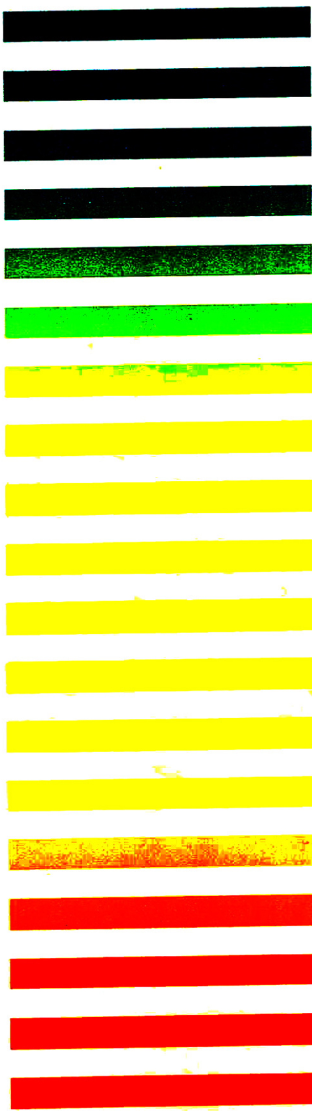

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A NEW CHROMATOGRAPHIC INSTRUMENT FOR MEASURING TRACE CONCENTRATIONS OF BREATH-HYDROGEN

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(First received August 31st, 1981; revised manuscript received December 23rd, 1981)

SUMMARY

A new instrument has been developed which offers many advantages over instruments presently utilized for the measurement of breath-hydrogen used to evaluate the intestinal absorption of sugars. The gas analyzer has a solid-state sensor which is more specific for hydrogen than most conventional chromatographic detectors. Air can be used as the carrier gas and can be circulated with a small internal pump, thereby eliminating large carrier gas tanks and pressure regulators. The intersample time is approximately 2 min, allowing rapid serial analysis of breath samples. A unique feature allows a short-term memory circuit to recall the signal and present it on a digital panel meter in parts per million. Recorder terminals on the back permit the generation of a permanent record, if desired. The gas analyzer is small, lightweight and simple to operate. Its application to the serial measurement of hydrogen in alveolar air after ingestion of sugars is demonstrated.

INTRODUCTION

The intraintestinal production of hydrogen gas results from fermentation of nonabsorbed sugars by intestinal bacteria. Some of this hydrogen is then absorbed and excreted by the lungs [1, 2]. These processes have led to the development of hydrogen breath-analysis tests to diagnose lactose, sucrose, glucose and D(—)-xylose malabsorption, to detect bacterial overgrowth and to measure intestinal transit time.

Gas chromatography (GC) has been most frequently used for the measurement of breath-hydrogen, with the detectors based on thermal conductivity or helium ionization. Instrument development and modifications have permitted sensitive analyses of hydrogen in unconcentrated breath samples from a single exhalation [3, 4], but several disadvantages have been obvious with the application of conventional chromatographic techniques to the measurement: (1) lack

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of portability; (2) requirements for high-pressure carrier gas tanks and regulators; (3) occasional baseline drift; (4) long elution times; and (5) a requirement for technical expertise in instrument operation. In an effort to simplify the measurement of breath-hydrogen, a new instrument has been developed. It is basically a gas chromatograph, but it has been redesigned to eliminate many of the disadvantages of a conventional system applied to the measurement of breath-hydrogen.

Description of instrument

The key element in this redesign is the use of a solid-state sensing device [5] for the detection and measurement of breath-hydrogen. This sensor consists of an N-type semiconductor material (sintered SnO_2) which exhibits a decrease in electrical resistance when combustible or reducing gases are adsorbed on the sensor surface. These resistance changes are relatively large for samples with low reducing-gas concentrations, thus enabling the sensor to accurately and reliably detect low concentrations of such gases. The sensor exhibits a non-linear change with varying concentrations of hydrogen; however, because it is a unimodal (exponential) relationship, the nonlinearity is easily corrected with solid-state analogue circuitry utilized in conjunction with the sensor.

Inasmuch as the detector is essentially insensitive to nonreducing gases, the major gases in expired air (oxygen, nitrogen, argon, carbon dioxide) and in room air (oxygen, nitrogen, argon) produce little or no response from the detector. Thus, the system can utilize room air as the carrier gas, and the molecular sieve column need only separate hydrogen from other gases which might interfere with the measurement, such as carbon monoxide. The specificity of the detector allows the molecular sieve column length to be reduced by a factor of approximately 10, thereby shortening the elution time and lowering the intersample interval to approximately 2 min. The shorter column and the ability of the system to utilize room air as the carrier gas permit the use of a small internal air pump and eliminate the need for large tanks of carrier gas and high-pressure regulators. The overall reduction in size of the instrumentation (approximately 23 × 29 × 37 cm) and its light weight (approximately 7 kg) allow it to be transported, when desired, to field laboratories or outpatient clinics. A digital panel meter has been incorporated into the system to display the linearized detector output, thus eliminating the need for a strip-chart recorder. In addition, an analogue track-hold circuit has been included in the circuit to be used with the panel meter for temporary storage of the linearized detector output. These modifications simplify the calibration procedure and allow the meter to display hydrogen concentration directly in parts per million (ppm). In addition, the linearized detector output signal is available as a separate buffered output signal for connecting to a chart recorder if hard copy is desired.

Gas-flow pattern

Fundamentally, the gas-flow pattern (Fig. 1) is similar to that of a standard thermal conductivity gas chromatograph. The carrier gas (which is room air that is supplied by an internal pump) is initially dried with a molecular sieve pellet column (to protect the GC column) and carried through the sampling

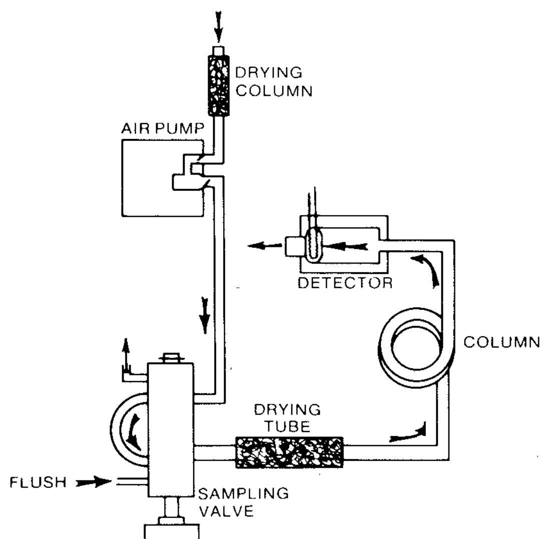

Fig. 1. Gas-flow pattern through the new instrument for measuring breath-hydrogen.

valve and sample loop (8 ml volume). The carrier gas transports the gas sample through an integral drying tube filled with indicating Drierite® to remove moisture from the sample, and then carries it through the GC molecular sieve column. This column separates the hydrogen gas from other combustible gases which might be present in expired air, and allows the hydrogen to be detected without interference. The chromatographic column consists of 5A molecular sieve (60—80 mesh) in 50 cm of 0.635 cm diameter aluminum tubing. The air carrier gas flow-rate is approximately 75 ml/min, delivered at a pressure of about 34 kPa. At this flow-rate, the peak signal for hydrogen is generated at the detector within 11—13 sec after the sample injection. The meter reading returns to baseline (zero) in approximately 100 sec. These time intervals are somewhat variable and depend on column density (packing), system flow-rate and delivery pressure.

Circuit operation

The circuit used to detect and process the signal from the sensor is shown in Fig. 2. The entire system, including the digital panel meter, is powered by a network of +12 V, —12 V, and +5 V power supplies. The sensor has a built-in heater which raises its operating temperature to a range of 200—400°C. The power for this heating element, which is buried in the sensor, is provided by the +5 V regulated power supply. It must be well regulated because the sensing material is considerably affected by small temperature changes.

The +5 V supply also provides power to the sensing element and its series dropping resistance supplied by RPI (Fig. 2). The increase in conductance associated with an increasing concentration of hydrogen provides a voltage signal to amplifiers A1—A2. This sensor output signal is then routed to the linearizer circuit which performs the function $V_{out} = V^{R}{}_{in}$ where R is related to a resistor value selected (for a particular sensor) to produce the most accurate linearizing of the signal. This linearized signal is then summed with a

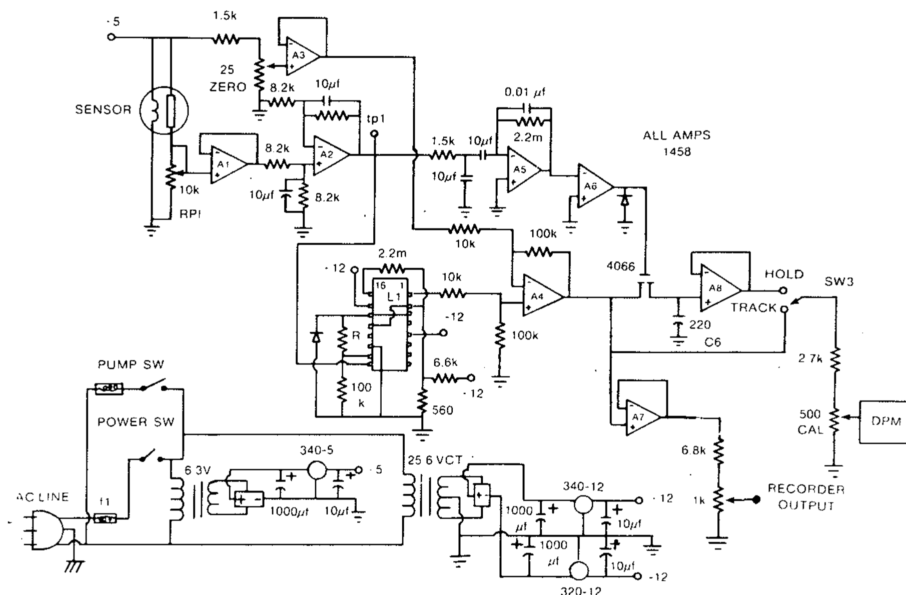

Fig. 2. Electronic circuit diagram for the detector, its amplifier, the linearizing element, and the track-hold system for the new hydrogen-measuring instrument.

zero reference signal (supplied by A3) and amplified A4. The output of A4 is fed into the output buffer amplifier A7 and the track-hold combination of A8—SW3 which supplies the signal to the digital panel meter. The amplifier pair A5—A6 provides a differentiator-driver for the track-hold (short-term memory element). This track-hold element (switch 4066, capacitor C6 and buffer amplifier A8) is used to track the signal to its peak and hold this peak value for the digital panel meter.

This arrangement simplifies the calibration procedure by allowing the digital panel meter to be adjusted to the value of the calibrating gas (by using the 500 Ω calibrate potentiometer) in the hold mode. It will then present the concentration of hydrogen in the unknown sample in ppm.

Performance of the gas analyzer

The solid-state gas sensor used in the hydrogen analyzer exhibited a nonlinear relationship between its conductance and the concentration of hydrogen in the sample at the low concentrations measured for the lactose intolerance test (generally below 100 ppm). Although there is some variability among sensors, Fig. 3 demonstrates a typical curve for sensor conductance and hydrogen concentration. The ordinate represents the voltage drop across a fixed resistor in series with the gas sensor. It is proportional to sensor conductance. The abscissa represents hydrogen gas concentration, with the range achieved by proportioning a known calibration gas (118 ppm hydrogen in air) with room air.

Fig. 4 presents a curve based on the output from the linearizer circuit plotted against hydrogen concentration. Six replicate measurements were made during a 4-h period in which the instrument was set at zero with air and at 118

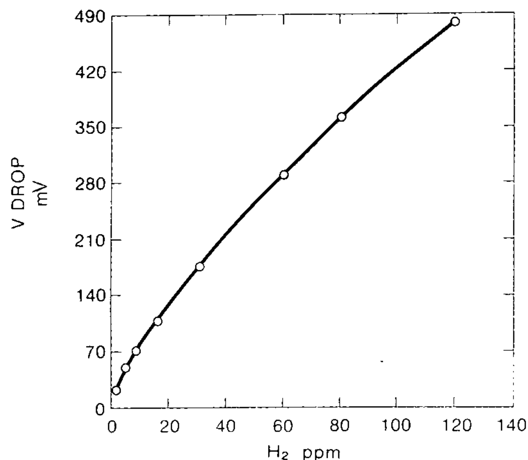

Fig. 3. Conductance change in the sensor (represented by the voltage drop across a fixed resistance in series with the sensor) related to a change in hydrogen concentration in air from 0 to 118 ppm.

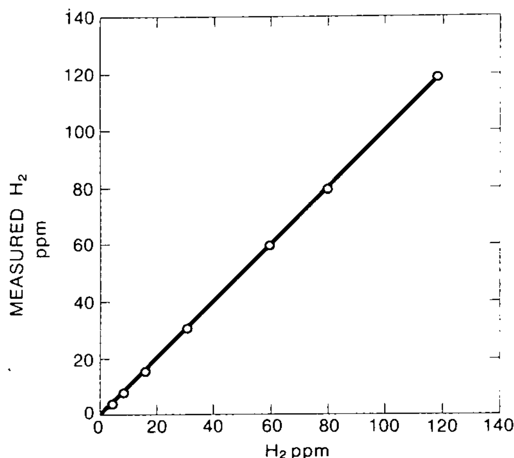

Fig. 4. Relationship between panel meter reading, reflecting the output signal from the linearizer circuit, and the hydrogen concentration in the sample introduced into the instrument. Each point is the mean of six measurements made during a 4-h period.

ppm with the reference gas. The reference gas was then serially diluted with room air to 60, 50, 25, 12.5, 6.25 and 3.12% of its initial hydrogen concentration; the diluted samples were analyzed with the gas analyzer. Both scales on the calibration curve are expressed in ppm hydrogen, with the values on the abscissa calculated to the first place after the decimal from the dilution factor, and the values on the ordinate taken from the digital panel meter display without decimals. The equation for the curve, calculated by the least squares method from the six calibration procedures, was $y = 1.005x - 0.031$. The standard error of estimate for the slope was 0.236 ppm and the standard deviation for the intercept was 0.081 ppm. Thus, the data indicated that the linearity and reproducibility (when calibrated prior to the analysis) were within 0.5 ppm

hydrogen for the range from 0 to 118 ppm. Since the meter readout was in ppm (without decimals), more precision is without meaning.

Because the gas sensor requires a relatively high operating temperature (200—400°C), there is a somewhat extended stabilization period when the instrument is powered up. Fig. 5 shows a typical curve for such stabilization. Conductance decreases with time as the temperature increases. A period of slightly over 2 h is required for equilibration of the system in an average room temperature, as seen in Fig. 5. Linearity will be affected during the stabilization period so that accuracy may be compromised unless adequate warm-up time is allowed. If enough time cannot usually be allowed, it may be more satisfactory to leave the unit on overnight. By using a drying column of molecular sieve

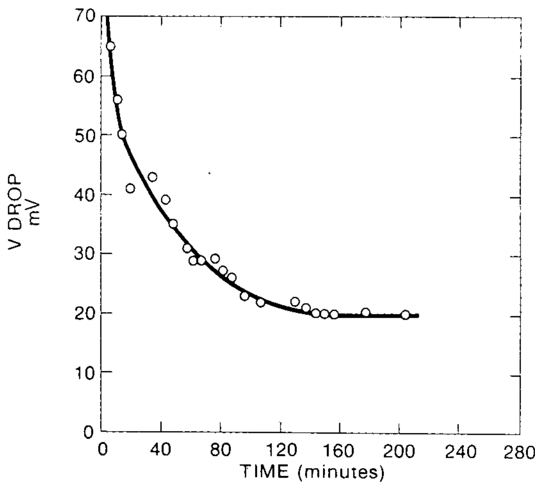

Fig. 5. Changes in conductance of the sensor with time during warm-up, to indicate that a 2-h period is required to assure equilibration and linearity of the system to measure breath-hydrogen.

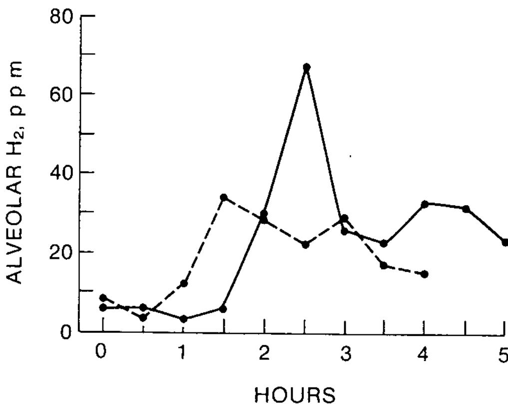

Fig. 6. Serial measurements of breath-hydrogen following sugar ingestion [6]. Solid line is from a lactose-intolerant patient following the ingestion of 18 g lactose (whole cow's milk). Broken line is from a normal subject after the ingestion of 12 g raffinose in 360 ml tomato juice.

pellets on the inlet air supply line, the column will be protected from room air humidity. If it is not protected, the column will be inactivated within a short period of time. The molecular sieve pellets can be reactivated in a drying oven at 300°C for 1–2 h, thereby permitting their use to be cost-effective in allowing continuous availability of the hydrogen gas analyzer.

Fig. 6 shows breath-hydrogen analyses [6] from two subjects to demonstrate the application of the instrument. Alveolar air samples were analyzed in duplicate at 0.5-h intervals following the ingestion of sugar. The broken line represents data following the ingestion of raffinose, an undigestible trisaccharide, which may be used to measure intestinal transit time since it undergoes bacterial fermentation in the large intestine. The solid line represents the hydrogen response following lactose ingestion by a lactose-intolerant patient.

If desired, the output response curves can be recorded with a potentiometric strip-chart recorder. The signal presented to the recorder terminal is from the output of the linearizing circuit and does not represent the track-hold circuit which is accessible to the digital panel meter. The recorded curve can be made a permanent part of the patient's record if needed and, when combined with the response curve from a reference gas, can be used to calculate hydrogen concentration by the conventional chromatographic technique.

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SAMPLE PURIFICATION USING A C_{18}-BONDED REVERSED-PHASE CARTRIDGE FOR THE QUANTITATIVE ANALYSIS OF CORTICOSTEROIDS IN ADRENAL CELL CULTURES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OR GAS CHROMATOGRAPHY—MASS SPECTROMETRY*

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SUMMARY

Quantitative extraction and subsequent purification of small biological samples often involve cumbersome procedures. We have devised a short and efficient method for the quantitative extraction of the corticosteroid and the 20α reduced steroid series from culture medium containing 20% sera in a single, pure fraction with separation from cholesterol. Passage through a C_{18}-bonded reversed-phase Sep-Pak® cartridge of the acidified culture medium and subsequent extraction of the steroid fraction with methanol yields a single fraction containing all steroids in 90% recovery and reduced quantities of cholesterol down to 30%. The extract can then be used without further purification for quantitative analysis by high-performance liquid chromatography or derivatized and analyzed by gas chromatography and gas chromatography—mass spectrometry.

INTRODUCTION

The quantitative analysis of small biological samples is often difficult due to the minute quantities of the compounds to be assayed mixed together with large quantities of interfering materials. The isolation of the compounds to be identified and quantified is usually done by methods of low resolution but which can handle large amounts of materials, separating compounds into groups. The individual separation which allows identification and quantitation is done by methods of low capacity but of high efficiency. While many advances have been achieved in the methods of separation and detection, the

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methods of extraction remain either laborious with minimal recovery or yielding samples of insufficient purity.

The most common extraction methods used for steroids from biological fluids are solvent partitioning and chromatography, using adsorbents such as Amberlite XAD-2 or Sephadex derivatives [1, 2]. Solvent partitioning gives fairly good steroid yield, but usually necessitates some other type of purification such as paper chromatography, thin-layer chromatography (TLC) or some form of column chromatography. Adsorption chromatography is usually done as a multi-column step to yield well-separated, pure samples.

Recently, Sep-Pak® cartridges have been available from Waters Assoc. These are small, pre-packed columns of various supports which have a large variety of uses. The C_{18}-bonded support packed cartridges are especially useful for biological applications. Among other applications they have been used by Hartwick et al. [3] for extraction of serum nucleotides. Very recently, Shackleton and Whitney [4] have described a method using these cartridges for extraction of urinary steroids.

In our laboratory newborn rat adrenal cells grown in culture have been shown to secrete steroid hormones under adrenocorticotropic hormone (ACTH) stimulation, for several weeks [5, 6]. The studies leading to the biochemical definition of this system as well as the evaluation of the effect of hormonal stimulators or inhibiting drugs need a sensitive, accurate analysis of steroid hormones down to the nanogram level. The method used until now has been the classical solvent extraction method, after deproteinization and delipidation, followed by TLC which fractionates the sample into three main fractions [7]. The least polar fraction contains pregnenolone, progesterone, 20α-dihydroprogesterone plus other $C_{21}O_2$ steroids. An intermediate fraction contains deoxycorticosterone and other mono-oxygenated progesterones of the general formula $C_{21}O_3$. The most polar fraction contains 18-hydroxy-11-deoxycorticosterone, corticosterone and other $C_{21}O_4$ steroids. Although cholesterol migrates with the solvent front, it tends to overload the plate and is thus present in all the fractions. This thin-layer purification step yields pure samples, serves to concentrate a compound or a family of compounds into a single fraction, and allows fast analysis of metabolites of radioactive precursors by radioscanning. However, it presents the disadvantage in quantitative analysis of differential steroid extraction according to their polarity. Another inconvenience is the length of this extraction—purification method.

We have developed a one-step extraction procedure using a C_{18} Sep-Pak cartridge system, which allows the processing of numerous samples in a very short period of time, with accurate, high yields. The samples so extracted in a single fraction are pure enough to be analyzed directly by high-performance liquid chromatography (HPLC), gas chromatography (GC), or gas chromatography—mass spectrometry (GC—MS).

MATERIALS AND METHODS

Steroids and reagents

Most of the authentic steroids were purchased from Makor Chemicals (Jerusalem, Israel) and Steraloids (Wilton, NH, U.S.A.); 18-hydroxy-11-deoxycorti-

costerone* was from Searle (Naucalpan, Mexico) and aldosterone from Sigma (St. Louis, MO, U.S.A.).

Labeled [7n-^{3}H] pregnenolone (specific activity, 21 Ci/mmol), [7n-^{3}H] cholesterol (specific activity, 15 Ci/mmol), [4-^{14}C] progesterone (specific activity 51.6 mCi/mmol) and [4-^{14}C] pregnenolone (specific activity, 59 mCi/mmol) were obtained from the Commissariat à l'Énergie Atomique (Saclay, France).

[4-^{14}C] Deoxycorticosterone (specific activity, 55 mCi/mmol) and [4-^{14}C]-cholesterol (specific activity, 59 mCi/mmol) were obtained from New England Nuclear (Boston, MA, U.S.A.). [4-^{14}C] Corticosterone (specific activity 50 mCi/mmol) and [4-^{14}C] cortisol (specific activity, 50 mCi/mmol) were obtained from The Radiochemical Centre (Amersham, Great Britain). All solvents were of the pure for analysis quality from Merck (Darmstadt, G.F.R.). The following reagents were used: O-methoxyamine hydrochloride (MO, Cl) from Pierce Chemical Co. (Rockford, IL, U.S.A.), bis(trimethylsilyl)trifluoroacetamide (BSTFA), trimethylchlorosilane (TMCS) and hexamethyldisilazane (HMDS) from Supelco (Bellefonte, PA, U.S.A.).

Cell cultures

The method was adapted from the one described by Maume and Prost [5]. Adrenals were taken from newborn rats of Wistar US/Commentry (INRA, Dijon, France) strain. They were trypsinized by a solution of trypsin (B Grade, 3000 IU/mg) from Calbiochem (San Diego, CA, U.S.A.) in Ham's F10 medium without Ca^{2+} and Mg^{2+} salts, at a concentration of 1.75 mg/ml in a cell-stir vial. The trypsin action was stopped by the addition of foetal calf serum and the trypsinized fractions were centrifuged. The cell pellets were isolated and suspended in cell-culture medium of the following composition: Ham's F10 medium (Gibco, Paisley, Great Britain), penicillin (10 U/ml) and streptomycin (10 μg/ml), 10% of foetal calf serum and 10% of newborn calf serum. The total cholesterol content in this combination of sera is about 260 μg/ml of culture medium; of this, approximately 62 μg/ml is unesterified cholesterol. Each fraction was poured into a Cooper tissue-culture dish (Falcon, Oxward, CA, U.S.A.) of 5 ml capacity (bottom area 20 cm^2). At confluency the cell monolayer attached to the dish bottom contained between 2 and 3 million adrenocortical cells.

The dishes were kept in a culture oven at 37°C and under an air—CO_2 (95 : 5, v/v) atmosphere saturated with water. When the cultures were seven days old, ACTH from Choay (Paris, France) was added to the dishes in 0.1 ml of medium without serum.

*The following code and trivial names are used: pregnenolone = 3β-hydroxy-5-pregnen-20-one; pregnanediol = 5α-pregnane-3β,20α-diol; progesterone = 4-pregnene-3,20-dione; pregnanolone = 20α-hydroxy-5α-pregnan-3-one; deoxycorticosterone = 21-hydroxy-4-pregnene-3,20-dione; deoxycorticosterone-21-acetate = 21-acetoxy-4-pregnene-3,20-dione; corticosterone = 11β,21-dihydroxy-4-pregnene-3,20-dione; corticosterone-21-acetate = 21-acetoxy-11β-hydroxy-4-pregnene-3,20-dione; deoxycortisol = 17α,21-dihydroxy-4-pregnene-3,20-dione; cortisol = 11β,17α,21-trihydroxy-4-pregnene-3,20-dione; 18-hydroxy-11-deoxycorticosterone = 18,21-dihydroxy-4-pregnene-3,20-dione; 20α-dihydroprogesterone = 20α-hydroxy-4-pregnen-3-one; 2α-hydroxyprogesterone = 2α-hydroxy-4-pregnene-3,20-dione; 11β-hydroxy-20α-dihydroprogesterone = 11β,20α-dihydroxy-4-pregnen-3-one; trilostane (WIN 24540) = 4,5α-epoxy-17β-hydroxy-3-oxo-2α-androstane carbonitrile.

Incubations

Cell-culture medium was usually removed each 24 h. The steroid precursors were added to the dishes in 5 or 10 μl of absolute ethanol. The culture media were gathered and kept at −30°C until steroid extraction was performed.

Reversed-phase chromatography

The steroid extraction was performed on Sep-Pak C_{18} columns from Waters Assoc. (Milford, MA, U.S.A.). These are cartridges, about 1 cm in diameter and 1.5 cm high, with octadecylsilane-bonded packing between two filters. The Sep-Pak columns were first rinsed with 4 ml of methanol, then with 4 ml of 0.01 *M* acetate buffer (pH 4.5). The cell-culture medium buffered to pH 4.5 was then passed through the column at a rate of one drop per sec (3 ml/min). The column was rinsed by 6 ml of 0.01 *M* acetate buffer (pH 4.5) and the steroids were eluted from the column by 3 ml of methanol at a rate of 1.5 ml/min. The sample was dried under a nitrogen stream and the humidity removed by adding a drop of benzene and evaporating again. Extraction yield was measured by adding a radioactive steroid to the culture medium, measuring the radioactivity before extraction and again in the methanol extract.

Delipidation, deproteinization and TLC method

This method, previously described [8], was used as a comparison to the Sep-Pak method. The culture media were deproteinized and delipidated by precipitation with methanol—water (7:3, v/v) at −30°C, followed by centrifugation at −20°C. The methanol was evaporated and the steroids extracted from the remaining aqueous solution three times with ethyl acetate and three times with dichloromethane. The organic phases were pooled, dried over anhydrous magnesium sulfate and concentrated to approximately 100 μl at 65°C under a stream of nitrogen. The steroid extracts were purified and fractionated by TLC on silica gel (60 F 254) with fluorescent indicator (Merck). They were run first with diisopropyl ether and then with diisopropyl ether—acetone (6:4, v/v). The silica gel of the different fractions was scraped off and the steroids extracted by methanol and dichloromethane using an ultra-sonicator. The supernatants were pooled after centrifugation to separate the silica gel. The extraction yields were measured by radioactive counting before extraction and on each fraction extracted from TLC on a Packard Tri-Carb 460C liquid scintillation counter.

Derivative formation

The MO-TMS derivatives were prepared according to a previously described method [9]. The dry extract, to which 1 μg of cholesteryl butyrate was added as internal standard, was taken up in 100 μl of a solution of O-methoxyamine hydrochloride in dry pyridine at 16 mg/ml and allowed to react for 3 h at 65°C. The solution was evaporated to dryness under nitrogen and the residue taken up in 100 μl of BSTFA—TMCS (4:1, v/v) which was then allowed to react at 65°C for 16 h.

Lipidex purification of derivatized samples

The purification on the Sep-Pak cartridge does not remove a number of volatile, low-molecular-weight impurities, which in themselves do not interfere

with the sample analysis. In time, however, these tend to cause deterioration of the capillary columns. Excess reagents and these polar residues were separated from the steroid MO-TMS derivatives by chromatography on Lipidex 5000 (Packard Instrument Co., Downers Grove, IL, U.S.A.) following the method of Axelson and Sjöval [10]. The reaction mixture is passed through a 3-cm Lipidex column using hexane—pyridine—HMDS (98:1:1, v/v) concentrated under nitrogen and taken up in 100 μl of the same solvent mixture or a mixture of BSTFA—pyridine (8:2, v/v). Standard mixtures tested in this system give close to 100% recovery for all steroids tested, measured against a cholesteryl butyrate internal standard.

Gas chromatography and gas chromatography—mass spectrometry

Gas chromatography was carried out on 25-m long SE-30 wall-coated open tubular columns (0.21 mm I.D.) made by Spiral (Dijon, France). A Packard Model 427 chromatograph equipped with an all-glass solid injector and a flame ionization detector was used. Injector temperature was 260°C, column temperature was programmed from 240°C to 295°C at 1°C/min, the detector temperature was 300°C. The flow-rate of nitrogen was 0.8 ml/min.

Determination of methylene unit (MU) values was achieved by simultaneously running the steroids under study and C_{28} to C_{34} *n*-alkanes (Fluka, Buchs, Switzerland). Quantitative measurements by GC have been done by using response coefficients calculated by comparing the height of the peak of each adrenal steroid with that of cholesteryl butyrate [7]. This response coefficient was then used to quantify the steroids in the samples using cholesteryl butyrate as internal standard.

A Finnigan 3300-6100 quadrupole mass spectrometer (Finnigan Corp., Sunnyvale, CA, U.S.A.) coupled with a glass capillary column was used with helium as carrier gas. The capillary column was directly connected to the mass spectrometer source by a silanized glass line. The instrument was equipped with a chemical-ionization source. The reagent gas was methane, ion source pressure was 0.8 Torr, electron energy 130 eV, and filament current 0.25 mA.

Characterization and quantitation of steroids by GC and GC—MS

The characterization of steroids is accomplished by comparing their MU values with those of reference compounds already published [7] and by verification of the similitude of their mass spectra in the chemical-ionization mode to those of authentic samples. For the quantitation a test mixture containing the reference steroids and the internal standard is processed with each sample series and run with it. In this manner there is constant control of the possible changes in the response coefficients to cholesteryl butyrate. The changes which we have found to affect this coefficient are incomplete derivatization, humidity in the sample and selective adsorption on the needle due to the presence of impurities, or on the column if reactivated.

High-performance liquid chromatography

A Varian 5000 Liquid Chromatograph System (Varian Assoc., Palo Alto, CA, U.S.A.), equipped with a UV detector (at 254 nm), a column heater and a solvent programmer was used. A stainless-steel column (30 cm × 4 mm I.D.)

prepacked with octadecylsilane (C_{18}) bonded to 10-μm silica particles, Micropak from Varian was used. The column temperature was 40°C and the solvent gradient was programmed linearly from 30% to 70% (v/v) acetonitrile in water in 30 min at a flow-rate of 0.8 ml/min.

Characterization and quantitation of steroids by HPLC

Steroids are characterized by their elution times relative to corticosterone-21-acetate and deoxycorticosterone-21-acetate, which are used as internal standards. Response coefficients towards these two steroid derivatives have been calculated by comparing their peak heights at different concentrations. A steroid test mixture is run before each series and analyzed to ensure proper functioning of the system.

RESULTS AND DISCUSSION

Comparative yield of extracted steroids

Steroids of varying polarity were extracted from the cell-culture incubation

TABLE I

RECOVERY OF RADIOLABELED STEROIDS FROM CULTURE MEDIUM IN DIFFERENT FRACTIONS OF SEP-PAK® EXTRACTION

Each experiment was performed on 6 ml of culture medium with 10% foetal calf serum and 10% newborn calf serum. Quantities added corresponded to both ^{14}C-labeled (specific activity, 50 mCi/mmol) and unlabeled steroids. The quantity of [^{3}H] cholesterol (4 Ci/mmol) added was less than 10 ng. Free cholesterol in the culture medium came from the sera and was measured by capillary GC. Values are expressed as the percentage of initial radioactivity added.

Steroid	Quantity (μg)	Percentage in buffered medium	Percentage in buffered wash			Percentage in methanol eluate		
			2 ml	+ 2 ml	+ 2 ml	2 ml	+ 2 ml	+ 2 ml
Pregnenolone*	0.001	2	0	0	0	92	0.4	0
Cholesterol	720	51	3.9	1.3	1	27	11	3
Pregnenolone	0.5	4.4	1.1	0.3	0	92	1	0
Cholesterol	720	31	11	3	1	29	9	2.3
Pregnenolone	50	3.4	0.4	0	0	91	0.5	0.3
Cholesterol	720	31	12	1.2	0.4	30	11	3
Progesterone	0.02	0	0	0	0	94	0	0
Cholesterol	720	46	4.1	1.1	0.5	24	13	0
Progesterone	1	3	0.1	0	0	94	1.7	0.1
Cholesterol	720	32	10	0.5	0.1	30	10	1
Progesterone	50	2.5	0.3	0	0	93	1	0
Cholesterol	720	30	14	1	0.2	30	3.5	0.6
Corticosterone	0.02	7	0.3	0	0	86	0.3	0
Cholesterol	720	39	8	4	1	20	13	0
Corticosterone	0.2	3.8	0.1	0	0	89	0.2	0
Cholesterol	720	27	2.4	0.1	0	36	19	0.6
Corticosterone	50	6	0.4	0	0	88	2	0.2
Cholesterol	720	47	19	6	2	17	4	0.4
Cortisol	0.02	6	0.8	0.2	0.4	88	0.6	0
Cholesterol	720	47	2.2	0.8	0.5	18	4	0.5
Cortisol	0.3	4.4	0.1	0	0	93	1	0
Cholesterol	720	22	1.3	0	0	38	9.4	1.5
Cortisol	50	3.3	0.1	0	0	91	0.3	0
Cholesterol	720	45	3.8	0.1	0.1	24	11	0.3

*In this experiment labeled steroids were [^{3}H] pregnenolone (21 Ci/mmol) and [^{14}C] cholesterol (50 mCi/mmol).

medium using the Sep-Pak method. [^{3}H]Cholesterol plus the ^{14}C-labeled steroid to be tested were added to the culture medium and separated on a Sep-Pak cartridge. The results (Table I) show that the yield of pregnenolone, progesterone, corticosterone and cortisol in the first 2 ml of methanol eluate are all approximately equal to about 90% and that this yield is independent of the quantity of steroid present; that is 1 ng to 50 μg of pregnenolone and 20 ng to 50 μg of the others. There seems to be slightly less retention of the more polar steroids such as cortisol and corticosterone in the passage of the medium, but not enough to change the final percentage recovery in the methanol fraction. Although 2 ml of methanol suffice to recover the bulk of the steroid, we usually collected 3 ml to ensure maximum recovery.

The yields obtained when the same steroids are extracted from the culture medium with the deproteinization, delipidation, TLC method are not as good (Table II). Although the total steroid extraction by ethyl acetate and methylene chloride is 86%, the individual steroid extractions from TLC are very different depending on their polarity. While there is 61% of the original pregnenolone extracted, only 50% corticosterone and 33% cortisol is extracted from the silica plate. The steroid recoveries from TLC only, also seen in Table II, show that the differential extraction occurs at this step.

Removal of interfering cholesterol

The sera which make up 20% of the culture medium (10% foetal calf serum and 10% newborn calf serum) contain an average 1.3 mg/ml total cholesterol of which approximately 0.3 mg/ml is unesterified cholesterol. The TLC step is performed in order to purify the steroid fractions and separate them as much

TABLE II

RECOVERY OF RADIOLABELED STEROIDS FROM CULTURE MEDIUM IN SUCCESSIVE STEPS OF DELIPIDATION, DEPROTEINIZATION, SOLVENT EXTRACTION AND TLC

Each experiment was performed on 6 ml of culture medium with sera as indicated in Table I. Steroids added were 4-^{14}C-labeled (specific activity, approximately 50 mCi/mmol) and unlabeled compounds: cholesterol was ^{3}H-labeled (4 Ci/mmol). Cholesterol content of the culture medium supplemented with sera was approximately 720 μg. In the TLC extraction only recoveries were calculated in a separate experiment after chromatography of the pure steroids. Recoveries by solvent extraction were 85—87% for all ^{14}C-labeled steroids and 82—85% for [^{3}H]cholesterol. Values are expressed as percentage of initial radioactivity added.

Steroids	Initial quantity (μg)	Solvent extraction and TLC extraction		TLC extraction only	
		Steroid (%)	Cholesterol (%)	Steroid (%)	Cholesterol (%)
Pregnenolone	0.5	61	15	62	11
Corticosterone	0.2	50	15	55	4
Cortisol	0.3	33	15	37	5
Pregnenolone	50	75	35	—	—
Corticosterone	50	52	35	—	—
Cortisol	50	34	35	—	—

as possible from this cholesterol. Attempts to analyse directly on GC the steroid fraction extracted by ethyl acetate and methylene chloride show a large amount of cholesterol which overloads the capillary columns and makes quantitation of steroids impossible. As can be seen in Table II, there always remains some cholesterol in all the fractions from TLC since it overloads the plate and tails. With the Sep-Pak method all the esterified cholesterol is removed as well as 64—83% of the unesterified cholesterol (Table I), so that quantitation is always possible.

Extraction of steroid mixture from the culture medium

A mixture of the standard steroids used as a test mixture for quantitation was added to 6 ml of culture medium and passed through a Sep-Pak cartridge as described in the Methods section. Recovery was calculated both by using [^{14}C]-corticosterone to monitor extraction and by adding the appropriate internal standards after extraction.

Figs. 1 and 2 show representative analyses of the test mixture before and after Sep-Pak extraction. Fig. 1a shows the separation of the mixture by HPLC with the internal standards deoxycorticosterone-21-acetate and corticosterone-

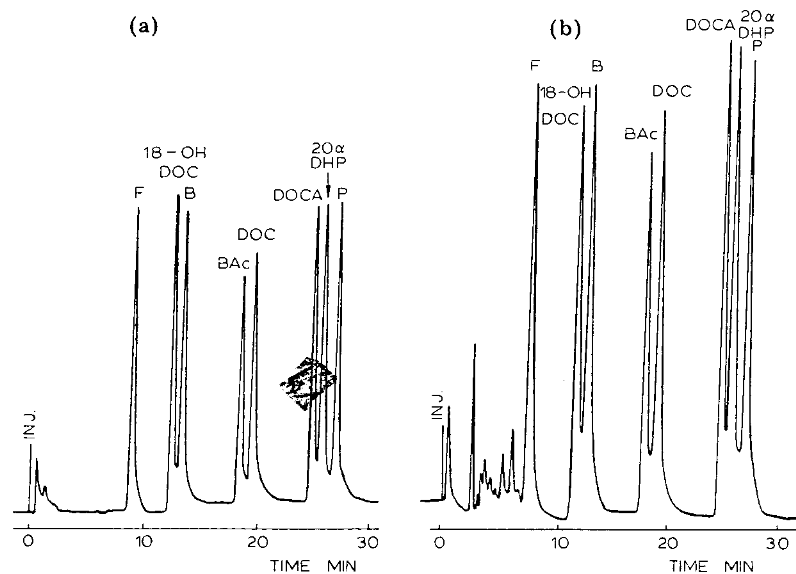

Fig. 1. Separation by HPLC of reference steroid test mixture extracted by Sep-Pak® cartridge. (a) Test mixture containing reference steroids run on a C_{18}-bonded reversed-phase column in the HPLC system. (b) Same test mixture after extraction from the cell-culture medium in identical conditions to incubation mixtures. The internal standards corticosterone-21-acetate and deoxycorticosterone-21-acetate were added after extraction. Chromatographic conditions: prepacked C_{18}-bonded, stainless-steel column (30 cm × 4 mm I.D.); 7200 theoretical plates; column temperature 40°C; solvent gradient programmed linearly from 30% to 70% (v/v) acetonitrile—water in 30 min at a flow-rate of 0.8 ml/min; UV detection at 254 nm. Abbreviations: F = cortisol (1 μg); 18-OH DOC = 18-hydroxy-11-deoxycorticosterone (1 μg); B = corticosterone (1 μg); BAc = corticosterone-21-acetate (1 μg); 20α-DHP = 20α-dihydroprogesterone (1 μg); P = progesterone (1 μg); DOC = deoxycorticosterone (1 μg).

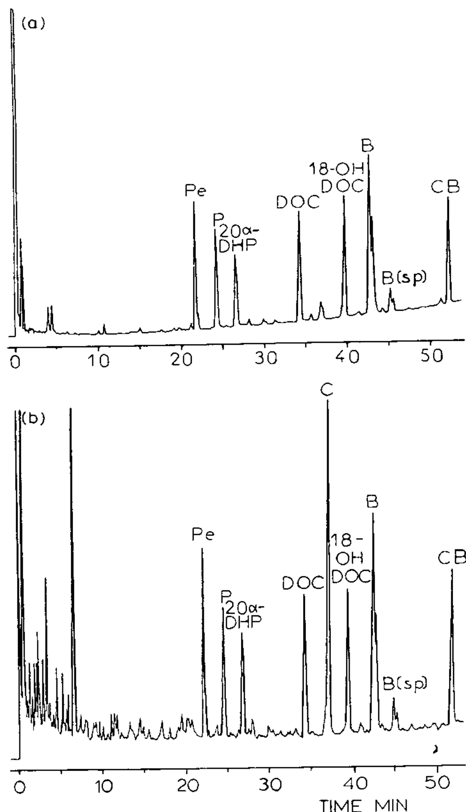

Fig. 2. Separation by GC of reference steroid test mixture extracted by Sep-Pak® cartridge. (a) MO-TMS derivatives of a test mixture containing reference steroids run on an SE-30 capillary column in the GC system. (b) Same test mixture after extraction from the cell-culture medium in identical conditions to the incubation mixtures. The internal standard cholesteryl butyrate was added after extraction and before MO-TMS derivatization. Chromatographic conditions: all-glass solid injection system; SE-30 coated glass capillary column (25 m × 0.21 mm I.D.); 75,000 theoretical plates; temperature programming from 240°C at 1°C/min. Abbreviations: Pe = pregnenolone (10 ng); P = progesterone (10 ng); 20α-DHP = 20α-dihydroprogesterone (10 ng); DOC = deoxycorticosterone (20 ng); C = cholesterol; 18-OH DOC = 18-hydroxy-11-deoxycorticosterone (40 ng); B = corticosterone (40 ng); B(sp) = secondary peak of corticosterone; CB = cholesteryl butyrate (10 ng).

21-acetate added. Using the response coefficients measured in this chromatogram the recoveries of each steroid from the culture medium are calculated from the chromatogram shown in Fig. 1b. The recoveries are between 88% and 95%. This is in agreement with the respective recovery of the radioactive standard. Previous experiments show that no interfering impurities are extracted from the culture medium.

The GC traces of these test mixtures are shown in Fig. 2a (pure standards) and Fig. 2b (standards extracted from culture medium). The recoveries, as determined by comparing these two chromatograms by their response coefficients to cholesteryl butyrate, were also between 88% and 95%. Note the fairly clean solvent front and the relatively small amount of cholesterol in comparison with its high ratio towards others steroids in the culture medium itself.

Stability of 18-hydroxylated steroids

The formation of methyl and ethyl ketals of 18-hydroxy-11-deoxycorticosterone catalyzed by an acid medium has been previously described [11]. Since C_{21} 18-hydroxylated steroids are among the steroids to be measured, the method was tested to see if the methyl ketal of 18-hydroxy-11-deoxycorticosterone could be detected. This method uses a final concentration of 0.01 *M* acetate buffer in the aqueous phase and then pure methanol (final pH of the residue in water approximately 4.5). After evaporation the sample is taken up in absolute ethanol (pH after evaporation, 5.5). Thus the acetic acid concentration in methanol or ethanol is much lower than that (0.1 *M*) used by Roy et al. [11] to produce the ketal in a short period of time. Therefore, methyl or ethyl ketal formation in the methanolic solution of 18-hydroxy-11-deoxycorticosterone during Sep-Pak elution, or during storage of its ethanolic solution at −30°C is unlikely. Nevertheless, a supplementary precaution is to add 0.1% triethylamine [4, 11] or pyridine to the alcolhol extracts.

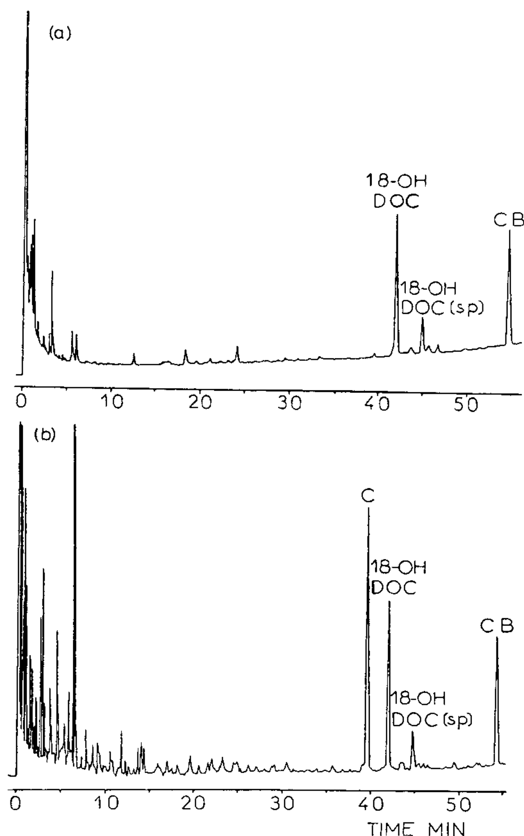

Fig. 3. Analysis by GC of 18-hydroxy-11-deoxycorticosterone extracted by Sep-Pak® cartridge. (a) MO-TMS derivative of 18-hydroxy-11-deoxycorticosterone run on an SE-30 capillary column in the GC system. (b) Same steroid after addition to and extraction by Sep-Pak cartridge from the cell-culture incubation medium. The internal standard, cholesteryl butyrate (1 µg), was added after extraction and before MO-TMS derivatization (10 ng injected). Chromatographic conditions, abbreviations and steroid quantities: same as in Fig. 2, except 18-OH DOC(sp) = secondary peak of 18-hydroxy-11-deoxycorticosterone.

A sample of this steroid added to the culture medium and extracted with the described Sep-Pak method is shown in Fig. 3b. The recovery here is 96% and there was no methyl ketal detected by mass chromatography of the protonated molecular ion m/z 462 of the MO-TMS derivative. Furthermore, there is no degradation of 18-hydroxy-11-deoxycorticosterone by the acid-catalyzed Beckman degradation as shown in Fig. 3b.

Analysis of endogenous steroid production using two methods of extraction

A comparison of the GC separations obtained through the deproteinization, delipidation, extraction and TLC method and the method using a single Sep-Pak column is shown in Fig. 4. As can be seen from the chromatograms, both methods are well adapted for the quantitation of steroids from one cell-culture dish, where we can easily detect 100—200 ng of a single steroid in a complex mixture with a limit down to 10--20 ng per culture dish.

In both cases we analyzed the endogenous steroid production by newborn rat adrenal cultured cells after treatment with 100 mU of ACTH. Fig. 4a and b show the two fractions eluted from the TLC plate. The samples are fairly pure and quantitation of the steroids through their response coefficients to cholesteryl butyrate is possible. Note there are large amounts of squalene in both fractions as well as cholesterol.

The analysis performed by Sep-Pak extraction is shown in Fig. 4c. It is a

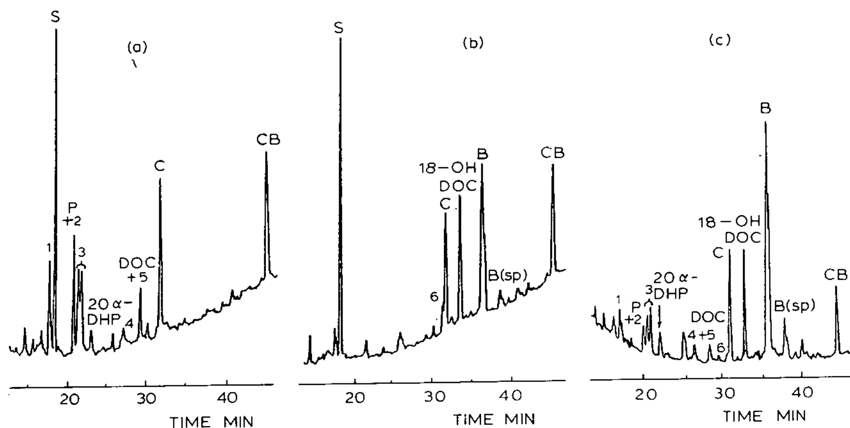

Fig. 4. Separation by GC of endogenous steroids produced in two cell cultures using two different methods of extraction. Culture 1, (a) and (b), was extracted by the deproteinization, delipidation, solvent extraction method and chromatographed on TLC. It was divided into two fractions, one containing the least polar steroids and some of intermediate polarity such as deoxycorticosterone and 18-hydroxy-20α-dihydroprogesterone (a), and the other containing the most polar steroids, together with others of intermediate polarity (b). Both fractions contain squalene and cholesterol. Culture 2 was extracted by the Sep-Pak® method and was chromatographed directly after MO-TMS derivatization. Cholesteryl butyrate (1 μg) was added to all three samples before MO-TMS derivatization, as internal standard. Chromatographic conditions: same as in Fig. 2. Abbreviations: 1 = 5α-pregnane-3α,20α-diol; S = squalene; 2 = 5α-pregnane-3β,20α-diol; 3 = 20α-hydroxy-5α-pregnan-3-one; 4 = 18-hydroxyprogesterone and 11-oxo-20α-dihydroprogesterone; 5 = 18-hydroxy-20α-dihydroprogesterone; 6 = 11β-hydroxy-20α-dihydroprogesterone; CB = cholesterylbutyrate (10 ng injected); other abbreviations are the same as in Fig. 2.

single fraction, as free of impurities as the TLC fractions, with no squalene and little cholesterol. Quantitation of all steroids produced is also possible, though mass spectrometry was necessary in both cases to identify mixed peaks.

The interference of cholesterol with the quantitation of 2α-hydroxyprogesterone, 11β-hydroxy-20α-dihydroprogesterone and deoxycortisol still remains, since they have similar MU values and are eluted together [7]. But the reduced amounts of cholesterol make an approximate quantitation possible. When the analysis necessitates mass fragmentography the reduced amounts of cholesterol are especially helpful.

The range of excretion of the individual steroids is between 0.2 and 30 μg per dish per day (up to 70 μg in maximum stimulation conditions). Each analysis is done on one hundredth of the sample. We can increase the sensitivity of the detection to 1 ng or less. By increasing the sample concentration and the volume injected on the column, both possible due to the purity of the sample, one tenth of the sample can be analyzed. A total production of less than 10 ng per dish per day can be measured. Of course with GC—MS and especially using mass fragmentography one can quantify much smaller amounts.

Analysis of exogenous steroid production by both HPLC and GC

An incubation of an exogenous steroid using an inhibitor was analyzed by the Sep-Pak method. A single culture dish was incubated with 10^{-4} *M* trilostane (as an inhibitor of endogenous steroid production), 50 μg of deoxycorticosterone and 500 mU of ACTH, for 24 h. The recovered medium was processed through a Sep-Pak cartridge and analyzed both by HPLC and GC. The resulting chromatograms are shown in Fig. 5.

The HPLC trace (Fig. 5a) shows a very clean scan with only corticosterone and 18-hydroxy-11-deoxycorticosterone as products. There is no remaining deoxycorticosterone. The most polar peaks, those that elute near the solvent front, have been identified as arising from the medium itself. The peak which elutes before 18-hydroxy-11-deoxycorticosterone is always present when a culture incubation with trilostane is analyzed and has not been identified. Trilostane has not been detected since it does not possess any function with intense UV absorption at 254 nm. This UV detection method does not allow the steroid products that do not possess a 3-oxo-4-ene ring A structure to be seen. Thus, pregnenolone, the pregnanediols and any of the ring A reduced metabolites will not be detected. Therefore, it is often of interest to analyze the same sample by GC.

The corresponding GC trace (Fig. 5b) shows the efficiency of the sample cleaning. There are some impurities at the solvent front and several peaks in the area of pregnenolone and pregnanediol, which are present after all culture incubations. The large amount of pregnenolone (14.9 μg per dish per 24 h) is due to the inhibition of 3β-hydroxysteroid dehydrogenase by trilostane. As seen on the chromatogram, there is no remaining deoxycorticosterone detected and the only products are 18-hydroxy-11-deoxycorticosterone and corticosterone. GC—MS analysis confirmed the identify of the steroids. Only the peak eluting before cholesterol with a protonated molecular ion at *m/z* 429 has not been identified. It has been seen in all culture incubations with trilostane and might be a metabolite, though its structure is not known. Indeed, it does not corre-

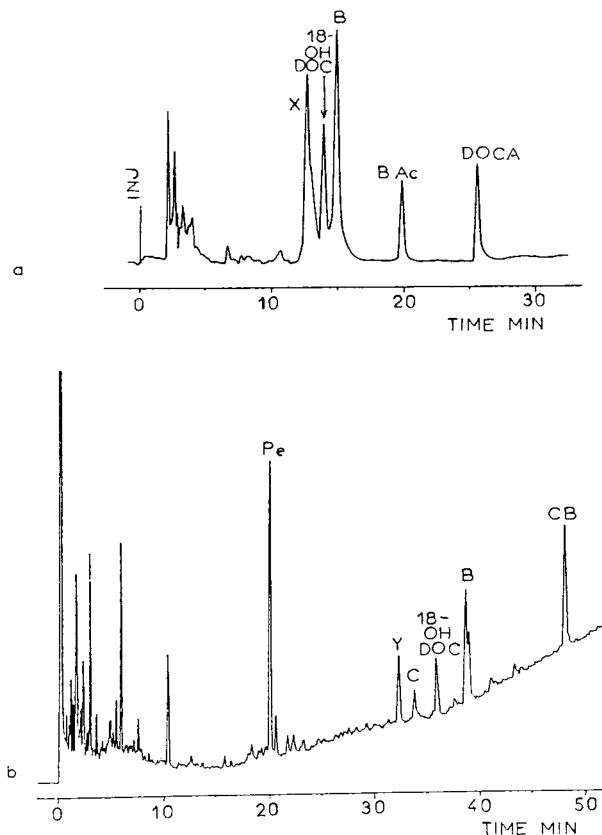

Fig. 5. Separations by HPLC and GC of exogenous steroid products by a single cell culture. An incubation using deoxycorticosterone as substrate and trilostane as inhibitor of endogenous steroid production was extracted by the Sep-Pak® method and analyzed by both HPLC and GC. (a) HPLC analysis with BAc (corticosterone-21-acetate, 1 μg) and DOCA (deoxycorticosterone-21-acetate, 1 μg) as internal standards. (b) GC analysis with cholesteryl butyrate (CB) as internal standard (10 ng injected). Both methods show no interfering impurities and are well adapted for quantitation. Pregnenolone is not detected by the UV detector at 254 nm in the HPLC system. Chromatographic conditions: same as in Figs. 1 and 2. Abbreviations: X or Y = unknowns; other abbreviations are the same as in Figs. 1 and 2.

spond to trilostane itself, which has a very low response coefficient to cholesteryl butyrate having four peaks as the MO-TMS derivative: the major one at 29.86 MU (0.034 response coefficient), the second largest at 29.28 MU (0.012 response coefficient) and two other very minor peaks at 29.58 and 30.99 MU. None of these peaks corresponds to the unknown.

Corticosterone and 18-hydroxy-11-deoxycorticosterone were quantitated using both methods (Table III). Deoxycorticosterone-21-acetate and corticosterone-21-acetate were added to the sample before HPLC analysis as internal standards. For the GC quantitation cholesteryl butyrate was added prior to MO-TMS derivatization. Both methods show similar results with 27.4 and 30.8 μg corticosterone per dish measured by HPLC using deoxycorticosterone-21-acetate and corticosterone-21-acetate as internal standards, respectively, and 26.4 μg per dish with the GC analysis. The results for 18-hydroxy-11-deoxy-

TABLE III

QUANTITATION OF STEROIDS PRODUCED BY AN ADRENAL CELL CULTURE: COMPARISON OF HPLC AND GC METHODS

Internal standards: HPLC A method = corticosterone-21-acetate, HPLC B method = deoxycorticosterone-21-acetate; GC method = cholesteryl butyrate. Incubation conditions: deoxycorticosterone (50 μg) as exogenous substrate, trilostane (10^{-4} *M*) as inhibitor of 3β-hydroxysteroid dehydrogenase, ACTH (500 mU per culture dish), incubation time = 24 h. Results are expressed as μg per dish per 24 h.

	HPLC methods		GC method
	A	B	
Pregnenolone	—	—	14.9
18-Hydroxy-11-deoxycorticosterone	15.8	16.7	16.1
Corticosterone	27.4	30.8	26.4

corticosterone are even closer, with 15.8 and 16.7 μg per dish with HPLC and 16.1 μg per dish with the GC methods. The agreement between the two different internal standards in the HPLC method indicates that there are no interfering substances in either measurement. There is also very good agreement between the two different quantitation methods, showing that there is a fairly general removal of interfering impurities by the Sep-Pak extraction.

CONCLUSION

This Sep-Pak extraction method is short and simple, saving not only analysis time but also materials, since there is a very limited amount of solvents and glassware necessary. In addition, this method provides a single, pure fraction, with excellent recoveries of all steroids in biological samples. The purification requires a single step and the samples are ready for analysis by HPLC or derivatization and analysis by GC, with no further work-up. Quantitation is reproducible and accurate down to 50—100 ng of steroid by HPLC, to 10 ng by GC and GC—MS methods and even more sensitive by mass fragmentographic methods.

ACKNOWLEDGEMENTS

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CHROMBIO. 1201

SIMULTANEOUS MEASUREMENT OF PREDNISONE, PREDNISOLONE AND 6β-HYDROXYPREDNISOLONE IN URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY COUPLED WITH A RADIOACTIVITY DETECTOR

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SUMMARY

We describe the first method for routine measurement of prednisone, prednisolone and 6β-hydroxyprednisolone concomitantly in urine. Urine (3 ml) is extracted with ethyl acetate, washed with base and separated by high-performance liquid chromatography on a silica column with a solvent system of hexane—diethyl ether—ethanol—tetrahydrofuran—glacial acetic acid (59.9:31:2.3:6.5:0.3, v/v). The steroids are detected at 254 nm. Because no conventional internal standard was found, 6β-[^{3}H]hydroxycortisol and [^{3}H]prednisolone are added to urine prior to extraction; ^{3}H is monitored by a radioactivity detector coupled with the chromatograph. The assay exhibits linearity from 200 to 7500 ng and an inter-day variability of $<$ 11.4% (C.V.).

INTRODUCTION

Metabolic studies of prednisolone, the most widely used synthetic glucocorticoid, require specific analytical techniques. The radioimmunoassay technique for prednisolone suffers from poor specificity [1] and poor reproducibility [2]. Recently, specific and reproducible high-performance liquid chromatographic (HPLC) techniques have been developed for prednisolone determination in plasma [3—5]. Furthermore, these methods allow the simultaneous determination of prednisone and prednisolone in plasma. The method of Rose et al. [4, 6] has also been shown to be suitable for measuring prednisolone and prednisone in urine. No method is currently available for routine measurement of 6β-hydroxyprednisolone. This report describes an HPLC method for the simultaneous assay of prednisolone, prednisone and 6β-hydroxyprednisolone in urine, with [^{3}H]prednisolone and 6β-[^{3}H]hydroxycortisol as the internal standards (Fig. 1).

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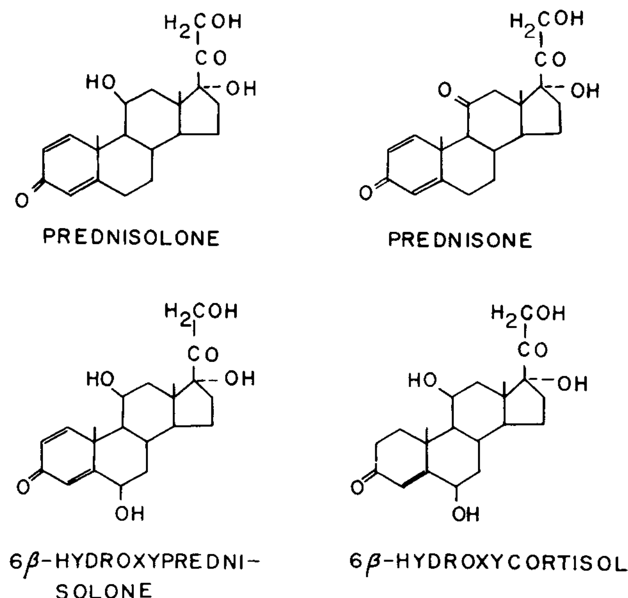

Fig. 1. Structure of prednisolone, prednisone, 6β-hydroxycortisol and 6β-hydroxyprednisolone.

MATERIALS AND METHODS

Reagents and standards

Prednisone and prednisolone were purchased from Sigma (St. Louis, MO, U.S.A.); 6β-hydroxyprednisolone and 6β-hydroxycortisol were obtained from Steraloids (Wilton, NH, U.S.A.). Radioactive [6,7(*n*)-^{3}H] prednisolone (specific activity 41 Ci/mmol) and 6β-hydroxy-[1,2-^{3}H] cortisol (specific activity 43 Ci/mmol) were purchased from The Radiochemical Centre (Amersham, Great Britain). Reagents used were of analytical grade, solvents of HPLC grade (Fluka, Buchs, Switzerland). Prednisone and prednisolone were initially dissolved in methanol—glacial acetic acid (99.9:0.1, v/v) (10 μg/ml); 6β-hydroxycortisol and 6β-hydroxyprednisolone were dissolved in ethyl acetate (10 μg/ml). Similarly, the internal standards [^{3}H] prednisolone and 6β-[^{3}H] hydroxycortisol were prepared; the final solution used contained 100,000 dpm per 100 μl of each of the two steroids. To prepare standard curves, the steroids were added to normal human urine.

The absorption maximum of prednisone, prednisolone and 6β-hydroxyprednisolone was determined by UV spectrophotometry (Fig. 2).

All glassware was cleaned with acetone, decontaminated with Deconex 11 (Borer Chemie, Solothurn, Switzerland), ultrasonicated for 15 min, washed with distilled water and dried at 80° C.

Extraction procedure

Saturate 0.5—3 ml of urine containing 100,000 dpm of [^{3}H] prednisolone and 100,000 dpm of 6β-[^{3}H] hydroxycortisol as internal standards with sodium

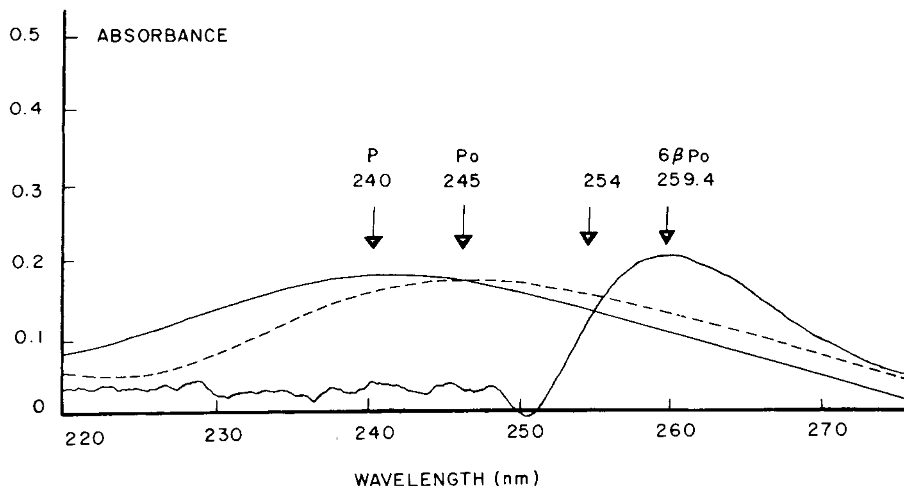

Fig. 2. UV absorbance spectra of prednisone (P), prednisolone (Po) and 6β-hydroxyprednisolone (6βPo). Prednisolone and prednisone were dissolved in methanol—glacial acetic acid (99.9 : 0.1, v/v) (10 μg/ml) and 6β-hydroxyprednisolone was dissolved in ethyl acetate (10 μg/ml). The arrows indicate the absorbance maxima. The UV detector used for the HPLC assay measures the absorbance at 254 nm.

sulfate for 10 min at 37°C. Extract with 9 ml of ethyl acetate using a mechanical shaker. Transfer the organic phase into a 16 × 160 mm glass tube and wash twice with 0.25 mol/l sodium hydroxide saturated with sodium sulphate. Remove the organic layer and evaporate it to dryness under a stream of nitrogen gas. Dissolve the residue in 3 ml of ethanol, mix for 20 sec, transfer into a new glass tube and evaporate to dryness. Dissolve the residue in 150 μl of ethyl acetate. For clinical samples inject 150 μl onto the column. Use a mobile phase consisting of hexane (55.9%), diethyl ether (31%), tetrahydrofuran (6.5%), ethanol (2.3%) and glacial acetic acid (0.3%). The observed pressure on the column is 186 MPa at a flow-rate of 1.8 ml/min. Set the UV detector at 0.004 A full scale and the double-pen recorder at 50 mV and 500 mV.

Apparatus

The HPLC pump was a Model 6000 A from Waters Assoc. (Milford, MA, U.S.A.), the UV detector an LC-15 from Perkin-Elmer (Norwalk, CT, U.S.A.) and the radioactivity detector an LB 504 with glass scintillator cell (0.5 ml) from Laboratorium Prof. Dr. Berthold (Wildbad, G.F.R.). The linear double-pen recorder was from Tegal Scientific (Martinez, CA, U.S.A.). The HPLC column, 250 × 3.2 mm, silica (SI-60), 5 μm average particle diameter was from Altex, (Berkeley, CA, U.S.A.). The UV spectrophotometer was an Aminco DW-2_a^{TM} (Division of Travenol Laboratories Inc., Silver Spring, MD, U.S.A.).

RESULTS

The chromatogram of pure prednisone, prednisolone, 6β-hydroxycortisol

and 6β-hydroxyprednisolone assessed by the UV detector at 254 nm and the radioactive peaks of the internal standards [^{3}H]prednisolone and 6β-[^{3}H]-hydroxycortisol assessed by the radioactivity monitor LB 504 are given in Fig. 3. The retention times for prednisone, prednisolone, 6β-hydroxycortisol and 6β-hydroxyprednisolone were 11, 14, 17, and 21 min, respectively. The ratio of the peak heights (cm) obtained by UV absorbance and radioactivity monitoring (counts) was used for internal standardization. 6β-[^{3}H]Hydroxycortisol was used as an internal standard for 6β-hydroxyprednisolone, and [^{3}H] prednisolone as an internal standard for prednisone and prednisolone. The analytical recovery for 6β-hydroxyprednisolone, (n = 12) and for [^{3}H]prednisolone (n = 12) was 70 ± 2% ($\overline{X}$ ± S.D.) and 85 ± 3%, respectively. The standard curves for all three steroids exhibit good linearity ($r^2 > 0.96$) from 200 to 7500 ng.

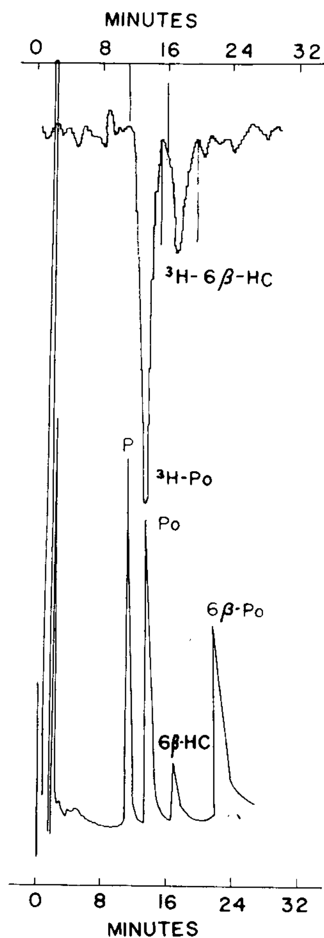

Fig. 3. The lower trace delineates the UV absorbance of injected standards of prednisone (P), prednisolone (Po), 6β-hydroxycortisol (6β-HC) and of 6β-hydroxyprednisolone (6β-Po). The upper trace depicts the radioactivity measurements of the internal standards [^{3}H]prednisolone (^{3}H-Po) and 6β-[^{3}H]hydroxycortisol (^{3}H-6β-HC). In this trace the negative mark indicates the start, and the positive mark indicates the end, of the integration of a peak.

Chromatograms of extracts of a blank urine and of urine containing 6β-hydroxyprednisolone, prednisolone and prednisone from the same subject are depicted in Fig. 4a and b. The blank urine shows no interference with the three steroids. The chromatogram in Fig. 4b was obtained by extracting 1 ml of urine after a dose of 50 mg of prednisolone was given. For routine analysis of 1 ml of urine, 200—7500 ng of each of the three steroids can be measured.

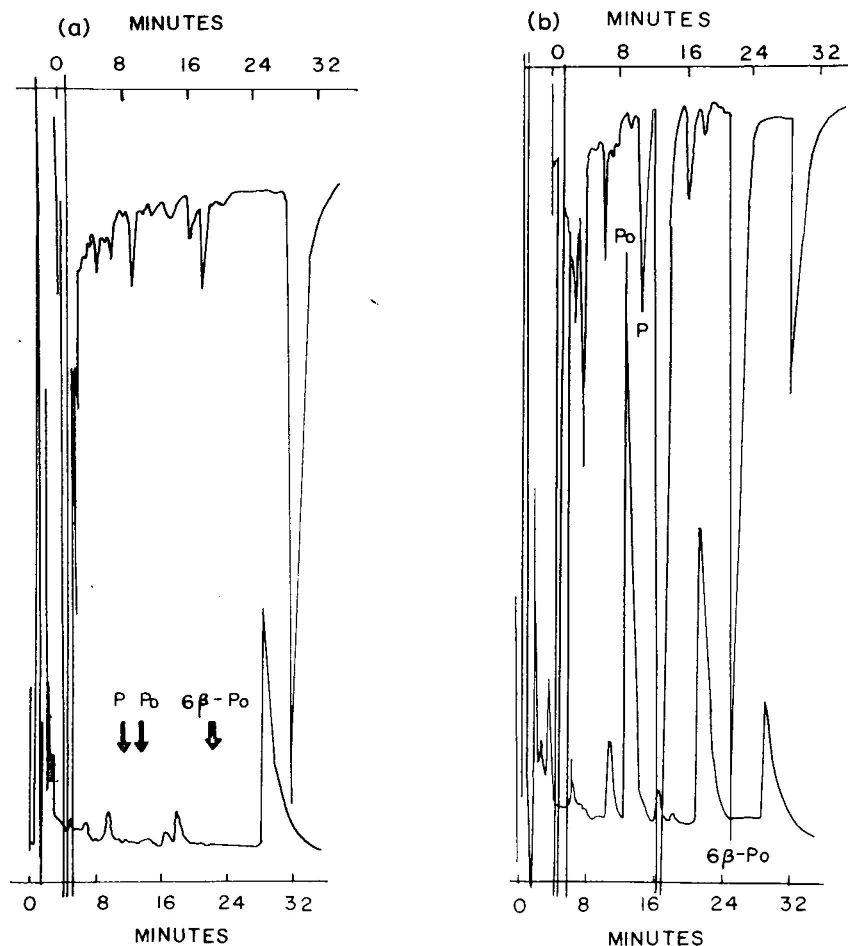

Fig. 4. (a) Dual-pen recording of chromatogram for blank human urine extract. The attenuation of the lower pen is 2.5 times that for the upper pen recording. P = prednisone; Po = prednisolone; 6β-Po = 6β-hydroxyprednisolone. (b) Dual-pen recording of chromatogram for urine sample from the same subject, treated with prednisolone.

The intra-day and inter-day variabilities for twelve measurements for each steroid are given in Table I. The urine used for these measurements was obtained from a patient on chronic prednisone treatment (0.7 mg/kg per day). To determine the stability of the three steroids, urine from a patient with a nephrotic syndrome treated with prednisone was stored at 4°C for four months; 0.5 ml of that urine was extracted and analyzed repeatedly (Fig. 5).

TABLE I

INTRA- AND INTER-DAY VARIABILITY OF THE METHOD

	C.V. (%)	
	Intra-day variability (n = 12)	Inter-day variability (n = 12)
6β-Hydroxyprednisolone	10.3	11.1
Prednisolone	5.5	4.5
Prednisone	10.9	11.6

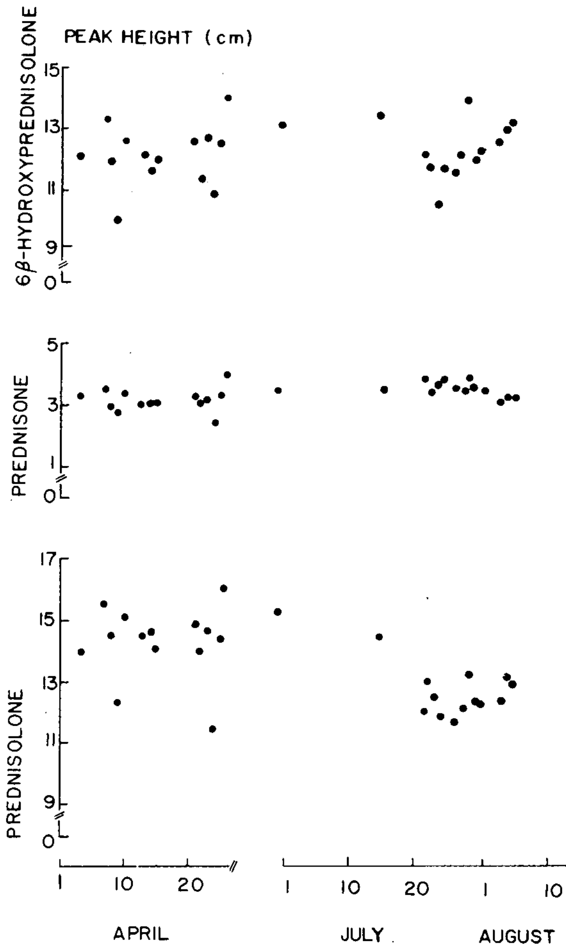

Fig. 5. Stability of prednisone, prednisolone and 6β-hydroxyprednisolone when urine of a patient treated with prednisone is stored at 4°C. On the y-axis the peak height of each of the three steroids measured by the UV detector is given.

Prednisone and 6β-hydroxyprednisolone are stable, while the UV absorbance attributable to prednisolone decreases by about 15% over a time period of four months.

Potential interference by 33 drugs commonly used was investigated by chromatographing extracts of urine obtained from patients treated with these drugs (Table II).

TABLE II

POTENTIAL INTERFERENCE* OF DRUGS WITH PREDNISONE, PREDNISOLONE AND 6β-HYDROXYPREDNISOLONE

Drug	Prednisone	Prednisolone	6β-Hydroxyprednisolone
Allopurinol	—	—	—
Amiodarone HCl	—	—	—
Atenolol	—	—	—
Calcitriol	—	—	—
Captopril	—	—	—
Chlorpromazine	—	—	—
Cimetidine	—	+	—
Clomethiazole	—	—	—
Clonidine HCl	—	—	—
Digoxine	—	—	—
Dihydralazine	—	—	—
Disulfiram	—	—	+
Ethinylestradiol—Linestrenol	—	—	—
Etofylline	—	—	—
Flucloxacilline	+	+	—
Furosemide	—	—	—
Heparin	—	—	—
Indapamide	—	—	—
Isosorbide dinitrate	—	—	—
Lynestrenol—Ethinylestradiol	—	—	—
Medigoxin	—	—	—
L-α-Methyldopa	—	—	—
Nifedipine	—	—	—
Norgestrel—Ethinylestradiol	—	—	—
Phenazone	—	—	—
Phenobarbital	—	—	—
Phenprocoumon	—	—	—
Phenytoin	—	—	—
Propranolol HCl	—	—	—
Pyridoxine	—	—	—
Sulfinpyrazone	—	+	—
Theophylline	—	—	—
Triameterene	—	—	—

*—, no interference; + interference.

Normal volunteers were given intravenous prednisolone and the amount of prednisolone, prednisone and 6β-hydroxyprednisolone in the urine was determined (Table III). For comparison, prednisolone and prednisone were measured by the method detailed in this paper and by our HPLC assay published previously [3]. Both methods provide similar results (Table III).

TABLE III

URINARY EXCRETION OF PREDNISOLONE, PREDNISONE AND 6β-HYDROXYPREDNISOLONE FOLLOWING INTRAVENOUS DOSING OF PREDNISOLONE IN FIVE NORMAL SUBJECTS

Subject	Dose (mg)	Prednisolone (mg per 24 h)	Prednisone (mg per 24 h)	6β-Hydroxyprednisolone (mg per 24 h)
1	52.5	15.5 (17.4)*	1.26 (1.68)	1.96
2	55.7	19.0 (17.6)	1.58 (1.25)	1.95
3	45.9	9.4 (9.0)	0.76 (0.83)	2.71
4	52.5	17.4 (19.6)	1.17 (1.28)	3.80
5	57.4	12.3 (13.8)	1.56 (1.63)	3.80

*Values in parentheses are the amounts obtained using the method of Frey et al. [3].

DISCUSSION

With the assay described we can simultaneously measure prednisone, prednisolone and 6β-hydroxyprednisolone in urine. The three steroids are extracted with ethyl acetate. Ethyl acetate was chosen for two reasons: (1) Frantz et al. [7] used ethyl acetate for the extraction of 6β-hydroxycortisol, a compound with close structural similarities to 6β-hydroxyprednisolone (Fig. 1), and (2) Morrison et al. [8] extracted prednisone and prednisolone with ethyl acetate. In our procedure the analytical recovery of 6β-[^{3}H]hydroxycortisol and [^{3}H]-prednisolone is higher than 70%. The analytical recovery of prednisone and 6β-hydroxyprednisolone was not determined accurately beause no labelled compound was available. However, comparison of the peak heights of these two steroids following direct injection with the peak heights following the extraction procedure indicates an analytical recovery of a similar magnitude.

The solvent system we previously described for measuring prednisone and prednisolone in plasma [3] allows the separation of urinary prednisone, prednisolone and 6β-hydroxyprednisolone on a silica column; however, the retention time for 6β-hydroxyprednisolone is about 1 h and no suitable internal standard was found. Roots et al. [9] determined 6β-hydroxycortisol by a normal-phase chromatographic procedure using a solvent system consisting of hexane (470 ml), methylene chloride (410 ml), ethanol (112 ml), and water (15 ml); likewise, these authors found no internal standard [9]. We tried more than 30 modifications of different solvent systems for separating the three steroids and presumptive internal standards. The solvent system described in the Methods section allows the separation of prednisone, prednisolone and 6β-hydroxyprednisolone; however, no internal standard was found. Therefore, we decided to add commercially available [^{3}H]prednisolone and 6β-[^{3}H]hydroxycortisol for internal standardization of the structurally related prednisone, prednisolone and 6β-hydroxyprednisolone. The radioactivity was monitored by a recently improved radioactivity detector coupled with the HPLC system. The solvent system described in our assay procedure does not contain the most commonly used constituent for glucocorticoid extraction and separation,

namely methylene chloride [3—5, 10]. This has the advantage that the radioactivity of tritium can be detected with the usually available monitors coupled with HPLC requiring scintillator fluid for detection. The latter detectors cannot be used for measuring radioactivity of tritium-labelled compounds, since the chlorinated solvents quench the radioactivity. Note that the recently developed high-efficiency glass scintillator cells allow the detection of tritium radioactivity also when chlorinated solvents are used.

There are several hints that our chromatographic peaks are attributable to prednisone, prednisolone and 6β-hydroxyprednisolone. Changing the retention times by changing the composition of the solvent system did not uncover additional peaks and/or induce differences in retention times between the pure compound injected directly on the column and the corresponding compound assessed in urine following glucocorticoid dosing. Prednisone and prednisolone amounts measured in urine by two different extraction procedures and separated by two different assay systems were of similar magnitude (Table III). For the clinical studies in progress, we are aware of potential chromatographic interference due to commonly used drugs (Table II); there is the possibility of using our assay system previously published [3] in the case of interference with prednisone and prednisolone. Possible interferences due to commonly used drugs were not reported for the method published by Rose and Jusko [4].

The renal excretion of prednisone measured in five healthy volunteers is within the range reported by Rose et al. [6]. The fraction of the dose of prednisolone excreted by our volunteers is higher than the fraction of the dose excreted by the volunteers of Rose et al. This is probably due to the higher dose of prednisolone given to our volunteers [6]. The increase in renal clearance of prednisolone with increasing dose of prednisolone administered, may best be explained by the non-linear binding of prednisolone to plasma proteins [11]. No data are available from the literature for comparison of the amount of 6β-hydroxyprednisolone excreted in urine, because no practical assay is available. The influence of disease states on the excretion of prednisone, prednisolone and 6β-hydroxyprednisolone, and the relevance of abnormal excretion of these steroids for prednisone dosing, will be reported in subsequent papers.

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CHROMBIO. 1202

DETERMINATION OF FREE AND ESTERIFIED CHOLESTEROL IN HUMAN SERUM BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND OPTICAL ACTIVITY DETECTION

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SUMMARY

A separation and detection scheme is presented for the determination of free, esterified and total cholesterol in human serum. Separation is accomplished by reversed-phase high-performance liquid chromatography and the eluate is monitored by the laser-based optical activity detector. The method is simple, accurate and has the advantage of specificity and selectivity when compared with the many methods commonly used.

INTRODUCTION

Cholesterol is a major component of all mammalian plasma membranes. Though it is vital to cell growth and survival [1, 2], there is a statistically significant correlation between elevated serum cholesterol levels and cardiovascular diseases in general and atherosclerosis [3] in particular. Cholesterol is found in two forms in the serum: either as free cholesterol, or esterified with long-chain fatty acids such as palmitic acid. Typically about 75% of the serum cholesterol is esterified [4]. This ratio has been shown to be related to hormones [5], diet [6], toxic conditions such as ethanol poisoning [7], and disorders like familial lecithin:cholesterol acyltransferase deficiency [8].

Numerous analytical procedures are available and are being used for the measurement of serum cholesterol because of its clinical significance. The most commonly used methods are photometric and are based on the color reaction of cholesterol with acid reagent [9]. The Libermann–Burchard method or a modification of it [10] has been the most popular. Disadvantages of the colorimetric methods include temperature dependence, light sensitivity, color instability as well as the manipulation of corrosive reagents [4]. The first step in the newer enzymatic method involves the catalyzed hydrolysis of th

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cholesterol esters by cholesterol ester hydrolase to generate free cholesterol for subsequent analysis [11]. This method is highly accurate but is mainly for total serum cholesterol determination.

Chromatographic methods are capable of separating free and esterified cholesterol. Gas—liquid [12, 13], thin-layer [14, 15], and, most recently, high-performance liquid chromatography (HPLC) using a variable-wavelength UV detector [16] are now available. Although cholesterol can be detected at 200 nm where double bonds and other functional groups absorb energy, the choice of solvents becomes severely limited due to the transparency requirement in this region. Also more interfering compounds would absorb and be detected at this short wavelength. A particularly serious problem is the interference from triglycerides [16]. This makes good separation and accurate quantitation difficult.

We describe here an alternative procedure for the reversed-phase HPLC separation and determination of free and esterified cholesterol as it is found in human serum by on-line monitoring of the optical rotation of the eluate. The combined use of simple extraction, direct sample injection, HPLC and optical activity detection offers a sensitive and specific method for the profiling and quantitative analysis of serum cholesterol and its esters.

EXPERIMENTAL

Chromatography

Separation was performed on a 25 cm × 4.6 mm, 10 μm C_{18} column (Alltech, Deerfield, IL, U.S.A.). Samples were eluted with a tetrahydrofuran—water (76:24, v/v) mobile phase at a flow-rate of 0.5 ml/min and a pressure of about 4.5 MPa. All injections were made through a 200-μl sample loop at a conventional injection valve (Rheodyne, Berkeley, CA, U.S.A., Model 7010). To reduce any pressure fluctuations caused by the pump (Milton Roy, Riviera Beach, FL, U.S.A., Model 196-0066-001) at the detector, we used a commercial pulse-dampener (Handy & Harman, Norristown, PA, U.S.A., Model Li-Chroma-Damp II) in conjunction with a pressure gauge (Alltech, Model 9228).

Materials

Cholesterol and cholesteryl esters were purchased from Sigma (St. Louis, MO, U.S.A.). Reagent-grade tetrahydrofuran was obtained from Fisher Scientific (Fair Lawn, NJ, U.S.A.). Water was deionized locally. Leder Norm normal clinical chemistry control serum was obtained from Lederle Diagnostics (American Cyanamid, Pearl River, NY, U.S.A.). Reference serum for automated procedures was purchased from Sigma (Cat. No. R3626). The serums from the two commercial sources were reconstituted with diluents as instructed by the manufacturers. Fresh serum from a healthy individual was obtained locally. For each 2.4 ml of serum sample, 7.6 ml of tetrahydrofuran was added drop by drop with vigorous stirring. The mixture was then centrifuged for 20 min at 11,000 *g* in a clinical centrifuge. The clear and yellowish liquid phase was thus separated from the residue and was used as the sample without further treatment. The prepared samples were then injected directly using the chromatographic and detection conditions described in this section.

Optical activity detection

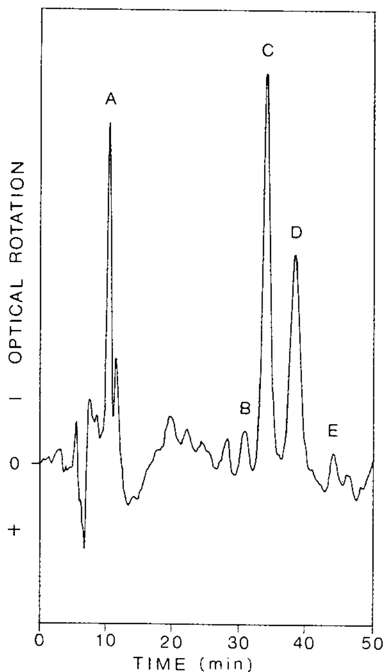
The basic arrangement for an optical activity detector for HPLC has been reported earlier [17, 18]. In this work the laser was operated at 514.5 nm. The laser power was maintained at about 20 mW at the flow cell by replacing one of the mirrors with a partially reflective optical flat. The system was operated without laser intensity stabilization. Instead, a longer time constant of 10 sec was used at the lock-in-amplifier without sacrificing performance. A flow cell with an internal volume of 200 μl was used. Optical alignment was made easier by the 1.6 mm diameter cell, which is about 1.5 times the size of the laser beam.

Laser heating causes changes in birefrigence in the polarizing crystals as well as the cell windows. Also, when the two polarizers are crossed, the light entering the second polarizer is rejected almost totally. The rejected light heats up the surrounding cement which holds the polarizing crystal in position. The thermal relaxation in the cement causes the crystal to rotate minutely and randomly deviates from the best extinction. Furthermore, with the flow cell in place the beam diverges due to the thermal lens effect [19] in the organic mobile phase. The expanded beam size reduces the effectiveness of extinction. These three effects combine to increase the residual depolarized light through the crossed polarizers and in turn the noise level. Since both heating and thermal lensing effects are laser-power dependent, lower laser powers are most desirable, up to the limit of photon statistics. So, in this work we used reduced power at 20 mW which proved to be satisfactory, and not the high powers used in the earlier work [17, 18].

Calculation of the concentrations of free and esterified cholesterol was straightforward using the procedures described in previous work [18], which eliminates the need for having available high-purity standards. In using the simple triangular approximation, the accuracy was limited by the measurement of peak base which was defined through extrapolation. Coupled with uncertainties in the specific rotations (α) at this laser wavelength and this solvent, a maximum error of ± 10% was assessed on our calculated results. This is confirmed by injecting single-component solutions of known concentration for each compound studied.

RESULTS AND DISCUSSION

Figs. 1 and 2 show chromatograms from the reconstituted serum and the freshly extracted natural serum, respectively, analyzed by the above procedure. Test solutions of individual cholesterols in tetrahydrofuran were used to determine the retention times under the same experimental conditions. HPLC retention times, the sign as well as the magnitude of the individual specific rotation, the possible distribution and structural considerations were the criteria for peak identification. Five major cholesterol peaks were identified: (A) cholesterol and cholestanol; (B) cholesteryl linolenate and arachidonate; (C) cholesteryl palmitoleate and linoleate; (D) cholesteryl palmitate and oleate; (E) cholesteryl stearate. In general the order of elution followed that obtained in earlier work using reversed-phase separation [16]. Obviously, other unidentified peaks are present in the chromatograms, but their signal levels are substantially below those of the main peaks.



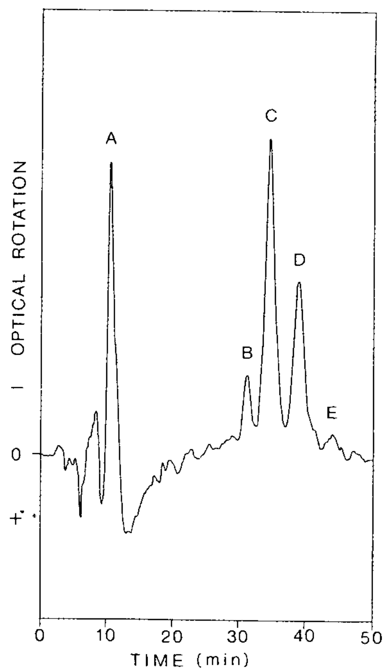


Fig. 1. Separation of cholesterol and cholesterol esters in reconstituted serum (Leder Norm). Peaks: A, cholesterol and cholestanol; B, cholesteryl linolenate and arachindonate; C, cholesteryl palmitoleate and linoleate; D, cholesteryl palmitate and oleate; E, cholesteryl stearate. Mobile phase, tetrahydrofuran—water (76:24, v/v); flow-rate, 0.5 ml/min.

Fig. 2. Separation of cholesterol and cholesterol esters in natural human serum (local). Peaks and conditions as in Fig. 1.

The structural difference between cholesterol and cholestanol is the presence of an isolated double bond between carbons 5 and 6. The small structural and molecular weight differences between these two compounds do not lead to a significant difference in the partition coefficients to permit a clean HPLC separation in our isocratic elution system. The dip (Fig. 1) and shoulder (Fig. 2) on the descending side of peak A are due to the appearance of cholestanol during the elution of cholesterol. This identification was established by the retention times and the signs of the optical rotations. The larger negative rotation of cholesterol ($[\alpha]_D^{20}$ = —39.5) [20] was partially offset by the smaller positive rotation of cholestanol ($[\alpha]_D^{20}$ = +24.2) [20]. A similar situation existed with three other pairs, peaks B, C, and D. Within each pair, despite the structural difference between the fatty acid chains, the change in polarity still did not manifest sufficient difference in partition coefficients for good separation. Between the two components in the same peak, the polarity increase due to one additional double bond was effectively offset by the decrease in polarity caused by the existence of two more methylene groups in the fatty acid chain.

Admittedly, the selected experimental conditions did not allow clear separation of every individual component. Further, not all the literature values of individual specific rotation, the common detector response factor, are available

for the cholesteryl esters. It is still possible to quantitate the combined contribution of those esters. Because the chiral centers are all in the cholesterol part, it is fair to assume similar values for the specific rotations of those cholesteryl esters considering the structural resemblance between them. The identical value of —24 is listed [21] for the specific rotation of cholesteryl palmitate and oleate. We thus take the liberty of using the same value for all components in peaks B, C, and D. The calculated serum cholesterol level is shown in Table I, using the method of triangular approximation discussed earlier. Again, the reliability of this procedure is confirmed by injecting standard solutions of the individual compounds.

The percentage of free cholesterol relative to the total amount of cholesterol is within the range of 17—39% reported in previous studies [22, 23]. For the purpose of quantitative comparison, an analysis of reference serum (Sigma, Cat. No. R3626) was performed. The total cholesterol determined by our HPLC method using optical activity detection was 135 mg/dl. A 130 mg/dl total cholesterol value based on classical Liebermann—Burchard method was provided on the data sheet for this sample. This indicates that our results are at least comparable to those determined colorimetrically. The total serum cholesterol determined by simple summation of the individual cholesterols is well within the normal range of 150—250 mg/dl [2].

The fatty acids of cholesterol esters have been separated and quantitated by various chromatographic methods [24]. Approximate distribution of the fatty acids of the cholesterol esters of normal serum, expressed as percent of total, is shown in Table II. The fatty acids of the seven cholesterol esters identified in our work make up about 96% of the total. Assuming the total cholesterol esters determined in this work to be only 96% of the total and calculating the approximate distribution of the cholesterol esters as percent of total according-

TABLE I

CONCENTRATION OF FREE AND ESTERIFIED CHOLESTEROL IN SERUM

Peak	Compound	Fatty acid*	Concentration in serum (mg/dl)**	
			Leder Norm	Local
A	Cholesterol Cholestanol		47	38
B	Cholesteryl linolenate Cholesteryl arachidonate	18:3 20:4	7	20
C	Cholesteryl palmitoleate Cholesteryl linoleate	16:1 18:2	76	86
D	Cholesteryl palmitate Cholesteryl oleate	16:0 18:1	56	58
E	Cholesteryl stearate	18:0	8	8
	Total cholesterol		194	210
	Free cholesterol (% of total)		24	18
	Esterified cholesterol (% of total)		76	82

*n:m refers to fatty acid with n carbons and m double bonds.
**All concentrations are ± 10%.

TABLE II

COMPARISON OF THE APPROXIMATE DISTRIBUTIONS OF CHOLESTEROL ESTERS AND THE FATTY ACIDS OF CHOLESTEROL ESTERS

Esterified fatty acid	Percentage of total fatty acid*		Percentage of total esters	
			Leder Norm	Local
Linolenic	4	10	5	11
Arachidonic	6			
Palmitoleic	6	49	50	48
Linoleic	43			
Palmitic	10	34	36	33
Oleaic	24			
Stearic	3	3	5	5
Others	4	4	(4)**	(4)**

*From ref. 24.
**By difference.

ly, Table II can be constructed for comparison. It is clear that the distribution of the cholesterol esters closely matches that of their corresponding fatty acids detached from the parent esters as expected. With some work, it should be possible to separate peaks B—D into the individual components, by for example introducing silver ions. However, unless future clinical studies show different implications for saturated versus unsaturated cholesterol esters, separation may not be necessary.

One major advantage of optical activity detection is its inherent selectivity. Compounds that do not possess optical activity simply will not interfere with analysis even if they elute with analytes we are interested in. This is especially useful when physiological fluids are being analyzed. Triglycerides are present in serum in relatively high concentration ranging from 29 to 134 mg/dl [24], and tend to interfere with HPLC serum cholesterol analysis when UV detection was monitored at 200 nm [16]. About 60% of the triglycerides are simple triglycerides [25] which do not possess asymmetric carbon and will elude optical activity detection. Among the 40% mixed triglycerides, only those with different fatty acid substituents on carbons 1 and 3 have the possibility of showing optical rotation since the carbon 2 then becomes asymmetric. Since the majority of the fatty acid substitutents come from long-chain fatty acids with similar chain lengths, the small structural differences among the three substituents surrounding the asymmetric carbon should only cause insignificant, if any, optical rotation. The combined effect of low optical activity and small quantity in their distribution makes their interference negligible in the region of interest. The numerical results presented verified this assumption. Small amounts of mono- and diglycerides (less than 2% of the total glycerides [24]) have appreciable polarity because of their free hydroxyl group and were eluted before cholesterol. 7-Dehydrocholesterol has been identified in human serum and apparently is present in concentration ranging from 5 to 40 mg/dl with 20 mg/dl as the median. This optically active compound is a potential interference for free cholesterol. Since about 80% is esterified [24], the spreading

of the peaks by gradient elution should greatly reduce its interference. Even as is, deconvolution techniques can provide individual concentrations for cholesterol and 7-dehydrocholesterol, since the two have retention times that are distinctly different, though only slightly. Δ7-Cholesterol comprises about 1% of total cholesterol, therefore its interference on free cholesterol is within the possible error estimated in our method. Through the use of a higher efficiency column and gradient elution, improved separation could be achieved and potential interferences reduced.

In summary, we report here a straightforward procedure for the simultaneous determination of free and esterified cholesterol in human serum. Simple extraction, direct injection, HPLC and the use of an absolute standard d.c. Faraday cell [18], offer great convenience in cholesterol determination. The results indicate that this scheme appears to be accurate and precise and to suffer less from interfering substances than colorimetric, enzymatic or UV detection. We expect this technique to be of major importance in the calibration of other analytical methods in clinical studies.

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MEASUREMENT OF CATECHOLAMINES IN BIOLOGICAL FLUIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

A COMPARISON OF FLUORIMETRIC WITH ELECTROCHEMICAL DETECTION

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SUMMARY

An improved method for the determination of catecholamines in biological fluids, by reversed-phase high-performance liquid chromatography (HPLC) with fluorimetric detection is presented. The pH titration previously employed in the alumina extraction was abandoned in favour of the use of a molar excess of pH 8.5 Tris—HCl buffer. A novel lyophilisation step serves to concentrate the catechols and by reconstituting in mobile phase, chromatography disturbances are minimised. The addition of 2 m*M* octanesulphonic acid to a citrate—phosphate mobile phase at pH 6.0 gave optimal resolution and sensitivity.

That HPLC separation can improve the specificity of the trihydroxyindole reaction, to the extent of providing a reliable analytical method, has been demonstrated and validated by the technique of HPLC with electrochemical detection. A correlation coefficient of 0.98 was obtained between the two techniques as applied to the measurement of urinary catecholamines. The HPLC—fluorimetric method was sensitive enough to measure 0.1 ng/ml of noradrenaline or adrenaline at a signal-to-noise ratio of 2.0. Application of the method to the quantitative determination of catecholamines in human urine, plasma and rat brain homogenates is demonstrated.

INTRODUCTION

The measurement of catecholamines in biological fluids has always proved a difficult task, because of their low concentrations, susceptibility to oxidation

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and the complex methods for their analysis. Despite these problems, the investigation of adrenergic pathophysiology in man has led to the development of diverse methods for the quantitation of catechols.

The most sensitive techniques available — gas chromatography—mass spectrometry (GC—MS) [1—3] and radioenzymatic assay [4—8] — have been limited to those workers with the considerable funds and expertise necessary. This situation has led to the recent upsurge in the use of high-performance liquid chromatographic (HPLC) separation of the catecholamines, followed by ultraviolet [9,10], fluorimetric [11—15] or electrochemical detection [16—24].

One of us recently described a fluorimetric method for the estimation of urinary catecholamines [25] which required a 5-ml sample of urine to achieve a detection limit of 5 μg/l. We now report on an improved extraction procedure and chromatography in which catecholamines can be measured down to 0.1 μg/l using only 500 μl of urine or 2—4 ml of plasma. This can be achieved using fluorimetric detection after a post-column trihydroxyindole reaction or alternatively by direct electrochemical detection.

We also describe the application of the HPLC—fluorimetric technique to the measurement of the decrease in the noradrenaline content of selected areas of rat brain, after chemical sympathectomy with 6-hydroxydopamine.

EXPERIMENTAL

Apparatus

A Model RR/015 solvent delivery system (HPLC Technology, Cheshire, Great Britain) equipped with an extra pulse dampener was used for chromatography. Samples were introduced through a Rheodyne 7125 injection valve, fitted with a 100-μl loop. The column used was a 250 × 4.6 mm I.D. Hypersil ODS, 5 μm particle size (Shandon Southern, Cheshire, Great Britain) protected by a guard column of Co:Pell ODS, 50 × 2.1 mm I.D. (Whatman, Maidstone, Great Britain).

The electrochemical detection system comprises a Faraday cage enclosing a Model TL-3 thin-layer cell packed with CP-O paste and a Model LC-2A electronic controller (BioAnalytical Systems, Lafayette, IN, U.S.A.).

The post-column trihydroxyindole reaction was achieved by combining the column eluent with a mixture of sodium acetate and potassium ferricyanide followed by a mixture of sodium hydroxide and ascorbic acid, in a continuous-flow AutoAnalyser (Technicon Instruments, Basingstoke, Great Britain). The manifold details are as given previously [25] except that the concentration of ascorbic acid was increased to 0.20% and air delivery increased to 0.42 ml/min. The fluorescent derivatives were detected by means of a Locarte Mark VI fluorimeter (Locarte, London, Great Britain) fitted with a flow-through cuvette and filters providing excitation at 380 nm and emission at 510 nm. The signal from the fluorimeter was considerably amplified by the Linseis chart recorder, itself fitted with a 4-μF capacitor in series to reduce noise.

Reagents

Noradrenaline, adrenaline, dopamine, 3,4-dihydroxybenzylamine, 6-hydroxydopamine, isoprenaline and α-methyldopa were purchased from Sigma (Poole,

Great Britain). The octanesulphonic acid was obtained from Fisons (Loughborough, Great Britain). All other chemicals including the alumina (Brockmann grade 1, neutral) were from BDH (Enfield, Great Britain). Catecholamine standards were prepared at 10 μg/ml in a mixture of 0.010 *M* hydrochloric acid and 0.00260 *M* sodium metabisulphite. Working concentrations were achieved by subdilution in 0.30 *M* acetic acid prior to use.

Sample collection

Human urine samples were collected into plastic bottles containing 15 ml of freshly prepared 0.26 *M* sodium metabisulphite solution. When completed, the volume and time were recorded and a portion of the urine frozen at −20°C.

Blood samples were obtained from sitting subjects by venipuncture and transferred to lithium heparin tubes containing 100 μl of 0.25 *M* sodium metabisulphite as preservative. The separated plasma was then stored at −20°C for up to eight weeks [26].

Rats were sacrificed by decapitation, their brains were removed and frozen on solid carbon dioxide. The cut sections were then weighed and homogenised in an ice-cold mixture of 1 *M* sodium metabisulphite (1 ml), 0.1 *M* disodium EDTA (1 ml) and 0.05 *M* perchloric acid (27 ml) in the ratio of 3 volumes per g tissue. The homogenate was then centrifuged at 11,000 *g* at 4°C for 15 min and the supernatant transferred to a ground-glass stoppered tube for storage at −20°C until required.

Chromatography

All mobile phases were citrate—phosphate buffers containing methanol as the organic modifier and were pumped at flow-rates of 1—2 ml/min. Retention of the catechols was altered by changing the concentration of the ion-pairing agent and the methanol content, and in the case of methyldopa, changing the pH.

Extraction

Prior to extraction, alumina was acid washed in 0.3 *M* acetic acid followed by several water washes and finally activation was accomplished by heating overnight at 200°C. Once activated the alumina remained potent for several months in a tightly capped dark glass container.

A 500-μl sample of urine (or aqueous standard) was added to a 12-ml plastic tube, contained in an ice-bath. Then 100 mg of activated alumina and 500 μl of 12 ng/ml dihydroxybenzylamine (internal standard) were added followed by 2 ml of distilled water and 3 ml of 1 *M* Tris buffer, pH 8.5. The tubes were stoppered and gently mixed for 10 min on a rotary mixer. The alumina was allowed to settle and the liquid aspirated at the sink, followed by three washes with 0.0050 *M* acetate buffer, pH 7.0. The wash water was removed as thoroughly as possible and then the catechols were eluted from their binding sites on the alumina, by increasing the pH with a 200-μl addition of 0.30 *M* acetic acid. The tubes were mixed for 2 min on a vortex mixer and then centrifuged at 2200 *g* at 4°C for 15 min to compact the alumina. Finally the upper acid layer was transferred into a 1.5-ml Eppendorf tube and 20—100 μl injected into the HPLC system. In the case of plasma samples 2—4 ml plasma

or standard were extracted with only 200 μl of 12 ng/ml dihydroxybenzylamine. For brain homogenate samples 1 ml homogenate or standard was extracted with 50 μl of 0.2 μg/ml α-methyldopa as internal standard, in place of dihydroxybenzylamine which does not undergo the trihydroxyindole reaction. A further concentration of the catechols was achieved by freezing the alumina extracts in a mixture of solid carbon dioxide and acetone, lyophilising and reconstituting in 50 μl of the mobile phase.

RESULTS AND DISCUSSION

Reversed-phase chromatography systems suitable for the separation of catecholamines have been extensively studied by several groups [27—29]. The measurement of urinary catecholamines in alumina extracts has proved difficult with reversed-phase systems because of void volume interferences [30,31]. This has led to the development of alternative extraction techniques based on ion exchange and boric acid gels [30]. However these interferences can be eliminated by using ion-pairing agents such as alkylsulphonic acids, to selectively increase the retention of the catechols so that they are separated from the more polar void volume peaks (Fig. 1). The chromatography solvent

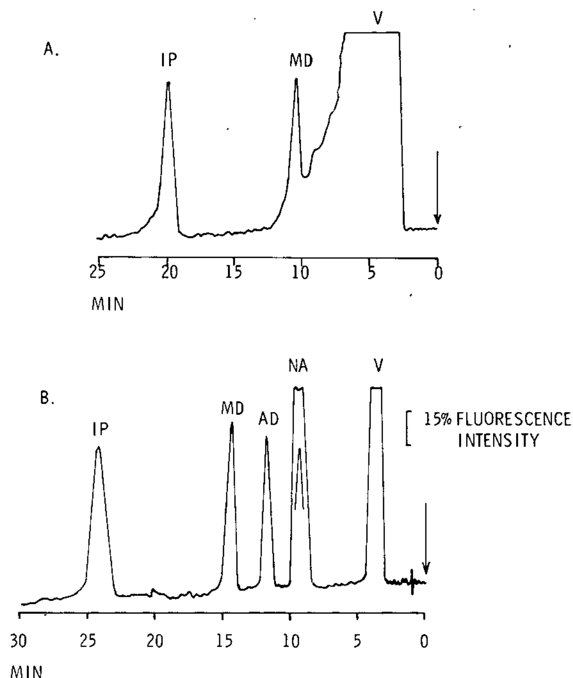

Fig. 1. Chromatograms of urinary catecholamines measured by HPLC with fluorimetric detection. (A) The void volume interference in the absence of ion-pairing agent; (B) the elimination of this interference by the ion-pairing agent 1-octanesulphonic acid at 2 m*M* concentration. Peaks: V = void volume peak, MD = methyldopa, IP = isoprenaline, NA = noradrenaline, AD = adrenaline. Mobile phase, citrate—phosphate buffer (pH 3.1) with 10% methanol, flow-rate, 1 ml/min; temperature, ambient; detector, Locarte fluorimeter with excitation at 380 nm and emission at 510 nm.

system outlined in Fig. 1B is analogous to that in our previous paper [25] but the sensitivity on the column was improved to 100 pg. However, this system could not be used successfully with plasma extracts, or in combination with electrochemical detection. It appears that the void volume contaminants are electroactive and present to a greater extent in plasma than in a 24-h urine specimen. A further problem noticed with extracted standards was that α-methyldopa and dopamine had similar retention. Changing the pH of the mobile phase from 3.1 to 6.0 reduced the retention of methyldopa, with little effect on the retention of the other amines, and had the added bonus of providing more stable baselines with both detectors. To eliminate the void volume interference in plasma extracts it was necessary to increase the ion-pair concentration to 2 m*M* (Fig. 2). This gave a mobile phase composed of 300 ml 0.1 *M* citric acid, 150 ml 0.1 *M* disodium hydrogen phosphate, 100 ml methanol, 20 ml 0.1 *M* octanesulphonic acid and 20 ml 0.1 *M* disodium EDTA per litre, adjusted to pH 6.0 with 5 *M* sodium hydroxide.

With electrochemical detection all three endogenous catecholamines could be measured in urine using dihydroxybenzylamine as internal standard. The separation of standards and a typical urine extract is shown in Fig. 3.

Rat brain homogenate extracts gave exceptionally clean chromatograms (Fig. 4); HPLC-fluorimetric quantitation was achieved using α-methyldopa as internal standard, calibration curves for noradrenaline (NA) being linear over the range 0.1—200 ng/ml.

In order to demonstrate the ability of the HPLC—fluorimetric assay to detect concentration changes after pharmacological manipulations, we administered 6-hydroxydopamine intravenously at a dose of 300 μg per 30 μl of saline (containing 0.2 mg/ml ascorbic acid as antioxidant). Controls were injected with the same volume of vehicle. The results (Table I) show the expected fall in NA after 6-hydroxydopamine, but the degree of adrenergic neurotransmitter depletion varied with the region of the brain. The midbrain was most sensitive to 6-hydroxydopamine and the medulla-pons the least sensitive. In the control animals NA levels were about five times higher in the

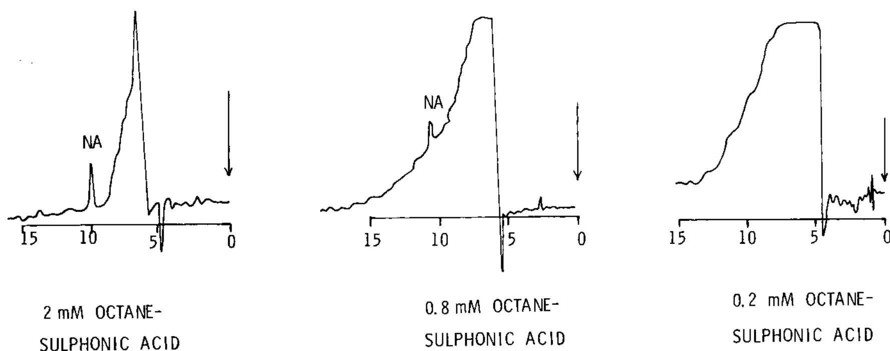

Fig. 2. Chromatograms of plasma catecholamines measured by HPLC with fluorimetric detection. The effect of 1-octanesulphonic acid is shown. Mobile phase, citrate—phosphate buffer (pH 6.0) with 10% methanol and 2 m*M* disodium EDTA; flow-rate, 1 ml/min; temperature, ambient; detector, Locarte fluorimeter with excitation at 380 nm and emission at 510 nm.

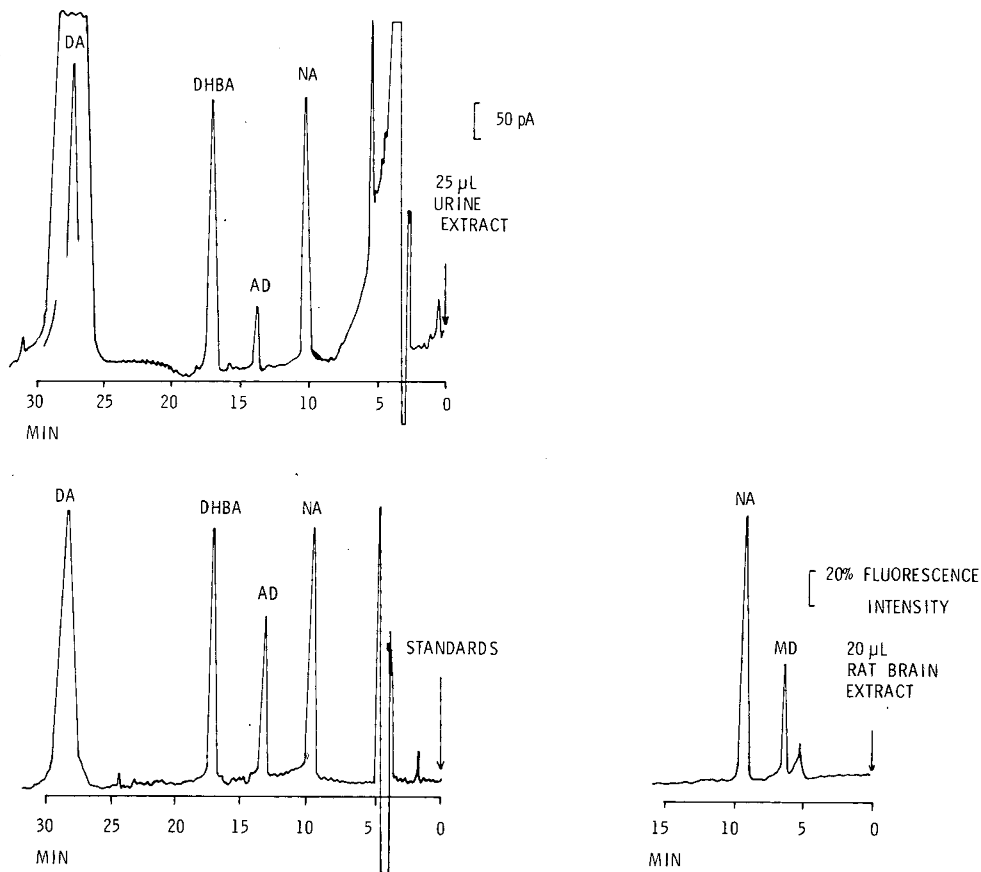

Fig. 3. Chromatograms of urinary catecholamines and extracted standards measured by HPLC with electrochemical detection. Peaks: DHBA = 3,4-dihydroxybenzylamine (internal standard), DA = dopamine. Urinary catecholamine concentrations NA = 39 µg/24 h, AD = 9 µg/24 h, DA = 190 µg/24 h. Mobile phase, citrate—phosphate buffer (pH 6.0) with 10% methanol, 2 m*M* 1-octanesulphonic acid and 2 m*M* disodium EDTA; flow-rate, 1 ml/min; temperature, ambient; detector, BioAnalytical Systems LC-2A held at +0.55 V vs. Ag/AgCl.

Fig. 4. Chromatogram of rat brain homogenate noradrenaline measured by HPLC with fluorimetric detection. Mobile phase, citrate—phosphate buffer (pH 6.0) with 10% methanol, 2 m*M* 1-octanesulphonic acid and 2 m*M* disodium EDTA; flow-rate, 1.5 ml/min; temperature, ambient; detector, Locarte fluorimeter with excitation at 380 nm and emission at 510 nm.

midbrain and medulla-pons than in the cerebellum and cortex. These results using HPLC—fluorimetry are in general agreement with those obtained by the alternative technique of radioenzymatic assay [32] and GC—MS [33].

The combination of improved extraction and chromatography meant that urine catecholamines could be measured by HPLC—fluorimetry without the need for internal standards, thus reducing the analysis time. The percentage recoveries of 1.0 µg of NA and adrenaline (AD) added to urine samples were 70% and 68%, respectively (n=12). Extracted aqueous standards were linear over the range 0.05—250 ng/ml and the coefficients of variation for the

TABLE I

THE EFFECTS OF 6-HYDROXYDOPAMINE ON RAT BRAIN NORADRENALINE CONCENTRATION, AS DETERMINED BY HPLC WITH FLUORIMETRIC DETECTION

All values are mean wet weight ± S.E.M., n = 3 for each determination.

Brain region	Control (ng/g)	6-Hydroxydopamine (ng/g)
Cortex	206 ± 19.6	53 ± 17.1
Cerebellum	132 ± 22.8	26 ± 3.5
Midbrain	445 ± 73.4	28 ± 2.2
Medulla-pons	546 ± 9.7	472 ± 25.1

catecholamines determined in a pooled urine were NA 8.3% (n=9) and AD 10.5% (n=9). The method was sensitive enough to measure 0.1 ng/ml of both amines at a signal-to-noise ratio of 2.0 and so well able to cope with typical urines, which were found to have levels of NA = 43.4 ± 7.03 μg per 24 h (mean ± S.E.M., n = 10) and levels of AD = 15.15 ± 3.592 μg per 24 h (mean ± S.E.M., n = 10) by this method.

The introduction of a lyophilisation step in the extraction has enabled the measurement of NA in 2 ml of human plasma (or serum) by HPLC—fluorimetry; and although the method is still not sensitive enough to accurately measure AD in normal resting subjects, it can be determined in patients with phaeochromocytoma where levels are frequently several times the upper limit of normal [30] and in subjects undergoing maximal exercise.

Using the HPLC—fluorimetric assay we examined the effect of treadmill exercise to exhaustion on the plasma catecholamine levels of ten male subjects, mean age 35 years. Post-exercise values were NA = 1.74 ± 0.993 ng/ml, AD = 0.23 ± 0.10 ng/ml (mean ± S.E.M.) and compared with the basal values of NA = 0.365 ± 0.041 ng/ml, AD $<$ 0.1 ng/ml (mean ± S.E.M.).

In order to measure NA and AD in plasma from resting subjects it was necessary to utilise the increased sensitivity afforded by the oxidation of the catecholamines (at a fixed potential difference) in a thin-layer graphite electrochemical cell [23]. The routine use of the electrochemical detector to measure plasma catechols requires very careful attention to experimental details, particularly as one has to work near to the limit of the instrument's sensitivity, i.e., 0.5 nA full scale. The elimination of noise by use of a Faraday cage, electrical earthing of metal parts, inclusion of disodium EDTA in the mobile phase to chelate metal ions, passivation of the pump and column when new (using acid), use of highly polished electrode surface [34], and maintaining the system on a continuous rather than daily basis, are all important factors in ensuring operation at high sensitivity. For urine work the more reliable glassy carbon electrode (TL 4/5) is adequate and can be used in place of the paste electrode. In contrast to the trihydroxyindole reaction the electrochemical detector is very sensitive to dopamine and since urinary dopamine is present in the order of 100—600 μg per 24 h, it gives a very large peak in the chromatogram which has to be off-scaled on the chart recorder to enable its accurate quantitation. This presence of a dopamine peak also means that sample throughput is

reduced for urines since dopamine has a longer retention time than the other catecholamines.

In order to compare the two methods, the concentrations of NA and AD in twelve urine samples were determined by HPLC—fluorimetry and HPLC with electrochemical detection. The HPLC—fluorimetric method gave values of NA = 56.5 ± 12.9 μg per 24 h and AD = 27.9 ± 14.2 μg per 24 h (mean ± S.E.M.), while the HPLC—electrochemical method gave corresponding values of NA = 59.25 ± 14.2 μg per 24 h and AD = 29.6 ± 14.8 μg per 24 h (mean ± S.E.M.). No significant difference between the two techniques was found ($P > 0.05$, paired t-test) and they were shown to be well correlated ($r = 0.98$).

The improved sensitivity of the HPLC—fluorimetric method presented, derives largely from the introduction of a novel lyophilisation step, a citrate—phosphate ion-pairing HPLC system and buffering of the sample to pH 8.5 with a high-molarity buffer. The lyophilisation procedure serves to concentrate the catechols and because reconstitution is in mobile phase, injection creates the minimum of disturbance to the chromatography. The use of a molar excess of pH 8.5 buffer overcomes the need for an indicator in the extraction. This is desirable since the alkaline end-point of thymol blue is difficult to judge, being a pale blue colour, and the use of 0.5 M sodium carbonate is also not ideal as it could easily create local hot spots of high pH and hence degrade the catechols.

The classical manual or automated fluorimetric methods employing the trihydroxyindole reaction have fallen into disrepute mainly because of their notorious lack of specificity. That HPLC separation can improve the specificity of the trihydroxyindole reaction, to the extent of providing a reliable analytical method, has been demonstrated and validated by the widely accepted technique of HPLC with electrochemical detection.

In our hands the improved HPLC—fluorimetric method was not as sensitive as the electrochemical approach, but it was less problematic in setting up and running on a routine basis and proved quite satisfactory for the measurement of urinary catecholamines and plasma NA. Since most of the difficulties associated with electrochemical detection derive from the thin-layer cell, it is likely that improved design such as the rotating disc electrode, which is reported to have increased sensitivity and performance, because it is insensitive to the condition of the electrode surface [35] will favour the technique in future.

ACKNOWLEDGEMENTS

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CHROMBIO. 1193

THE DETERMINATION OF UROPORPHYRINOGEN DECARBOXYLASE IN TISSUES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY COUPLED TO FLUORESCENCE DETECTION

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SUMMARY

High-performance liquid chromatography coupled to fluorescence detection was utilized for the separation and quantitation of porphyrins as methyl esters. The method (developed for biochemical investigation of porphyrias) permitted quantitation down to 0.2 nanograms of porphyrins per sample. One of the possible applications is the study of the enzyme uroporphyrinogen decarboxylase. No significant difference was found between two methods of methylation and extraction of the samples prior to chromatography.

INTRODUCTION

Porphyrins are tetrapyrrolic compounds synthesized in a variety of biological tissues. They differ in the type and number of carboxylic side-chains.

Disturbances of porphyrin metabolism attributed to drugs, environmental contaminants and genetic errors [1—3] have been described. In many of these situations it is necessary to measure the porphyrin content of biological fluids and tissues.

High-performance liquid chromatography (HPLC) of porphyrin mixtures, both methylated [4—6] and as free acids [7], has been developed with advantages of accuracy and time required when compared with the widely used thin-layer chromatographic (TLC) separation of porphyrin methyl esters. The detection is usually by a UV detector, so sensitivity remains a major problem because in many experimental models using tissue culture [8] or human biopsy material [9], the porphyrins present are in the nanogram range. In these situations radioactive methods were often used [9].

This paper reports an analytical technique utilizing HPLC with fluorometric detection, which permits the quantitation of porphyrins in subnanogram

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amounts. The method, developed for the biochemical investigation of porphyrias, was applied to the determination of the activity of the enzyme uroporphyrinogen decarboxylase which is markedly affected in the liver of patients with porphyria cutanea tarda and of animals intoxicated with porphyrogenic compounds [2, 9].

EXPERIMENTAL

Chemicals

Mesoporphyrin IX dimethyl ester, protoporphyrin IX dimethyl ester, coproporphyrin III tetramethyl ester, pentacarboxylporphyrin I pentamethyl ester, hexacarboxylporphyrin I hexamethyl ester, heptacarboxylporphyrin I heptamethyl ester, and uroporphyrin III octamethyl ester were purchased from Porphyrin Products (Logan, UT, U.S.A.). Ethyl acetate and *n*-heptane (reagent grade) were obtained from Farmitalia Carlo Erba (Milan, Italy). Solvents were filtered under vacuum through a polycarbonate membrane (0.4 μm) (Nucleopore, Pleasanton, Canada) before use. Chloroform (Aristar grade) was purchased from BDH (Poole, Great Britain) and was always washed with distilled water before use.

Apparatus and conditions

The high-pressure liquid chromatograph was a Model Series 3 microprocessor-controlled pump module (Perkin-Elmer, Norwalk, CT, U.S.A.) equipped with a Rheodyne 7105 injector supplied with 175-μl sample loop injector (Rheodyne, Berkeley, CA, U.S.A.). The column (25 $\times$ 0.26 cm I.D.) was packed with Silica A 10 μm (Perkin-Elmer).

A Model 3000 fluorescence spectrometer (Perkin-Elmer) was used with the excitation wavelength set at 404 nm (slit width 10 nm) and the emission wavelength at 623 nm (slit width 10 nm). The attenuation of the fluorescence spectrometer was varied according to the expected porphyrin concentration. The UV detector was a Model LC 56 B (Perkin-Elmer) with the detection wavelength set at 400 nm.

Separation of the porphyrins was obtained by multilinear gradient elution. The initial conditions were 30% ethyl acetate in *n*-heptane for 6 min after injection; then the percentage of ethyl acetate was raised to 45% within 1 min. Over the next 10 min the ethyl acetate percentage was raised linearly up to 80% and this condition was held for 6 min. Elution was completed with a 3-min purge period at 80% of ethyl acetate. Between two analyses the column was reconditioned for 10 min with the starting solvent mixture. The flow-rate was kept constant at 0.8 ml/min.

Sample preparation from tissues

Porphyrinogens formed as reaction products of the in vitro uroporphyrinogen decarboxylase activity were oxidized to porphyrins, adsorbed on Zerolit FF (ip) resin (BDH), methylated for 48 h with methanol—sulphuric acid and extracted into chloroform as described by Smith et al. [10]. Alternatively, the methylation was performed with boron trifluoride—methanol (14%, Merck-Schuchardt) as described by Smith and Francis [11] with slight modifi-

cations: to 2 ml of boron trifluoride—methanol, 4 ml of chloroform were added; the tubes were mixed on a Vortex for 40 sec and centrifuged at 5090 *g* for 10 min. This procedure was repeated twice with a mixture of chloroform—methanol (6 ml in the ratio 2 : 1 and 2 ml in the ratio 1 : 1). The pooled extracts were combined and washed twice with distilled water. After the second washing the chloroform layer was taken, mixed with ethanol (10 ml) and evaporated under a stream of nitrogen at 37°C. Precautions were taken during the whole procedure to avoid contact with direct light, and samples were kept in the dark at −20°C until injected. Immediately before the HPLC analysis, samples were dissolved with a chloroform solution of mesoporphyrin dimethyl ester (0.5 nmol/ml). This compound, not present in biological tissues, was used as internal standard.

Calibration curves

Porphyrin methyl esters of known concentrations for the calibration curve were prepared from individual porphyrin methyl esters dissolved in chloroform. The concentrations of these original solutions were determined spectrophotometrically by measuring the absorption at the Soret band and using the extinction coefficients reported by Falk [12] for meso-, proto-, copro- and uroporphyrin and by Doss [13] for pentacarboxylic, hexacarboxylic and heptacarboxylic porphyrin. The standard mixtures were prepared ready to dissolve shortly before use with 2.5 ml of a cloroform solution of mesoporphyrin dimethyl ester (0.5 nmol/ml). When dissolved, they were stable for at least one week if stored in the dark at −20° C.

RESULTS

The multilinear gradient elution for the separation of mesoporphyrin IX, protoporphyrin IX, copro-, pentacarboxylic, hexacarboxylic, heptacarboxylic and uroporphyrin methyl esters obtained using a mixture of pure compounds is shown in Fig. 1. No interfering peak(s) were found in extracts from liver, spleen, kidneys and red blood cells; occasionally, additional peaks were observed, quite likely due to the formation of trace amounts of the zinc complex of the porphyrins.

Absolute quantitation of porphyrins present in samples was performed by comparison with a calibration curve obtained by plotting the peak area ratio of each porphyrin to mesoporphyrin versus the concentration of the porphyrin standard solutions. The calibration was linear for all the porphyrins considered within the concentration range 20—2000 ng/ml. Fig. 2 shows the part of the curves up to 1000 ng. The relative correlation coefficients (which also take into account the 2000 ng/ml concentration point) were all greater than 0.999. The calibration curves sloped differently depending on the specific fluorescence of each porphyrin. The best sensitivity was obtained for coproporphyrin tetramethyl ester. On some occasions, the HPLC system was coupled to the UV detector and the two methods of detection were compared. Fig. 3 presents the profiles obtained using a UV or a fluorometric detector after injecting the same sample containing the reaction products of a preparation of human red blood cell uroporphyrinogen decarboxylase. In this example, the chromatogram was

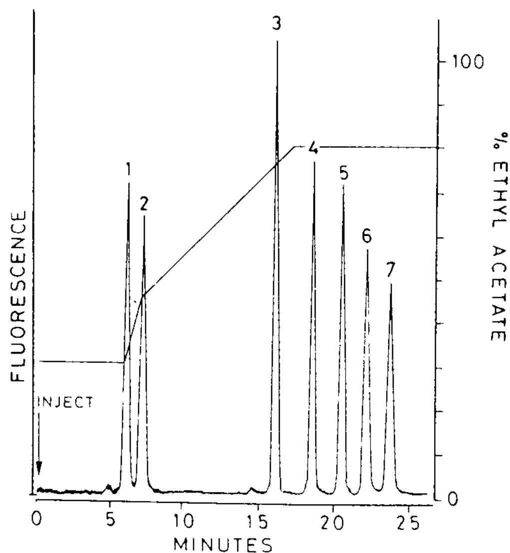

Fig. 1. HPLC separation of standard mixtures of porphyrin methyl esters. Peaks: 1 = mesoporphyrin dimethyl ester; 2 = protoporphyrin dimethyl ester; 3 = coproporphyrin tetramethyl ester; 4 = pentacarboxylporphyrin pentamethyl ester; 5 = hexacarboxylporphyrin hexamethyl ester; 6 = heptacarboxylporphyrin heptamethyl ester; 7 = uroporphyrin octamethyl ester. Conditions of elution are as described in the text.

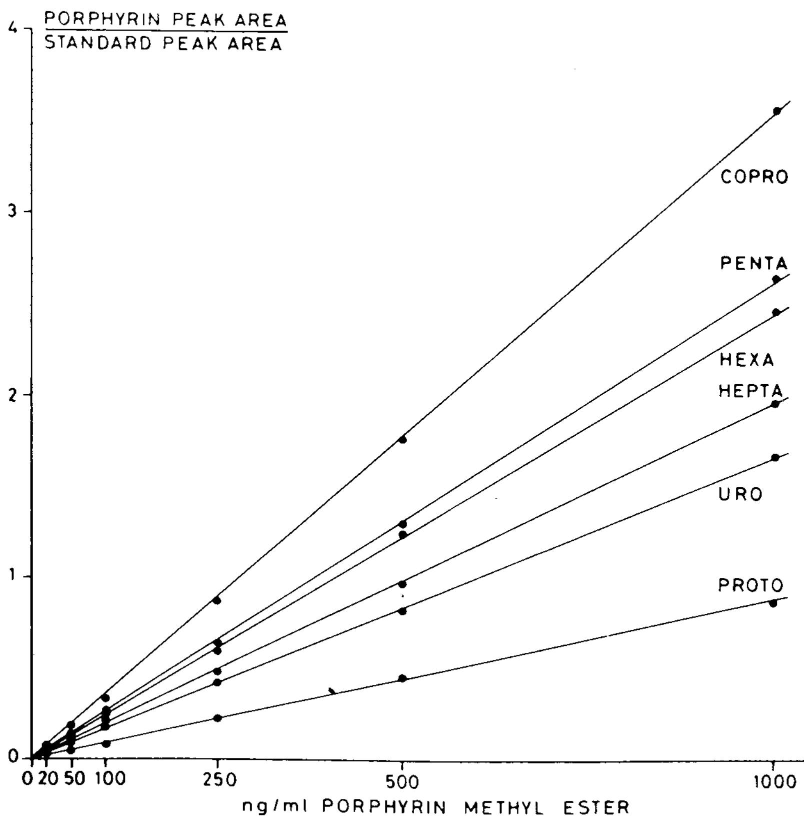

Fig. 2. Calibration curves for standard porphyrin esters.

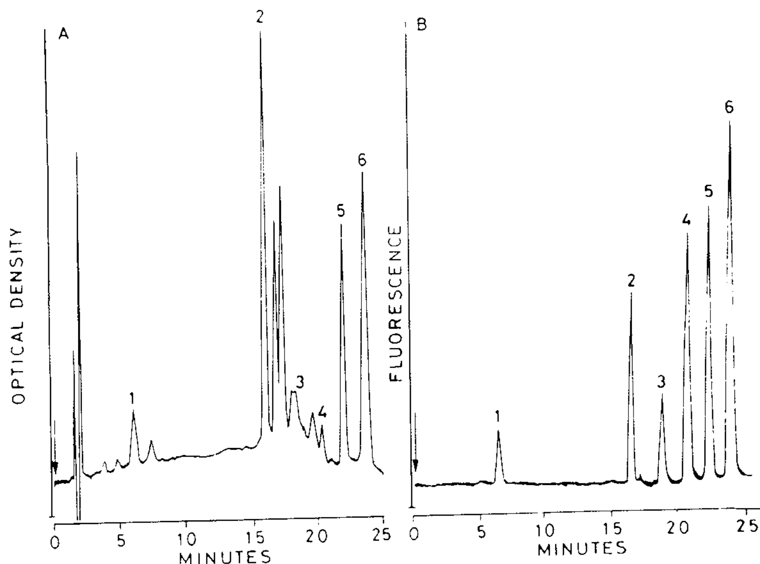

Fig. 3. Comparison of the profiles obtained with (A) UV and (B) fluorometric detectors of a sample containing the reaction products of a preparation of human red blood cell uroporphyrinogen decarboxylase. Peaks: 1 = mesoporphyrin dimethyl ester; 2 = coproporphyrin tetramethyl ester; 3 = pentacarboxylporphyrin pentamethyl ester; 4 = hexacarboxylporphyrin hexamethyl ester; 5 = heptacarboxylporphyrin heptamethyl ester; 6 = uroporphyrin octamethyl ester. Conditions of elution are as in Fig. 1.

obtained at the highest sensitivity of the UV detector (0.02 a.u.f.s.) (Fig. 3A). With the fluorometric detector (Fig. 3B), sensitivity could be further enhanced 5—10 times, depending upon the porphyrin, without changes in the signal-to-noise ratio.

Tables I and II report the results of recovery experiments on the effectiveness of two methylation and relative extraction procedures, one with methanol—sulphuric acid and one with boron trifluoride—methanol. Known amounts of porphyrins (copro-, pentacarboxylic, hexacarboxylic, heptacarboxylic and uroporphyrin) were adsorbed as free acids on Zerolit FF resin and then methylated and extracted as previously described.

TABLE I

RECOVERY OF PORPHYRINS AFTER METHYLATION WITH METHANOL—SULPHURIC ACID

Values represent the mean ± S.E. of two determinations.

Amount of each porphyrin added to the incubation mixture (nmol)	Recovery (%)				
	Copro-	Penta-	Hexa-	Hepta-	Uro-
0.083	90 ± 5	83 ± 5	74 ± 5	65 ± 1	106 ± 1
0.160	90 ± 2	81 ± 1	73 ± 3	61 ± 3	102 ± 8
0.800	93 ± 1	84 ± 1	77 ± 1	67 ± 0	103 ± 1
1.600	98 ± 2	90 ± 1	84 ± 5	68 ± 1	107 ± 2

TABLE II

RECOVERY OF PORPHYRINS AFTER METHYLATION WITH BORON TRIFLUORIDE

Values represent the mean ± S.E. of two determinations.

Amount of each porphyrin added to the incubation mixture (nmol)	Recovery (%)				
	Copro-	Penta-	Hexa-	Hepta-	Uro-
0.083	83 ± 3	73 ± 1	67 ± 3	58 ± 6	89 ± 6
0.160	88 ± 3	77 ± 3	67 ± 6	57 ± 6	90 ± 6
0.800	88 ± 3	81 ± 3	73 ± 3	61 ± 5	98 ± 3
1.600	86 ± 6	75 ± 10	69 ± 6	58 ± 5	95 ± 8

In both procedures, no interfering peaks were visible in the chromatograms and recovery was not influenced by changes in the amount of porphyrin adsorbed on the resin. The efficiency of methylation and extraction, relative to each porphyrin, increased in the order: uro- > copro- > penta- > hexa- > hepta-. The recovery was always higher than 57%.

As an example of the applicability of the method, Table III reports the results of determination of the activity of uroporphyrinogen decarboxylase in animal and human tissues. The decarboxylated products (copro-, penta-, hexa-, and heptacarboxylic porphyrinogen) obtained with 1 h of incubation of the enzymatic preparation at 37°C under nitrogen, were oxidized to porphyrins with light and then methylated with methanol—sulphuric acid. The amount of uroporphyrin present at the end of incubation represents the residual substrate.

With all the tissues considered, it was possible to measure quantitatively all the reaction products simultaneously.

DISCUSSION

The present method measured porphyrins in tissues or formed in in vitro incubations down to a concentration of 0.2 ng/sample. This sensitivity was achieved by the use of fluorescence detection which is more sensitive than the more commonly used UV detection [4—6]. The amount of sample in the optimal range for analysis with fluorescence detection is about one tenth that required with UV detection. Another major advantage of using a fluorometric detector is its specificity, which makes it easy to obtain a clean baseline in the chromatogram, eliminating many interfering substances present in biological samples which absorb light but do not fluoresce in the same range as porphyrins. Furthermore, the gradient elution program described here efficiently separates all the porphyrins without the need for a second derivative system coupled to the detector as described by other authors [14].

HPLC methods of separation of free porphyrins are still in the developmental stage and have been applied mainly to analysis of urines [7, 15]. As a consequence, methylation of the porphyrins is still a necessary step in sample preparation, especially when the amounts present are fairly low. We have

TABLE III

ACTIVITY OF UROPORPHYRINOGEN DECARBOXYLASE IN ANIMAL AND HUMAN TISSUES

Values represent the mean ± S.E. of two samples. Each sample was done in duplicate. The enzymatic preparations had a protein concentration of 2.5—4.6 mg/ml, except blood (11.4 mg/ml).

Tissue	Uroporphyrinogen added (nmol)	Porphyrins recovered (nmol)					Recovery (%)	Uroporphyrinogen decarboxylase activity (pmol per min per mg protein)
		Copro-	Penta-	Hexa-	Hepta-	Uro-		
Rat liver	8	1.223 ± 0.14	0.190 ± 0.01	0.459 ± 0.04	1.820 ± 0.14	3.933 ± 0.16	95	13.209 ± 1.16
Rat kidneys	8	0.455 ± 0.04	0.116 ± 0.00	0.314 ± 0.01	1.336 ± 0.03	4.778 ± 0.20	87	12.724 ± 0.43
Rat spleen	8	0.311 ± 0.02	0.103 ± 0.00	0.296 ± 0.01	1.394 ± 0.04	5.910 ± 0.02	100	9.162 ± 0.40
Human liver	8	0.051 ± 0.00	0.033 ± 0.00	0.100 ± 0.00	0.671 ± 0.07	5.997 ± 0.59	86	10.490 ± 0.47
Human blood	8	0.304 ± 0.02	0.088 ± 0.00	0.270 ± 0.01	1.964 ± 0.17	3.074 ± 0.31	71	3.820 ± 0.04

shown that two commonly used procedures of methylation do not seem to differ significantly in efficiency.

A somewhat higher percentage of recovery and better reproducibility were obtained with the methanol—sulphuric acid method, but the boron trifluoride—methanol method has the advantage of a considerably shorter reaction time.

Finally, we have shown that the use of HPLC coupled to fluorescence detection provides a technique suitable for the biochemical investigation of porphyrias; for example, measurement of the activity of uroporphyrinogen decarboxylase. By our method all the reaction products can be determined simultaneously with advantages in specificity in comparison to radioactive assays, and in sensitivity and accuracy in comparison to TLC separation coupled to spectrophotometric quantitation previously described by other authors [9—11]. This should facilitate the characterization of the various steps of this enzymatic process and of its response to stimuli of different origin.

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CHROMBIO. 1195

MICROPROCEDURE TO DETERMINE THE POLYMORPHIC FORMS OF ACID α_1-GLYCOPROTEIN IN PLASMA

APPLICATION TO DEPRESSIVE PATIENTS TREATED WITH AMITRIPTYLINE

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SUMMARY

The polymorphic forms of α_1-acid glycoprotein have been determined by isoelectric focusing of small samples of whole plasma, without prior isolation of the protein. The results obtained by this technique confirm the microheterogeneity of this glycoprotein, which is not due to artefacts. Densitometric measurements of the polymorphic forms of this protein, which binds antidepressive drugs, have been performed in twelve depressive patients.

INTRODUCTION

The general biological and clinical significance of glycoproteins has been reviewed extensively [1, 2]. In psychopharmacology, some research has been focused on the α_1-acid glycoprotein. Indeed, the most commonly used anti-depressive drugs are bound in blood to this protein [3].

α_1-Acid glycoprotein (AAG) is heterogeneous in the native state [4]. On starch gel electrophoresis, the native protein exhibits four different patterns, with 5, 6, 7 or 8 bands [5]; the same patterns are observed on polyacrylamide gel isoelectric focusing (PAGIF) [6]. This heterogeneity is due to different linkages of sialic acids to galactose residues of the oligosaccharide side-chain and to different loci of the oligosaccharide chains linked to the protein [4]. These different linkages modify the p*I* of the protein and produce the polymorphic forms of AAG. This polymorphism appears to be genetically transmitted and different races exhibit different percentages of the polymorphic forms [7]. Until now AAG had to be isolated from large volumes of

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plasma prior to PAGE or PAGIF analysis; moreover, it is only recently that the polymorphic forms have been quantitated [6].

The present paper describes a simple method for the determination of polymorphic forms by direct analysis in only 20—50 μl of total plasma, without prior isolation of AAG. Results on the distribution and the quantitation of polymorphic forms in depressive patients are presented.

MATERIAL AND METHODS

Acrylamide (twice crystallised), N,N′-methylene bisacrylamide (twice crystallised) and Coomassie Brilliant Blue R-250 were from Serva (Heidelberg, G.F.R.). Ampholines were purchased from LKB (Bromma, Sweden). All other chemicals were of analytical grade and obtained from E. Merck (Darmstadt, G.F.R.).

Polyacrylamide gel slabs with a pH gradient of 2.5—4.2 are prepared according to ref. 8. PAGIF is performed using an LKB Multiphor, with a constant power of 15 W and a run time of 3 h.

A 20—50 μl volume of heparinised blood is applied at the cathodic end of the gel using small pieces of filter paper, which are removed after 1 h of focusing.

At the end of the analysis the pH gradient is measured across the gel using a surface electrode (Metrohm, Herisau, G.F.R.) at 15°C.

The gels are fixed, stained and preserved according to ref. 8, with a modification to the staining period which is lengthened to 1 h instead of 10 min.

Densitometric measurements are performed using a Beckman Densitometer R-112.

Subjects

The development of the method has been carried out with plasma obtained from the Swiss Red Cross (Lausanne, Switzerland) and with blood samples from healthy subjects. Twelve depressive patients treated for three weeks with 150 mg of Laroxyl® (amitriptyline) participated in a clinical study. The pharmacological treatment started on day 1 after the first blood sampling.

Preliminary experiments have been performed according to those proposed by Giannazza and Righetti [9] in order to detect an eventual presence of artefacts.

RESULTS AND COMMENTS

Artefacts

Four tests were performed to detect the possible presence of artefacts: (1) PAGIF with and without prefocusing; (2) PAGIF with different Ampholine concentrations; (3) PAGIF in 8 *M* urea; and (4) two-dimensional PAGIF.

Identical results are obtained when PAGIF is performed, with the same sample, according to tests 1—3, demonstrating the absence of artefacts. Two examples illustrate experiment 1 (Fig. 1a and b) and experiment 2 (Fig. 2a and b).

The results of two-dimensional PAGIF (experiment 4) (Fig. 3) clearly show

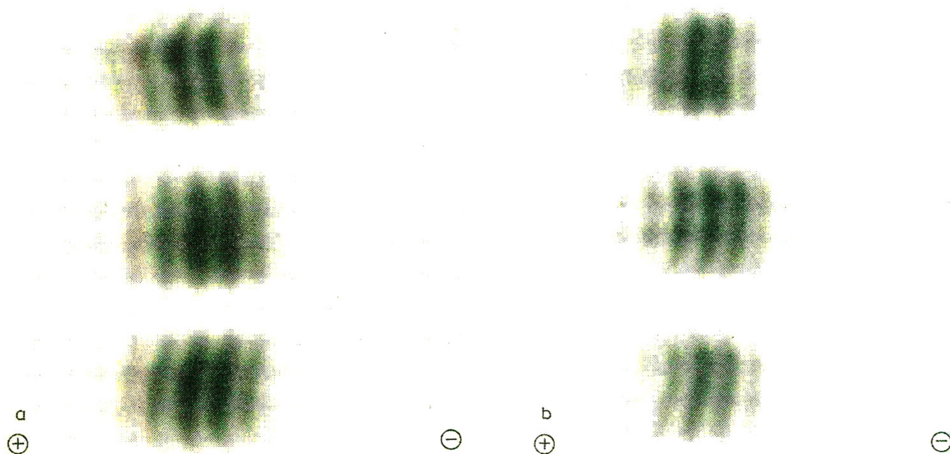

Fig. 1. Test for artefacts. Comparison of two PAGIF analyses of the same plasma sample, without prefocusing (a) and with prefocusing for 1 h (b). Conditions: 3 h focusing at 10° C, or 1 h prefocusing and 2 h focusing, with a constant power of 15 W.

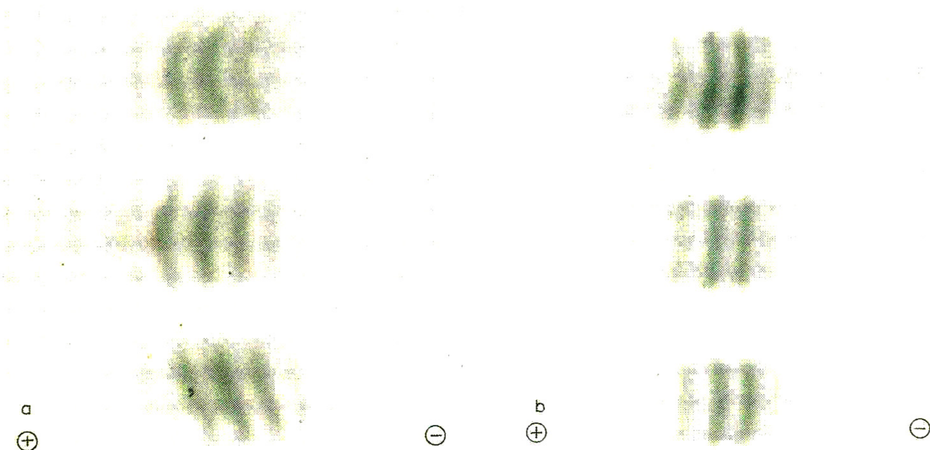

Fig. 2. Test for artefacts. The same plasma sample is focused with two different Ampholine concentrations: 5% Ampholine (a) and 2.5% Ampholine (b). Conditions: 3 h focusing at 10° C, with a constant power of 15 W.

the absence of artefacts. If artefacts were present, some bands in the second dimension would not be aligned diagonnally with the others (cf. ref. 9).

pH gradients

Experimental gradients are usually 0.1—0.8 unit higher than the theoretical ones. This difference is not constant along the whole length of the gel slab; there might be differences of 0.1—0.3 unit between the gradients measured at the top and those measured at the bottom of the gel (Fig. 4). Therefore, in all

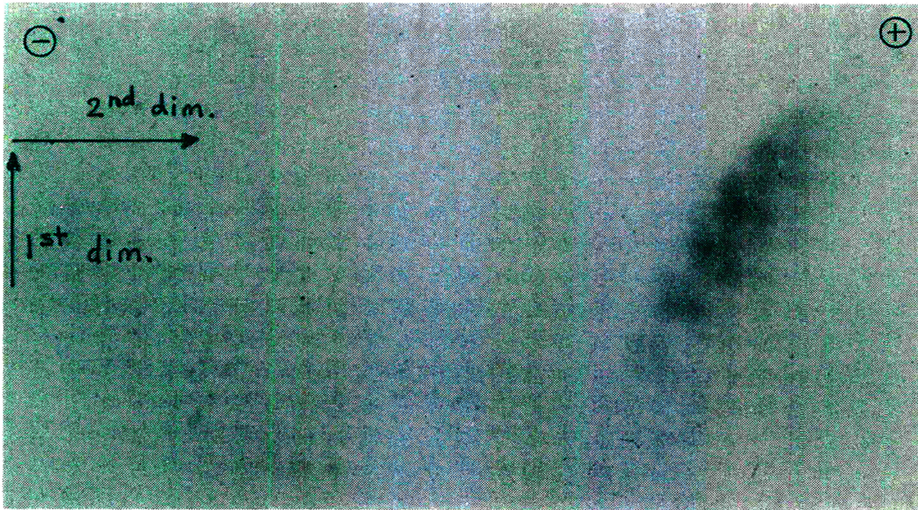

Fig. 3. Test for artefacts. Polymorphic forms of AAG after two-dimensional PAGIF. Conditions: 2 h focusing in the first dimension and 2 h in the second at 10° C, with a constant power of 15 W.

subsequent experiments, at least three sets of pH measurements will be performed, usually at 5, 12 and 20 cm from the top of the gel. For each set, ten pH measurements are performed every cm across the gel.

Polymorphic forms of AAG

Polymorphic forms have been determined in twelve depressive patients in a three-week study.

Fasting blood samples were drawn at days 1, 8, 15 and 22, and the total plasma samples were analysed by PAGIF. For each patient, the p*I* values and the relative intensity of the individual bands were determined.

The patients can be classified in three groups from the number of bands of the polymorphic patterns: group 1 — two subjects with 6 bands; group II — four subjects with 7 bands; group III — six subjects with 8 bands. An example of each of the three phenotypes is presented in Fig. 4.

In each patient, the number of bands and the p*I* of the individual bands are constant for all four determinations. In Fig. 5 the average p*I* values of polymorphic forms for the twelve subjects are shown. It seems from Fig. 5 that it is not possible to decide which bands are identical interindividually. For this reason, no means of the p*I* values were calculated within the group.

The average relative intensities of the bands for the twelve subjects are represented in Table I; as for the p*I*, the intensities are constant for the four analyses. This is consistent with the assumption that the polymorphism of AAG is genetically determined, and that the polymorphic forms are synthesised in the same ratios.

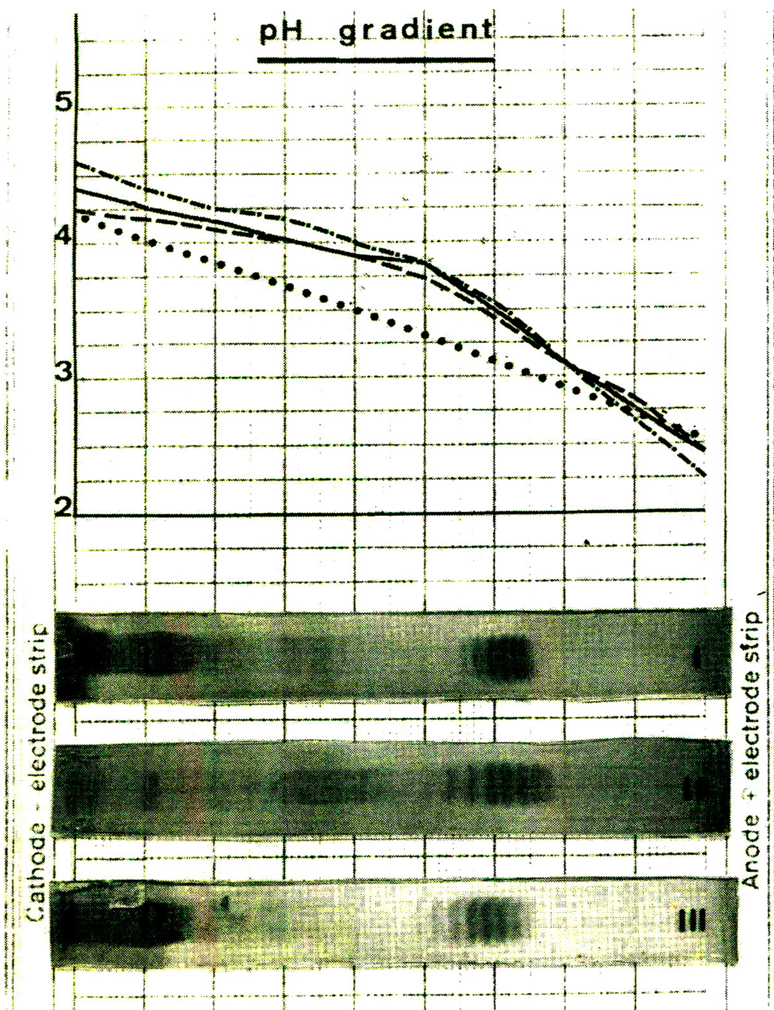

Fig. 4. Experimental pH gradient and polymorphic forms of AAG. Above: the pH is measured every cm with a surface electrode, from the top of the gel: ——, gradient at 5 cm; — · —, gradient at 12 cm; — — —, gradient at 20 cm, · · ·, theoretical gradient. Below: comparison of the three polymorphic forms of AAG: I = pattern with 6 bands; II = pattern with 7 bands; III = pattern with 8 bands.

CONCLUSION

PAGIF has proved to be a very valuable technique for the determination of polymorphic forms of AAG in total plasma samples. It is shown that AAG needs not to be isolated from plasma prior to focusing; its very acidic p*I* allows its complete separation from other plasma proteins on a gel with a pH gradient of 2.5—4.2.

With home-made gels the formation of the pH gradient is highly reproducible. Moreover, the deviation from theoretical gradient never exceeds

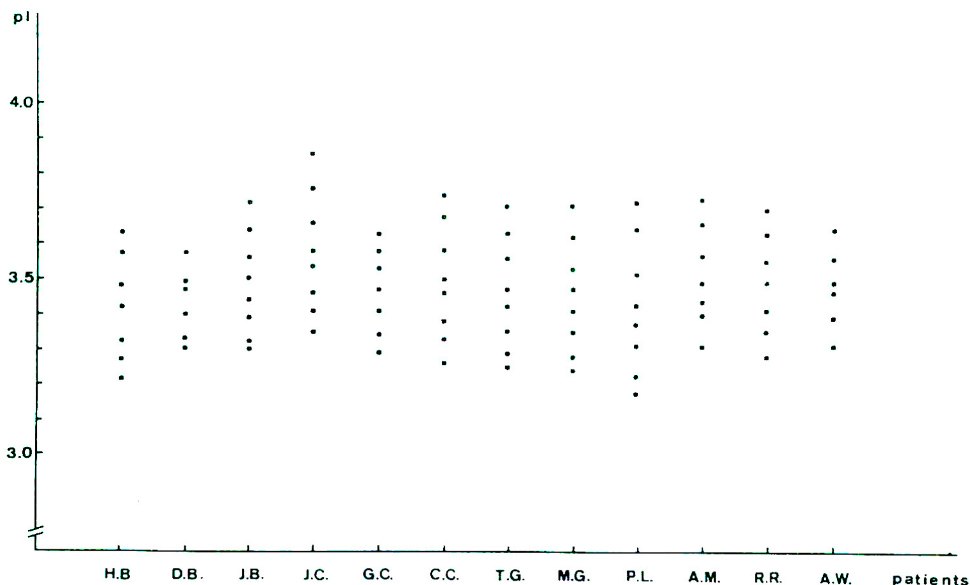

Fig. 5. Average p*I* values of the polymorphic forms of AAG in twelve depressive patients.

TABLE I

AVERAGE RELATIVE INTENSITIES (%) OF POLYMORPHIC FORMS OF AAG IN TWELVE DEPRESSIVE PATIENTS

Patient	No. of bands							
	1	2	3	4	5	6	7	8
H.B.	4.2	6.9	11.3	19.9	24.5	20.1	14.5	
D.B.	3.6	8.4	15.3	25.3	27.5	19.9		
J.B.	2.7	4.6	8.8	18.2	21.4	24.7	14.3	5.8
J.C.	3.8	5.0	10.2	16.9	20.5	23.3	14.4	6.1
G.C.	3.0	5.9	11.5	23.1	27.1	17.8	11.8	
C.C.	4.2	5.0	7.8	19.8	23.9	24.9	11.5	3.5
T.G.	2.8	6.7	11.8	16.9	25.0	20.2	11.2	5.1
M.G.	3.6	6.5	9.8	17.2	24.9	20.3	11.0	6.7
P.L.	1.1	3.4	8.3	15.2	21.8	23.6	17.0	
A.M.	1.3	4.5	11.6	21.0	25.3	23.0	15.1	
R.R.	3.6	9.0	19.8	30.7	21.6	11.2	4.1	
A.W.	3.3	9.2	19.0	30.6	26.0	29.0		

0.8 pH unit, i.e. 15%, which is satisfactory for home-made gels. Experimental pH gradients are always more basic than in theory, except at the anode where they are more acidic; the maximum anodic deviation is 0.34 pH unit, i.e. 16%. Other experiments would be necessary in order to determine if this deviation is due to cathodic drift [10] or if it is due to an incomplete formation of the pH gradient, which would not be achieved in 3 h.

The experimental pH gradients are not linear along the whole gel; it is there-

fore necessary to perform at least three series of pH measurements, one at the top, one at the middle and one at the bottom of the gel.

Tests for artefacts show that none are formed during PAGIF of total plasma samples.

The determination of polymorphic forms in twelve patients during a 3-week period reveals no intraindividual differences in the number and in the relative concentrations of polymorphic forms. For each subject, the polymorphic pattern and the relative intensities of bands are conserved, even when total AAG plasma levels rise [12]. This observation confirms that the polymorphic forms of AAG are genetically determined.

The present results complete earlier findings, where qualitative analysis gave evidence of at least seven polymorphic forms with quantitative evaluation [11]. It is difficult to compare our densitometric data with those of Berger et al. [6], as comparative measurements of the p*I* values of the corresponding bands are missing. Until a method exists for a more complete chemical characterization of the bands, assignment of a number to the bands is rather arbitrary.

With regard to the binding of the antidepressive drug amitriptyline to AAG, nothing is known about the possibility that one polymorphic form would bind this drug more than another. If this were the case, interindividual differences could occur in the binding of antidepressive drugs as the number of bands vary between subjects.

ACKNOWLEDGEMENTS

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DETERMINATION OF GLYCERYL TRINITRATE IN HUMAN PLASMA BY GAS CHROMATOGRAPHY—NEGATIVE ION CHEMICAL IONIZATION—SELECTED ION MONITORING

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SUMMARY

A specific, sensitive and accurate quantitation method for glyceryl trinitrate was developed using gas chromatography—negative ion chemical ionization—selected ion monitoring with dichloromethane as a reagent gas. $[^{15}N_3]$ and $[^{2}H_5, {}^{15}N_3]$ variants were synthesized from non-labelled or $[^{2}H_8]$glycerol and $[^{15}N]$nitric acid. The former variant was used for preventing adsorption of glyceryl trinitrate onto active sites on column materials and the latter was used as an internal standard for quantitation of glyceryl trinitrate in biological fluids by selected ion monitoring. The quantitation limit of this method is 0.1 ng/ml of human plasma. When glyceryl trinitrate was administered intravenously in the dose of 4 μg/kg to patients receiving hypotensive anesthesia for surgical operation, the plasma levels exhibited a biexponential decay. The mean and standard deviation of half-lives of the α and β phases were found to be about 0.41 ± 0.13 and 5.34 ± 1.60 min, respectively.

INTRODUCTION

Glyceryl trinitrate (GTN) has been used effectively to decrease peripheral vascular resistance in patients in heart failure and after acute myocardial infarction. Recently, it has been recognized that GTN is useful as a hypotensive drug during general anesthesia for surgical operation. However, there are no

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reports on the pharmacokinetic data of GTN in patients receiving hypotensive anesthesia with it. Previous studies on the metabolism of GTN in experimental animals using radioisotope technique [1, 2] indicated that the plasma level is extremely low because GTN is rapidly metabolized by endogenous esterases to glyceryl dinitrates, glyceryl mononitrates and glycerol. It is therefore essential to determine exactly plasma GTN levels in patients in order to establish a dose schedule which enables favourable blood pressure during hypotensive anesthesia.

Several analytical methods for the microdetermination of GTN in plasma have been published, in which gas chromatography (GC) with an electron-capture detector [3—5] was used. However, there are no methods that use an internal standard to compensate completely for the losses of GTN during sample preparation.

GC—mass spectrometry (MS) enables the use of an ideal internal standard labelled with stable isotopes. It is known that in the electron impact ionization mode GTN gives rise to NO_2^+ as a base peak [6]. With the positive ion chemical ionization (PICI) mode using various reagent gases, there were no prominent ions reflecting the molecular weight in the high mass region [7]. The use of the above fragment ions for monitoring GTN in biological fluids may be unsuitable because of poor specifity due to lack of information on its molecular weight. On the other hand, there has been only one report [8] that $[M+H]^+$ could be observed in the PICI mode when the temperature of the ion source in a direct inlet system was kept at less than 60°C by a special temperature-control device.

Negative ion chemical ionization (NICI)-MS provides not only enhancement of the sensitivity of the detection limit but also enough information to confirm the molecular weight of the sample in comparison with PICI-MS [9]. Consequently, this technique has been applied to the micro-analysis of compounds with high electron capture [10—12], suggesting that it may be applicable to the determination of trace amounts of GTN in biological fluids. This paper deals with a sensitive and specific method for the quantitation of GTN in human plasma by GC—NICI—SIM using the $[^2H_5, {}^{15}N_3]$ variant as an internal standard.

EXPERIMENTAL

Materials

All reagents and solvents used in this study were of analytical grade and were used without further purification.

GTN used as the standard material was obtained by several washings of the medicinal grade with water until nitrate ions, glycerol, glyceryl mononitrates and glyceryl dinitrates were completely removed. Its purity was checked by the high-performance liquid chromatographic method of Crouthamel and Dorsch [13]. $[^{15}N_3]$ and $[^2H_5, {}^{15}N_3]$ variants were synthesized in our Explosives Research Laboratory, using $[^2H_8]$glycerol (Merck Sharp & Dohme Canada Ltd., Quebec, Canada) and/or $[^{15}N]$nitric acid (Merck Sharp & Dohme Canada Ltd.). The concentration of GTN and the $[^2H_5, {}^{15}N_3]$ variant in ethanol solution (50 ng/ml) was determined according to the procedure described in the Pharmacopeia Japonica (9th edition).

Sephadex LH-20 (25—100 μm) was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden) and silica gel (70—230 mesh) from Merck (Darmstadt, G.F.R.). An Extube® No. 1003 was purchased from Analytichem International (CA, U.S.A.).

Gas chromatography—mass spectrometry

A Shimadzu-LKB 9000A GC—MS system modified for detection of negative ions and equipped with a data processing system was employed. The column was 1.0 m × 2.0 mm I.D. glass, packed with 3% OV-3 (Ohio Valley Speciality Chemical Co., Marietta, OH, U.S.A.) on Gas-Chrom Q 80—100 mesh (Applied Science Labs., State College, PA, U.S.A.). The temperature of the column oven was maintained at 130°C. The temperature of the injection port and separator was 180°C, and the ionization source was kept at 190°C. The flow-rate of helium gas was 30 ml/min. The accelerating voltage was —3.5 kV. The ionization energy and emission current were 500 eV and 500 μA, respectively.

Selected ion monitoring

Prior to the determination of GTN plasma levels, the column was loaded with three 1-μl injections of the [$^{15}N_3$] variant in ethanol solution (1 μg/ml) to saturate all binding sites: then the sample solutions were subjected to selected ion monitoring (SIM).

The peak heights of chlorinated molecular ions $[(M+Cl]^-$ of GTN and the [$^2H_5$, $^{15}N_3$] variant used as an internal standard were monitored at *m/z* 262 and 270. The ratio of the peak heights of these two ions was calculated and compared with a calibration curve to determine the plasma levels.

Administration of GTN

Six patients, average age 52 years (range 36—73 years), received hypotensive anesthesia for surgical operation under the supervision of Drs. K. Hanaoka and K. Nishitatsuno (Department of Anesthesiology, Medical School, Tokyo University, Tokyo, Japan). Hypotension was induced during the operation by bolus injection of GTN aqueous solution (0.5 mg/ml) in the dose of 4 μg/kg. Blood samples were obtained at 0.5, 1, 2, 5, 10 and 15 min after injection of the GTN solution, using a heparinized syringe.

Sample preparation

The blood sample was immediately centrifuged at 1910 *g* for 15 min at 0°C and the plasma was collected. The internal standard solution (40 ng/ml) was added to each 2.0-ml aliquot of plasma. This procedure was carried out in a ice-bath in order to avoid the enzymatic degradation of GTN. The plasma samples were transferred directly onto the Extube No. 1003 (solid-phase extraction tube). After the Extube had been allowed to stand for about 5 min, the GTN was eluted with 25 ml of *n*-hexane. This eluate was transferred to a column (50 × 5 mm) packed with silica gel in *n*-hexane. The column was washed with 15 ml of *n*-hexane and eluted with benzene—*n*-hexane (1:6). The fraction of 20—50 ml was collected and concentrated to approximately 0.1—0.2 ml. The residue was redissolved with 0.5 ml of *n*-hexane—chloroform—methanol (10:10:1) and applied to a column (100 × 5 mm) packed with

Sephadex LH-20 in *n*-hexane—chloroform—methanol (10:10:1). The column was washed and eluted with the same solvent described above. The fraction of 4—8 ml was collected and concentrated to approximately 5—10 μl. The residue was redissolved with 30 μl of benzene and an aliquot of this solution was subjected to GC—NICI—SIM.

RESULTS AND DISCUSSION

NICI-MS provides the negative molecular ion yielded by compounds possessing electron-capture ability. Contrary to our expectations, however, when methane, isobutane or ammonia was used as a reagent gas in the NICI mode, GTN gave rise to the nitrate ion $[NO_3]^-$ as a base peak, and there were no negative ions in the high mass region, whereas, when dichloromethane was used as a reagent gas, it has been reported that the chlorinated molecular ion $[M+Cl]^-$ was often observed as a prominent ion [6]. Thus, NICI-MS of GTN using dichloromethane was examined.

Fig. 1A shows the NICI mass spectrum of GTN with dichloromethane as a reagent gas. The chlorinated molecular ion $[M+Cl]^-$ was confirmed by the presence of the doublet ion at *m/z* 262 and 264, due to the characteristic intensities of the chlorine atom. The adduct ion with nitrate ion yielded by the GTN molecule was observed at *m/z* 289. Although it has been reported that the NICI mass spectrum of dichloromethane is characterized by the appearance of Cl^-, HCl_2^- and $CH_2Cl_3^-$ ions [14], the expected $[M + HCl_2]^-$ and $[M + CH_2Cl_3]^-$ ions could not be observed in the spectrum of GTN. The fragment ions ap-

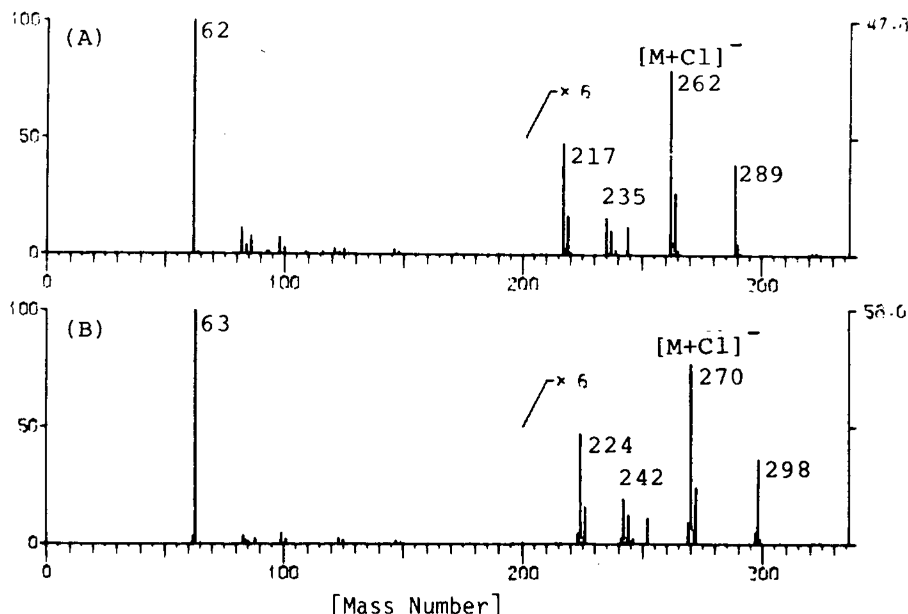

Fig. 1. NICI mass spectra of glyceryl trinitrate (A) and its [$^2H_5$, $^{15}N_3$] variant (B) using dichloromethane as a reagent gas.

pearing at m/z 217 and 235 were considered to be produced by the loss of $NOCl_2$ and HNO_3 from the unidentified ion $[M+HCl_2]^-$, because, when $C_2{}^2H_2Cl_2$ was used as a reagent gas, the ion at m/z 217 was shifted by one mass unit to m/z 218, whereas the ion at m/z 235 remained without any shift, and the ions corresponding to those of m/z 217 and 235 were observed at m/z 224 and 242 in the mass spectrum of the $[^2H_5, {}^{15}N_3]$ variant (Fig. 1B).

On the other hand, it has already been reported [4, 5, 15] that the GC column should be loaded with GTN by several injections prior to analysis, to prevent loss due to adsorption of GTN onto possible active sites on the column materials. However, if the GTN adsorbed to active sites on the column material was labile, it might be released gradually and lead to the appearance of a "memory" peak, resulting in the over-estimation of GTN. In order to clarify this problem, it was necessary to examine whether the adsorbed GTN was released by the subsequent injection of another sample solution containing GTN. Thus, 2 μl of the $[^{15}N_3]$ variant in ethanol (1 μg/ml) used as a tracer were injected three times to saturate all binding sites on the column materials, and then 1 μl of the $[^2H_5, {}^{15}N_3]$ variant in ethanol (50 μl/ml) was injected. Then the release of the $[^{15}N_3]$ variant coated onto the column materials was monitored using the $[M+Cl]^-$ ion of m/z 265 in the SIM mode, but there was no corresponding peak by this monitoring. When a mixture of GTN and the $[^2H_5, {}^{15}N_3]$ variant in the ratio 1:2 was injected ten times in succession, it was found that the initial ratio calculated by measuring the peak height of these two peaks was maintained for every subsequent injection, as shown in Table I, indicating that GTN adsorbed onto column materials was not replaced by the following injection of GTN. These results show that preinjection of the $[^{15}N_3]$

TABLE I

REPRODUCIBILITY OF THE PEAK HEIGHT RATIOS IN TEN SUCCESSIVE INJECTIONS OF A 1:2 MIXTURE OF GLYCERYL TRINITRATE AND ITS $[^2H_5, {}^{15}N_3]$ VARIANT

A 1-μl aliquot of the mixture of GTN and its $[^2H_5, {}^{15}N_3]$ variant in ethanol solution (0.5 and 1.0 μl/ml) was injected. Prior to the determination, ion intensities of chlorinated molecular ions $[M+Cl]^-$ of m/z 262 and 270, which appeared with an intensity ratio of about 1:2, were made equal by adjusting a gain controller in the multiple-ion detector.

Injection	Ratio of peak heights
1	1.09
2	0.98
3	0.99
4	1.01
5	1.06
6	1.04
7	0.98
8	0.99
9	0.96
10	1.04
Mean ± C.V. (%)	1.01 ± 3.27

variant in ethanol solution may be a useful means of covering the active sites on the column materials.

It is well known that GTN is rapidly degraded enzymatically in plasma [16]. Thus some enzyme inhibitors were added to prevent loss due to the enzymatic degradation of GTN. For instance, Yap et al. [17] reported that this enzymatic degradation of GTN could be inhibited for at least 1.5 h by the addition of silver nitrate in a 0.05% final concentration and the residual amount of GTN after 12 h was found to be 80%. When human blood with a suitable amount of GTN added was allowed to stand at room temperature, GTN was degraded with a half-life of 3.5 h, as shown in Fig. 2. Whereas the degradation of GTN could be inhibited significantly by cooling the blood sample immediately in a ice-bath, practically quantitative recovery of GTN was obtained if fresh plasma was kept below 0°C by immersion in a ice-bath and analyzed within 12 h. Thus, the loss of GTN before the addition of the internal standard solution could be eliminated completely by the above simple treatment.

Solid-phase extraction using an Extube and non-polar organic solvent made it possible to extract GTN from its polar metabolites such as denitration products and their glucuronide conjugates. The recovery of GTN from human plasma by extraction using Extube No. 1003 was more than 95%, whereas the polar metabolites were retained on this column. This method provides not only reproducible and excellent recovery but also simple and rapid extraction and purification of GTN from plasma. The fraction obtained was further purified in order to eliminate interfering substances and to prevent overloading the column with endogenous substances in the sample, since the signal-to-noise ratio may be affected by endogenous substances with physicochemical properties similar to those of the compounds of interest.

The [$^{2}H_5$, $^{15}N_3$] variant was synthesized for use as an internal standard in the quantitation of GTN in human plasma by GC—NICI—SIM. As shown in Fig. 1B, the NICI mass spectrum of the [$^{2}H_5$, $^{15}N_3$] variant was similar to that of the non-labelled compound except for the moderate mass unit shift in some ions, i.e. the chlorinated molecular ion was observed at *m/z* 270 with a shift of eight mass units from *m/z* 262 in the non-labelled compound. The relative abundance of the chlorinated molecular ion cluster agreed well with that

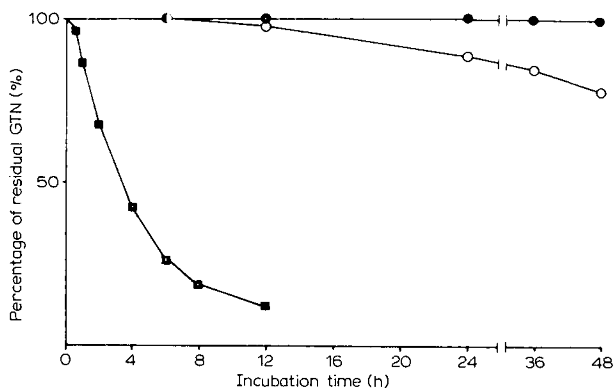

Fig. 2. Stability of glyceryl trinitrate in human plasma at −20°C (●), 0°C (○) and 37°C (■).

calculated from the enrichment of [^{15}N]nitric acid (isotope purity ≈ 96%), suggesting that no loss of the deuterium atom from the carbon—deuterium bond in [$^{2}H_{8}$]glycerol occurred during nitration. MS analysis revealed that the labelled compound synthesized as an internal standard was a mixture of the [$^{2}H_{5}$, $^{15}N_{2}$] and [$^{2}H_{5}$, $^{15}N_{3}$] variants, and the isotopic purity of the [$^{2}H_{5}$, $^{15}N_{3}$] variant was estimated to be 89.6%.

To clarify the maximum permissible amount of internal standard that could be added to human plasma, the accurate ratio of non-labelled GTN to the [$^{2}H_{5}$, $^{15}N_{3}$] variant in the internal standard was determined by SIM. The recording of ion intensities at m/z 262 and 270 indicated that the ratio of non-labelled GTN to the [$^{2}H_{5}$, $^{15}N_{3}$] variant was less than 0.1%. This result confirms that it is permissible to add the internal standard to the extent of 10—100 times the amount of GTN in plasma.

The calibration curve constructed for GTN using the [$^{2}H_{5}$, $^{15}N_{3}$] variant as an internal standard gave a good linearity in the concentration range 1—20 ng/ml of plasma. Fig. 3 shows a representative selected ion recording obtained when a 1-μl aliquot of plasma extract containing approximately 300 pg of GTN and 1 ng of the [$^{2}H_{5}$, $^{15}N_{3}$] variant was analyzed. The retention times of these compounds were approximately 1 min.

Drug-free control plasma showed no interfering peaks at m/z 262 and 270, suggesting that the quantitation of GTN by the present method can be performed without interference from endogenous substances in plasma. A known amount of GTN was added to the drug-free plasma together with an aliquot of the internal standard solution and then extracted, purified and analyzed as described in Experimental. The analytical data and recovery are

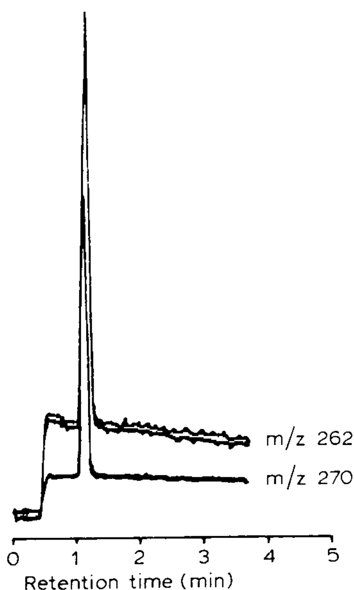

Fig. 3. Selected ion recording of glyceryl trinitrate and its [$^{2}H_{5}$, $^{15}N_{3}$] variant extracted from human plasma of a patient receiving hypotensive anesthesia for surgical operation. Apparatus: Shimadzu-LKB 9000A with multiple-ion detector. Column: 3% OV-3 on Chromosorb W HP. Column temperature: 130°C. Reagent gas: dichloromethane.

TABLE II

RECOVERY OF GLYCERYL TRINITRATE FROM DRUG-SUPPLEMENTED HUMAN PLASMA

Added (ng/ml)	Recovery (%)			Analysis of variance*	
	X_1	X_2	Mean ± S.D.	$\bar{\sigma}_E$	$\bar{\sigma}_S$
1.0	98.3	91.5			
	94.1	89.3			
	108.3	106.2	97.9 ± 6.9	3.07	6.34**
	104.1	101.5			
	95.2	91.5			
5.0	106.0	99.7			
	104.1	102.2			
	92.2	97.3	98.8 ± 4.4	3.01	3.42
	98.3	97.1			
	97.6	93.4			

*$\bar{\sigma}_E$ = estimated value of standard deviation for SIM process; $\bar{\sigma}_S$ = estimated value of standard deviation for sample preparation process.
**Statistically significant at the 5% level.

shown in Table II. The recoveries for the two series of drug-supplemented plasma were found to be 97.9 ± 6.9% and 98.8 ± 4.4%, and there were no significant differences in drug level. The analytical data Table II were submitted

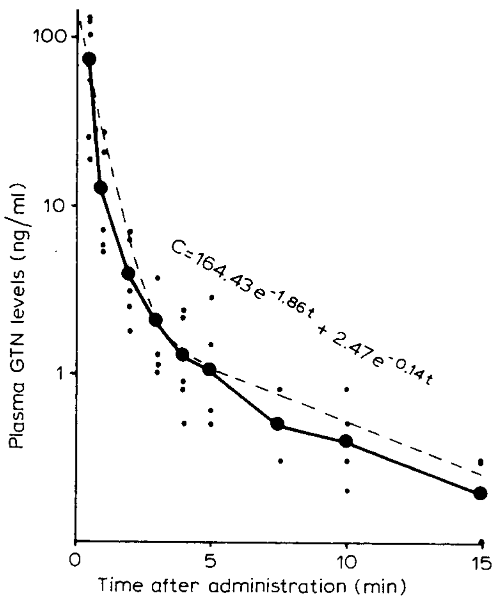

Fig. 4. Average plasma glyceryl trinitrate levels after intravenous administration of a single dose (4 μg/kg) in patients receiving hypotensive anesthesia for surgical operation. (•) Observed value; (•——•) curve for averaged value; (— — —) curve calculated by fitting a two-compartment model to the data.

TABLE III

TWO-COMPARTMENT DISPOSITION CONSTANTS* FOR GLYCERYL TRINITRATE FOLLOWING INTRAVENOUS ADMINISTRATION (4 μg/kg) IN SIX PATIENTS UNDERGOING HYPOTENSIVE ANESTHESIA FOR SURGICAL OPERATION

Patient	α (min^{-1})	β (min^{-1})	k_{12} (min^{-1})	k_{21} (min^{-1})	k_{10} (min^{-1})	V_1 (l)	Cl (l min^{-1})
A	3.09	0.22	0.58	0.28	2.46	0.60	1.48
B	1.76	0.16	0.23	0.19	1.50	4.68	7.02
C	1.54	0.16	0.18	0.18	1.33	1.65	2.19
D	1.47	0.11	0.43	0.16	0.99	8.44	8.36
E	2.14	0.10	0.10	0.11	2.03	0.62	1.26
F	1.16	0.10	0.22	0.13	0.91	6.78	6.17
Mean	1.86	0.14	0.29	0.18	1.54	3.80	4.41
S.D.	0.68	0.05	0.18	0.06	0.61	3.35	3.13

* α = elimination rate constant of the α phase.
β = elimination rate constant of the β phase.
k_{12} = apparent first-order intercompartment transfer rate constant from the central compartment to the peripheral compartment.
k_{21} = apparent first-order intercompartment transfer rate constant from the peripheral compartment to the central compartment.
k_{10} = apparent first-order elimination rate constant from the central compartment.
V_1 = apparent volume of the distribution in the central compartment.
Cl = clearance.

to the statistical analysis of one-way lay out [18, 19] in order to divide the total variation in this experiment into the variations of the sample preparation and the SIM measurement. As shown in the last column of Table II, the estimated standard deviations for sample preparation and SIM process in this recovery experiment were calculated to be 6.34 and 3.07 at the 1 ng/ml GTN level and 3.42 and 3.01 at the 5 ng/ml GTN level, respectively. This result indicates that the loss of GTN through the process of sample preparation may be compensated for completely by the use of internal standard.

Fig. 4 shows the average of the plasma GTN concentration—time curve observed after intravenous bolus administration of a GTN aqueous solution in the dose of 4 μg/kg to six patients receiving hypotensive anesthesia with GTN for surgical operation. The plasma levels exhibited a biexponential decrease and then the decay curve was fitted in a two-compartment open model. The average and standard deviation of the half-lives of the α and β phases were 0.41 ± 0.13 and 5.34 ± 1.60 min, respectively. Table III lists the pharmacokinetic parameters in the two-compartment model calculated from the above data. These pharmacokinetic data may be useful for the estimation of GTN levels suitable for maintaining a favourable hypotension during hypotensive anesthesia.

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QUANTITATIVE DETERMINATION OF THE β-ADRENOCEPTOR STIMULANT FORMOTEROL IN URINE BY GAS CHROMATOGRAPHY MASS SPECTROMETRY

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SUMMARY

A method for the quantitative determination of the β-stimulant formoterol in urine, using a gas chromatograph—mass spectrometer, is described. Formoterol can be analyzed after the addition of a deuterium-labelled internal standard and conversion to a mixed bis-pentafluoropropionyl-methyl derivative for selected ion monitoring. The detection limit was 5 ng/ml.

Urinalysis after the oral administration of formoterol fumarate, using a combined enzymic hydrolysis method, revealed that the drug was conjugated with glucuronic acid in rats, dogs and humans.

INTRODUCTION

A catecholamine analogue formoterol fumarate, 2-hydroxy-5-[(1*RS*)-1-hydroxy-2-{[(1*RS*)-2-(*p*-methoxyphenyl)-1-methylethyl]amino}ethyl]-formanilide fumarate dihydrate, has been introduced as a potent β-adrenoceptor stimulant with high selectivity for β_2-receptor [1—3]. Because its effective dose is small (0.5—10 μg/kg), we can expect formoterol concentrations to be very low in biological samples. For drug measurements we employed selected ion monitoring. Our method involves the detection of nanogram levels of urinary formoterol as the corresponding bis-pentafluoropropionyl-methyl derivative, using a deuterium-labelled internal standard.

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EXPERIMENTAL

Chemicals

Formoterol fumarate was synthesized in our laboratory by the method of Murase et al. [1]. Deuterium-labelled formoterol used as internal standard was prepared by the same method, except that for some raw materials we substituted deuterium compounds as follows. *p*-Methoxyphenylacetone, which was the source of the 2-(*p*-methoxyphenyl)-1-methylethyl function of formoterol, was boiled in a mixture of 1 *N* NaO^2H in ^2H_2O and $C^2H_3O^2H$ (1:1) and then extracted with diethyl ether. The *p*-[$^2H_5$]methoxyphenylacetone obtained was mixed with benzylamine in $C^2H_3O^2H$ and the solution was hydrogenated in the presence of platinum. In this step, deuterium from $C^2H_3O^2H$ was partially introduced into the product and a mixture of 2-benzylamino-1-(*p*-methoxyphenyl)-[1,1,3,3,3-$^2H_5$]propane and -[1,1,2,3,3,3-$^2H_6$]-propane was obtained. The mixture was subjected to the next process. For the reduction of the ketone to the alcohol with $NaBH_4$ [1], we used NaB^2H_4 instead, and finally obtained a mixture of [$^2H_6$]- and [$^2H_7$]formoterol. 2-Hydroxy-[3-^3H]-5-[(1*RS*)-1-hydroxy-2-{[(1*RS*)-2-(*p*-methoxyphenyl)-1-methylethyl]amino}ethyl]-formanilide fumarate dihydrate ([^3H]formoterol fumarate, radiochemical purity > 99%) was synthesized by Shinloihi Co. (Kanagawa, Japan).

Pentafluoropropionic anhydride (PFPA) was purchased from Tokyo Kasei (Tokyo, Japan). The ethereal solution of diazomethane was prepared from *p*-toluenesulfonyl-N-methyl-N-nitrosamide (Tokyo Kasei). β-Glucuronidase and arylsulphatase were from Boehringer Mannheim Yamanouchi (Tokyo, Japan).

Gas chromatograph—mass spectrometer

An Hitachi RMU-6MG gas chromatography—mass spectrometry (GC—MS) system fitted with an accelerating voltage alternator was used. Gas chromatography was on a glass column (50 cm × 3 mm I.D.) packed with 3% OV-1 on Chromosorb W (AW DMCS, 80—100 mesh) with a helium flow-rate of 30 ml/min. The temperatures were 260°C for the injection and interface, and 240°C for the column. The operating conditions were: electron energy, 20 eV; emission current, 80 μA; ion source temperature, 160°C; accelerating voltage, 2.2—2.4 kV; entrance and collector slits, 0.4 mm. Mass spectra were obtained with the GC—MS system in the scanning mode under the above conditions, except that the entrance and collector slits were adjusted to 0.1 mm and the accelerating voltage was 1.5 kV.

Procedures

To each 2 ml urine sample were added 2 ml of an aqueous solution containing 200 ng of internal standard and 0.5 g of sodium hydrogen carbonate; the mixture was extracted with 4 ml of ethyl acetate. The organic layer was then extracted with 3 ml of 0.1 *N* hydrochloric acid and aspirated off. After adding 0.8 g of sodium hydrogen carbonate, the aqueous layer was extracted with 4 ml of ethyl acetate. The organic layer was evaporated to dryness under reduced pressure. To the residue were added 10% pyridine in methylene chloride (100 μl), and 25% PFPA in methylene chloride (250 μl). After 30 min

at room temperature, the solvent and reagent were evaporated under a nitrogen stream; subsequently, 0.8 g of sodium hydrogen carbonate, 4 ml of distilled water and 4 ml of diethyl ether were added to the residue. The mixture was shaken thoroughly for 30 sec, centrifuged and the ether layer was evaporated to dryness. The residue was admixed with 100 μl of ethereal diazomethane and allowed to stand for 5 min at room temperature. After eliminating the solvent and reagent, the residue was dissolved in 50 μl of ethyl acetate, then 1—2 μl of the solution were injected for GC—MS. The molecular ions of the bis-PFP-methyl derivative of formoterol and [$^2H_7$]formoterol (m/z 604 and m/z 611, respectively) were monitored, and the peak height ratio was used to calculate the amount of formoterol in each sample by referring to the standard curve, prepared by subjecting control urine spiked with known amounts of formoterol (5—100 ng/ml) to the above procedure.

Extraction recoveries

Control urine samples containing 20 ng/ml [^3H]formoterol (0.01 μCi/ml) were extracted with ethyl acetate after adjusting the pH to between 1 and 10, and the radioactivity in the organic layer was counted on a Packard liquid scintillation counter (Model 3255). Concurrently, the urine samples spiked with [^3H]formoterol were carried through the assay procedure and the radioactivity extracted in each step of the procedure was measured.

Animal and human experiments

After an overnight fast, two male rats (200 and 210 g) and two male beagles (12 and 13 kg) received an oral dose of 60 and 500 μg/kg formoterol fumarate, respectively, in an aqueous solution; the 0—8 h urine was then collected. In the human study, three male volunteers (50—62 kg) received 40 μg of formoterol fumarate diluted with lactose after an overnight fast; their 0—6 h urine was collected. The urine samples (2 ml) were incubated at 37°C for 20 min, either with β-glucuronidase (9000 units, *p*-nitrophenyl glucuronide as substrate) at pH 7.0, or with arylsulphatase (7500 units, *p*-nitrophenyl sulphate as substrate) at pH 6.2. To inhibit the activity of contaminating β-glucuronidase, before incubation 10^{-3} *M* saccharo-1,4-lactone was added to the arylsulphatase medium. Control samples were analyzed without enzymic hydrolysis.

RESULTS AND DISCUSSION

Derivatization of formoterol for GC—MS

Formoterol has many reactive hydrogen atoms available for substitution (Fig. 1, I). The trimethylsilylation of formoterol with various silylating reagents did not give uniform products, owing to incomplete silylation of the amino or amido group. When N-trimethylsilylimidazole (TSIM) and N,O-bis-(trimethylsilyl)acetamide (BSA) were used as silylating reagents, the products obtained were a major bis-TMS derivative with a minor tris-TMS derivative, and a mixture of almost equal amounts of bis-TMS and tris-TMS derivatives, respectively. When a mixture of TSIM, BSA and trimethylchlorosilane (3:3:2), one of the most active combinations of silylating reagents, was used, a mixture of tris-TMS and tetrakis-TMS derivatives was obtained. Moreover, the ratio of

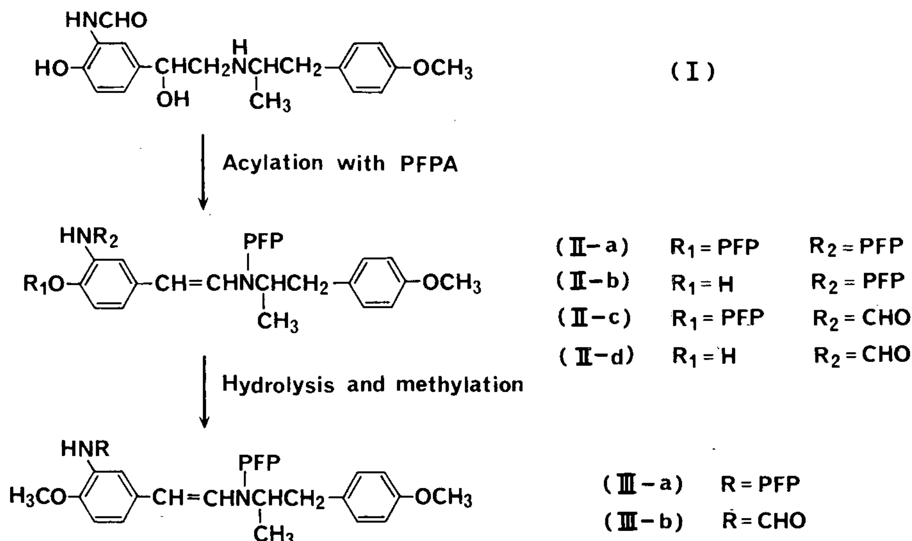

Fig. 1. Derivative-forming reaction of formoterol. Acylation of formoterol (I) with pentafluoropropionic anhydride (PFPA) in the presence of pyridine produces II-a and II-b resulting, after hydrolysis and methylation, in a single product (III-a). When formoterol is acylated with PFPA alone, the reaction is thought to proceed as I → II-c and/or II-d → III-b.

these derivatives was affected by the temperature of the sample; it also varied with the time course.

When formoterol was acylated with PFPA in the presence of pyridine and injected into a GC column (OV-1 3%, oven temperature 220°C), a sharp peak at the retention time of 1.7 min and a broad peak at 2.6 min were obtained. MS analysis revealed that the former peak was the tris-PFP derivative with a molecular ion at m/z 736 (Fig. 1, II-a) and the latter was the bis-PFP derivative with a molecular ion at m/z 590 (Fig. 1, II-b). Thus, acylation of formoterol did not produce a uniform product either. However, in alkaline solution, the phenolic ester group of II-a was preferentially hydrolyzed thereby converting II-a to II-b. Moreover, treatment of the unified derivative II-b with diazomethane gave bis-PFP-methyl formoterol (Fig. 1, III-a) which produced a well-shaped peak on GC. The derivative, dissolved in ethyl acetate, was stable for at least five days at room temperature. At the acylation step of I to II, the benzylic hydroxy group was lost; this type of hydroxy group of catecholamines has been acylated with PFPA [4]. Whether the loss of the hydroxy function occurs during the reaction, or thermally in the GC—MS system, is uncertain; however, dehydroxylation by perfluoroacylation has been reported by Knapp et al. [5]. The optimal acylation condition is shown in Fig. 2 and was verified by selected ion monitoring. When 10 μl of pyridine were added to the reaction mixture, recovery of III-a was highest and the yield reached a plateau after 30 min at room temperature (ca. 22°C). In the absence of pyridine, the yield of III-a was less than 10% of that in the presence of 10 μl of pyridine. We examined the side-reaction when pyridine was absent by thin-layer chromatography (TLC). Formoterol (20 μg) was reacted with PFPA without adding pyridine, hydrolyzed and methylated as described above and then subjected to TLC on silica gel 60 F_{254} plates (Merck, Darmstadt, G.F.R.), using the solvent system n-hexane—ethyl acetate—benzene (5:2:2). The product gave a major spot of

R_F 0.62 with a minor spot of R_F 0.74; acylation of formoterol with 10 μl of pyridine gave a major spot of R_F 0.74. The derivatives of R_F 0.74 and 0.62 were, as was suggested from mass spectra, III-a and the mono-PFP-methyl derivative (Fig. 1, III-b), respectively. Thus, pyridine causes deformylation and the main route of reaction without pyridine is thought to be I → II-c and/or II-d → IIIb.

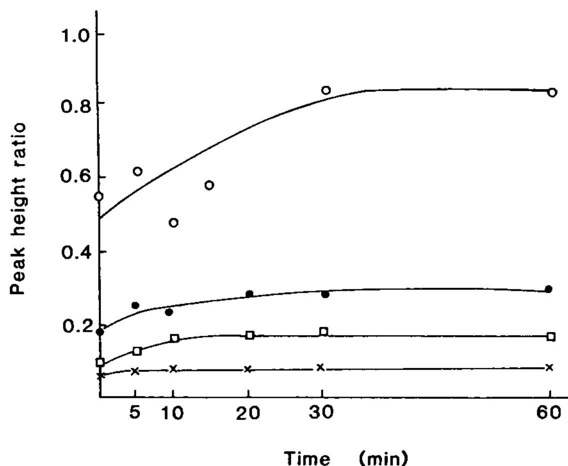

Fig. 2. Peak height ratio of III-a formed to internal standard versus the acylation time course. Acylation was at room temperature with 50 μl (●), 10 μl (○), and 2 μl (□) of pyridine and without pyridine (×). The mixture was admixed with acylated deuterium-labelled formoterol and analyzed by GC–MS as described in the text.

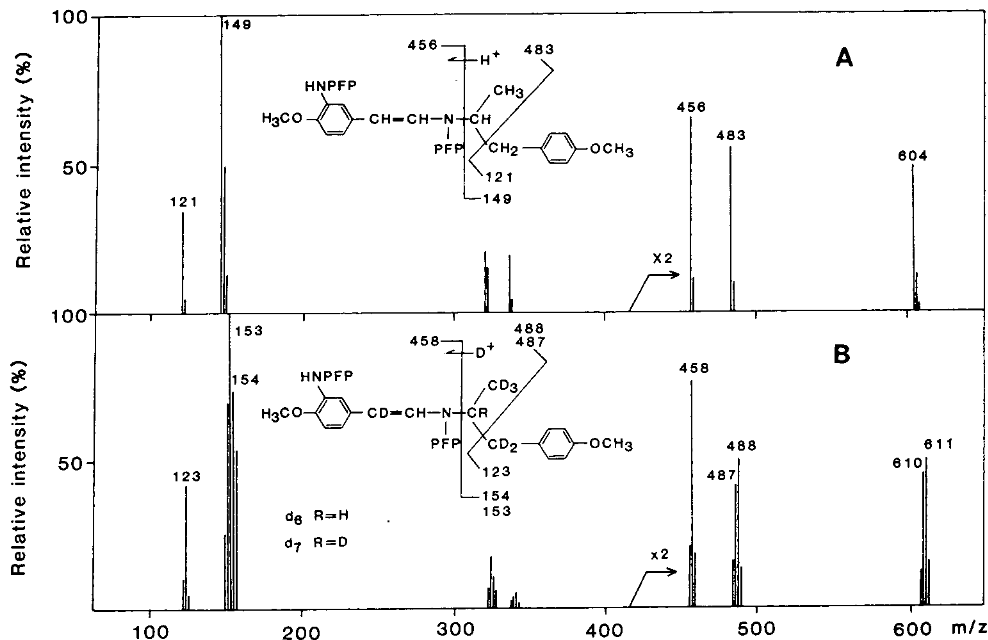

Fig. 3. Mass spectra of III-a (A) and of deuterium-labelled formoterol (B).

Fig. 3 shows the mass spectra of III-a and of deuterium-labelled formoterol. The molecular ions at m/z 604 were used for quantitation with selected ion monitoring. Although the ions at m/z 483, 456 and 149 were more intense than that at m/z 604, the latter was chosen for analysis because background ions at m/z 604 were much smaller than the others. As internal standard, we monitored the molecular ions from [$^{2}H_{7}$]formoterol at m/z 611. Initially, the acylation of formoterol was examined using trifluoroacetic anhydride (TFAA) as the acylating reagent; exactly the same acylation pattern as with PFPA was obtained. However, the molecular ions of the bis-TFA-methyl derivative (m/z 504) coincided in mass number with high background ions rising from the dimethyl silicone phase of the GC column [6], resulting in an extreme reduction of analytical sensitivity.

Assay procedure

The extraction of formoterol with ethyl acetate was pH-dependent. Urinary formoterol was extracted efficiently at pH 8—9 and remained about 90% unextracted in the aqueous layer at pH values below 5 (Fig. 4). Therefore, we extracted formoterol under alkaline conditions and back-extracted with diluted hydrochloric acid. To simplify the procedure of making the urine or the acid layer alkaline, we added excess sodium hydrogen carbonate and achieved a good result. The pH values at each step are listed in Table I with the recovery of urinary formoterol determined by radioactive counting. After the three-step extraction, the extract was derivatized for GC–MS by the method described above. As shown in Fig. 5, no significant interference was observed on the chromatograms obtained from control human urine and human urine to which 25 ng/ml formoterol and 200 ng of internal standard had been added. About 10% of the added [^{3}H]formoterol was lost by back-extraction with hydrochloric acid (Table I), and when this procedure was omitted, no interfering peaks were seen on the chromatograms. However, the background level increased with repeated sample injection, necessitating the occasional cleaning of the column and the ion source when a large number of samples was run.

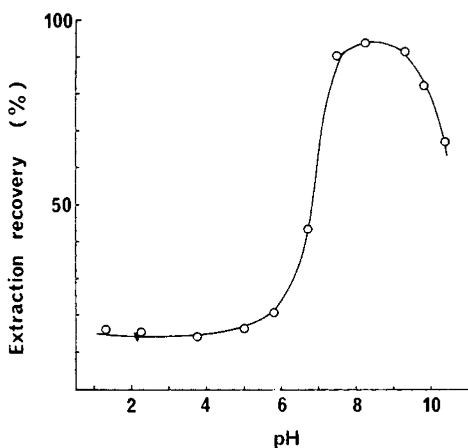

Fig. 4. Effect of pH on the extraction of formoterol from urine.

TABLE I

RECOVERY TEST OF [^{3}H]FORMOTEROL FROM URINE

The recoveries are expressed as the mean ± S.E.M. from three experiments.

	pH of aqueous layer	Recovery of [^{3}H]formoterol (%)
Initial extraction with ethyl acetate	8.1	94.0 ± 0.2
Back-extraction with hydrochloric acid	1.3	84.8 ± 0.6
Re-extraction with ethyl acetate	8.0	83.1 ± 1.3
After derivatization	—	57.7 ± 1.8

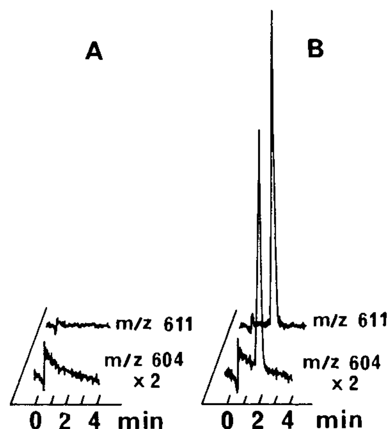

Fig. 5. Chromatograms obtained from (A) control human urine, and (B) human urine spiked with 25 ng/ml of formoterol and 200 ng of internal standard.

The precision of our method at formoterol concentrations from 0 to 100 ng/ml is shown in Table II. Regression analysis of these data gave a slope of 0.013, an intercept of 0.032 and a correlation coefficient of 0.9985. As the internal standard contained a small percentage of protium formoterol, the calibration graph did not pass through the origin. At a concentration of 5 ng/ml, the formoterol peak was about three times higher than in the control; this concentration apparently represents the limit of detection. The signal-to-noise ratio at that concentration was about five.

Specificity

Since deformylation and dehydration of formoterol occur during analysis, deformylated or dehydrated formoterol can be expected to decrease the specificity of the method if these compounds are present in the sample as metabolites. Our study in which we used [^{3}H]formoterol in rats and dogs showed that the only major metabolite was a highly polar conjugate; it was not extracted with ethyl acetate at any pH value examined. However, further study on the metabolism of formoterol in humans is necessary.

Application of the method

The nature of conjugated formoterol was investigated using the present

TABLE II

PRECISION AND REPRODUCIBILITY OF THE METHOD

The results are from four experiments.

Standard concentration of formoterol (ng/ml)	Ratio of peak height of formoterol to peak height of internal standard		C.V. (%)
	Mean	± S.E.M.	
0	0.032	0.004	22.2
5	0.093	0.003	6.0
10	0.157	0.006	7.9
25	0.350	0.006	3.3
50	0.684	0.011	3.2
100	1.300	0.029	4.5

TABLE III

ANALYSIS OF URINE SAMPLES WITH AND WITHOUT ENZYMIC HYDROLYSIS AFTER ORAL ADMINISTRATION OF FORMOTEROL TO RATS, DOGS AND HUMANS

	Urinary concentration of formoterol (ng/ml)						
	Rat samples		Dog samples		Human samples		
	A	B	A	B	A	B	C
Control	69.8	61.6	250.7	227.0	19.5	16.8	6.8
Plus β-glucuronidase (9000 units)	334.6	343.0	676.1	757.9	41.8	34.3	19.9
Plus arylsulphatase (7500 units)	67.2	54.5	221.7	262.7	19.5	13.2	7.0

method. For the treatment of asthma, various sympathomimetic amines have been synthesized, taking adrenaline as a prototype [7]. Among these drugs, isoproterenol, in which the catechol function is not substituted, was conjugated with glucuronic acid in the rat and with sulphate in the dog and human beings [8—10]. As pointed out by Conway et al. [9], this conjugation pattern is the same as that of orally administered adrenaline or some other related catecholamines. However, salbutamol, which is a salicyl alcohol derivative, instead of catechol, was conjugated with glucuronic acid in the dog and the rat and with sulphate in human beings [11,12]. A non-catechol β-stimulant, procaterol, was primarily conjugated with glucuronic acid in all three species [13,14]. It was of interest for us to determine whether these species utilize glucuronidation or sulphation in the metabolism of the non-catechol β-stimulant formoterol. As shown in Table III, formoterol was conjugated with glucuronic acid in rats, dogs and humans.

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CHROMBIO. 1197

GAS CHROMATOGRAPHIC DETERMINATION OF ISOSORBIDE DINITRATE IN HUMAN PLASMA AND URINE

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SUMMARY

A rapid, simple and sensitive method for the specific determination of isosorbide dinitrate at concentrations down to 0.5 ng/ml in human plasma and urine is described. Following extraction (with or without internal standard) of isosorbide dinitrate into toluene, the compound is determined by gas chromatography using a ^{63}Ni electron-capture detector.

INTRODUCTION

Isosorbide dinitrate (ISDN) is an organic nitrate vasodilator. Many methods have been already proposed for its quantitative assay in biological fluids. Concentrations of [^{14}C]ISDN [1] were measured in blood after thin-layer chromatography and determination of radioactivity, the limit of accurate measurement being 2 ng/ml.

Many gas chromatographic (GC) methods have been described [2—10]. Some methods lack sensitivity [2, 3, 6, 7] and require a large sample volume: between 4 and 10 ml. Several procedures use a laborious extraction [4, 7, 10] or a chromatography which takes around 30 min [4, 8]. In two procedures, no internal standard is required [6, 7]. In the others, nitroglycerin [4, 8, 9], dinitrobenzene [2, 3] or isomannide dinitrate [10] are used as internal standard. In most of the published assays, the internal standard is dissolved in a solvent before extraction [8, 9] or is added just before injection [2—4] and the incomplete extraction recovery of ISDN is not corrected. The methods cited above have a sensitivity between 0.5 and 50 ng/ml.

Owing to the volatility of ISDN, it is advisable to avoid evaporation of the extraction solvent, which is required in most of the above-mentioned procedures.

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This paper describes a rapid and sensitive procedure which permits the determination of ISDN (with or without isomannide dinitrate as internal standard) down to 0.5 ng/ml (without internal standard) in human plasma and urine using GC and an electron-capture detector. This technique is faster than the existing assay procedures, it requires a one-step extraction and avoids solvent concentration. The method is particularly well suited for routine determinations of large numbers of samples with convenient sensitivity.

EXPERIMENTAL

Chemicals and reagents

ISDN on lactose was supplied by Sanol (Monheim, G.F.R.). Its content is determined following the colorimetric method of USP XIX [11]. The ISDN solutions are prepared in acetone. Isomannide dinitrate (IMDN) was synthesized from isomannide in our laboratories according to the method of Jackson and Hayward [12]. Isomannide was obtained from D-mannitol according to the method of Hockett and Fletcher [13]. Toluene (Pestipur, SDS, Peypin, France) is of analytical grade. The methanolic solution of internal standard contains 10 ng of isomannide dinitrate per 25 μl. The ISDN calibration solutions contain 0.5—500 ng per 25 μl of acetone.

Equipment

All the glassware (flasks, glass tubes) is pretreated to prevent adsorption. It is immersed in toluene containing hexamethyldisilazane, trimethylchlorosilane and pyridine (1% v/v each) for 15 min and rinsed with methanol. The treatment is repeated every month. Between such treatments, the glassware is cleaned as usual and rinsed with methanol.

A Hewlett-Packard Model 5713A gas chromatograph, equipped with a Hewlett-Packard electron-capture detector (Model 18 713A) is used. The temperature controllers of detector and injection port have a temperature range from 100 to 400°C in 50°C steps but have been modified for a continuous adjustment of temperature. The peak areas are given by a Hewlett-Packard computing integrator (Model 3388A). The column is operated at 147°C, the injector temperature is 165°C and the detector is set at 215°C with argon—methane (90:10) at a flow-rate of 75 ml/min. The glass column (1 m × 2 mm I.D.) is washed [14], and packed with 3% QF-1 on Gas-Chrom Q (80—100 mesh) (Supelco, Bellefonte, PA, U.S.A.). The column temperature is held overnight at 150°C and after some injections of ISDN the column is ready for use.

Extraction

Twenty-five microlitres of internal standard solution (if an internal standard is used) are measured into a 5-ml glass centrifuge tube. Then, 1 ml of the sample (plasma or urine) and 250 μl of toluene are added. The tube is stoppered and shaken mechanically (Infors shaker) for 10 min at 200 rpm, then centrifuged for 5 min at 2500 *g*.

Gas chromatography

A 3-μl aliquot of the organic layer is injected into the gas chromatograph.

The ISDN content is calculated from the peak area (peak-area ratio if an internal standard is used) by reference to a calibration curve. This curve is obtained by extraction of plasma or urine spiked with increasing amounts of ISDN (from 0.5 to 500 ng/ml) and a constant amount of internal standard (10 ng/ml plasma or urine if an internal standard is used). A calibration curve is prepared every day.

Study in man

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

RESULTS AND DISCUSSION

Extraction procedure and internal standard

Without internal standard, numerous samples spiked with ISDN in plasma and urine (within-day and day-to-day) have been determined for the validation of the method. The one-step extraction with toluene was reproducible. But it is possible to use isomannide dinitrate as internal standard. The chromatographic separation of ISDN and IMDN is difficult at 147°C; a better resolution is obtained at 138°C but the sensitivity limit of the method will be lower and the retention time of ISDN longer.

TABLE I

RECOVERY IN THE WITHIN-DAY DETERMINATION OF ISOSORBIDE DINITRATE IN SPIKED PLASMA AND URINE SAMPLES WITHOUT INTERNAL STANDARD

Amount added (ng/ml)	Mean amount found in plasma (ng/ml) (n = 6)	Recovery in plasma (%)	Mean amount found in urine (ng/ml) (n = 6)	Recovery in urine (%)
0.50	0.51	102.0	0.51	102.0
0.75	0.74	98.7	—	—
1	0.97	97.0	0.93	93.0
5	5.0	100.0	5.3	106.0
25	25.0	100.0	25.7	102.8
50	51.2	102.4	51	102.0
100	—	—	98	98.0
250	262	104.8	—	—
500	529	105.8	—	—
Mean		101.3		100.6
C.V. (%)		± 2.9		± 4.5

Precision and recovery without internal standard

The within-day reproducibility of the method was checked in plasma and urine by determining six samples spiked with several different concentrations. Table I gives the results obtained when the described procedure was applied to spiked plasma and urine. The day-to-day reproducibility was checked in plasma and urine by determining during one week, on each day, two concentrations in duplicate: 1 and 50 ng/ml ISDN. Table II shows the day-to-day reproducibility

TABLE II

PRECISION AND RECOVERY IN THE DAY-TO-DAY DETERMINATION OF ISOSORBIDE DINITRATE IN SPIKED PLASMA AND URINE SAMPLES WITHOUT INTERNAL STANDARD

Amount added (ng/ml)	In plasma			In urine		
	Mean amount found (ng/ml) (n = 8)	Standard deviation (±)	Recovery (%)	Mean amount found (ng/ml) (n = 8)	Standard deviation (±)	Recovery (%)
1	0.95	0.098	95.0	1.05	0.081	105.0
50	52.4	3.6	104.8	49.8	3.0	99.6

of the method in plasma and urine. These tables demonstrate the good reproducibility of the assay without internal standard down to concentrations of 0.5 ng/ml of plasma or urine. This concentration (0.5 ng/ml) may be taken as the quantitation limit of the method; lower concentrations could still be detected.

Precision and recovery with internal standard

Table III gives the results obtained when the described procedure with internal standard was applied to spiked plasma samples. As seen in the table, a good reproducibility was obtained with concentrations down to 1 ng/ml.

TABLE III

RECOVERY IN THE WITHIN-DAY DETERMINATION OF ISOSORBIDE DINITRATE IN SPIKED PLASMA WITH ISOMANNIDE DINITRATE AS INTERNAL STANDARD

Amount added (ng/ml)	Mean amount found (ng/ml) (n = 8)	Recovery (%)
1	1.05	105.0
5	5.03	100.6
25	24.0	96.0
Mean		100.5
C.V. (%)		± 4.5

Plasma and urine interference

Fig. 1.1 shows chromatograms of an extract of human plasma and of the same extract spiked with 25 ng of ISDN. Fig. 1.2 shows chromatograms of a human plasma extract and of the same extract spiked with 25 ng of ISDN and 10 ng of internal standard. No interference from normal plasma components was recorded. ISDN and the internal standard are well separated from the normal components of the urine extract.

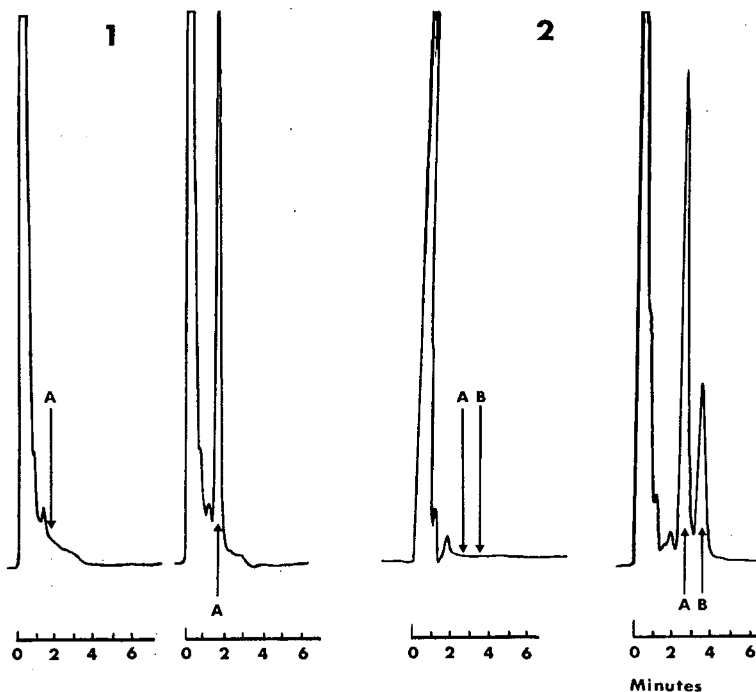

Fig. 1. Examples of chromatograms. (1) Human plasma blank (1 ml plasma), and same plasma spiked with 25 ng of isosorbide dinitrate (A); the column temperature is 147° C. (2) Human plasma blank (1 ml plasma), and same plasma spiked with 25 ng of isosorbide dinitrate (A), and 10 ng/ml internal standard (B); the column temperature is 138° C.

Speed of analysis

The analytical technique is fast: one single extraction is needed (15 min) before chromatography, which takes about 5 min.

Selectivity

The two known isosorbide mononitrate metabolites were injected under the same conditions as isosorbide dinitrate. These compounds were detected with retention times of 1 min and 1.5 min, respectively, for the 2-mononitrate and the 5-mononitrate, the retention time of ISDN being 3 min. ISDN is clearly separated from its two known mononitrate metabolites.

The procedure described here does not permit the simultaneous determination of ISDN and its mononitrate metabolites because these metabolites are poorly extracted by toluene under the conditions described above.

Storage stability of ISDN in human plasma

Table IV shows a decrease of around 15% in the ISDN content (5 ng/ml and 100 ng/ml) which was observed in plasma samples when stored frozen for 3 months at —20° C; this decrease reaches 30% after 9 months at —20° C.

TABLE IV

STORAGE STABILITY OF ISOSORBIDE DINITRATE IN HUMAN PLASMA AFTER 9 MONTHS AT —20° C

Duration of storage at —20° C	Amount of isosorbide dinitrate added to plasma (ng/ml)	
	5.0	100
	Amount of isosorbide dinitrate found (average of two assays) (ng/ml)	
1 day	4.8	96
8 days	4.8	103
15 days	4.4	98
1 month	4.6	90
3 months	4.2	85
6 months	4.3	80
9 months	3.8	69

Application

The technique was used to study the elimination of ISDN after oral administration to one healthy subject. Fig. 2 shows the curve obtained from the

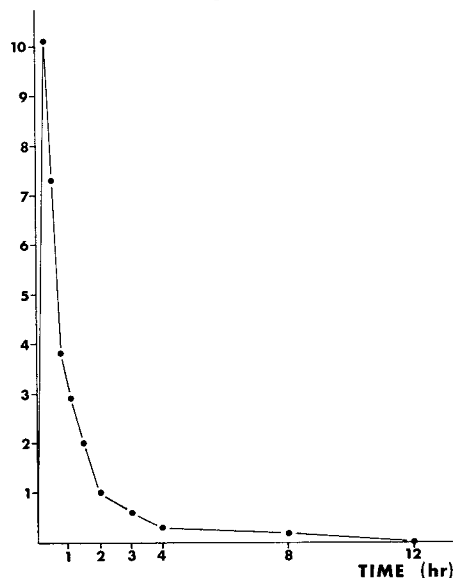

Fig. 2. Isosorbide dinitrate plasma concentrations obtained in one healthy subject after oral administration of 5 mg as one Isoket® tablet.

plasma samples of the subject given 5 mg of ISDN as one Isoket® tablet. ISDN was detected below the limit of quantitation in the 12—24 h urine only.

CONCLUSION

This paper describes a simple and sensitive GC technique for the determination of isosorbide dinitrate in human plasma and urine. This technique is faster than the existing assay procedures. The use of internal standard is not recommended because the one-step extraction is reproducible and solvent concentration is avoided.

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CHROMBIO. 1203

SIMULTANEOUS DETERMINATION OF TRIAZOLO-BENZOPHENONE [2′,5-DICHLORO-2-(3-GLYCYLAMINOMETHYL-5-METHYL-4H-1,2,4-TRIAZOL-4-YL)-BENZOPHENONE] AND ITS MAJOR BLOOD METABOLITE, TRIAZOLAM, IN MONKEY PLASMA BY ELECTRON-CAPTURE GAS—LIQUID CHROMATOGRAPHY

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SUMMARY

A gas—liquid chromatographic method for the simultaneous determination of triazolo-benzophenone [2′,5-dichloro-2-(3-glycylaminomethyl-5-methyl-4H-1,2,4-triazol-4-yl)-benzophenone, TB] and its major blood metabolite, triazolam, 8-chloro-6-(*o*-chlorophenyl)-1-methyl-4H-*s*-triazolo[4,3-*a*] [1,4] benzodiazepine (TZ), in monkey plasma was developed. Decomposition of TB was observed during gas—liquid chromatography. In alkaline medium, TB in plasma was submitted to ring closure reaction to yield triazolo-aminoquinoline, [4-amino-7-chloro-5-(2-chlorophenyl)-1-methyl-4H-*s*-triazolo[4,3-*a*]quinoline (TAQ), while TZ remained unaffected, and TAQ and TZ in the benzene extract were assayed by gas—liquid chromatography using an electron-capture detector. The concentration ranges studied were from 5 to 40 ng of TB per 0.5 ml of plasma and from 2 to 20 ng of TZ per 0.5 ml of plasma. This method could be applied to the determination of the plasma levels of TB and TZ in monkeys following intravenous administration of a single 0.2 mg/kg dose of TB.

INTRODUCTION

Triazolo-benzophenone [2′,5-dichloro-2-(3-glycylaminomethyl-5-methyl-4H-

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1,2,4-triazol-4-yl)-benzophenone, TB] is a member of a new series of sleep inducers recently developed in our laboratory [1], and is expected to be a pro-drug of triazolam (TZ) as well as peptido-aminobenzophenone which was defined as a pro-drug of 1,4-benzodiazepine in previous papers [2—6].

The peptido-aminobenzophenone was assayed in a previous study [5] by a cyclization reaction in alkaline medium in which the peptido-aminobenzophenone was converted into the thermostable aminoquinolone derivative having good chromatographic properties, since the peptido-aminobenzophenone decomposed during gas—liquid chromatography (GLC). But a simultaneous determination of the parent drug, peptido-aminobenzophenone, and its 1,4-benzodiazepine metabolites was not successful because the latter were readily hydrolyzed in the alkaline medium.

In the case of TB, it would be cyclized to 4-amino-7-chloro-5-(2-chlorophenyl)-1-methyl-4H-*s*-triazolo[4,3-*a*]quinoline (TAQ) in the alkaline medium by the same mechanism as peptido-aminobenzophenone. Further, the simultaneous determination of the parent drug and the metabolite TZ would become feasible, as TZ was stable in the alkaline medium [7].

The metabolism of TZ in several species [8—10] has already been reported and the major blood metabolites in rats and dogs were 1'-hydroxytriazolam (1'-HT) and 4-hydroxytriazolam (4-HT) [9, 10]. Therefore, gas chromatographic differentiation between the above metabolites and TAQ derived from the cyclization of TB would be required. Ultra-micro determinations of TZ have been reported for the radioimmunoassay [11], high-performance liquid chromatography [12], and capillary gas chromatography with electron-capture (EC) detector [13].

This paper describes the simultaneous determination of TB and TZ in monkey plasma by the EC-GLC method in order to investigate the metabolic conversion of TB into TZ following administration of TB.

EXPERIMENTAL

Chemicals and reagents

TB, TZ, 1'-HT, 4-HT, TAQ and 3-amino-6-chloro-5-(2-chlorophenyl)-1-propylquinolin-2-one (PAQ) were synthesized in our laboratory. The solvents used were of a special grade for EC-GLC (Wako Industries Co., Osaka, Japan) while other chemicals were of reagent grade and used without further purification.

Gas—liquid chromatography

A Shimadzu (Kyoto, Japan) Model 4APE gas chromatograph equipped with a 10 mCi ^{63}Ni electron-capture detector was used with a 0.5 m × 2 mm I.D. glass column filled with 3% OV-17 on 100—120 mesh Gas-Chrom Q. The column temperature was kept at 290°C after conditioning with a nitrogen flow-rate of 20 ml/min for 48 h at 330°C. The detector and injection port temperatures were 320°C. The nitrogen flow-rate was 70 ml/min, and the pulse mode was of a 100-μsec frequency with a 2-μsec width.

The trimethylsilyl (TMS) derivative of 1'-HT was obtained by treatment with bis(trimethylsilyl)acetamide in acetone at room temperature.

TABLE I

RETENTION TIMES AND R_F VALUES OF TRIAZOLO-BENZOPHENONE RELATED COMPOUNDS

	TZ	TAQ	1'-HT	4-HT	PAQ
Retention time (min)	2.75	4.15	2.58*	5.00	1.10
R_F	0.20	0.40	0.16	0.12	0.60

*Retention time of the TMS derivative of 1'-HT.

The retention times and R_F values of the compounds in this assay are listed in Table I.

Thin-layer chromatography

Thin-layer chromatography (TLC) plates of silica gel F_{254} (250 μm; E. Merck, Darmstadt, G.F.R.) were used; the solvent system was chloroform—acetone—ethanol (8:1:1). The chromatograms were visualized under UV light.

Preparation of standard solutions

For preparation of the calibration curve, solutions containing 0.04—0.4 μg/ml TZ, 0.05—0.5 μg/ml TAQ, and 0.15 μg/ml PAQ as an internal standard were prepared in benzene. Samples of 2 μl each were subjected to gas chromatography.

For recovery study, solutions containing both 0.1—0.8 μg/ml TB and 0.04—0.4 μg/ml TZ were prepared in 0.1% aqeuous solution of ethanol; 50 μl of the solutions were added to 0.5 ml of monkey control plasma.

Procedure for determination of TB and TZ in plasma

To a 12-ml centrifuge tube containing 0.5 ml of plasma, 0.5 ml of 2 *M* potassium hydroxide was added and the mixture was heated on a water bath at 91 ± 1°C for 90 min. After cooling, the mixture was extracted with 5 ml of benzene containing 6 ng of the internal standard (PAQ) on a mechanical shaker. The benzene extract was separated and evaporated to dryness in vacuo at 40°C. The residue was dissolved in 0.1 ml of solvent (benzene—acetone, 1:1), and a 5-μl sample was subjected to gas chromatography. Calculations were done using a calibration curve prepared by the peak height method.

Procedure for determination of TZ in plasma

Determination of TZ following its administration was performed without alkaline hydrolysis as follows. To a 12-ml centrifuge tube containing 0.5 ml of plasma, 0.5 ml of water was added and the mixture was extracted with 5 ml of benzene containing 12 ng of the internal standard (PAQ) on a mechanical shaker. The extract was evaporated to dryness in vacuo at 40°C, the residue was dissolved in 0.2 ml of benzene, and a 5-μl sample was subjected to gas chromatography. Calculations were done using a calibration curve prepared by the peak height method.

Animal protocol

Animal experiments were carried out according to the cross-over design of experiment with Latin Square. The male rhesus monkeys used (5.7—7.7 kg) were made to fast overnight before being given the drug.

TB was given intravenously at a dose of 0.2 mg/kg as a 0.04% solution of TB monohydrochloride monohydrate in 0.9% aqueous sodium chloride. TZ was given intravenously to the same four monkeys being used for TB dosing at a dose of 0.2 mg/kg as a 0.04% solution of TZ in 0.9% aqueous sodium chloride containing 40% propylene glycol.

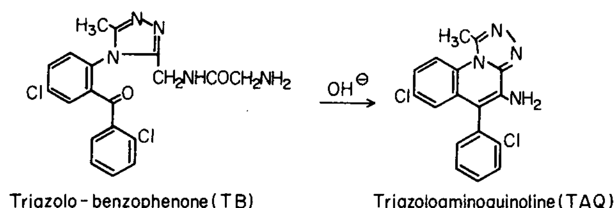
Blood samples (2.5 ml) were collected at 5, 10, 20, 30, 45 min, and 1, 2, 3, 5, 7, 24 and 48 h post-dosing, then immediately centrifuged at 1500 *g* for 15 min. The plasma samples separated were stored at $-20°$ C until analysis.

RESULTS AND DISCUSSION

Derivatization of TB

TB was pyrolized during GLC and gave no remarkable peaks. Therefore, the derivatization of TB into a compound which possessed good chromatographic properties was attempted.

As well as in the analysis of the peptido-aminobenzophenone, when TB was heated in 1 *M* potassium hydroxide solution at 92°C for 1 h, colorless crystals were precipitated. The precipitate was removed by filtration and was subjected to TLC and GLC, which gave a single spot with an R_F value of 0.40 and a single peak with a retention time of 4.15 min, respectively. Furthermore, the mass spectrum of this compound showed a molecular ion peak at *m/e* 342 and was identical to that of authentic TAQ. Thus, TB was found to be converted into TAQ in an alkaline medium, as shown below.



The reaction conditions were examined further in various alkaline solutions such as 0.1 *M*, 0.5 *M*, 1 *M*, 2 *M*, 3 *M* potassium hydroxide and 5% potassium carbonate. The reaction in 1 *M* potassium hydroxide gave the most satisfactory results with respect to yield and by-product; the result of a time—course study under the same conditions is shown in Fig. 1. The reaction proceeded to completion with a yield of 86% after 90 min. A by-product of the reaction, which appeared on TLC with an R_F value of 0.20 and on GLC with a retention time of 2.75 min, was derived in low yield. This compound possessed the same retention time and R_F value as TZ, and was identified as TZ by mass spectrometry.

To simplify the procedure for the determination of TB in plasma, the above reaction was performed in plasma solution containing 1 *M* potassium hydroxide

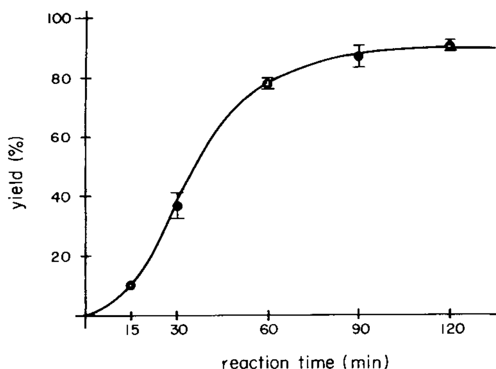


Fig. 1. Time—course for the cyclization reaction of triazolo-benzophenone into triazolo-aminoquinoline in 1 *M* potassium hydroxide solution.

without preliminary extraction of TB according to the same procedure etablished for the analysis of peptido-aminobenzophenone.

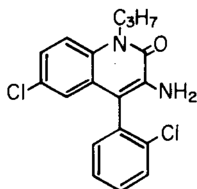
Interference

No endogenous component of plasma interfered with this assay, as shown in Fig. 2. Moreover, 1′-HT and 4-HT, the possible metabolites of TB, and the compounds to be analyzed could be perfectly separated from each other as shown in Table I. In this table, 1′-HT is shown as its TMS derivative because decomposition of the intact compound was observed during GLC. But the by-product, TZ, resulting from the cyclization reaction for the plasma TB interfered with the TZ determination.

Accordingly, the yield of the by-product TZ from the cyclization reaction was estimated from the specific peak height ratio which was obtained by dividing the peak height of TZ by that of TAQ. The specific peak height ratio obtained was 0.086 as the mean (n = 6, C.V. = 3.0%) at the TB concentration of 40 ng per 0.5 ml of plasma. Consequently, the net plasma levels of TZ in the presence of TB were estimated by subtracting 8.6% of the peak height of TAQ from that of TZ.

Choice of an internal standard

Because of the strong alkalinity of the reaction medium, stability of the internal standard is the foremost requirement. Estazolam and PAQ were selected as they possessed adequate GLC properties. PAQ was considered the preferable internal standard, considering the interferences by the metabolites of TB which might appear at longer retention times than that of PAQ in GLC.



N-Propylaminoquinolone (PAQ)

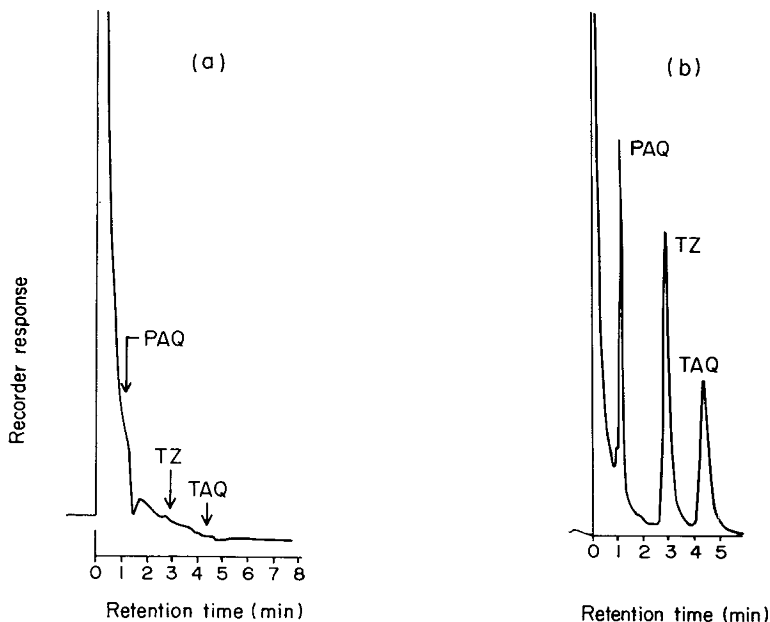


Fig. 2. Gas chromatograms of (a) control plasma and (b) sample plasma.

Calibration curve

The ratios of the peak height of TZ and TAQ to that of PAQ were plotted against the concentrations of TZ and TAQ in the range of 0.04—0.4 μg/ml and 0.05—0.5 μg/ml, respectively. The calibration curve obtained gave satisfactory linearity for each of the two compounds.

Extraction solvent

The recoveries of TZ and TAQ were examined with benzene, ethyl acetate and *n*-hexane, and were 102%, 88% and 37% for TZ and 100%, 96% and 88% for TAQ, respectively. Although ethyl acetate gave sufficient recoveries, the peaks and the baseline were affected by a small amount of water dissolved in the extract. *n*-Hexane gave an unsatisfactory recovery for TZ. Accordingly, benzene was selected as the extraction solvent.

Metabolites

General 1,4-benzodiazepines such as diazepam were readily hydrolyzed by alkali [5] and acid [14], but the metabolite TZ remained unaffected. The recovery of TZ following the cyclization reaction under the assay conditions was 97% as the mean in the range 0.2—1.0 μg/ml TZ.

Recovery studies

Solutions containing both 5—40 ng of TB and 2—20 ng of TZ were prepared in 0.5 ml of heparinized monkey plasma. Each solution was analyzed according to the procedure described above. The man percentage recoveries of TB and TZ, calculated from a total of 27 analyses, were 67.5% (S.D. = 4.3) and 112% (S.D. = 7.5), respectively.

Plasma levels of TB and TZ in monkeys

The plasma levels of TB and TZ were determined in monkeys following a single intravenous dose of 0.2 mg/kg, as shown in Fig. 3. The plasma level of the intact drug TB was 0.2 μg/ml at 5 min after dosing (0.3 μg/ml as the mean for the four monkeys), declined rapidly by apparent first-order elimination with a half-life of 20 min (16 min as the mean), and was below the detection limit of the assay at about 1 h post-dosing. The plasma level of the metabolite TZ was immediately detectable after dosing and disappeared more slowly than TB with apparent first-order elimination kinetics with a half-life of 68 min (67 min as the mean). The plasma half-lives of TB and TZ after intravenous 0.2 mg/kg dosing of TB are shown in Table II. The values were obtained from estimation by the least-squares method of each β-phase of the plasma concentration—time curve.

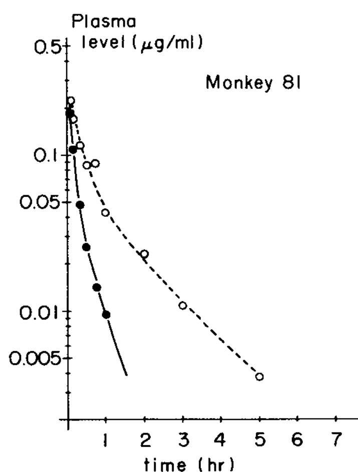


Fig. 3. Plasma levels of triazolo-benzophenone (•) and triazolam (○) following 0.2 mg/kg intravenous administration of triazolo-benzophenone to a monkey.

TABLE II

PLASMA HALF-LIVES OF TRIAZOLO-BENZOPHENONE AND TRIAZOLAM IN MONKEYS FOLLOWING 0.2 mg/kg INTRAVENOUS ADMINISTRATION OF TRIAZOLO-BENZOPHENONE

Monkey No.	Plasma half-life (min)	
	TB	TZ
79	16	77
81	20	68
92	12	48
99	14	74
Mean (S.D.)	16 (3)	67 (13)

On the other hand, the area under the plasma concentration—time curve (AUC) of TZ following the intravenous administration of TZ was significantly larger ($P < 0.05$) than that after TB dosing (Table III). However, the AUC ratio of the latter to the former, calculated from AUC values normalized to an equimolar dose basis, was 0.82. Therefore, the conversion of TB into TZ was at least 82%. Thus, TB seemed to be rapidly metabolized to TZ with high efficiency.

TABLE III

AREA UNDER THE PLASMA CONCENTRATION—TIME CURVES (AUC) OF TRIAZOLAM AFTER INTRAVENOUS 0.2 mg/kg DOSE OF TRIAZOLO-BENZOPHENONE OR TRIAZOLAM

Values are expressed as $\mu g \cdot h \cdot ml^{-1}$. AUC was estimated by the trapezoidal rule. TB/TZ ratios were calculated from AUC values normalized on an equimolar dose basis.

	Monkey No.				Mean (S.D.)
	79	81	92	99	
TB	0.195	0.176	0.118	0.166	0.164 (0.033)
TZ	0.247	0.353	0.324	0.223	0.287 (0.062)*
TB/TZ	1.09	0.685	0.501	1.02	0.824 (0.279)

*$P < 0.05$.

CONCLUSION

The determination of TB after the cyclization reaction with alkali proved to be applicable to general peptido-aminobenzophenone as described in the previous paper [5]. In addition, simultaneous determination of the parent drug TB and the metabolite TZ was successful. This method should be useful in further studies on the metabolism of TB.

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CHROMBIO. 1213

CHROMATOGRAPHIC MICRO-PROCEDURE FOR TRACE DETERMINATION OF PHENOBARBITAL IN BLOOD SERUM

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SUMMARY

Volumes of 100 µl of serum were sufficient for the determination of therapeutic levels of phenobarbital. The isolation procedure was performed using a column method with a hydrophobic adsorbent, graphitized carbon black (Carbopack B). With this method the quantitative (98.1%) recovery of phenobarbital was measured. By suitable choice of experimental conditions, a highly selective purification of the drug can be obtained, thus eliminating various sources of error during quantitation due to the presence in the final samples of endogenous compounds. For the quantitation procedure, another type of graphitized carbon black (Carbopack C) suitably modified was used for gas chromatography. Calibration curves showed no chemisorption effect along the column even on injecting 5 ng of phenobarbital. Some practical aspects of the procedure for improving the reliability of the results are discussed.

INTRODUCTION

Determination of the blood concentrations of barbiturates, and in particular of phenobarbital, is of importance in the treatment of epilepsy and in the diagnosis of acute poisoning. For this purpose, gas chromatographic (GC) and, more recently, high-performance liquid chromatographic (HPLC) procedures have been a great aid in clinical laboratories since they meet the requirements for rapid identification and accurate quantitation.

Many of the GC methods currently available succeed in the analysis of drug overdoses, but they fail in the case of therapeutic monitoring, where the blood drug concentration is much lower. Chemisorption of acidic drugs occurring along the chromatographic column and/or in the injection port is often responsible for this failure. Recently, it has been reported [1] that a typical chromatographic column used for drug analysis, i.e. phenylsilicone supported by an H_3PO_4-deactivated siliceous material, is inadequate to determine

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accurately phenobarbital concentrations lower than 15 μg/ml, due to partial irreversible adsorption of phenobarbital on the column.

Chemisorption occurring on the injection port is likely caused by insufficient isolation of drugs from endogenous biological molecules, such as peptides, proteins, nucleotides, etc. These molecules are denatured on the injection port and can act as centers of chemical adsorption of acidic compounds [2].

This drawback can be eliminated by derivatization procedures prior to GC [3—5] and/or by clean-up procedures following extraction of drugs from biological fluids [6, 7]. These additional steps in the analytical scheme are time-consuming, however, and can provoke loss of the sample due to excessive manipulation.

HPLC has proved to be valid for the analysis of drugs and their metabolites in biological fluids [8, 9]. Nevertheless, even in this case a complete isolation of drugs from matrix material in clinical samples is necessary to avoid degradation of the column to a point where the system becomes unusable.

Graphitized carbon black (Carbopack) has been successfully used as packing material in GC of very polar compounds [10—13]. Recently, this adsorbing material has shown to be effective in the purification and concentration of pesticides and other organic molecules in water [14, 15].

In this paper, an accurate and simple micro-method for the determination of phenobarbital in serum at a few μg/ml is described. A small (100 μl) volume of the biological specimen was purified using a column filled with Carbopack B. Phenobarbital was desorbed with methanol as mobile phase. Analytical recoveries ranged from 96.1 to 99.7% for phenobarbital in serum. The quantitation procedure was performed using a GC column packed with acid-washed Carbopack C modified with Apiezon and polyethylene glycol (PEG) 20M. The advantages of using the adsorption method over solvent extraction for the purification of acidic compounds in biological samples are demonstrated.

MATERIALS AND METHODS

Chemicals

Phenobarbital, cyclohexenylbarbital and cycloheptenylbarbital were obtained from Fluka (Buchs, Switzerland). Carbopack B and C in the 80—120 mesh range were kindly supplied by Supelco (Bellefonte, PA, U.S.A.). PEG 20M and Apiezon N were purchased from Carlo Erba (Milan, Italy). 1,3,5-Tricarboxybenzene (trimesic acid) was obtained from Fluka.

Gas chromatography

A Carlo Erba Model GI gas chromatograph equipped with a flame ionization detector was employed. Integration of peak areas was performed by a Hewlett-Packard 3352 data system. The column, a coiled glass tube (1 m × 1.5 mm I.D.), was washed with trimesic acid in methanol (ca. 1 mg/ml) in order to deactivate basic centers of glass and dried under vacuum. Subsequently, it was packed with acid-washed Carbopack C modified with PEG 20M (0.3%, w/w), Apiezon N (0.28%, w/w) and trimesic acid (0.1%, w/w). This latter is added to increase the thermal stability of PEG 20M. Trimesic acid was deposited on the Carbopack C surface from methanol, PEG 20M and Apiezon N were deposited

from methylene chloride. The procedures of acid-washing, coating and packing have been reported elsewhere [16, 17]. No glass wool was used to plug the injection end of the column. The column was conditioned at 250°C overnight under flow.

For the quantitation procedure, the column was operated at 235°C with hydrogen as carrier gas, which offers advantages over nitrogen [18]. With a dead-time of 6 sec, phenobarbital was eluted within 12 min. The retention time was longer (16 min) by operating with nitrogen (dead-time 10 sec) at 240°C. Under the former experimental conditions, the chromatographic column was perfectly stable. A 3% variation of the retention time for phenobarbital was measured after 72 h of use at 240°C. This variation did not modify the separation factors of barbiturates and it was minimized by maintaining the column temperature at 150°C when not in use. With this precaution, the column could be used continuously for three months.

During chromatographic analysis, the injection port temperature was maintained at 250°C to ensure instantaneous vaporization of barbiturates. At lower temperatures, a certain peak broadening for these compounds was noted.

Procedure

The column for isolating phenobarbital from serum was prepared by suspending 0.6 g of Carbopack B in water and pouring the slurry into a 14 × 0.6 cm glass column with a PTFE stopcock. The adsorbent was packed by tapping the column while water passed through it. This operation was stopped when the level of carbon reached a height of 6 cm. The dead volume of the column was 1.2 ml; this was calculated by passing 1 *M* HCl through the column and measuring the acidity of the effluent. A 0.1-ml volume of serum containing phenobarbital was added to the head of the column and allowed to drain into the column. When the level of serum reached that of the adsorbent, 4 ml of 1 *M* HCl in water were passed through the column to denature proteins which are eliminated from the column as precipitate. After the acidic solution was passed into the column, the adsorbent was washed with 1.5 ml of a methanol—water (1:1) mixture to eliminate the major part of the acidic water. Methanol was used to desorb phenobarbital from the carbon surface. During elution, the flow-rate was maintained at 0.5—0.7 ml/min. The first 6.8 ml of the effluent of the column were discarded and the following 1 ml containing phenobarbital was collected. The discarded fraction consists of 1.2 ml of dead volume, 0.1 ml of serum and 5.5 ml of washings. Then 10 μl of a methanolic solution of cycloheptenylbarbital (0.05 mg/ml), used as internal standard, were added to the collected fraction. After this, the fraction was submitted to a stream of nitrogen at room temperature to remove methanol completely from co-eluted water. A final volume sample of about 6—8 μl was obtained. This sample was then analyzed by injecting aliquots of 1.5 μl into the GC column.

RESULTS AND DISCUSSION

Column

In order to ascertain whether some chemisorption of acidic drugs can occur in the GC apparatus, calibrated test solutions containing phenobarbital

TABLE I

RESPONSE FACTOR FOR PHENOBARBITAL vs. OCTADECANE AT VARIOUS CONCENTRATIONS

Concentration (μg/ml)	Mean ± S.D. (n = 6)
5.00	0.517 ± 0.030
12.5	0.509 ± 0.029
25.0	0.491 ± 0.018
50.0	0.493 ± 0.021
100.0	0.507 ± 0.018

dissolved in chloroform at concentrations ranging from 5 to 100 μg/ml, and octadecane, as internal standard, were prepared. For each solution, 1-μl aliquots were repeatedly injected into the column. Partial chemisorption of a given compound by the chromatographic column can be adequately recognized by injecting variable amounts of the compound examined together with an inert eluate and measuring changes of the response factor at any given concentration. Results are shown in Table I. As can be seen, by injecting phenobarbital solutions at increasing concentrations no regular increase of the response factor was observed. This demonstrates that even by injecting a few nanograms of phenobarbital into the column a complete, linear elution is obtained. Also, a well-defined peak for phenobarbital, whose area could be measured with an uncertainty not higher than 4%, was obtained by injecting 5 ng of phenobarbital (Fig. 1).

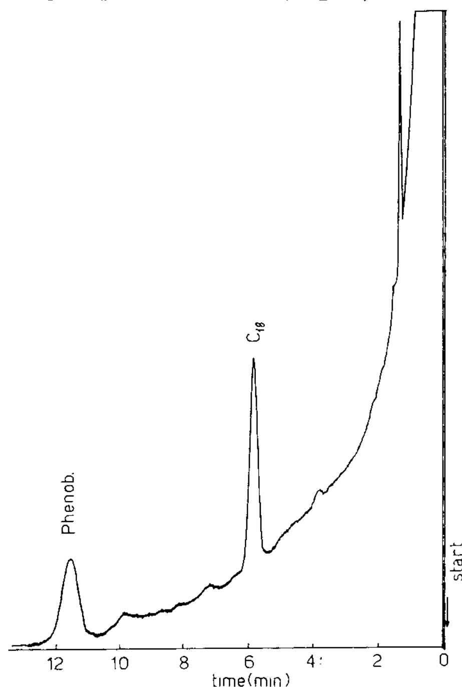

Fig. 1. Chromatogram of a methanol solution containing phenobarbital (5 μg/ml) and C_{18} (2.5 μg/ml) at 235°C and with hydrogen as carrier gas.

Even after injecting a large number of biological samples, the column packing did not show any need to be deactivated or restored or substituted. This is also because liquid-modified Carbopack C was very tolerant to injections of the aqueous sample containing drugs. The advantage of injecting water over organic solvents is that a much smaller baseline drift is obtained.

Recovery

Recovery studies were performed by adding specified quantities of phenobarbital to pooled sera known to be drug-free and contained in glass tubes with PTFE-lined screw caps. Then 0.1-ml sample aliquots were submitted to the purification procedure and 10 μl of the methanolic solution of cycloheptenylbarbital (0.05 mg/ml), used as internal standard, were added to the purified sample. After concentration, replicate injections of the sample were made. The peak areas of the extracted drug relative to those of the internal standard were compared with the relative peak areas obtained by injecting standard solutions prepared by adding to water known amounts of phenobarbital and internal standard. Recoveries using the adsorption column method on six determinations varied between 96.1 and 99.7% with a mean of 98.1 ± 1.1% for concentrations of phenobarbital in serum ranging from 4 to 20 μg/ml.

Specificity

The analytical procedure discussed here can be used for the determination of barbiturates other than phenobarbital in blood. The analytical column was only slightly modified with respect to one used previously, which was able to separate the most commonly used barbiturates [19].

Under the experimental conditions chosen for the isolation procedure, no interference from endogenous sample constituents was observed. This was made evident by the fact that on analyzing twenty drug-free human serum samples no measurable peaks with retention times equal to those of barbiturates appeared. Fatty acids and cholesterol contained in serum are not co-eluted from the adsorption column, provided methanol is used as the mobile phase. Selective elution from the column filled with Carbopack B is lost when methylene chloride and, to a lesser extent, acetone are used as mobile phases. In this case, fatty acids contained in blood interfere with some barbiturates in the quantitation procedure when using our GC column. Cholesterol did not interfere in the analysis because its retention time is far higher than that of cycloheptenylbarbital, which is the last of the barbiturates to be eluted. However, the presence of cholesterol did reduce the rate at which these analyses could be performed.

Limits of sensitivity

It has been shown [20] that the limit of sensitivity towards phenobarbital is influenced by chemisorption which may occur on-column. As mentioned above, this problem does not occur when using acid-washed Carbopack C suitably modified. The chromatographic system permitted us to measure accurately peaks for phenobarbital contained in standard aqueous solutions at the 5 μg/ml level. Considering that after the isolation and concentration procedures the final sample volume is about 6—8 μl (note that this sample volume

allows replicate 1.5-μl injections), it can be deduced that blood phenobarbital concentrations of about 0.5 μg/ml can be still measured with fair accuracy. Alternatively, considering that phenobarbital concentrations achieved during therapy are usually in the range 3—8 μg/ml, very small (10—20 μl) serum volumes may be used without affecting the quality of the analysis. An example of the chromatogram normally obtained on analysis of phenobarbital in human serum (100 μl) at the 5 μg/ml level is given in Fig. 2.

Comparison with the results obtained using the extraction method

The isolation method under discussion was evaluated by comparison with results from the solvent extraction method. An extraction procedure similar to that commonly reported in the literature was followed. To 1 ml of acidified serum 10 μl of a methanol solution of phenobarbital and cylcohexenylbarbital, used as internal standard, (1 mg/ml each) were added. Then, 2 ml of chloroform were used for extraction by shaking for 2 min and centrifuging for 10 min. Subsequently, a 1.5-ml portion of the chloroform phase was obtained with a syringe fitted with a 9-cm needle, taking care not to withdraw denatured proteins present at the interphase. After this, the organic solvent was evaporated with a stream of nitrogen at 40°C. For the GC analysis, the extract was reconstituted with about 20 μl of chloroform and 2-μl aliquots of the extract were injected ten times into the GC apparatus followed by 1 μl of 0.1 *M* formic acid in water. As can be seen in Fig. 3a, by injecting acidified water two "ghost" peaks having retention times equal to those of phenobarbital and cylcohexenylbarbital appeared. This effect can be explained by the

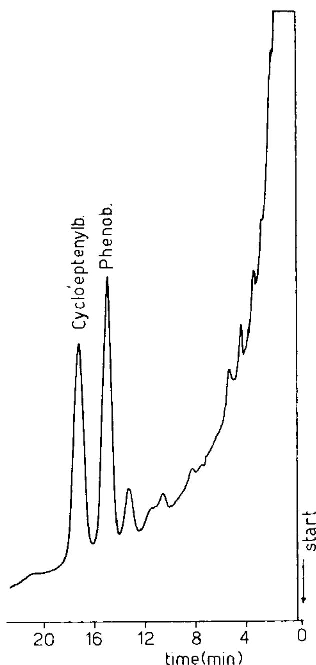

Fig. 2. Analysis of purified serum (100 μl); 1.5 μl injection. The serum concentration of phenobarbital was 5 μg/ml.

consideration that endogenous substances co-extracted with the two drugs are thermally denatured and accumulate in the injection port, provoking chemisorption of acidic compounds. Acidic drugs can be removed from the initial part of the column by injections of a stronger acidic compound. Selective chemisorption towards the compounds injected can give rise to erratic results to a greater or lesser extent depending upon the nature of the compound chosen as internal standard. It can be also expected that a prolonged number of analyses increases the degree of contamination of the injection port, thus increasing the effect of chemisorption to a point where the chromatographic system is unusable.

By using solvent extraction for isolating barbiturates and the GC system discussed here, another limiting factor is that co-extracted fatty acids are interfering compounds for the quantitation and identification of some barbiturates.

In Fig. 3b is shown a chromatogram obtained by injecting formic acid in water just after 40 injections of samples purified with the adsorbent column method following the procedure described above. The complete absence of "ghost" peaks makes it evident that this purification method is effective in isolating drugs from endogenous compounds. It should be pointed out, however, that for any class of compounds the column method using Carbopack necessitates a careful choice of the procedure to be followed in order to obtain the maximum degree of purification of biological samples. In our instance, methylene chloride was not found suitable as mobile phase since barbiturates are co-eluted with fatty acids. Moreover, washing the column with acidified water before using methanol was found necessary to remove from the column substances that are eluted with methanol and are responsible for chemisorption of acidic compounds on the injection port.

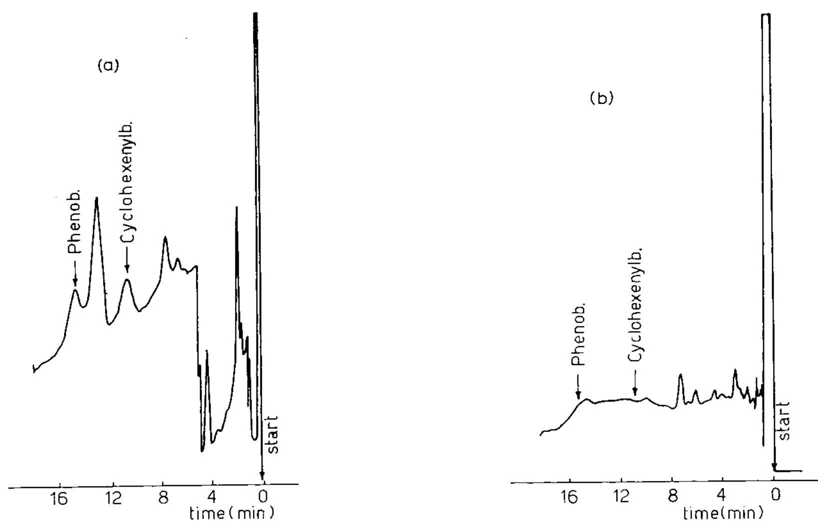

Fig. 3. (a) Chromatogram showing the appearance of two "ghost peaks" for injection of acidic water following injections of an extract of serum. (b) No ghost peaks for injection of acidic water following injections of serum purified by using Carbopack B.

CONCLUSION

The procedure described here shows the advantages of minimal sample, high sensitivity, good accuracy and reproducibility for analyzing trace concentrations of phenobarbital in serum. The isolation method proposed by us can be very selective, thus eliminating interferences from endogenous compounds. Another virtue of this method is its flexibility, since any kind of solvent or mixtures of these can be used to elute selectively the compounds of interest.

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CHROMBIO. 1190

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF PLATINUM COMPLEXES ON SOLVENT GENERATED ANION EXCHANGERS

III. APPLICATION TO THE ANALYSIS OF CISPLATIN IN URINE USING AUTOMATED COLUMN SWITCHING

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(First received August 11th, 1981; revised manuscript received December 2nd, 1981)

SUMMARY

Platinum complexes are retained on solvent generated anion exchangers, prepared by coating reversed-phase (C-18) supports with a monolayer of hexadecyltrimethylammonium bromide. The retention mechanism is described in terms of ion—dipole interactions in the stationary phase, reinforced by a hydrophobic effect. The high degree of ligand selectivity exhibited by these systems arises from the use of purely aqueous mobile phases which maximize the differences in solute dipole and hydrophobic surface area. By using stationary phases of different surface characteristics and the application of automated column switching, the technique is applicable to the clinical analysis of cisplatin in urine. After chromatography, the purified cisplatin fractions are determined by atomic absorption spectrophotometry. The recovery of cisplatin from urine is 101.1% with a relative standard deviation of 3.6% and the limit of detection is 2 μg/ml.

INTRODUCTION

The discovery that platinum complexes are useful in the treatment of many solid tumors [1] has led to the increased interest in the analysis of such species in pharmaceutical formulations [2, 3] and biological fluids [1, 4—10]. At present the greatest interest surrounds the analysis of cisplatin (CDDP) which is widely used clinically [1]. It is estimated [1] that over 1100 analogues of cisplatin have been synthesized in an attempt to reduce toxicity and increase potency. Fig. 1 shows the structures of three cisplatin analogues currently undergoing clinical trials.

Although X-ray fluorescence [4] and atomic absorption spectrophotometry [6] provide the necessary sensitivity for monitoring platinum levels in

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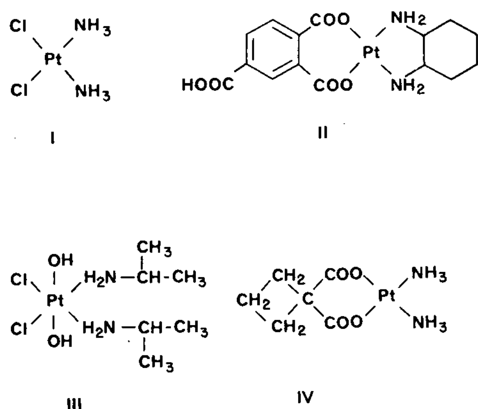

Fig. 1. Structures of some clinically investigated platinum complexes. I = *cis*-dichlorodiammine platinum(II) (cisplatin, CDDP); II = *cis*-1,2-cyclohexyldiamine-4-carboxyphthalate platinum(II); III = *cis*-dichlorodiiospropylamine-*trans*-dihydroxy platinum(IV) (CHIP); IV = *cis*-diammine-1,1-cyclobutyldicarboxylate platinum(II) (CBDCA).

clinical samples, they suffer from matrix effects when applied to urine analysis. Additionally, these techniques respond only to total levels of platinum and cannot differentiate between platinum complexes and their respective degradation and biotransformation products. The present primary need is a method for the determination of cisplatin in urine. Two high-performance liquid chromatography (HPLC) methods have been described [9, 10] for the analysis of cisplatin in urine. However, these methods require derivatization with diethyldithiocarbamate to enhance detection and respond only to total platinum. Selective HPLC methods [1, 5] have been reported for the analysis of cisplatin in plasma, involving the use of chemically bonded anion-exchange stationary phases and aqueous methanol mobile phases. The high methanol concentrations required for adequate retention of cisplatin are, however, incompatible with injections of urine. Additionally, poor peak shape, low selectivity and column instability are associated with these systems.

Very recently we have reported that solvent generated anion exchangers [11, 12] offer significant advantages over the chemically bonded stationary phases for the retention of cisplatin. These advantages include good column stability, high column efficiency and the possibility of using purely aqueous mobile phases.

The present study is concerned with the application of solvent generated anion-exchange systems to the analysis of cisplatin in urine. In addition, the effect of ligand substitution on selectivity is discussed using a functional group approach.

EXPERIMENTAL

Apparatus

The basic liquid chromatograph comprised of a Model 110A Altex pump (Beckman Instruments, Berkeley, CA, U.S.A.) a fixed-wavelength UV detector (280 nm, Altex Model 153) and an Altex injector (Model 210) fitted with a

20-μl loop. Three columns, Hypersil (5 μm, 50 mm × 4.6 mm I.D., Shandon Southern, Sewickley, PA, U.S.A.), ODS Hypersil (5 μm, 200 mm × 4.6 mm, I.D.) and ODS Ultrapak (10 μm, 150 mm × 4.6 mm, I.D., Beckman) were slurry-packed using standard procedures [13, 14].

For the analysis of cisplatin in urine, a microprocessor (SLIC Systec, New Brighton, MN, U.S.A.) controlled column switching system was used in conjunction with the Hypersil and ODS Ultrapak columns, a WISP 710 autoinjector (Waters Assoc., Milford, MA, U.S.A.) and a fraction collector (Model 273, Instrumentation Specialities, Lincoln, NE, U.S.A.) (Fig. 2). The two Altex pumps were modified by Systec and interfaced with the microprocessor. The flow-cell of the Altex detector was modified by replacing all the internal tubing with PTFE microbore tubing to minimize dead volume. The two columns were linked by a high-pressure six-port valve (Rheodyne, Cotati, CA, U.S.A.) interfaced with the microprocessor, so that fractions eluting from column 1 could either run to waste or be passed to column 2. Fig. 3 summarizes the program used for the separation and collection of the cisplatin fractions which were subsequently determined by atomic absorption.

Platinum determinations were made with a Varian Techtron Model 175B atomic absorption spectrophotometer (Palo Alto, CA, U.S.A.) coupled with a CRA-90 carbon rod atomizer. The platinum line was monitored at 265.95 nm with a constant lamp current of 10 mA. A three-stage heating program of 95°C for 45 sec, 1400°C for 15 sec and 2300°C for 0.5 sec was used with a ramp rate of 600°C sec^{-1}.

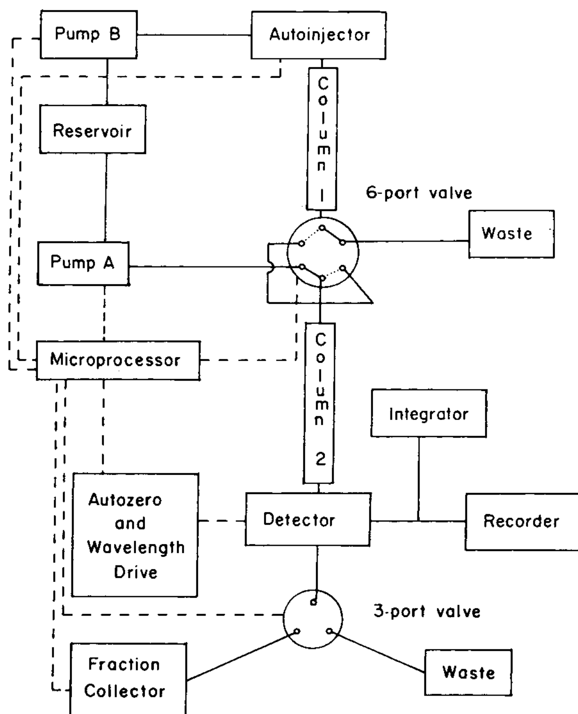

Fig. 2. Diagram of the automated chromatography system (see text for explanation).

Reagents

Reagent-grade chemicals and glass distilled water were used throughout. Crystalline samples of cisplatin (CDDP), *cis*-dichlorodiisopropylamine-*trans*-dihydroxyplatinum(IV) (CHIP), *cis*-diammine-1,1-cyclobutyldicarboxylate platinum(II) (CBDCA) and *cis*-1,2-cyclohexyldiamine-4-carboxyphthalate platinum(II) were obtained from the National Cancer Institute (Bethesda, MD, U.S.A.) and used as received.

Liquid chromatography

Solvent generated anion exchangers were prepared by pumping 0.5% (w/v) hexadecyltrimethylammonium bromide (HTAB) through the columns until a rise in baseline attributed to equilibration of the system, was observed. Mobile phases were filtered through 0.45-μm Millipore filters (Millipore, Bedford, MA, U.S.A.) and degassed in vacuo. Solute capacity ratios, k, were determined at least in duplicate according to eqn. 1 using ^2H_2O for the determination of t_0.

$$k = (t_r - t_0)\, t_0^{-1} \tag{1}$$

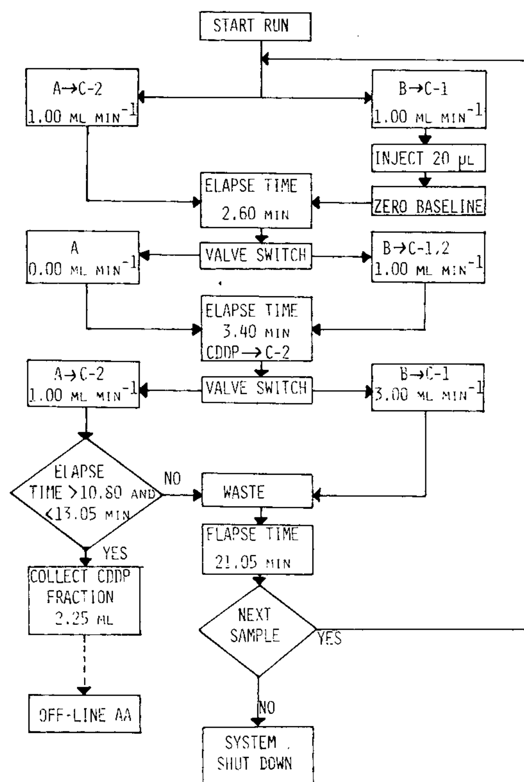

Fig. 3. Flow diagram of column switching program for the HPLC separation of cisplatin in urine. A, B, C-1 and C-2 refer to pumps A and B, column 1 (Hypersil) and column 2 (ODS Ultrapak), respectively. Arrows indicate the flow through the columns. The elapse times are from the point of injection. For further details see text and Fig. 2.

Divalent platinum complexes

Aqueous solutions containing mixtures of $[Pt(NH_3)_2Cl_2]$, $[Pt(CH_3)_2ClY]$ and $[Pt(NH_3)_2Y_2]$ (where Y = H_2O, Br, I, N_3) were prepared by incubating cisplatin in water and aqueous solutions of NaBr, NaI and NaN_3 (at 0.1 mol dm^{-3}) at 30°C for 2 h [15].

Urine analysis

Clinical urine samples (5 ml) were frozen rapidly and stored over solid carbon dioxide prior to analysis. The samples were thawed, sonicated for 2 min, filtered through 3-μm Millipore filters and chromatographed immediately. The purified cisplatin fractions, collected from the chromatograph were subsequently determined by atomic absorption spectrophotometry. Each sample was determined in duplicate and compared with calibration curves prepared by treating standard solutions of cisplatin in 0.1 mol dm^{-3} sodium chloride in an identical manner.

RESULTS AND DISCUSSION

Ligand selectivity

Previously the application of solvent generated anion exchanger HPLC has been restricted to cisplatin and no precedent exists for predicting the chromatographic behavior of cisplatin analogues and their biotransformation products. Accordingly, we have investigated the effect of ligand substitution on retention using divalent platinum complexes as model solutes and applying solvophobic theory [16] and a functional group approach [17—23] to the data. For this study a solvent generated anion exchanger consisting of 2.09 μmol m^{-2} HTAB adsorbed onto ODS Hypersil was used in conjunction

TABLE I

CHROMATOGRAPHIC RETENTION DATA FOR SOME DIVALENT PLATINUM COMPLEXES

Stationary phase: ODS Hypersil + 2.09 μmol m^{-2} HTAB; mobile phase: 10^{-4} mol dm^{-3} HTAB.

Solute	k^{*}	τ^{**}
cis-$[Pt(NH_3)_2Cl_2]^0$	1.05	—
trans-$[Pt(NH_3)_2Cl_2]^0$	0.30	—0.54
cis-$[Pt(NH_3)_2ClH_2O]^+$	0.10	—1.02
cis-$[Pt(NH_3)_2Br\,Cl]^0$	2.15	0.34
cis-$[Pt(NH_3)_2Br_2]^0$	4.63	0.64
cis-$[Pt(NH_3)_2Cl\,I]^0$	8.00	0.88
cis-$[Pt(NH_3)_2I_2]^0$	49.0	1.67
cis-$[Pt(NH_3)_2Cl\,N_3]^0$	3.00	0.46
cis-$[Pt(NH_3)_2(N_3)_2]^0$	4.55	0.63

$^{*}k = (t_r - t_0)t_0^{-1}$ (eqn. 1).

$^{**}\tau = \log(k_j \cdot k_i^{-1})$ (eqn. 2), where k_i = $Pt(NH_3)_2Cl_2$.

with a mobile phase of 10^{-4} mol dm^{-3} HTAB in water (Table I). The functional group contribution term, τ, is related to the chromatographic selectivity factor, α, by

$$\tau = \log \alpha = \log (k_j \cdot k_i^{-1}) \quad (2)$$

where the subscripts j and i refer to substituted and unsubstituted molecules, respectively. In the present study, cisplatin is taken as the reference compound (i.e., j = $[Pt(NH_3)_2ClY]$ or $[Pt(NH_3)_2Y_2]$ and i = $[Pt(NH_3)_2Cl_2]$).

The relationship for τ according to solvophobic theory is given by [16, 18, 19, 21, 22]:

$$\tau = [(a_j - a_i) + \gamma(\Delta A_j - \Delta A_i)] \, [2.3\,RT]^{-1} \quad (3)$$

where $(a_j - a_i)$ represents the differences in overall effects of dipole, charge and polarizability of the solutes, $(\Delta A_j - \Delta A_i)$ is the difference in hydrophobic surface area (HSA) of the solutes and γ is the mobile phase surface tension. The high selectivity of solvent generated anion exchanger systems for divalent platinum species (Table I) presumably arises from the use of a purely aqueous eluent which maximizes these differences in addition to providing a high surface tension (γ_{H_2O} = 72.0 mNm^{-1}). The retention order of the halogenated complexes indicates that hydrophobic differences play the major role in governing selectivity in these systems, the replacement of two chloride ions on cisplatin by two bromide ions produced a τ value which was approximately twice that obtained for the replacement of one chloride ion. Similarly, the τ value for the diiodo species was twice that obtained for the monoiodo species. In contrast, non-equivalency of the two azide groups was observed, indicative of different intramolecular interactions within one or both of the azide complexes, compared with cisplatin. The poor retention of the aquo species ($[Pt(NH_3)_2ClH_2O]^+$) arises primarily from electrostatic repulsion of the solute from the stationary phase by the adsorbed cationic surfactant. The weak retention of the *trans*-isomer of cisplatin arises from its lack of a molecular dipole and a reduced HSA.

In addition to rationalizing retention behavior, functional group analysis in HPLC has been used in drug design models [21, 23—30] and for the identification of drug metabolites and degradation products [23]. Consequently, this approach should prove useful in future studies of noble metal anti-tumor agents. Fig. 4 shows the preliminary results of the application of solvent generated anion exchanger HPLC to the analysis of three cisplatin analogues and demonstrates further the flexibility of this approach. With a purely aqueous mobile phase, III and IV were unretained, since presumably, they present a lowered HSA compared with cisplatin. In contrast the anionic complex, II, was strongly retained as a result of electrostatic interactions. The addition of 10^{-2} mol dm^{-3} citrate buffer (pH 7.0) enhanced the retention of III and IV due to an increase in the mobile phase surface tension [12, 13, 31] while decreasing the retention of II as predicted by conventional ion-exchange theory. The 4-carboxyphthalado complex, II, was found to be very unstable in aqueous solution and two major and two minor peaks were observed in a sample which was injected immediately after dissolution. The peak corresponding to

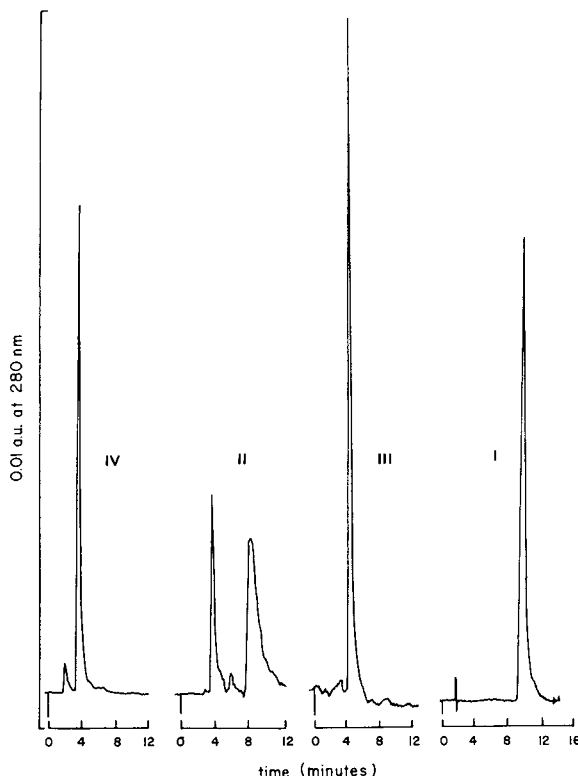

Fig. 4. Chromatograms of some clinically investigated platinum complexes. Stationary phase: ODS Hypersil + 2.09 μmol m^{-2} HTAB. Mobile phase: 10^{-2} mol dm^{-3} citrate buffer (pH 7.0)—10^{-4} mol dm^{-3} HTAB. Peak identification as Fig. 1.

II itself was broad and asymmetrical indicating significant degradation during chromatographic migration. Further studies at different wavelengths of detection have revealed several other degradation products with long retention times in solutions of this complex, II.

Nature of the stationary phase

A number of reversed stationary phases were investigated as supports for the adsorbed cationic surfactant (HTAB). Previous studies [11, 12] were performed on μBondapak C_{18}; however, the instability of this material with prolonged use and the considerable batch-to-batch variations between columns led to the subsequent use of ODS Hypersil and ODS Ultrapak. These spherical alkylsilicas having no residual silanols proved to be much more stable: for example, over 1000 injections of cisplatin in solutions of high ionic strength (0.5 *M*) were made on the ODS Hypersil column without any observable column deterioration. Additionally, ODS Ultrapak and ODS Hypersil gave similar uptakes of HTAB (ca. 2 μmol m^{-2}) which were higher than those observed for μBondapak C_{18} (ca. 1.3 μmol m^{-2}). This increased uptake of HTAB was associated with a proportional increase in the retention of cisplatin.

For the analysis of cisplatin in urine, a silica column (Hypersil) was used

for the primary clean up of the samples. Significant amounts of HTAB (0.63 μmol m^{-2}) were adsorbed onto the silica surface resulting in cisplatin retention. In the absence of adsorbed HTAB, cisplatin is not retained on silica gel [11] which is deactivated due to hydrogen bonding with water molecules. Thus, it appears that the silica column in this study is behaving primarily as a reversed-phase material with a reduced retention capacity [32].

Analysis of cisplatin in urine

Column switching. A priori, purely aqueous mobile phases are preferred for the analysis of cisplatin since they produce the potential for maximum selectivity combined with optimum solute retention. However, water is the weakest solvent in reversed-phase systems. As a result cisplatin co-eluted with several urine components from the solvent generated anion exchanger (ODS Ultrapak + HTAB) using a mobile phase of 10^{-4} mol dm^{-3} HTAB in a citrate buffer (10^{-2} mol dm^{-3}, pH 7.0). The problem was exacerbated by the high retention of several urine components which were presumably anionic in character. The use of a silica pre-column (Hypersil + HTAB) only partially solved the problem. However, it was observed that cisplatin was retained on the silica pre-column and co-eluted with different urine components, i.e., the two stationary phases had different selectivities. Therefore, by use of these two columns and the application of column switching it was possible to resolve cisplatin from the urine components (Fig. 5).

The switching was designed so that only the fraction containing cisplatin was transferred from column 1 (Hypersil + HTAB) to column 2 (ODS Ultrapak + HTAB). After transferring cisplatin to column 2, the remaining material was vented from column 1 to waste at a higher flow-rate (3.00 ml min^{-1}). The required time for this clean-up step was measured by monitoring the eluent from column 1 by UV spectroscopy at 280 nm and determined the overall analysis time, which was 20 min. Some of the urine components were irreversibly adsorbed onto column 1, which had to be repacked after about 50 injections of urine. The same ODS Ultrapak column was used throughout this study without overall changes in performance. This stability reflects the superior quality of the packing material and the fact that very little of the urine was actually transferred to column 2. The different selectivities of the two columns were such that cisplatin eluted through the detector after all the material with which it co-eluted from column 1.

The relationship between cisplatin peak height and concentration was linear; however, its low absorptivity (ϵ ca. 100, 280 nm) limited this method of measurement to samples containing more than about 100 μg ml^{-1}. Therefore, measurement of cisplatin by off-line atomic absorption was preferred. By means of the three-port switching valve positioned after the detector (and also under microprocessor control), the cisplatin was collected as a single fraction (2.25 ml) and measured subsequently by atomic absorption. Fig. 6 shows that there was little lag time and sample dilution between the detector and the fraction collector. Thus, the window of the fraction collector could be set accurately by observation of the UV detector output.

The relationship between the platinum absorbance (A) and the concentration of cisplatin injected onto the chromatograph was linear over the range 0–250 μg ml^{-1} (eqn. 4)

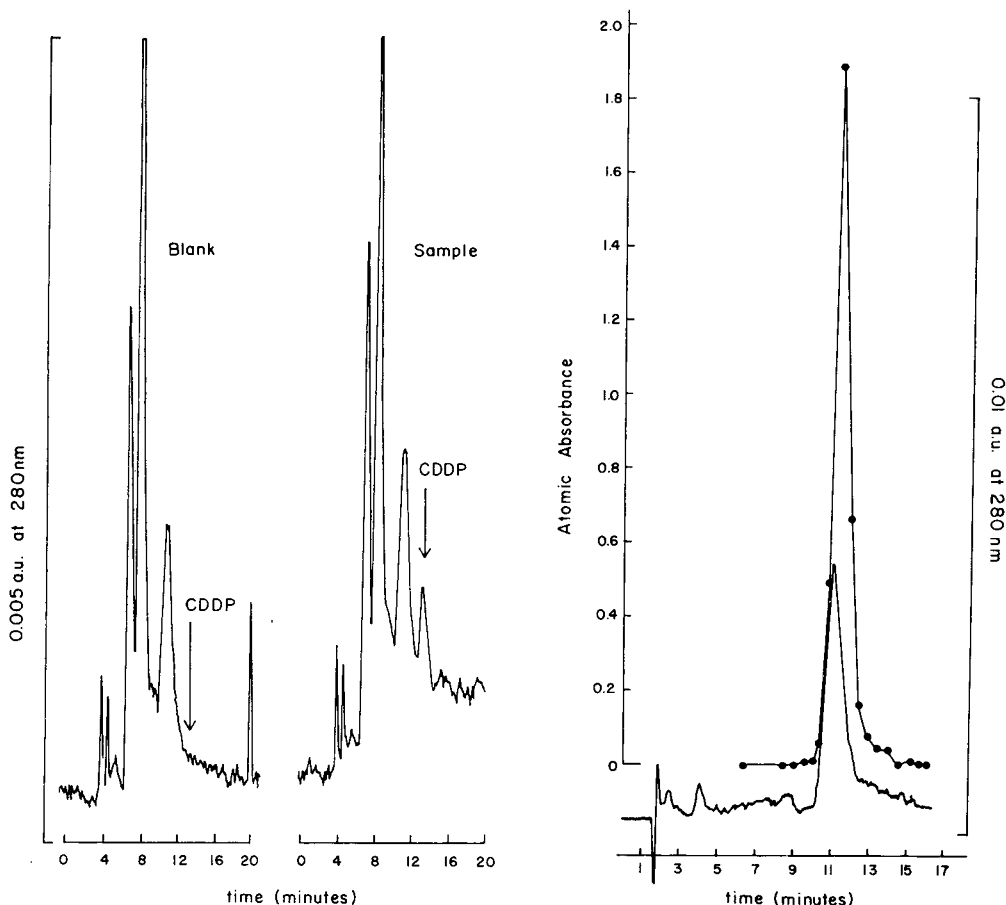

Fig. 5. Chromatogram of urine spiked with 100 μg ml^{-1} cisplatin (see text for conditions).

Fig. 6. Chromatograms of cisplatin obtained by on-line UV and off-line atomic absorption (●). The atomic absorption data were obtained by collecting 500-μl eluent fractions. CDDP concentration: 500 μg ml^{-1}.

$$A = (6.49 \cdot 10^{-4})\ [\mathrm{CDDP}] - 0.0011\ (r = 0.999, n = 6) \qquad (4)$$

At concentrations above 250 μg ml^{-1}, CDDP overloading of column 1 resulted in reduced efficiency of solute transfer from column 1 to column 2 and negative deviation from linearity. The limit of detection of cisplatin in urine injected onto the chromatograph was 2 μg ml^{-1} which could be measured by injecting 20 μl of the collected fraction onto the carbon rod atomizer of the atomic absorption spectrophotometer.

Cisplatin stability in urine

Cisplatin was found to be unstable when incubated in the urine of healthy volunteers at room temperature (ca. 22°C) over a 6—8-h period (Fig. 7). Considerable subject variation was observed (Fig. 7) and the amount of cisplatin lost varied between about 40% and 80%. The rate of loss of cisplatin on the

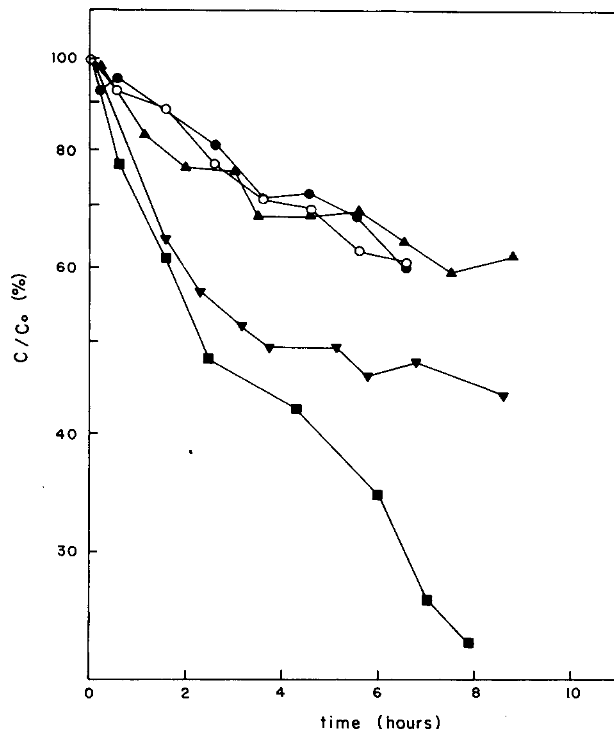

Fig. 7. The effect of subject variation on the loss of cisplatin from urine. Initial CDDP concentration: 200 μg ml⁻¹. The different symbols represent the data from samples of urine taken from four healthy volunteers. Open symbols (○), off-line atomic absorption measurements. Closed symbols (■, ▲) UV peak height measurements.

TABLE II

STABILITY OF CISPLATIN IN URINE AT −11°C AND −60°C

Temperature			
−11°C*		−60°C**	
Time (h)	CDDP concentration*** (μg ml^{-1})	Time (h)	CDDP concentration*** (μg ml^{-1})
0	51.6	0	46.2
11	41.4	1	45.8
23	36.2	2	45.4
30	32.6	3	48.4
48	32.1	4	48.4
		5	47.7
		8	46.2
		28	48.8
		48	43.8

*Refrigeration.
**Stored in solid carbon dioxide.
***Determined by HPLC and off-line atomic absorption.

other hand was independent of solute concentration. The data obtained from one subject was measured both by peak height and off-line atomic absorption (Fig. 7). There was no difference in the observed rate of loss of cisplatin by the two techniques but there was more scatter by peak height measurement, confirming off-line atomic absorption to be the method of choice for quantitation.

The loss of cisplatin in urine could not be totally arrested by storage at —11°C such that about 40% was lost after 48 h from a sample originally containing 50 $\mu g\ ml^{-1}$ (Table II). Cisplatin could only be successfully kept without significant degradation by storage over solid carbon dioxide (ca. —60°C). Statistical evaluation of the data in Table II revealed that the recovery of cisplatin from urine stored over solid carbon dioxide was 101.1% and the relative standard deviation of the assay was 3.6%.

Clinical samples

The developed methodology was applied to the determination of cisplatin in urine taken from three patients suffering from ovarian cancer. Cisplatin was administered (50 $mg\ m^{-2}$) by slow intravenous (i.v.) infusion at a rate of ca. 1 $mg\ min^{-1}$ after prior dosage with cyclophosphamide (ca. 600 $mg\ m^{-2}$ i.v.) and hydration with 0.45% sodium chloride and 5% dextrose (i.v.). Urine was voided before administration of cisplatin and the samples taken 30 min after completion of infusion. This procedure was designed to minimized the residence time of cisplatin in the bladder.

The urine concentration of unchanged cisplatin varied from 16.3 to 96.3 $\mu g\ ml^{-1}$ CDDP (equivalent to 10.6—62.6 $\mu g\ ml^{-1}$ Pt); however, the amounts and percentages of the total doses excreted were remarkably similar for each of the three patients (Table III). The total amount of platinum was determined by dilution of the sample (1:10 to 1:50 in water) to minimize matrix effects and direct atomic absorption spectrophotometry. It was found that between 75% and 95% of the drug was excreted unchanged in the urine (Table III).

CONCLUSIONS

The retention of platinum complexes on solvent generated anion exchangers is influenced by solute charge and ligand substitution. For neutral complexes, retention and selectivity are controlled by solute dipole and hydrophobic effects, whereas electrostatic effects dominate the retention of charged complexes such that cations are poorly and anions are strongly retained. The extrathermodynamic approach taken towards rationalizing the retention behavior of platinum complexes in reversed-phase systems employing secondary equilibria should prove useful in future studies, particularly in those aimed at identifying degradation and biotransformation products.

The inherent high selectivity of solvent generated anion exchanger systems towards platinum species may be attenuated by exposing the solutes to stationary phases of different surface properties during chromatographic migration. Furthermore, the use of this HPLC system together with a specific platinum detection system (off-line atomic absorption) produces a technique

TABLE III

URINE ANALYSIS OF THREE PATIENTS TREATED WITH CISPLATIN FOR OVARIAN CANCER

Patient	Total dose*,** (mg)	Urine analysis***						
		Concentration ($\mu g\ ml^{-1}$)		Amount (mg)		Percentage of dose		CDDP/Pt ratio (%)
		As CDDP**,§	Total Pt §§	As CDDP**,§	Total Pt §§	As CDDP	Total Pt	
1	39 (60)	10.6 (16.3)	13.4	4.7 (7.3)	6.2	12.2	16.1	75.8
2	65 (100)	39.1 (60.2)	41.0	8.8 (13.5)	9.2	13.5	14.2	95.1
3	58.5 (90)	62.6 (96.3)	75.6	6.2 (9.6)	7.6	10.6	13.0	81.5

*50 mg m^{-2} by i.v. infusion at ca. 1 mg min^{-1}.
**Values refer to amounts or concentrations of Pt. Values in parentheses refer to amounts or concentrations of CDDP.
***Urine collected ca. 30 min post infusion.
§ Determined by HPLC and off-line atomic absorption.
§§ Determined by direct atomic absorption.

capable of the unambiguous determination of cisplatin in urine. This technique requires minimal sample preparation and is easily automated.

The clinical results indicate that large amounts of cisplatin are excreted unchanged within the first 2 h of administration and that previous results [1] reporting low levels of cisplatin in urine, attributed to extensive biotransformation, may be due to degradation during storage either in the bladder or after sample collection. Finally, the presence of relatively high concentrations of cisplatin in urine may be related to the clinical findings of its utility in the management of bladder cancers [33].

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LIQUID CHROMATOGRAPHIC DETERMINATION OF CAFFEINE IN BIOLOGIC FLUIDS

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SUMMARY

A high-performance liquid chromatographic procedure was developed for the determination of caffeine in various biologic fluids and coffee. A reversed-phase column and UV detection at 254 nm were used to obtain a sensitivity of 0.1 μg/ml caffeine in serum and saliva using a sample volume of 0.1 ml. Caffeine metabolites and commonly ingested xanthines do not interfere with the assay. The within-day coefficients of variation were 9.8 and 9.9% at plasma caffeine concentrations of 2 and 10 μg/ml, respectively. The day-to-day coefficients of variation were 6.8 and 6.6% at plasma caffeine concentrations of 2 and 10 μg/ml, respectively. Serum and saliva caffeine concentrations were determined following a single oral dose of coffee and an intravenous infusion of caffeine in one subject. Computer estimates of caffeine pharmacokinetic parameters in one subject are in excellent agreement with previously published values.

INTRODUCTION

Caffeine is a commonly ingested drug present in foods and many beverages consumed throughout the world. Recently, the drug has been used therapeutically in the management of neonatal apnea [1]. Studies indicate that the

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pharmacologic and toxicologic effects of caffeine are incompletely understood [2,3]. For example, caffeine is teratogenic in rats [2] and as a consequence it has been recommended that pregnant women restrict caffeine intake until further studies can determine the applicability of this finding to humans [3]. Another study has shown that caffeine is a potent cardiovascular stimulant, but it is not known whether caffeine ingestion causes arrhythmias [4]. One study has demonstrated that among regular consumers of caffeine, even a brief abstinence from the drug may produce anxiety [5]. All of these findings suggest that caffeine is not an innocuous compound and that additional studies on the toxicology and pharmacology of caffeine are needed.

Pharmacologic and toxicologic studies of caffeine both in animals and man often require collection of multiple blood samples and detection of caffeine concentrations below the limit of sensitivity for many available assays [4,6]. It may also be necessary to determine the caffeine content of commonly ingested beverages for pharmacologic studies of caffeine. This report describes a sensitive and selective high-performance liquid chromatographic (HPLC) procedure for the analysis of caffeine in various biologic fluids and coffee. Application of the method to pharmacokinetic and protein binding studies of caffeine are demonstrated in one volunteer subject.

MATERIALS AND METHODS

Chemicals and reagents

Caffeine, theophylline and theobromine were purchased from Eastman Kodak (Rochester, NY, U.S.A.). Paraxanthine and 3-methylxanthine were purchased from Adams Chemical (Round Lake, IL, U.S.A.). β-Hydroxyethyl-theophylline (internal standard) was obtained from Sigma (St. Louis, MO, U.S.A.). Acetonitrile and chloroform (UV grade) were purchased from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Phosphoric acid and 2-propanol were obtained from Mallinckrodt (St. Louis, MO, U.S.A.). All reagents and chemicals were reagent grade or better.

Standard solutions

Donor human plasma was obtained from the University Hospital blood bank. Analysis of chromatograms obtained from extracts of blood bank plasma suggested that caffeine and caffeine metabolites were present. Consequently, four 25-ml quantities of plasma were placed in cellulose dialysis membranes (1.75 in. flat width, 24 Å pore size, VWR Scientific, Columbus, OH, U.S.A.) and suspended in 2 l isotonic phosphate buffer, pH 7.4, containing 2.5% activated charcoal. The buffer solution was stirred at 8°C for 40 h to remove any caffeine or caffeine metabolites from plasma. Volumes (10 ml) of plasma containing 0.5, 1.0, 2.0, 2.5, 5.0, 7.5 and 10.0 μg/ml caffeine were prepared. Aliquots of these plasma standards (0.5 ml) were stored in 1.5-ml polypropylene microcentrifuge tubes (VWR Scientific). An aqueous reference solution containing 33 μg/ml theophylline, theobromine, β-hydroxyethyl-theophylline and 20 μg/ml caffeine, and an aqueous internal standard solution containing 5 μg/ml β-hydroxyethyl-theophylline were prepared. Chromatographic resolution of caffeine, caffeine metabolites, and β-hydroxyethyl-theophylline was determined

daily by injecting 25 μl of the reference solution onto the column. All solutions were stored at −4°C.

Apparatus

Chromatography was performed with a Beckman Model 100A HPLC pump equipped with a Beckman Model 210 sample injection valve with a 50-μl sample loop capacity (Beckman, Berkeley, CA, U.S.A.). The mobile phase effluent was monitored with a Beckman Model 153 fixed-wavelength detector (254 nm) equipped with an 8-μl flow cell (Beckman). Specificity studies were performed with a Beckman Model 155-00 variable-wavelength detector equipped with a 20-μl capacity flow cell. A 5-μm particle size ODS column, 25 cm × 4.6 mm I.D. was used (Altex Scientific, Berkeley, CA, U.S.A.).

Extraction procedure

Volumes (0.1 ml) of serum, plasma, or saliva were placed in 16 × 125 mm PTFE-capped borosilicate culture tubes (Kimble, Vineland, NJ, U.S.A.) containing 100 μl 0.2 *N* hydrochloric acid and 75 μl internal standard solution. Twelve ml of 20% 2-propanol in chloroform were added and the mixture was shaken gently for 20 min. After centrifugation for 5 min the aqueous (top) layer was aspirated and the remaining organic layer was transferred to a clean disposable culture tube. The organic phase was evaporated at 50°C under a gentle stream of nitrogen. The residue was reconstituted with 100 μl mobile phase and 25 μl were injected onto the column.

Chromatography and standard curve

The mobile phase was prepared by diluting a mixture of 260 ml acetonitrile and 1 ml of 85% phosphoric acid to a final volume of 2 l with distilled water. The mobile phase flow-rate was 1.0 ml/min and the effluent was monitored at 254 nm with detector output recorded at 10 mV on a Beckman Model BD 40 chart recorder with the chart speed set at 0.25 cm/min. Peak heights of caffeine and internal standard were measured following the analysis of plasma standards and the ratio plotted against the respective plasma caffeine concentrations. Linear regression analysis was used to determine the slope and intercept values of the standard curve. Samples containing caffeine were compared with the standard curve using the caffeine:internal standard peak height ratio.

Recovery studies

Plasma was prepared as described above and spiked with caffeine to concentrations of 1.0 and 10.0 μg/ml. Volumes of plasma (0.1 ml) were extracted as described above with 12 ml 20% 2-propanol in chloroform and 6 ml of the organic phase were transferred to a clean disposable culture tube and evaporated to dryness. The residue was reconstituted with 100 μl mobile phase and 50 μl were injected onto the column. Peak heights of caffeine were measured and compared to those obtained following the injection of 25 μl of 1.0 and 10.0 μg/ml aqueous caffeine solutions.

Five coffee mixtures were prepared by dissolving 2-g quantities of instant coffee (Taster's Choice®, Nestlé, White Plains, NY, U.S.A.) in 200 ml water by gently heating to boiling. The mixtures were cooled to room temperature and

aliquots were diluted 1:100 and analyzed for caffeine as described above for plasma and serum. Recovery of caffeine from coffee was determined by spiking aliquots of each coffee mixture with an additional 100 μg/ml caffeine and then analyzing the resulting mixture to determine the caffeine content.

Precision

Replicate plasma specimens containing 2.0 and 10.0 μg/ml caffeine were analyzed each day for ten consecutive days to determine the day-to-day coefficient of variation of the assay. Ten plasma specimens containing 2.0 and 10.0 μg/ml caffeine were analyzed on the same day to assess the within-day coefficient of variation of the assay. These plasma specimens were stored at −4°C in glass vials between analyses.

Specificity studies

Four serum specimens were obtained from a human subject following intravenous caffeine administration. Samples were extracted and injected onto the column and the mobile phase effluent monitored at each of three wavelengths (254, 280 and 290 nm). Caffeine concentrations were determined from standard curves prepared following the analysis of plasma standards with effluent monitoring at the appropriate wavelengths.

Human study

A healthy, non-smoking volunteer received a single, intravenous dose of caffeine followed one month later by a single oral dose of coffee. The subject had refrained from ingesting any xanthine containing foods or beverages for at least one week prior to the studies. An intravenous infusion of caffeine benzoate (equivalent to 280 mg caffeine base, Eli Lilly, Indianapolis, IN, U.S.A.) was administered into an antecubital vein over 5 min with a sage syringe pump (Sage Instruments, Orion Research, Cambridge, MA, U.S.A.). Blood was obtained from a heparin-containing syringe placed in a vein in the contralateral arm. The oral dose consisted of 400 ml instant coffee (Taster's Choice) containing 160 mg caffeine (base). The dose was ingested over 10 min. Five ml blood and mixed saliva samples were obtained at various times over the subsequent 24-h periods. Caffeine concentrations in serum and saliva extracts were determined according to the present procedure. Blank serum for caffeine protein binding determination was dialyzed against an equal volume of phosphate buffer (pH 7.4) containing 2.0 and 10.0 μg/ml caffeine. Equilibrium dialysis was performed at 37°C in a plexiglass two-chambered apparatus of 1-ml capacity (Technilab Instrument Corp., Pequannock, NJ, U.S.A.) separated by a dialysis membrane (Spectrapor, Spectrum Medical Industries, Los Angeles, CA, U.S.A.). After 16 h of mild agitation, 0.1-ml volumes of serum and buffer were extracted for caffeine determination according to the present procedure.

Pharmacokinetics

Serum concentration—time data from the intravenous and oral doses were simultaneously fitted to a one-compartment open pharmacokinetic model with first order absorption following the oral dose [7]. A non-linear regression technique [8] was used to generate the least-squares computer estimates of the

elimination rate constant, K; the volume of distribution, V; the absorption rate constant, K_a; and the fraction of the oral dose absorbed, F. Areas under the total and free concentration—time curves were determined by trapezoidal integration with inclusion of the area from the last measured concentration to infinity. Clearance values were determined from the dose to area under the serum concentration—time curve quotient for free and total drug concentrations following the intravenous dose.

RESULTS

Column life was at least 3.5 months with almost daily use. Slight rises in pressure and loss of resolution were corrected by flushing the column overnight with a solution consisting of 500 ml methanol and 500 ml distilled water at a flow-rate of 0.2—0.3 ml/min. All of the dimethylxanthine metabolites of caffeine were well separated from caffeine and internal standard (Fig. 1). The retention times for caffeine, β-hydroxyethyl-theophylline, theophylline, paraxanthine, and theobromine were 8.8, 5.7, 5.2, and 4.0 min, respectively. Theophylline and paraxanthine elute with identical retention times on this chromatographic system. Caffeine and β-hydroxyethyl-theophylline are well separated from 3-methylxanthine which has a retention time of 2.4 min. Analysis of chromatograms from extracts of blood-bank plasma revealed peaks with retention times identical to those of theophylline, theobromine, and caffeine (Fig. 1). Serum extracts from coffee drinkers resulted in similar chromatograms. These peaks probably represent dietary xanthines and their

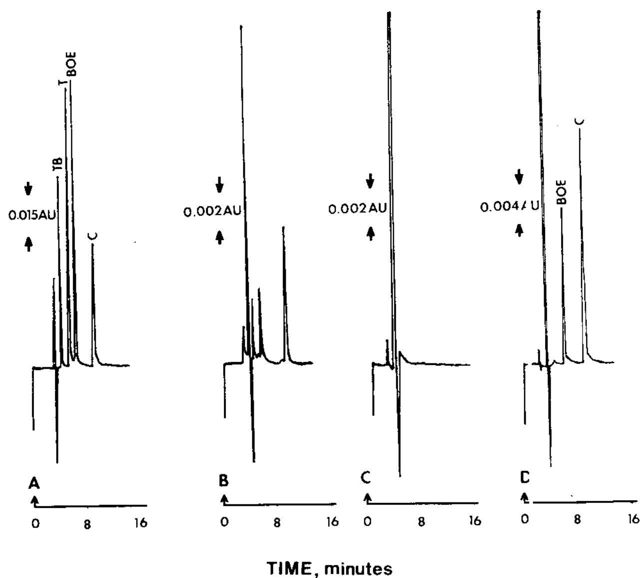

Fig. 1. Chromatograms of an aqueous standard mixture and donor plasma. (A) Standard mixture of the xanthines theobromine (TB), theophylline (T) and β-hydroxyethyl-theophylline (BOE) (0.825 μg), and caffeine (C) (0.5 μg); (B) extract of donor human plasma; (C) extract of stripped donor plasma; (D) extract of human plasma containing caffeine (C) (0.5 μg) with β-hydroxyethyl-theophylline (BOE) as the internal standard.

metabolites. Non-coffee drinkers had serum extracts which were free from any interfering substances. Dialysis of blood-bank plasma removed any interfering peaks (Fig. 1).

The caffeine:internal standard peak height ratios determined from the analysis of extracted samples were linearly related to caffeine concentration over the range of 0.5—50.0 μg/ml. The lower limit of detection for caffeine was 0.1 μg/ml allowing a signal-to-noise ratio of 4 and a sample volume of 0.1 ml.

The analytical recoveries of caffeine from five plasma specimens averaged 102 and 95% at plasma caffeine concentrations of 1.0 and 10.0 μg/ml, respectively. These were not significantly different using an unpaired *t*-test. Caffeine concentrations in five samples of instant coffee ranged from 238 to 258 μg/ml. The recovery of caffeine from spiked coffee averaged 92% for these five samples.

The day-to-day coefficients of variation of the assay were 6.8 and 6.6% at measured plasma caffeine concentrations of 1.98 and 10.1 μg/ml, respectively. The within-day precisions of the assay were 9.8 and 9.9% at measured plasma caffeine concentrations of 2.09 and 9.50 μg/ml, respectively. Serum specimens ranging in concentration from 0.6—5.5 μg/ml obtained following the intravenous dose were analyzed with mobile phase effluent monitoring at each of three different detection wavelengths. There was excellent agreement between the values obtained at 254, 280 and 290 nm (Table I). Linear regression analysis of concentrations determined at 290 and 280 nm (dependent variable) against concentrations determined at 254 nm (independent variable) resulted in slope and *y*-intercept values of 0.95 and 0.04 μg/ml, respectively. These values were not significantly different from unity (slope) and zero (intercept).

TABLE I

SERUM CAFFEINE CONCENTRATIONS (μg/ml) DETERMINED IN FOUR DIFFERENT SERUM SPECIMENS MEASURED WITH HPLC EFFLUENT MONITORING AT THREE DETECTION WAVELENGTHS

Similar concentration values at each detection wavelength indicate assay specificity.

254 nm	280 nm	290 nm
5.5	5.2	5.5
4.4	4.0	4.2
2.0	1.8	2.1
0.5	0.4	0.6

Fig. 2 shows the serum caffeine concentrations following the intravenous infusion and the oral dose. Serum caffeine concentrations continued to rise slightly for 1 h following the termination of the intravenous infusion due to some extravasation of the dose. The subject complained of some mild discomfort at the injection site at the end of the infusion. Serum caffeine concentrations declined in a log—linear fashion 1 h following the infusion. A peak serum caffeine concentration of 3.2 μg/ml occurred 1 h following the oral dose. There was an excellent correlation between observed and computer-predicted serum concentration—time values. The largest deviation between observed and computer-predicted values was only 13.6% and averaged 5.6% for all the experimental data. The fraction of free drug (unbound) in serum was 0.74 at total

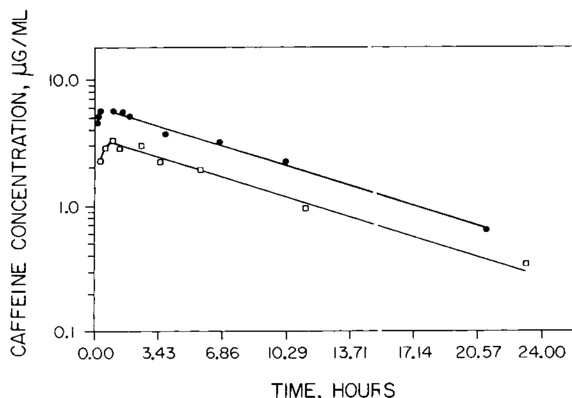

Fig. 2. Measured and computer predicted serum caffeine concentrations following an intravenous infusion of caffeine benzoate (●), and an oral dose of coffee (□).

(free and bound) post-equilibrium caffeine concentrations of 5.7 and 1.2 µg/ml.

The non-linear least-squares computer estimates of k_a, F, K and V were 4.21 h^{-1}, 0.93, 0.106 h^{-1} and 0.58 l/kg, respectively. Clearance of total (free and bound) and clearance of free (unbound) caffeine from serum was 1.0 ml/min/kg and 1.4 ml/min/kg, respectively.

There was considerable variability in the saliva caffeine concentration—time profile particularly after the oral dose. The saliva:unbound serum caffeine concentration ratios ranged from 0.93 to 1.46 and averaged 1.18 with a standard deviation of 0.16. Linear regression analysis of saliva (dependent variable) and unbound serum (independent variable) caffeine concentrations obtained from 1—24 h following the oral and intravenous doses gave a slope of 1.27 and a y-intercept value of −0.18 µg/ml with a correlation coefficient of 0.97 (n = 14).

DISCUSSION

The present procedure for the analysis of caffeine in biologic fluids and coffee is rapid, sensitive, and selective. The method employs a single extraction step suitable for detection of 0.1 µg/ml caffeine in 0.1 ml serum, plasma or saliva and can be used for caffeine determination in solutions of coffee. Previous gas—liquid chromatographic and HPLC methods for the analysis of caffeine in biologic fluids or beverages have been proposed [4,6,9—15]. This method offers some advantages over previously reported procedures. The small sample volume required and good sensitivity of the present method are suitable for pharmacologic studies requiring multiple blood samples in neonates and small laboratory animals. Other methods employ larger sample volumes (0.5—1.0 ml serum or plasma) to obtain similar sensitivity [4,6]. Some gas—liquid chromatographic procedures involve multiple extraction steps or lengthy derivatization steps for caffeine quantitation [12,13]. In addition, one HPLC procedure does not employ an internal standard making sample preparation, extraction, and sample reconstitution potential sources of variability [14]. Another HPLC method employs theobromine as an internal standard [15].

Theobromine is a dimethylxanthine metabolite of caffeine in dogs [14] and would be expected to interfere with the assay in this species. Furthermore, results from the present investigation indicate that theobromine is often present in human specimens due to dietary xanthine intake. The present procedure employs β-hydroxyethyl-theophylline as an internal standard. This compound is well resolved from caffeine and the dimethylxanthine metabolites of caffeine.

The results of the single-dose caffeine study agree well with the findings reported by other investigators. Desmond et al. [16] reported similar caffeine distribution volumes and slightly higher clearance values for caffeine compared to the values obtained in the present study, probably due to inclusion of smoking subjects in their study. Cigarette smoking can induce drug metabolising enzymes and as a result increase caffeine clearance [17].

Considerable variability was observed in the ratio of saliva to free (unbound) serum concentrations following the oral and intravenous doses. The saliva to unbound serum caffeine concentration ratio ranged 1.5-fold in the present study. Caffeine is a neutral compound and partitioning across biologic membranes should not be influenced by pH differences between blood and saliva. The drug is only 26% bound to plasma proteins and yet the saliva to unbound serum caffeine concentration ratio averaged 1.18. Active secretion of caffeine into saliva or non-specific binding of caffeine to the buccal mucosa would account for the high saliva to serum concentration ratio observed in the present study. The correlation between serum and saliva caffeine concentrations is much higher however than previously reported values in neonates [18]. Saliva caffeine concentrations obtained within 1 h of the oral dose were not included in the analysis because those values were several-fold higher than the corresponding serum concentrations, probably due to buccal adsorption of caffeine. A previous study of another xanthine compound, theophylline, has shown that drug concentrations in saliva are relatively higher than drug concentrations in serum 10—30 min following ingestion of a theophylline solution [19].

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QUANTITATIVE ANALYSIS OF PROCARBAZINE, PROCARBAZINE METABOLITES AND CHEMICAL DEGRADATION PRODUCTS WITH APPLICATION TO PHARMACOKINETIC STUDIES

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SUMMARY

Quantitative analytical methods are described for the analysis of the anticancer drug procarbazine and eight known metabolites including those known to have cytotoxic activity. A direct sample insertion mass spectrometric assay for procarbazine and the urinary excretion product, N-isopropyl-terephthalamic acid, has been developed. This method employs stable isotope labeled variants in a procedure that minimizes analytical errors that may be encountered in the quantitation of the chemically unstable parent drug. A liquid chromatographic method is described for the analysis of seven known procarbazine metabolites. Use of these methods is demonstrated by the analysis of procarbazine metabolism during incubation in a 9000-g rat liver homogenate preparation. Procarbazine disappearance and metabolite appearance are also monitored in rat plasma following intraperitoneal administration of a 150 mg/kg bolus dose. Applications to patient pharmacokinetics is demonstrated using the liquid chromatographic assay to follow the appearance of active procarbazine metabolites on the first and fourteenth day of an oral 250 mg/kg/day course of therapy of a patient being treated for cancer.

INTRODUCTION

Procarbazine, N-isopropyl-α-(2-methylhydrazino)-p-toluamide, Fig. 1, is a clinically useful antineoplastic agent for the treatment of Hodgkin's disease [1] and brain tumors [2,3]. Procarbazine is not an active molecule but may under-

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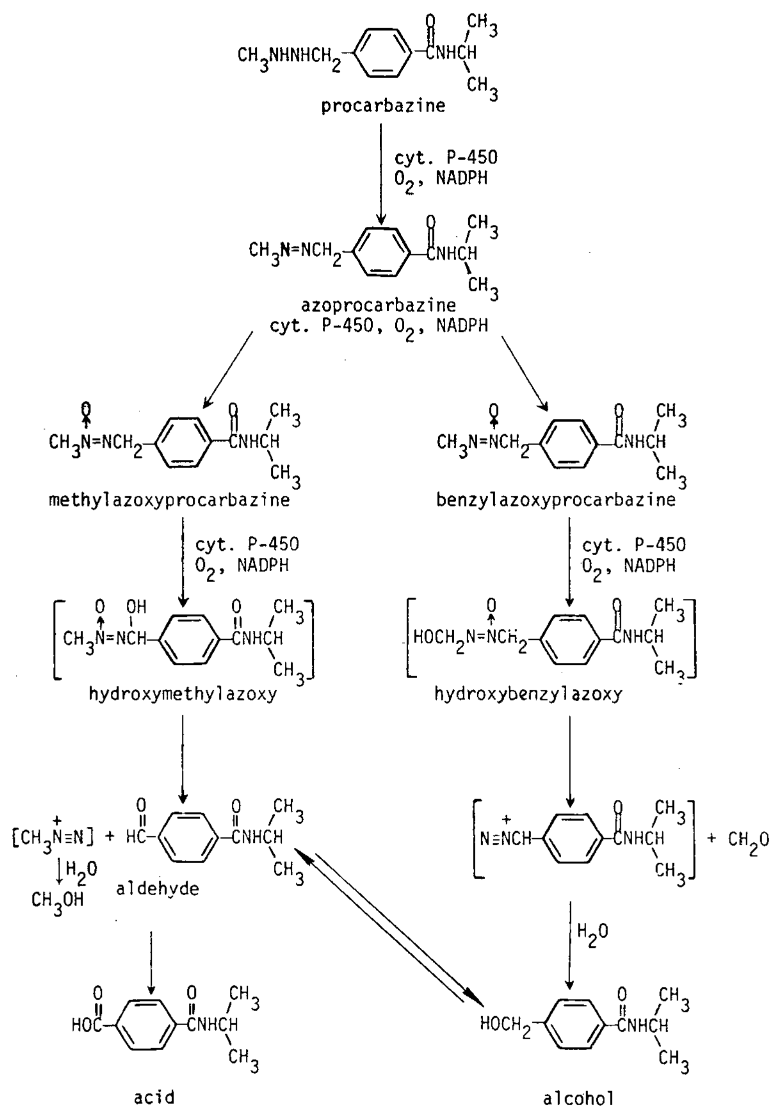

Fig. 1. The proposed pathway for the metabolic activation of procarbazine leading to the formation of alkylating intermediates. The scheme shows the formation of the known active metabolites azo-, methylazoxy- and benzylazoxyprocarbazine, as well as metabolic degradation products.

go metabolic conversion to cytotoxic alkylating intermediate(s). Investigations of procarbazine metabolism have identified three products that have antitumor activity in vivo [4] and are present in the plasma of animals after procarbazine treatment [5,6]. These include the initial procarbazine metabolite, N-isopropyl-α-(2-methylazo)-*p*-toluamide (azoprocarbazine) [7] and the isomeric azoxy compounds formed during azoprocarbazine metabolism, N-isopropyl-α-(2-methyl-ONN-azoxy)-*p*-toluamide (methylazoxy) and N-isopropyl-α-

(2-methyl-NNO-azoxy)-p-toluamide (benzylazoxy) [5]. Conversions of procarbazine to azoprocarbazine and of this metabolite to the azoxy isomers are mediated by cytochrome P-450 [8,9] and are stereoselective [5,8,9]. It has been suggested [5] that the azoxy isomers are converted to proximal active alkylating agent(s) by hydroxylation on the carbons adjacent to the azoxy function. The resulting hydroxy-azoxy intermediates may be chemically converted to active alkylating species in a reaction analogous to that proposed for 1,2-dimethylhydrazine [10]. The suggested scheme for metabolic activation of procarbazine is shown in Fig. 1.

In addition to the parent drug and the biologically active intermediates, there are a number of other chemical and metabolic reaction products. Reaction of the hydroxymethylazoxy intermediate may give a benzyldiazonium ion species that can react with water to give N-isopropyl-α-hydroxy-p-toluamide (alcohol). This product may also arise from the reduction of N-isopropyl-p-formylbenzamide (aldehyde) that may be formed by reaction of the isomeric hydroxy-azoxy intermediate [5]. Both of these metabolites may be converted to and excreted as N-isopropyl-terephthalamic acid (acid) which is the only identified urinary excretion product of procarbazine [11,12]. Azoprocarbazine may also isomerize chemically to N-isopropyl-p-formylbenzamide methylhydrazone [13] (hydrazone, structure not shown). Low-molecular-weight products, formaldehyde [14,15] and carbon dioxide [12,16,17] are also released during procarbazine metabolism.

Procarbazine is also converted in vivo to metabolic products that apparently result from free-radical reactions. These include methane [18,19] and N-isopropyl-p-toluamide [5] (methyl, structure not shown). Methane is expired while the methyl metabolite may undergo successive metabolic oxidation steps to give alcohol, aldehyde, and acid.

As indicated above, procarbazine is converted to at least eight chemical and metabolic products by reactions involving the methylhydrazine portion of the molecule. No metabolic alteration of the aromatic or N-isopropylcarboxamide groups has been observed. This paper reports mass spectral analytical methods for the analysis of procarbazine and the acid urinary excretion product, and high-performance liquid chromatographic (HPLC) procedures for the quantitation of the biologically active azo and azoxy metabolites, as well as the alcohol, aldehyde, hydrazone, and methyl degradation products.

The use of these assay procedures is demonstrated by the analysis of procarbazine disappearance and metabolite formation in rat liver homogenate preparations. Applications to pharmacokinetic studies are shown by the analysis of procarbazine disappearance and metabolite appearance in rat plasma following an intraperitoneal (i.p.) dose as well as the monitoring of active metabolites in the plasma of a patient who received orally administered procarbazine during a 14-day course of therapy.

EXPERIMENTAL

Instrumentation

Mass spectra were obtained with a Finnigan 3200 mass spectrometer equipped with a dual chemical ionization/electron impact source and operated in the

chemical ionization mode. Isobutane was used as a reagent gas at an ion chamber pressure of 0.75 Torr and temperature of 175°C. Samples were introduced by means of a direct sample insertion probe equipped with a ceramic tip. Samples were deposited onto the ceramic rod, introduced into the ion source, and evaporated from the probe tip as it was heated by induction from the ion source. The mass spectrometer was interfaced to a Data General Nova 830 computer capable of operating the instrument in the selected ion monitoring mode.

HPLC analyses were performed utilizing a Chromatronix Model 3500 pump and a Chromatronix Model 220 mixed-wavelength UV detector operating at 254 nm. A Waters Assoc. (Milford, MA, U.S.A.) 30 cm × 3.9 mm I.D. C_{18} μBondapak (10 μm) column was used at ambient temperature.

Reagents and materials

Procarbazine hydrochloride, NSC-77213, was obtained from Dr. W.E. Scott of Hoffmann-LaRoche (Nutley, NJ, U.S.A.). The procarbazine chemical and metabolic products were synthesized as previously described [5]. 4-Methylacetophenone was obtained from Aldrich (Milwaukee, WI, U.S.A.) and used as a standard in HPLC quantitation. N-(1,1,1,3,3,3-Hexadeuteroprop-2-yl)-terephthalamic acid (acid-$^2H_6$) and N-(1,1,1,3,3,3-hexadeuteroprop-2-yl)-α-(2-methylhydrazino)-*p*-toluamide hydrochloride (procarbazine-$^2H_6$ hydrochloride) were prepared using isopropylamine-$^2H_6$, which was synthesized in 27% yield from hydroxylamine hydrochloride (Eastman Kodak, Rochester, NY, U.S.A.) and acetone-$^2H_6$ (gold label, 99.5 atom %, Aldrich) according to the procedure of Colombini et al. [20].

Glass distilled methanol (Burdick and Jackson Labs., Muskegon, MI, U.S.A.) and triple distilled water were used as solvents in HPLC analyses.

Mass spectral analysis

Procarbazine and the acid metabolite were analyzed in in vitro enzyme preparations or plasma using mass spectral analysis of extracts without chromatographic separation. A 0.5–1.0 ml sample was acidified to pH 2 by addition of 5–7 drops of 1 *N* hydrochloric acid. Known amounts of internal standards, procarbazine-$^2H_6$ hydrochloride and acid-$^2H_6$ were prepared as 500 μg/ml stock solutions in ethanol and stored at −30°C. The acidified sample was extracted with 3 × 5 ml of diethyl ether to remove the acid and acid-$^2H_6$. The combined ether extracts were evaporated to dryness with a stream of nitrogen at ambient temperature in preparation for mass spectral analysis. The aqueous phase of the above extraction was made basic, pH 11, by addition of 1 *M* sodium hydroxide and immediately extracted with 3 × 5 ml of diethyl ether to remove procarbazine and procarbazine-$^2H_6$. The combined ether extracts were evaporated to dryness with a stream of nitrogen at ambient temperature in preparation for mass spectral analysis.

The residue containing acid/acid-$^2H_6$ and procarbazine/procarbazine-$^2H_6$ may be analyzed separately or simultaneously by redissolving the residue in 100 μl of ethanol or methylene chloride and placing an aliquot of this solution onto the ceramic tip of the direct sample insertion probe. Ion current produced during the evaporation of the sample into the ion source is monitored at *m/z*

222.2 and 228.2 for procarbazine and procarbazine-$^{2}H_6$ protonated molecular ions and at m/z 208.2 and 214.2 for acid and acid-$^{2}H_6$ protonated molecular ions, respectively. Sample evaporation times ranged between 60 and 120 sec.

Procarbazine and the acid metabolite were quantified by comparison of the ion current ratio for the unknown and standard compounds and calculation of the concentration of the amount of procarbazine or acid in the sample volume by reference to the respective standard curve.

High-performance liquid chromatographic analysis

The metabolic and chemical reaction products, azoprocarbazine, methylazoxyprocarbazine, benzylazoxyprocarbazine, alcohol, aldehyde, hydrazone, and methyl are analyzed by HPLC using 4-methylacetophenone as an internal standard. A 0.5—1.0 ml sample was extracted with 5 × 3 ml of cold diethyl ether. The combined ether extract was evaporated to dryness with a stream of nitrogen at ambient temperature. The residue was redissolved in 250—500 μl of methanol and 10 μl (6.7 μg) of a stock solution of 4-methylacetophenone in methanol (67 mg per 100 ml) was added. After thorough mixing using a vortex mixer (Vanlab, San Francisco, CA, U.S.A.) and centrifugation for 1 min at 12,000 *g* using an Eppendorf Micro centrifuge, approximately 15 μl of the sample were injected onto the reversed-phase liquid chromatographic C_{18} μBondapak column. Separation was achieved using a water—methanol (20 : 80) solvent at a flow-rate of 2.0 ml/min. The quantities of the above procarbazine products present in the sample aliquot were determined from the ratio of the respective peak area to the internal standard 4-methylacetophenone by reference to the appropriate standard curve.

In vitro procarbazine metabolism kinetics

Procarbazine disappearance and metabolite appearance were monitored during incubation in rat liver 9000-*g* supernate. The supernate was prepared using Fisher C-344 male rats according to the procedure of Fouts [21]. The incubation mixture containing 10 ml supernate, 1.5 ml of 18.7 m*M* procarbazine (final concentration 2.0 μ*M*), 2.0 ml of 0.1 *M* $MgSO_4$ (final concentration 14.3 m*M*), and 0.5 ml of 14.0 m*M* NADPH added every 20 min (final concentration 0.5 m*M*) was incubated with shaking at 37°C for 40 min. Two 1.0-ml aliquots of the incubation mixture were removed at 1, 10, 25, and 40 min. A 1.0-ml aliquot was analyzed for procarbazine and acid using the mass spectral assay and 1.0 ml was analyzed for metabolites using the HPLC assay.

In vivo procarbazine and metabolite kinetics in rat plasma after an i.p. dose

Fisher C-344 male rats weighing an average of 200 g received a 0.3-ml i.p. injection of procarbazine at a dose of 150 mg/kg body weight. The drug solution was prepared by dissolving 100 mg of procarbazine hydrochloride in 1 ml of 0.9% sodium chloride solution. At 10, 30, 60, and 90 min after administration of drug, two rats were anesthetized with diethyl ether and the blood removed by cannulation of the femoral artery. Blood was placed in a conical centrifuge tube and centrifuged at 1000 *g* for 10 min at 5°C. Equal aliquots of plasma from each of the two animals were pooled to represent a single post-injection time point. A 0.5- or 1.0-ml plasma aliquot was analyzed using the mass spectral and HPLC assays.

In vivo procarbazine metabolite kinetics in human plasma after an oral dose

A female patient undergoing chemotherapy for treatment of a malignant brain tumor received an oral dose of 250 mg procarbazine hydrochloride per kg body weight/day during a 14-day course of therapy. Plasma was assayed for circulating metabolites on the first and fourteenth day of therapy. At 20, 40, 60, 90, 120, 190, 250, and 360 min after administration of drug, a 3.0-ml aliquot of blood was removed and centrifuged at 12,000 *g* for 1 min. A 1.0-ml aliquot of plasma was removed and extracted with 3 $\times$ 4 ml of diethyl ether. The combined ether extract was evaporated with a nitrogen stream at ambient temperature and the residue was redissolved in 20 μl of methanol and 5 μl (3.4 g) of the 4-methylacetophenone standard stock solution were added. This solution was again centrifuged at 12,000 *g* for 1 min and 15—20 μl of the solution removed for analysis of procarbazine metabolites using the HPLC assay. Plasma concentrations for procarbazine and the acid metabolite were not obtained during this preliminary study.

RESULTS AND DISCUSSION

Procarbazine hydrochloride is reasonably stable in acidic solution, but the free hydrazine group is rapidly oxidized to azoprocarbazine when the solution is made basic. The rapid degradation reaction complicates the accurate analysis of this drug as significant decomposition may occur in the process of extracting small amounts of procarbazine from plasma into organic solvents. For this reason, we have selected to use the mass spectral analysis method in which an isotopic variant, procarbazine-$^2H_6$, is added to the sample aliquot. This establishes a ratio between intact procarbazine and the standard that is not altered by subsequent partial losses due to chemical oxidation or extraction. This procedure has an advantage over previously reported assays for procarbazine as a pure substance or in drug dosage forms [22] in that these methods are not suitable for analysis of small amounts of drug in plasma. Quantitative methods based on HPLC analysis of radiolabeled drug and metabolites are not readily applicable to patient pharmacokinetic studies [9]. A gas chromatography—mass spectrometry assay for procarbazine and metabolites has been reported [23]; however, results obtained using this assay method are not consistent with this report and others [5, 7—9, 13].

Standard curves for the analysis of procarbazine and the acid metabolite were found to be linear for sample/standard ion current ratios of 0.2—20. Recovery of procarbazine from human plasma averaged 95% and acid recovery averaged 103% over a range of plasma concentrations. The standard deviation of the procarbazine assay, determined from multiple analyses of known plasma doped samples, was 10% and the sensitivity limit was 5 nmol/ml plasma. Accuracy and sensitivity limits for the acid metabolite were comparable to those of procarbazine. The acid is assayed by the mass spectral method as a matter of convenience. The compound could be analyzed with HPLC, but the large difference in polarity between the acid and other procarbazine metabolites (Fig. 1) would have required a separate HPLC assay for the acid or the use of gradient elution for the simultaneous analysis of acid with the other metabolites.

Data obtained with this assay procedure are included in Figs. 2 and 3. Fig. 2 shows the partial disappearance of procarbazine during incubation in a 9000-*g* rat liver homogenate preparation. The acid is found to be a significant metabolite that is formed in the latter stages of the incubation. Fig. 3 shows procarbazine clearance from rat plasma following a 150 mg/kg i.p. bolus dose. Procarbazine plasma concentration decreases from 0.48 n*M* to 0.03 n*M*, four half-lives, within 90 min of administration. The acid appearance in plasma reaches a steady-state concentration after 20 min and is maintained at 0.10–0.18 n*M*.

An HPLC assay was developed for the simultaneous assay of azoprocarbazine, methylazoxy, benzylazoxy, alcohol, aldehyde, hydrazone, and methyl metabolites. A representative chromatogram of a mixture of these compounds and the 4-methylbenzophenone standard is shown in Fig. 4a. Good separation is obtained for all compounds except for the methylazoxy and hydrazone derivatives under conditions in which the eight compounds are eluted within 70 min. Table I shows the ratio of elution times for each metabolite relative to the standard. Normal elution times for 4-methylacetophenone averaged 60 min using 20% water in methanol solvent at a flow-rate of 2.0 ml/min. Standard curve slopes and *y*-intercepts are reported as are the standard deviations of

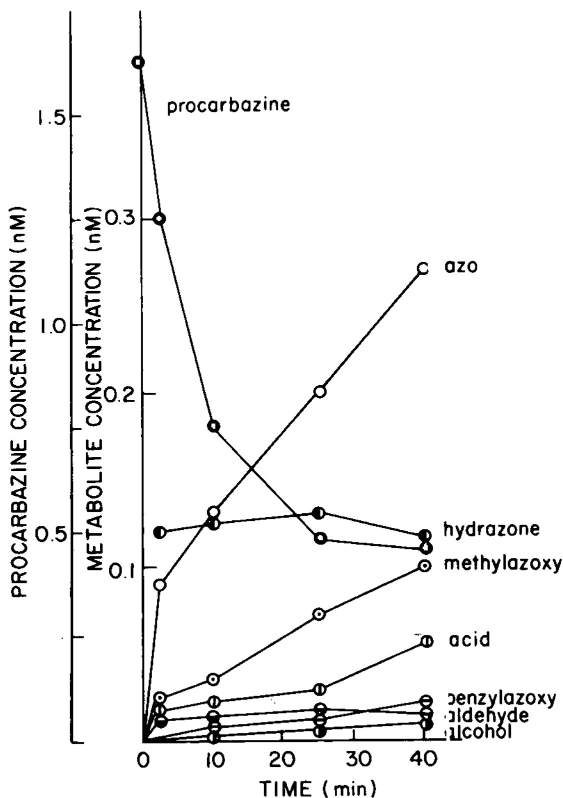

Fig. 2. Procarbazine disappearance and metabolite appearance monitored during a 40-min incubation of a 1.62 n*M* procarbazine solution in a 9000-*g* rat liver supernate preparation.

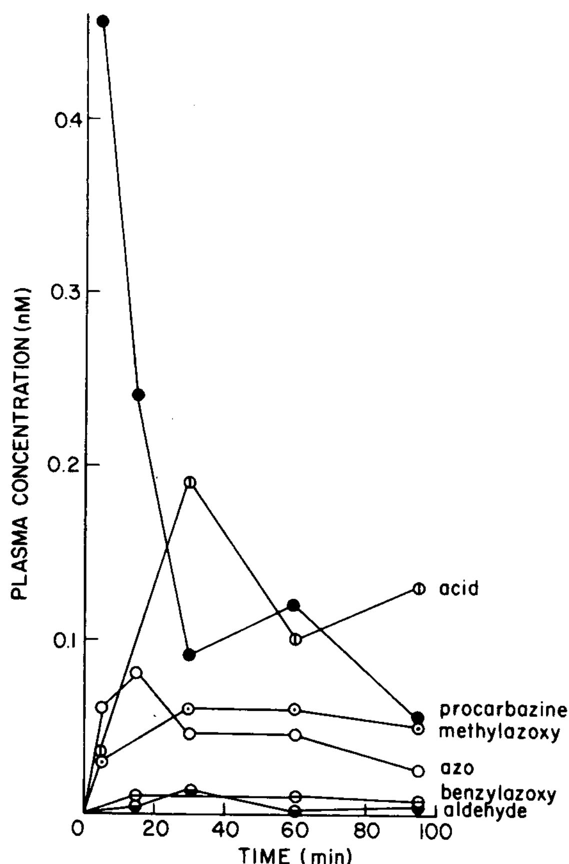

Fig. 3. Procarbazine disappearance and metabolite appearance in rat plasma following administration of a 150 mg/kg i.p. bolus dose of procarbazine.

TABLE I

HPLC ASSAY PARAMETERS OF PROCARBAZINE METABOLITES OBTAINED USING A C_{18} μBONDAPAK REVERSED-PHASE COLUMN AND 20% METHANOL IN WATER AS A SOLVENT

Compound	Elution ratio*	Slope**	y-Intercept	Recovery (%)	S.D.*** (%)
Aldehyde	0.27	2.81	-0.14	98	11
Benzylazoxy	0.39	9.31	-0.16	110	6
Methylazoxy	0.50	10.19	-0.40	107	5
Hydrazone	0.55	5.96	-0.18	98	5
Azo	0.67	3.91	0.10	99	2
Methyl	0.75	4.60	-0.09	117	8

*Retention time relative to 4-methylacetophenone = 1.00.

**Obtained from a plot of ratio of (sample/reference amounts added) (sample/reference peak area)$^{-1}$.

***Standard deviation of amount found from known was determined from nine or more measurements made over a range of concentrations of metabolite in human plasma.

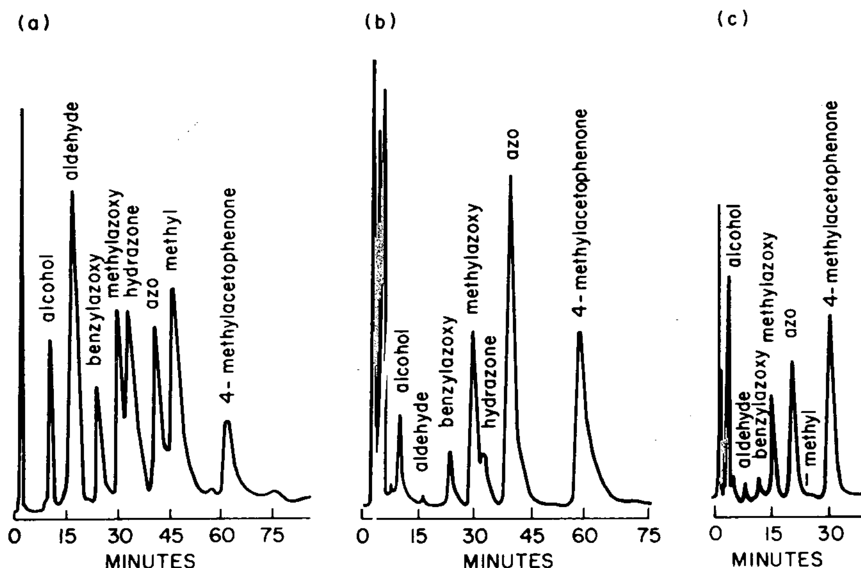

Fig. 4. HPLC chromatograms obtained using a C_{18} μBondapak reversed-phase column to separate (a) a standard mixture of synthetic procarbazine metabolites, (b) metabolites extracted from a 9000-g rat liver supernate incubation mixture, (c) metabolites extracted from rat plasma following administration of a 150 mg/kg i.p. bolus dose.

measured concentrations from known values of each agent added to human plasma. The concentration of each metabolite was calculated from the standard curves and the ratio of peak areas for the agent and internal standard. Peak areas were calculated from the peak height times the peak width at one-half peak height. Recovery of metabolites from plasma using the described ether extraction procedure was near 100%. Sensitivity limits for these HPLC assays were 2 nmol/ml or 100 ng injected onto the column.

This procedure was used to assay the metabolite formation in 9000-g rat liver supernate and the plasma concentration of metabolites in rat. Representative HPLC traces from the analyses are shown in Fig. 4b and c, respectively. The elution time for this assay may be shortened for convenience as in the in vivo plasma assay (Fig. 4c), where the hydrazone product was not detected.

The formation of procarbazine metabolites in vitro is shown in Fig. 2. Azoprocarbazine is the major metabolite after 40 min incubation. Methylazoxy is present in a higher concentration than the benzylazoxy isomer, and both compounds may be further metabolized to alcohol, aldehyde, and acid. Hydrazone is present at relatively high concentrations initially and does not increase during incubation. Hydrazone is probably present as an impurity or as a chemically generated artifact in this experiment.

Procarbazine metabolites may also be monitored in rat plasma (Fig. 3). Azoprocarbazine is less abundant in rat plasma than methylazoxyprocarbazine. Benzylazoxy and aldehyde are present in trace amounts. The alcohol was not monitored. Methyl metabolite, although sometimes observed, was not detected in these animals.

The plasma concentrations of procarbazine metabolites in man are shown in Fig. 5. Following a 250 mg/kg oral dose, the three active metabolites, azo, methylazoxy, and benzylazoxy are present in plasma in significant concentrations. Methylazoxyprocarbazine is the major circulating metabolite and the benzylazoxy isomer is present at a concentration approximately equal to its precursor, azoprocarbazine, on the first day of treatment. Since both methylazoxy and benzylazoxy procarbazine require further metabolic activation, the plasma concentration of these compounds may not be simply related to antitumor activity. The turnover, or amount of azoxy isomer formed and then converted to active product, may be more important than the steady-state plasma concentration of these intermediate metabolites. Data contained in Fig. 5 also demonstrate that a significant increase in the plasma concentration and a change in the relative concentration of metabolites occur by the fourteenth day of therapy. Studies are in progress to determine patient pharmacokinetics of circulating procarbazine metabolites using this assay procedure.

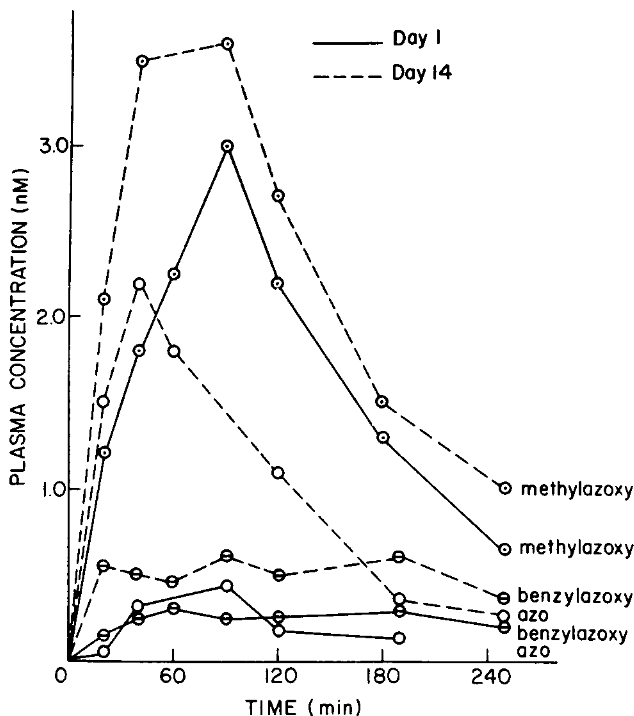

Fig. 5. Plasma concentrations of azo and azoxyprocarbazine metabolites are shown for a period of 250 min following the oral administration of a 250 mg/kg/day dose on days 1 and 14 of a 14-day treatment schedule.

CONCLUSION

Assay methods are described for the analysis of procarbazine and eight metabolic products. These procedures are useful for the analysis of in vitro metabolism and for the quantitation of plasma concentrations in rat and man.

The sensitivity of the HPLC assay is sufficient to permit pharmacokinetic studies of circulating active procarbazine metabolites in patients receiving normal therapeutic oral doses of this anticancer drug. This is the first procedure described that is applicable to clinical studies of procarbazine biodistribution.

ACKNOWLEDGEMENTS

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CHROMBIO. 1212

REVERSED-PHASE ION-PAIR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF MERCAPTOACETATE AND N-ACETYLCYSTEINE AFTER DERIVATIZATION WITH N-(1-PYRENE)MALEIMIDE AND N-(7-DIMETHYLAMINO-4-METHYL-3-COUMARINYL)MALEIMIDE

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SUMMARY

We have developed a high-performance liquid chromatographic system capable of resolving mercaptoacetate and N-acetylcysteine as their N-(1-pyrene)maleimide (PM) and N-(7-dimethylamino-4-methyl-3-coumarinyl)maleimide (DACM) derivatives. Good resolution was obtained by ion pairing with tetramethylammonium hydroxide and chromatography on reversed phase. The detection limits for the thiols were about 50 fmol as their DACM derivatives and about 400 fmol as their PM derivatives. The method is illustrated by chromatography of urinary thiols which indicates that the derivatization and chromatography procedures should be well applicable in bioanalytical work.

INTRODUCTION

Earlier methods for thiol determination such as the nitroprusside method, and iodometric, amperometric, spectrophotometric and colorimetric methods require several milliliters of sample and have detection limits of around 10 μmol/l [1]. Recently, Hannestad and Sörbo [2] published a gas chromatographic method for the determination of 3-mercaptolactate, mercaptoacetate and N-acetylcysteine in urine. The thiols were determined with a sensitivity of about 3 μmol/l using 5 ml of urine. When these thiols are to be determined in serum, for example, a much higher sensitivity is required. An attempt to estimate N-acetylcysteine in serum after oral ingestion of 400 mg of substance was performed by Maddock [3]. He used a high-performance liquid chromatographic (HPLC) method but did not give any chromatographic data. The method had a sensitivity of 1 μmol/l, which, however, was not sufficient to

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quantitate N-acetylcysteine in all sera. Recently, high sensitivity for the determination of biologically interesting thiols has been obtained by HPLC of the fluorescent sulfhydryl derivatives with N-(9-acridinyl)maleimide [4], with monobromobimane [5] and in HPLC methods with electrochemical detection [6].

The maleimide derivatives (for review see ref. 7) are by themselves not fluorescent, but they react with thiol-containing substances giving highly fluorescent compounds. These reagents have mainly been used as fluorescent probes in the study of thiol-containing proteins and enzymes [7]. However, they should also be applicable for the labeling of low-molecular-weight sulfhydryl compounds in chromatographic separation as recently shown [4] for HPLC of N-(9-acridinyl)maleimide derivatives with glutathione, homocysteine, coenzyme A and cysteine. Two maleimides, N-(1-pyrene)maleimide (PM) [8,9] and N-(7-dimethylamino-4-methyl-3-coumarinyl)maleimide (DACM) [10], are commercially available but to our knowledge their potential use in HPLC methods for low-molecular-weight thiol-containing substances has not been explored. This paper describes the ion-pair HPLC separation of mercaptoacetate and N-acetylcysteine derivatives obtained with these two maleimides.

EXPERIMENTAL

Materials

Mercaptoacetic acid was obtained from E. Merck (Darmstadt, G.F.R.) and N-acetylcysteine was from Sigma (St. Louis, MO, U.S.A.). N-(1-Pyrene)maleimide and N-(7-dimethylamino-4-methyl-3-coumarinyl)maleimide were from Fluka (Buchs, Switzerland). Tetramethylammonium hydroxide (10% in water) was from Merck. All other reagents were of reagent grade or better. The analytical column was a LiChrosorb RP-8, 5 μm (250 × 4.0 mm) from Merck.

Apparatus

The apparatus we used was a Constametric III pump from LDC (Riviera Beach, FL, U.S.A.), a Rheodyne (Berkeley, CA, U.S.A.) Model 7125 sample injector with a 100-μl sample loop, and a Perkin-Elmer (Beaconsfield, Great Britain) Model 3000 spectrofluorometer with an LC-cell accessory. With chromatography of PM derivatives the fluorometer was set at 342 nm excitation (band width 15 nm) and 396 nm emission (band width 20 nm), and with DACM derivatives the corresponding wavelengths were 400 and 480 nm.

Derivatization and chromatography of pure thiols

Stock solution of PM, 1 mmol/l, was prepared in ethanol—acetone (1:1). DACM, 0.5 mmol/l, was dissolved in acetone. The solutions were stored at 4°C and before use the reagents were diluted to a concentration of 20 μmol/l in ethanol—acetone and acetone, respectively.

Stock solutions (1 mmol/l) of mercaptoacetic acid and N-acetylcysteine were prepared in hydrochloric acid, 10 mmol/l, containing disodium EDTA, 2 mmol/l. These solutions were stored in the refrigerator at 4°C and were stable as controlled by thiol determination with the 5,5′-dithiobis-(2-nitrobenzoic acid) method [11]. Dilutions to the concentrations 100, 75, 50, 25 and 10

μmol/l were prepared freshly in the hydrochloric acid containing disodium EDTA and immediately before derivatization these thiol solutions were diluted 1:101 with carbonate buffer, 50 mmol/l, containing disodium EDTA, 10 mmol/l, pH 9.0.

The derivatization was performed in tubes with PTFE-lined screw-caps by reacting 5.05 ml of thiol solution of an appropriate concentration (less than 1 μmol/l) with 0.5 ml of PM or DACM, 20 μmol/l, which gave a final maleimide concentration of 1.8 μmol/l. After mixing, the tubes were placed in a water-bath at 37°C for approximately 20 h. The addition products initially formed will hydrolyze and the final derivatives are ready for chromatography. The derivatives obtained were stable at room temperature for at least four days. The solutions were diluted 1: 5 with the mobile phase and 100-μl samples were then loop-injected. The mobile phase was prepared from sodium phosphate buffer, 2 mmol/l, to which tetramethylammonium hydroxide (TMA) was added, and the pH adjusted to 7.4 with hydrochloric acid (6 mol/l). The amount of TMA added was calculated to give a final concentration of 10 mmol/l in the mobile phase after mixing the solution with methanol to give an appropriate methanol concentration; for details, see Results. All mobile phases were filtered through a 0.5-μm cellulose acetate filter, type EH (Millipore Corp., Bedford, MA, U.S.A.) and were thereby also deaerated.

Application for urinary thiols

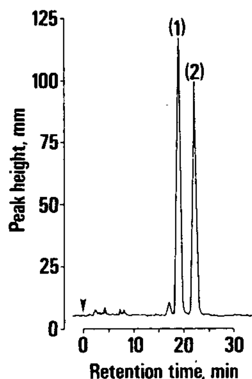
Urinary thiols were purified according to the description of Hannestad and Sörbo [2]. Thus, from freshly collected urine a 5-ml aliquot, mixed with EDTA and adjusted to pH 9.8—10.0, was reacted with thiopropyl-Sepharose, acidified with acetic acid, and after centrifugation the thiols were obtained in free form. From the supernatant, an amount of less than 2 μmol of thiols as measured according to Rootwelt [11] was adsorbed on an organomercurial adsorbent, *p*-acetoxymercurianiline—Sepharose 4-B. After washing, the thiols were eluted with cysteine. The eluate was then applied on a small cation-exchange chromatography column (AG 50 W) which retained the cysteine. The thiols of interest were obtained in the first hydrochloric acid eluate (4.0 ml) and 0.2 ml of disodium EDTA, 0.18 mol/l, was added. From this eluate, containing less than 0.5 mmol/l thiols, 250 μl were neutralized with 25 μl of sodium hydroxide, 0.1 mol/l. Then 5 ml of carbonate buffer, 50 mmol/l, containing disodium EDTA, 10 mmol/l, pH 9.0, were added, followed by 0.5 ml of maleimide, 0.5 mmol/l. This amount of maleimide was chosen in order to obtain an excess of maleimide in relation to the thiols. Hydrolysis and chromatography were performed as described above.

RESULTS

Chromatography

PM derivatives of mercaptoacetate and N-acetylcysteine each gave a single peak, as shown in Fig. 1.

The DACM derivative of mercaptoacetate gave a single peak, but N-acetylcysteine in addition to a major peak also gave a small peak with a shorter retention time. However, by choosing an appropriate methanol concentration in the mobile phase, these peaks could be baseline separated (Fig. 2).



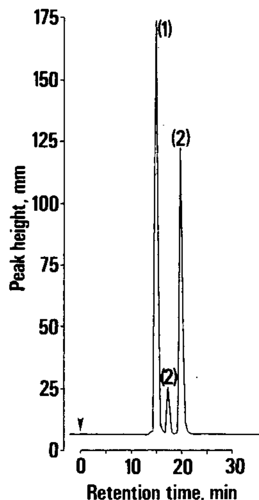


Fig. 1. Chromatographic separation of PM derivatives of 9 pmol mercaptoacetate (1) and 9 pmol N-acetylcysteine (2). Mobile phase: sodium phosphate buffer (2 mmol/l, containing TMA, pH 7.4)—methanol (70 : 30, v/v). Final TMA concentration 10 mmol/l. Flow-rate 0.75 ml/min, pressure 22 MPa, ambient temperature.

Fig. 2. Chromatographic separation of DACM derivatives of 9 pmol mercaptoacetate (1) and 9 pmol N-acetylcysteine (2). Mobile phase: sodium phosphate buffer (2 mmol/l, containing TMA, pH 7.4)—methanol (85 : 15, v/v). Final TMA concentration 10 mmol/l. Flow-rate 0.75 ml/min, pressure 18 MPa, ambient temperature.

Sensitivity and linearity

Both with mercaptoacetate and N-acetylcysteine the DACM derivatives gave higher detector responses than PM derivatives when peak heights were compared. For both types of fluorophores the sensitivity was high, and the lowest detection limit was obtained for the DACM derivatives (Table I).

Linearity of the analytical procedure, including the derivatization step and detector response, was evaluated for both derivatization reagents using injected amounts of 1.8—18 pmol. Linearity was found for the range tested (Fig. 3).

Chromatography of urinary thiols

To test the utility of the method for thiol determinations with the maleimides, a clean-up procedure [2] for urine was chosen which gives an extract containing acid and neutral thiols. Fig. 4 shows such a urinary chromatogram obtained with PM. Two major peaks corresponding to mercaptoacetate and N-acetylcysteine were found together with a number of hitherto unidentified peaks. Similar chromatograms were obtained with DACM. Reagent peaks with longer retention times were observed with the procedure for urinary thiols, causing interference with the PM derivatives. However, with the procedure for pure thiols only insignificant reagent peaks were obtained due to the lesser amount of maleimide used.

The precision of the method for urinary mercaptoacetate and N-acetylcysteine was estimated from duplicate analysis of urinary samples from sixteen

TABLE I

COMPARISON OF SENSITIVITIES OBTAINED WITH PM AND DACM DERIVATIZATION PRODUCTS

	Amount injected (pmol)	Peak height (mm)	Noise (mm)	Detection limit* (fmol)
PM derivatives				
Mercaptoacetate	1.8	49	4.3	320
N-Acetylcysteine	1.8	41	4.3	380
DACM derivatives				
Mercaptoacetate	1.8	160	1.7	38
N-acetylcysteine	1.8	115	1.7	53

*Detection limit was calculated as $\frac{2N}{S} \cdot A$, where N = noise, S = signal (peak height), and A = amount of substance injected.

healthy subjects. The range for the mercaptoacetate concentration was 3.5–9.5 μmol/l and for N-acetylcysteine it was 13.8–44.1 μmol/l. From the mean of 6.4 μmol/l and S.D. of 0.30 μmol/l (calculated from duplicate analysis) a coefficient of variation (C.V.) of 4.7% was obtained for mercaptoacetate. The mean value for N-acetylcysteine was 30.7 μmol/l (S.D. = 0.70 μmol/l) and the C.V. was 2.3%. The inter-assay variability was estimated from repeated analysis of a urinary sample during three weeks (n = 12). The results were for mercaptoacetate 5.9 ± 0.62 μmol/l (mean ± S.D.), C.V. = 10.5%, and for N-acetylcysteine the results were 35.3 ± 2.54 (mean ± S.D.), C.V. = 7.2%.

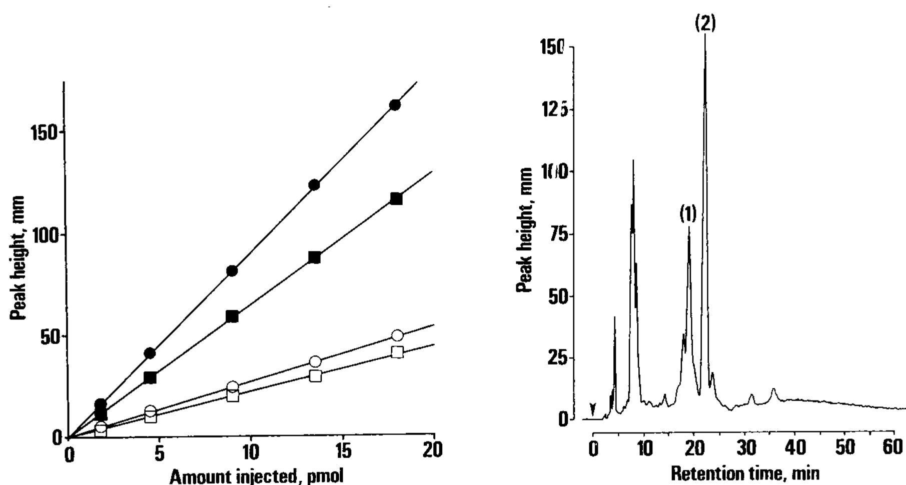


Fig. 3. Standard curves for mercaptoacetate (○, ●) and N-acetylcysteine (□, ■) derivatives with PM (open symbols) and DACM (filled symbols).

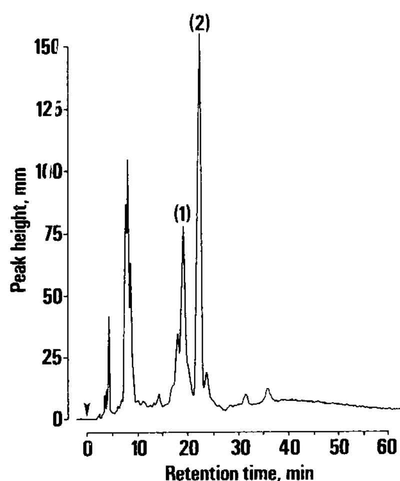


Fig. 4. Chromatography of urinary thiols. Chromatographic conditions are the same as in Fig. 1. Peaks 1 and 2 correspond to mercaptoacetate and N-acetylcysteine, respectively.

DISCUSSION

A number of maleimides which react with thiols have recently been synthesized [7]. When such a reagent is coupled to the thiol it will afterwards hydrolyze giving a carboxylic acid, and thus the products with mercaptoacetate and N-acetylcysteine will contain two carboxyl groups. We thus thought that these derivatives would be suitable for ion-pair chromatography. These expectations were fulfilled, and we have shown here that the substances could be well separated, both as the PM and the DACM derivatives. With the use of DACM two peaks appeared for N-acetylcysteine. This may be a drawback when this derivatization is used in the analysis of complex biological fluids. We considered the possibility that an impurity of the substance was responsible for the extra peak by analysing N-acetylcysteine from different sources. Identical results were obtained. Furthermore, corresponding peaks were found in urinary chromatograms with DACM. There is a possibility that the hydrolysis of maleimide adduct occurs in two different positions, giving rise to two different products. Such possibilities have been discussed both for derivatives with PM [9], DACM [12] and N-(9-acridinyl)maleimide [4].

In comparison with earlier thiol determinations [1], the sensitivity of the chromatographic—fluorometric detection of the derivatives was extremely high, with a detection limit of about 50 fmol for the DACM derivatives. These results also compare well with the detection limit of 2.5 pmol obtained for some monothiols in serum as described by Takahashi et al. [4]. They used N-(9-acridinyl)maleimide in a reversed-phase HPLC method. Recently, Newton et al. [5] published an HPLC method for analysis of biological thiols using the fluorogenic reagent monobromobimane for derivatization. They found a sensitivity of 2 pmol for cysteine which is in the same range.

It was previously shown [2] that the procedure used for clean-up of urine gave extracts containing both mercaptolactate, mercaptoacetate and N-acetylcysteine. With the present method the latter two thiols always gave well-defined peaks, but with mercaptolactate we obtained a broad peak with a retention time longer than that of N-acetylcysteine. The corresponding peak from urine may be observed in Fig. 4 with a retention time between 30 and 55 min. This chromatographic behaviour of mercaptolactate may depend on the interaction of its hydroxyl group with the silica matrix of the column.

As presented here the chromatographic method gave promising resolution and sensitivity for determination of urinary mercaptoacetate and N-acetylcysteine. The precision of the method is satisfactory for urinary mercaptoacetate and N-acetylcysteine in healthy subjects, and the values agree closely with those found in healthy subjects by gas chromatography [2]. Further studies, however, are needed in order to eliminate reagent peaks and to obtain satisfactory results for mercaptolactate.

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CHROMBIO. 1188

QUANTITATIVE THIN-LAYER CHROMATOGRAPHIC DETERMINATION OF DIHYDROERGOT ALKALOIDS

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SUMMARY

Direct, quantitative, thin-layer chromatographic methods for the determination of dihydroergot alkaloids are described, in particular the determination of dihydroergotamine with dihydroergokryptine as internal standard. The internal standard was added to plasma, which was extracted twice in dichloromethane; the organic phase was removed under nitrogen, the residue resolved in ethanol and applied on a silica gel G-60 plate. Dihydroergotamine and the internal standard can be measured directly by fluorescence, with excitation at 264 nm and with use of a Zeiss remission filter FL 39. The percentage recovery from this method is 49.17 ± 6.71% (plasma). These methods enable the determination of 10 pmol dihydroergotamine per ml of plasma (ca. 6.8 ng/ml) with a coefficient of variation of 10.3%. They have proved useful in biochemical and pharmaceutical applications.

INTRODUCTION

The majority of publications which have previously appeared concerning the determination of dihydroergot alkaloids [1—6] describe methods for quality control or special methodological aspects. Yet these methods are unsuitable for the quantitative determination of very small amounts in the nanogram range under controlled conditions. For example, in plasma or other biological fluids, these methods would be inappropriate for enzymatic [7] and biotransformation studies with these substances.

We have developed a sensitive method for the quantitative determination of dihydroergotamine with dihydroergokryptine as the internal standard and vice versa, based on the extraction of these pH basic drugs from alkaline biological solution with chloroform or dichloromethane, thin-layer chromatographic separation, and in situ fluorescence spectrometric determination on thin-layer plates. We believe that the application of unspecific spraying reagents such as

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o-phthalaldehyde [6] is not to be recommended with biological extracts because endogenous substances may possibly react with these reagents. On the other hand, the fluorometric excitation wavelengths of dihydroergot alkaloids are very specific. The combination of dihydroergotamine and dihydroergokryptine as test substances and internal standards is also of pharmacological relevance because in therapy both drugs are not administered simultaneously. Our investigations on the quantitative determination of dihydroergot alkaloids by gas—liquid and high-performance liquid chromatography have shown that the thin-layer chromatography (TLC) described here is the most suitable, sensitive and exact method.

EXPERIMENTAL

Chemicals

The dihydroergot alkaloids [mesylates, dihydroergotamine (DET), dihydroergocristine (DEC), dihydroergokryptine (DEK)] were obtained as gifts from Dr. Rentschler Arzneimittel GmbH & Co., Laupheim, G.F.R. These were used as standard solutions in ethanol ($2.5 \cdot 10^{-3}$, $2.5 \cdot 10^{-4}$ and $2.5 \cdot 10^{-5}$ mol/l). Other reagents and solvents were Merck (Darmstadt, G.F.R.) p.a. products. The TLC solvents were dried and distilled before use.

Thin-layer chromatography

TLC was carried out on commercially available Merck silica gel G-60 plates (20 × 20 cm) or Merck HPTLC-Kieselgel 60 plates (10 × 10 cm) using ethanol—benzene—chloroform (1:2:4), with the addition of 1 ml of aqueous conc. NH_3 per 200 ml as a mobile phase. The plates were activated for 30 min at 100°C and then kept for 15 min before use in an incubator, which was saturated with water vapour at 60°C.

Fluorescence measurements

Fluorescence excitation spectra on the TLC plates were measured using a Zeiss fluorescence densitometer KM 3 with deuterium or mercury lamp, an emission filter FL 39 and a Siemens compensation recorder.

Extraction procedure

Ethanolic standard solutions were added to a heparinized blood sample (5 ml), mixed, and immediately centrifuged (5000 *g*). Then 2 ml of the supernatant (plasma) were combined with 1 ml of 0.1 *N* sodium hydroxide and 3 ml of dichloromethane, mixed, and centrifuged (8000 *g*, 0°C); 2 ml of the organic phase were separated, and the aqueous medium was eluted a second time with 3 ml of dichloromethane (0°C). In total 4 ml of the organic phase were evaporated under nitrogen in darkness. The residue was resolved in ethanol and applied to a TLC plate by means of a Desaga Microdoser 80 or a Camag TLC-Nanoapplicator.

RESULTS AND DISCUSSION

For the direct in situ determination after TLC separation, it is necessary to

use optimal fluorescence excitation and emission conditions on the TLC plates. It may be seen that the region of the emission intensity for dihydroergot alkaloids ranges from 320 to 390 nm (see also ref. 1). We used the Zeiss FL 39 filter, which excludes wavelengths above 390 nm, for the thin-layer reflection measurements. Fluorescence excitation spectra were taken using a deuterium

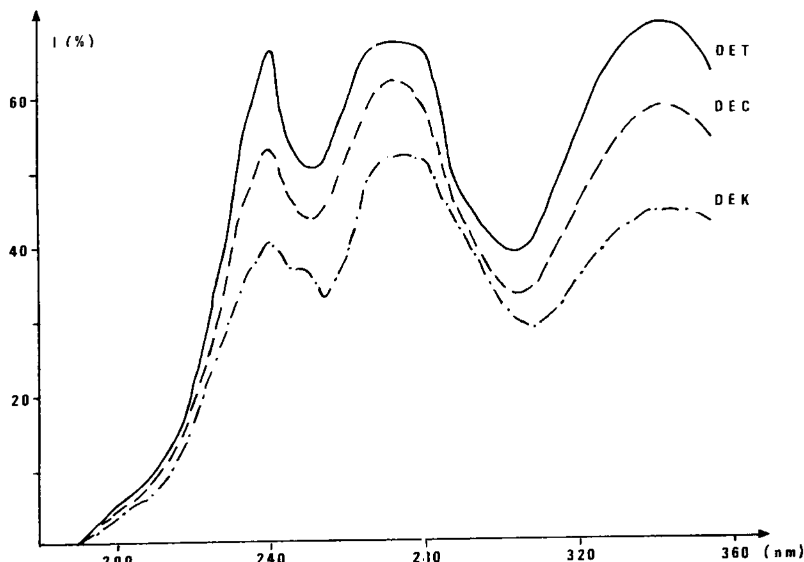

Fig. 1. Fluorescence excitation spectra of dihydroergotamine (DET), dihydroergocristine (DEC) and dihydroergokryptine (DEK) on TLC silica gel G-60 plates with deuterium lamp continuous excitation and FL 39 Zeiss remission filter. *I* = relative fluorescence intensity (%).

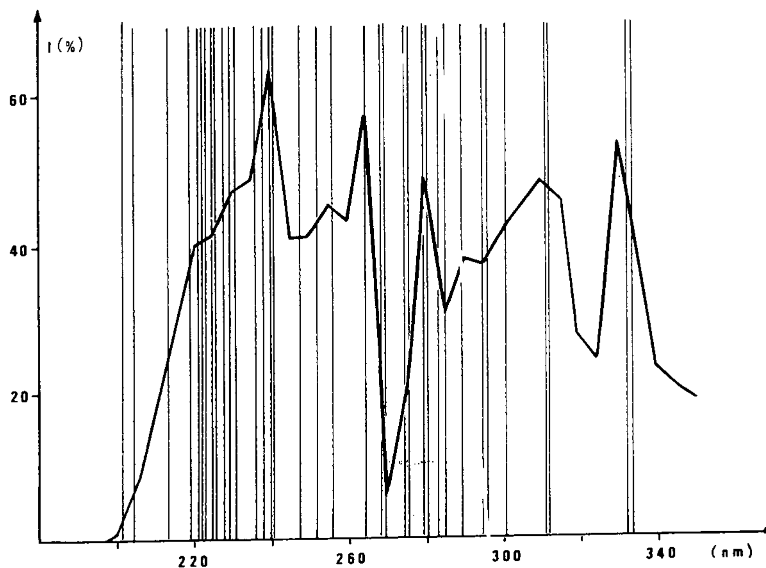

Fig. 2. Fluorescence excitation spectra of dihydroergotamine (DET) on TLC silica gel G-60 plates with mercury damp distinct line excitation and Fl 39 Zeiss remission filter. *I* = relative fluorescence intensity (%).

lamp (continuous spectrum) and a mercury lamp (distinct excitation lines). The two types of spectra for the dihydroergot alkaloids (DET, DEK, and DEC) on TLC silica gel G-60 plates are shown in Figs. 1 and 2, respectively. In conclusion, the optimal conditions for the fluorescence determination of the dihydroergot alkaloids on silica gel G-60 plates were found to be excitation through a mercury damp lamp of 264 nm and remission measurement with a Zeiss FL 39 filter.

The mobile phase for the TLC (ethanol—benzene—chloroform, 1:2:4, with 1 ml of conc. NH_3 per 200 ml of solvent mixture) is similar to that used by Reichelt [3]. The R_F values are 0.41 for DET, 0.51 for DEK, and 0.54 for DEC. Plasma extracts, eluted according to the procedure described in the Experimental section, showed no spots of endogenous origin in the vicinity of these R_F values (Fig. 3). An excitation wavelength of 264 nm is highly specific for dihydroergot alkaloids. At this wavelength, endogenous substances from plasma extractions show no emission in the R_F ranges of DET and DEK. Therefore, DET and DEK can be determined quantitatively under these

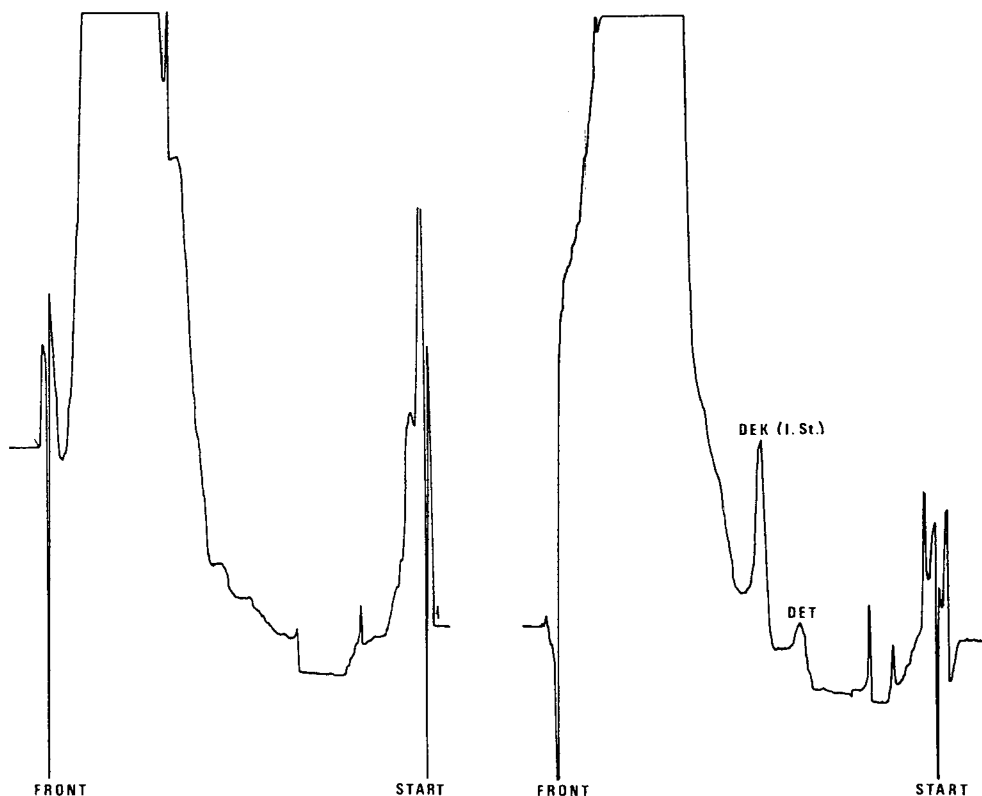

Fig. 3. TLC separation of a plasma extract (see text) without dihydroergot alkaloids. TLC plate, silica gel G-60 (Merck); mobile phase, ethanol—benzene—chloroform (1:2:4) with 1 ml of conc. NH_3 per 200 ml.

Fig. 4. TLC separation of a plasma extract with dihydroergotamine (DET) on silica gel G-60 plate (Merck). Internal standard: dihydroergokryptine (DEK). Mobile phase: ethanol—benzene—chloroform (1:2:4) with 1 ml of conc. NH_3 per 200 ml.

conditions (Fig. 4), and DET may be quantitated with DEK as the internal standard. Fig. 5 shows a plasma level determination of DET in a kinetic study with Wistar rats 2 and 3 h after oral application of 5 mg of DET (4 ml of plasma for extraction with 100 pmol of DEK as internal standard). The results here are 22 and 13 pmol/ml (13 and 8 ng/ml) DET.

The sensitivity and standardisation of the quantitative determination of DET with DEK as the internal standard is shown in Table I for standard solutions in ethanol of 250—1000 pmol DET contrasted with 500 pmol DEK as internal standard, and 50—200 pmol DET contrasted with 100 pmol DEK. Fig. 6 shows a standard curve for plasma extracts where analogous amounts of the dihydroergot alkaloids were used. Serum extractions often show unsatisfactory separation of the organic and aqueous layers. We therefore consider plasma extraction a superior method. The sensitivity of the determination extends to

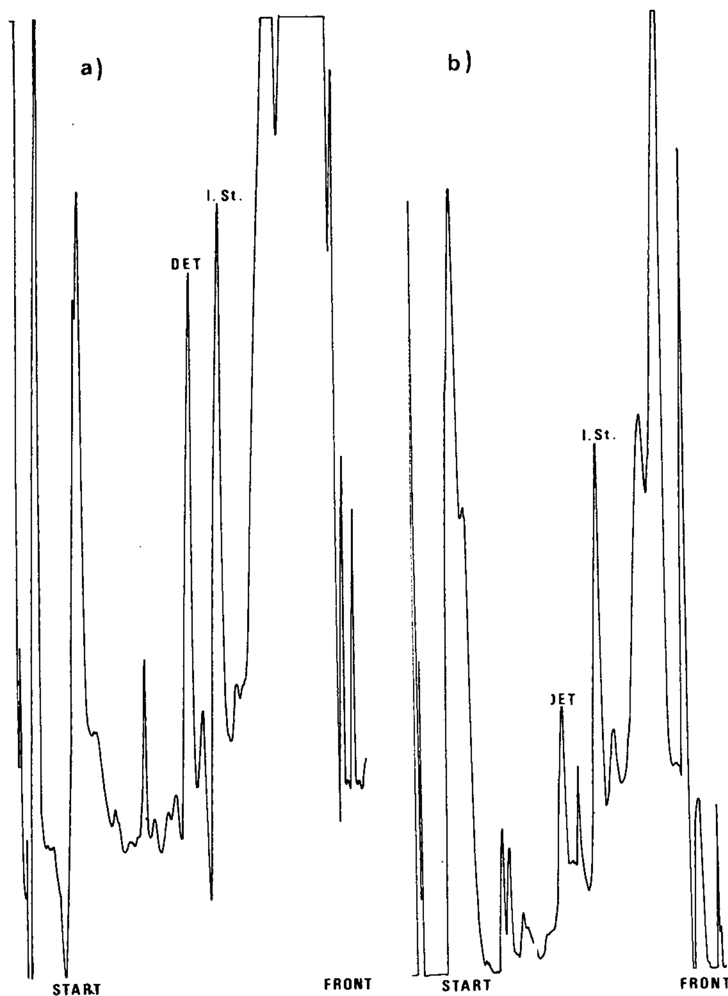

Fig. 5. Plasma level determination of dihydroergotamine (DET) in rat plasma. (a) 2 h, (b) 3 h after oral application of 5 mg of DET against 100 pmol of dihydroergokryptine as internal standard (added to the plasma before extraction).

TABLE I

STANDARD DEVIATIONS TO THE DIRECT TLC CALIBRATION AND THE STANDARD CURVES

m = (pmol dihydroergotamine)/(pmol dihydroergokryptine) applied to the TLC plates from 2 ml of plasma. Q_S = peak area quotient of the directly applied dihydroergot alkaloids. Q_E = peak area quotient of the dihydroergot alkaloids extracted from 2 ml of plasma.

m (pmol/pmol)	Q_S (DET/DEK)	s	C.V. (%)	n	Q_E (DET/DEK)	s	C.V. (%)	n
$\frac{25}{100} = 0.25$	0.12	0.01	8.3	6	0.29	0.03	10.3	6
$\frac{50}{100} = 0.5$	0.47	0.06	12.8	18	0.73	0.08	10.9	11
$\frac{100}{100} = 1.0$	1.00	0.11	11.0	21	1.20	0.13	10.8	11
$\frac{200}{100} = 2.0$	1.90	0.10	5.3	8	2.84	0.10	3.5	5
$\frac{100}{500} = 0.2$	0.19	0.01	5.3	6	0.19	0.02	10.5	6
$\frac{200}{500} = 0.4$	0.40	0.03	7.5	8	0.45	0.04	8.9	6
$\frac{500}{500} = 1.0$	0.98	0.03	3.1	8	1.00	0.06	6.0	6
$\frac{1000}{500} = 2.0$	1.93	0.14	7.2	10				

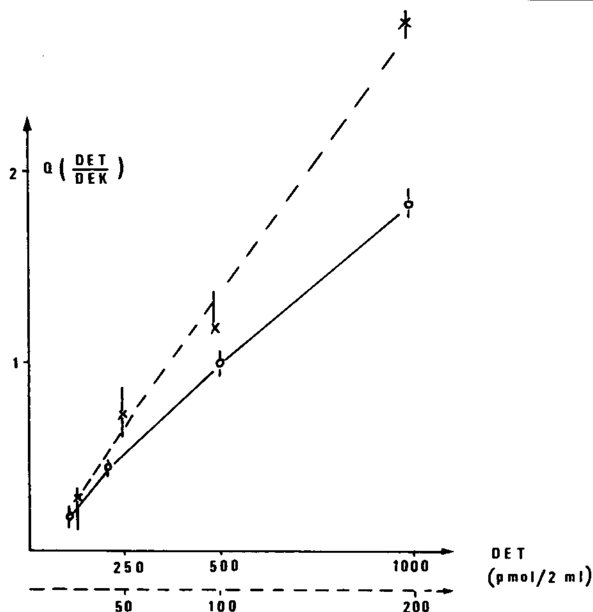

Fig. 6. Standard curves for the quantitative determination of dihydroergotamine (DET) in plasma extracts with dihydroergokryptine (DEK) as internal standard. Q = peak area quotient a_{DET}/a_{DEK}. Standard curves: o——o, with 500 pmol internal standard in 2 ml of plasma; x — — — x, with 100 pmol internal standard in 2 ml of plasma. For standard deviations see Table I.

10 pmol of DET per ml of plasma or test solution. The recovery of DET for extraction between 25 and 500 pmol per ml of plasma is 49.17 ± 6.71% (n = 15).

The standard deviations (Table I) can be minimized if the molar proportions of DET and the internal standard (DEK) in the plasma sample remain between 0.2 and 2.0. Therefore, within the range of 25—1000 pmol of DET, two standard curves are needed — one from 25 to 200 and another from 100 to 1000 pmol. The direct quantitative analysis carried out with pure substances in ethanol solution shows definite regression lines with correlation coefficients r = 0.9972 (range 1000 pmol) and r = 0.9939 (range 200 pmol). In the extraction procedure a slight difference in the partition coefficients of DET and DEK created the convex slopes in the standard curves (Fig. 6).

The quantitative determination of DEK with DET as the internal standard can be done in a manner analogous to the study given here. As a further example, these methods have been used in the determination of the specific radioactivity of di-[9,10-^{3}H]hydroergot alkaloids as synthesized from native ergot alkaloids catalytically hydrogenated with tritium [8].

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THIN-LAYER CHROMATOGRAPHIC DETERMINATION OF FUROSEMIDE AND 4-CHLORO-5-SULFAMOYL ANTHRANILIC ACID IN PLASMA AND URINE

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SUMMARY

A method is described for the assay of furosemide based on thin-layer chromatography and measurement of fluorescence directly on the plates. Conditions are specified for stabilizing fluorescence over the time of measurement. As little as 10 ng can be accurately measured and fluorescence is linear up to 160 ng. The metabolite or decomposition product 4-chloro-5-sulfamoyl anthranilic acid is well separated and measured quantitatively in the procedure. Application of the method to human plasma and urine is demonstrated.

INTRODUCTION

Furosemide is a widely used diuretic which is effective by oral or parenteral administration. It may now be marketed by any registered pharmaceutical manufacturer who obtains approval of the Food and Drug Administration. One of the requirements for approval is proof of bioavailability of the finished product, which requires measurement of plasma or urine concentrations in normal human subjects following a single low dose of the drug. Thus, there is a need for measurement of low levels of furosemide in plasma and urine. In addition, since it is primarily removed from the body by renal excretion [1], there is a need to assess plasma levels in patients where renal function is impaired. It is important that an assay of furosemide involves separation of 4-chloro-5-sulfamoyl anthranilic acid (CSA), since this has been identified in samples from patients given furosemide, and has been thought to be both a product of metabolism and of in vitro hydrolysis [2].

Furosemide has been measured in biological fluids by spectrofluorometry [3—5], gas chromatography [6], liquid chromatography [7—12] and thin-layer chromatography (TLC) [13—16]. Several of these methods require

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lengthy extraction procedures or large sample volumes because of the low concentrations found in plasma [3—6, 8, 10]. Other methods involve derivatization of the drug in the extract prior to measurement [6], thus extending the complexity of the assay. Some of the procedures do not separate the drug from the metabolite or decomposition product and may yield misleading results [3—5, 9]. One of the TLC methods [13] uses radioactive furosemide and the bands, corresponding to furosemide and CSA, are scraped from the plates for counting. Similar scraping of the plates is used for a spectrofluorometric method on thin layers [14]. A direct densitometric assay measures the color of the spots on the plates after reacting the drug with Ehrlich reagent [15]. The most recent TLC method [16] measures fluorescence of the drug and CSA with a spectrodensitometer directly on the plates. Applied to plasma containing furosemide and CSA, it deproteinates small volumes with methanol and brings them onto the chromatoplates. The failure to separate the drug and the metabolite from plasma proteins constitutes a troublesome weakness.

The present paper describes a method for extracting furosemide quantitatively, separating the furosemide and CSA by TLC, stabilizing the fluorescence on the plates and measuring the fluorescence directly by densitometry. The method was evaluated with spiked plasma and urine samples and by application to samples from volunteers and patients given furosemide.

EXPERIMENTAL

Materials and reagents

The TLC plates used were 20 × 20 cm Silica Gel 60 (E. Merck, Darmstadt, G.F.R.). Hamilton microliter syringes were used for spotting.

All solvents were A.C.S. spectranalyzed grade and the diethyl ether was distilled in glass prior to use. Phosphate buffer, 0.2 *M* (pH 2.2) was prepared from 0.2 *M* phosphoric acid and potassium dihydrogen phosphate.

The mobile phase consisted of chloroform—methanol—glacial acetic acid (89:6:5). The moisturizing solution for the plates contained 45 ml propyleneglycol, 130 ml water and 25 ml glacial acetic acid.

Samples of furosemide powder and 40-mg tablets were supplied by Hoechst-Roussel Pharmaceuticals (Somerville, NJ, U.S.A.); CSA reference standard was purchased from U.S. Pharmacopeial Convention (Rockville, MD, U.S.A.).

Stock solutions of furosemide and CSA were prepared to contain 100 μg/ml in methanol. These were diluted with water to spike plasma and urine and diluted with methanol to prepare standard solutions for the plates. Aqueous solutions were prepared fresh daily and methanol solutions were stored in the refrigerator.

Apparatus

TLC plates were divided into twenty 1-cm channels with a scoring device (SDA 320, Schoeffel Instrument). Samples of extracts and standards were applied simultaneously to the channels by means of an automatic spotter (Multi-Spotter, Analytical Instrument Specialities).

A scanning spectrodensitometer with fluorescent attachment was used for measurement of fluorescence on the plates (Model SD 300-1 UV-VIS Spectro-

densitometer, Schoeffel Instrument). This was attached to an integrator (M-2 Calculating Integrator, Perkin-Elmer) and a recorder (Honeywell OPTS 102 110). The system was used in the fluorescence mode with irradiation at 275 nm and an ultraviolet exclusion filter in the emission detector.

Procedure

To 0.1—0.5 ml of plasma or 0.05—0.25 ml of urine in a 15-ml screw-cap centrifuge tube with PTFE cap liner, are added 0.2 ml of phosphate buffer and 2 ml of diethyl ether, and the tube is rotated in a suitable mechanical rotator for 10 min. The tube is removed and centrifuged for 5 min to separate the phases. The ether layer is transfered to a conical 15-ml glass tube, the aqueous layer is extracted a second time with 2 ml of diethyl ether and the combined extracts are evaporated to dryness at 35°C under a stream of nitrogen. The residue is dissolved in 100 μl methanol and one or more 20-μl portions are spotted on the TLC plate. Along with each set of samples, at least two methanol standards are applied to the same plate. The plates are protected from light and developed in a glass tank with a solvent-saturated atmosphere. In this sytem the R_F value is 0.29—0.30 for furosemide and 0.13—0.15 for CSA. The solvent front is allowed to move about 15 cm, the plate is removed and the solvent allowed to evaporate. The plate is then dipped in moisturizing solution, contained in a suitable dip tray, placed in an oven at 35°C for 10 min, removed and allowed to stand at room temperature for 30 min. The plate is then scanned to record the fluorescence and integrate peak areas, making sure that fluorescence has stabilized before proceeding by reading the first channel at 2-min intervals until peaks are constant.

Amounts of furosemide and CSA are calculated by comparison of peak areas of samples to those of known standards on the same plate.

Linearity of response, recovery and application

To determine the range of linearity of response, standard solutions of furosemide and CSA were spotted on the plates, developed and measured.

Recovery was determined by assaying samples of plasma and urine spiked with varying concentrations of furosemide and CSA, then comparing the areas obtained with standard solutions spotted directly on the plate.

This method was applied to samples of plasma and urine of patients and volunteers and was found to be sensitive enough for measurement following a single 40-mg dose of furosemide.

RESULTS AND DISCUSSION

The solvent mixture used for the development of the TLC plates yielded clear separation of furosemide and CSA, as is demonstrated in Fig. 1. Standard curves of furosemide were linear in the range of 0—160 ng, but non-linear above 160 ng. For CSA the fluorescence—concentration curve was somewhat lower than that of the parent compound and was linear up to 200 ng per spot. These curves are shown in Fig. 2.

The presence of acid in the development fluid greatly enhanced the fluorescence of furosemide on the plates. This is demonstrated in Fig. 3, where

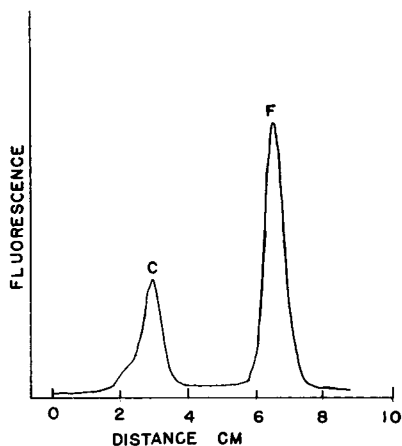

Fig. 1. Separation of furosemide (F) and CSA (C) isolated from spiked human plasma.

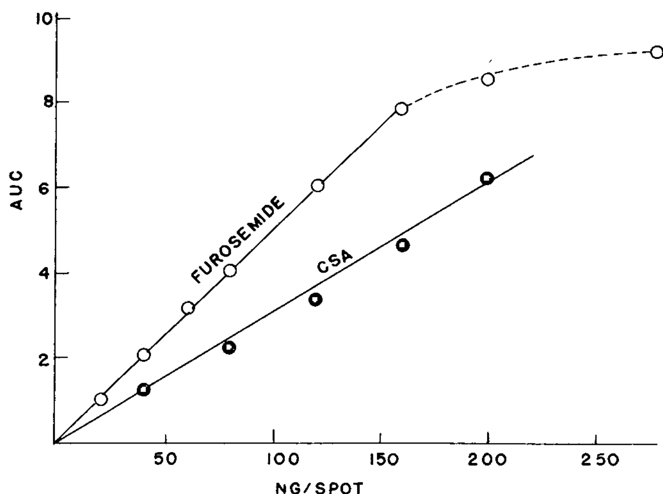

Fig. 2. Relationship of area under the curve (AUC) to concentration of furosemide and CSA.

peak areas of standards are shown following development in an acidic fluid (chloroform—methanol—glacial acetic acid, 89:6:5) and an alkaline fluid (*sec.*-propanol—butyl acetate—water—ammonium hydroxide, 50:30:15:5) [13].

Early in the development of the assay it was recognized that fluorescence of furosemide on the plates decreased in intensity with time, due to loss of moisture and that the decrease was more pronounced with alkaline fluids than with acidic ones. To minimize the fading of fluorescence, a moisturizing solution was selected which maintained moisture and acidity on the plates and yielded stable readings over the time required to scan all the channels on a given plate. This is illustrated in Fig. 4.

Recoveries of furosemide and CSA are shown in Table I. For furosemide it is 100.4 ± 3.2% from plasma and 101.6 ± 4.7% from urine; for CSA it averaged 97.3 ± 5.2% from plasma and 102.0 ± 1.0% from urine. The procedure as written is accurate for samples containing 0.5 μg/ml of furosemide, but much

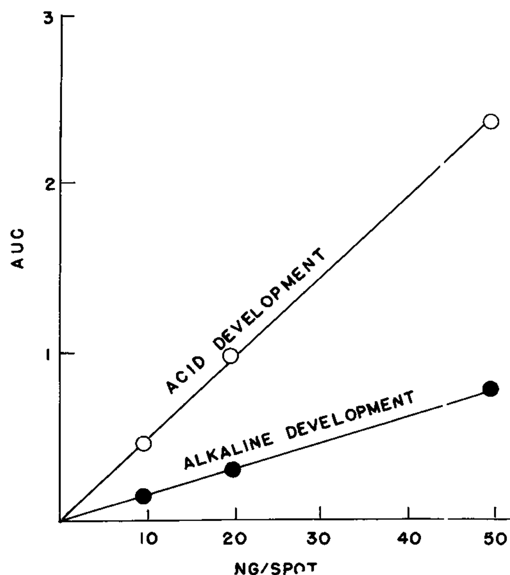

Fig. 3. Relationship of area under the curve (AUC) to concentration of furosemide in acidic (○) and alkaline (●) solvents.

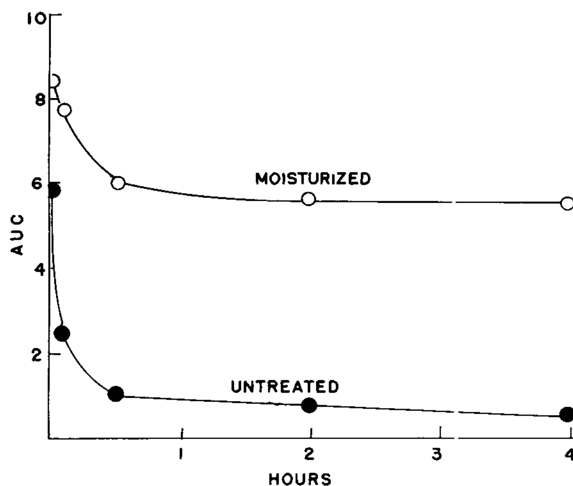

Fig. 4. Relationship of area under the curve (AUC) to concentration of furosemide on moisturized (○) and untreated (●) plates.

lower concentrations can be measured by dissolving the extraction residues in smaller volumes of solvent, since as little as 10 ng on a plate gives sufficient fluorescence. For example, by using 0.5 ml plasma, dissolving the extracted residue in a small volume of methanol and transferring it quantitatively to the plate, a concentration of 20 ng/ml in the sample could be accurately measured.

We have applied our method to plasma samples where the patient was given an intravenous (i.v.) dose of furosemide and plasma samples were taken at intervals up to 3 h. The results are illustrated in Fig. 5. It is seen that the

TABLE I

RECOVERIES OF FUROSEMIDE AND CSA FROM SPIKED HUMAN PLASMA AND URINE

Plasma			Urine		
Added (μg/ml)	Recovered (μg/ml)	Percent recovery ± C.V.	Added (μg/ml)	Recovered (μg/ml)	Percent recovery ± C.V.
*Furosemide**					
0.5	0.48	96.0 ± 2.1	1.0	1.11	111.0 ± 3.4
1.0	1.04	104.0 ± 8.2	2.5	2.48	99.0 ± 2.1
2.5	2.42	97.0 ± 4.0	5.0	4.89	98.0 ± 1.0
5.0	5.21	104.0 ± 0.6	10.0	9.99	100.0 ± 2.1
10.0	10.12	101.0 ± 7.1	20.0	20.02	100.0 ± 0.5
*CSA***					
1.0	0.92	92.0 ± 0.2	2.0	2.08	104.0 ± 3.9
2.0	1.81	92.0 ± 1.3	4.0	4.09	102.0 ± 2.4
4.0	4.07	102.0 ± 2.4	8.0	8.14	102.0 ± 1.0
8.0	8.24	103.0 ± 1.8	16.0	15.95	100.0 ± 0.8

*Each value represents four samples.
**Each value represents three samples.

plasma levels drop quite rapidly to a low level at first and then drop much more slowly subsequently. No CSA was detected in these or other patient plasma samples.

Results on urine samples are shown in Fig. 6 where data were plotted as cumulative amounts excreted over nearly 13 h. In these and urine samples from other patients small amounts of CSA were consistently found.

It was noted that no CSA has been detected in any plasma sample but it has

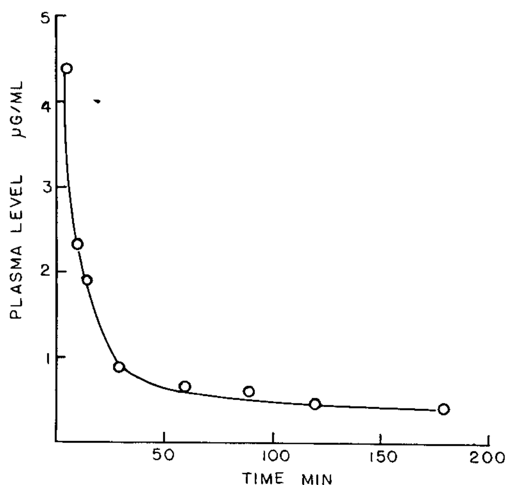

Fig. 5. Plasma levels (μg/ml) vs. time (min) of furosemide in a patient on i.v. dose of furosemide.

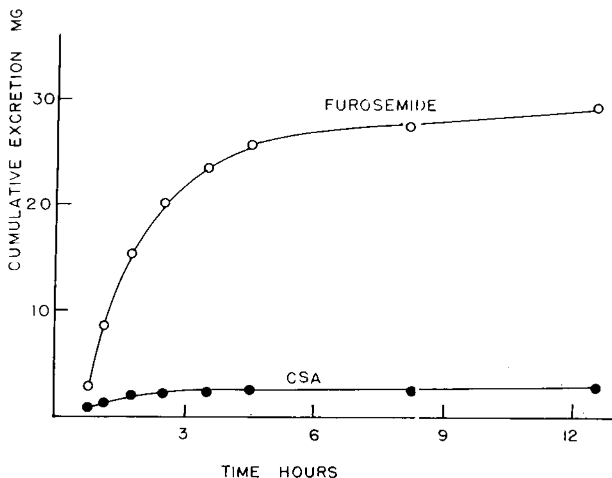

Fig. 6. Cumulative excretion of furosemide (○) and CSA (●) over time in a subject after a 40-mg oral dose of furosemide.

been found in all urine samples. To be certain that CSA was not being formed by chemical reaction in the assay process, urine samples spiked with furosemide were assayed, but no CSA was found. These facts suggest that CSA is a metabolite of furosemide which is filtered from the plasma as rapidly as it is formed and thus is found in urine only.

This method has advantages over previous ones in that it provides for separation and sensitive measurement of both the drug and the metabolite in concentrations that are present in biological fluids for several hours after a small dose. Thus it will afford improved measurement following single-dose administration of the drug as is required in bioavailability studies. Since each TLC plate serves for as many as fifteen samples (and two or three standards) this method is convenient for the assay of large numbers of samples.

This method has advantages over a similar TLC method [16] where preparation of samples for application to TLC plates involves simply addition of methanol to the plasma, centrifugation and filtration of the supernatant. The developing solvent was chloroform—ethyl acetate—formic acid, and the plates were air-dried for at least 3 h, then sprayed with a mixture of ehtyleneglycol and citric acid solution. Tests of this method in our laboratories revealed several problems and we were unable to reproduce the results. It was quickly recognized that substantial amounts of the drug were lost in the methanol protein precipitation procedure and it was suggested that this may be due to plasma protein binding. Further tests with diluted plasma did not show a clear relationship between plasma protein concentration and percentage of drug loss. These tests showed that the recovery of furosemide fluctuated within a wide range of 22.7—79.1% with a mean of 55.6% and standard deviation of ± 12.3, based on 79 samples. Also, the fluorescence was consistently lower with this developing solvent than with the system used in our procedure. Thus, there was still need for an assay which would yield more reproducible results.

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CHROMBIO. 1210

Note

Assay and stability of α-methyldopa in man using high-performance liquid chromatography with electrochemical detection

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Due to the important intersubject [1] and intrasubject [2] variation of the bioavailability of α-methyldopa, plasma monitoring of this antihypertensive drug is considered necessary. Previous measurements of α-methyldopa in biological fluids have been carried out by fluorimetric [1, 3] and gas—liquid chromatographic procedures [4]. More recently, two methods by high-performance liquid chromatography (HPLC) with electrochemical detection were proposed [5, 6]. All methods, with the exception of that of Cooper et al. [6], employ a sample clean-up step involving adsorption on alumina, a difficult and time-consuming procedure. In the latter method, however, the acid supernatant from the deproteinization with perchloric acid is chromatographed directly. Our preliminary experiments using this method (unpublished observation) did not give satisfactory results, possibly due to the instability of the drug and its metabolites: inconsistent results were obtained on the concentration profiles of this drug in plasma from 3—8 h following a single 250-mg dose administered to volunteers, when an apparent increase of its concentration was observed.

The addition of the antioxidant, sodium metabisulfite, to the evacuated blood collection tubes has been described by Kim and Koda [3] in connection with their fluorimetric procedure. However, most workers use heparinized tubes without further additives.

Perchloric acid has been used by Kwan et al. [1] for the release of α-methyldopa from its conjugated metabolites, but the rate of hydrolysis at room temperature (during the deproteinization step) has never been reported. The present study was undertaken to optimize conditions for the rapid analysis of α-methyldopa in biological specimens.

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EXPERIMENTAL

Reagents and standards

Perchloric acid, sodium bisulfite, disodium EDTA, citric acid and disodium hydrogen phosphate were reagent grade. Water was deionized and glass double distilled. The mobile phase was passed through a 0.2-μm filter (Millipore, Bedford, MA, U.S.A., FGLP 025 00) before introduction to the system. α-Methyldopa (Aldrich, Milwaukee, WI, U.S.A.) and sodium octanesulfonate (Eastman Chemicals, Rochester, NY, U.S.A.) were used without further purification. The stock solution of α-methyldopa (1 mg/ml) in perchloric acid (0.05 *M*) containing sodium bisulfite (0.1%) and disodium EDTA (0.005–0.05%) was freshly prepared every week. The working standards were prepared fresh each day by dilution of the stock solution with blank plasma (outdated plasma, Canadian Red Cross, Ottawa, Canada) or fresh whole blood from human volunteers. The protein precipitant solution was perchloric acid (1 *M*) containing sodium bisulfite (0.2 m*M*).

For blood collection commercially available evacuated containers with EDTA as anticoagulant (Vacutainers, 10 ml, Lavender stopper, B-D, Canlab, Ottawa, Canada) were used, to which 0.1 ml of 1 *M* sodium metabisulfite solution was added. The addition was done with a 26-gauge needle to prevent the loss of the vacuum.

Equipment

The chromatographic system consisted of a pump (Waters Assoc., Milford, MA, U.S.A., Model 6000A), an injection valve (Rheodyne, Berkeley, CA, U.S.A., Model 7125) with a 10-μl loop and a reversed-phase column (Waters Assoc., μBondapak alkyl phenyl column, mean particle size 10-μm, 30 × 0.39 cm). The detection system consisted of a thin-layer flow-through electrochemical cell with glassy carbon as the working electrode, and a silver—silver chloride reference electrode (Bioanalytical Systems, W. Lafayette, IN, U.S.A., Models TL5 and LC2A) and a recorder (Perkin-Elmer, Norwalk, CT, U.S.A., Model 56). The glassy carbon electrode was repolished once a week, or as required, using the polishing kit supplied with the detector.

Chromatographic conditions

The mobile phase consisted of a 2:1 mixture of 0.02 *M* citric acid and 0.02 *M* Na_2HPO_4 containing sodium octanesulfonate (0.1%) and sodium EDTA (0.055 *M*) adjusted to pH 3.05 with phosphoric acid. After warming to slightly above room temperature, the mobile phase was sparged with helium and pumped at a flow-rate of 2 ml/min. The effluent was passed through the detector cell and monitored at a potential of + 0.75 V versus the silver—silver chloride reference electrode.

Procedure

The standard curve was prepared by adding α-methyldopa, 0, 0.05, 0.1, 0.5 and 1.0 μg/ml, to commercial blank plasma or whole blood samples; four aliquots at each concentration were assayed as described below, and the recoveries were estimated by comparing the peak heights obtained to those observed

after direct chromatography of the stock solution. A calibration curve with working standards in the expected concentration range was prepared daily.

For analysis of plasma or whole blood samples a 100-μl aliquot was mixed with 200 μl of the perchloric acid precipitant, the sample was immediately agitated on a Vortex mixer for 30 sec and cooled in an ice bath for 5–10 min. After centrifugation at 2000 g at 4°C for 20 min, aliquots (10 μl) of the supernatant were chromatographed. The samples were kept on ice until the injection which was done within 30 min after the addition of perchloric acid.

Method validation

One healthy male volunteer (86 kg, 50 years old) was given an oral dose of 250 mg α-methyldopa with 100 ml water after an overnight fast. Two 10-ml blood samples were collected from the antecubital vein at 0, 0.5, 1, 2, 3, 5 and 8 h after the dose using the evacuated containers described above. One of each of the tubes was centrifuged immediately (600 g, 5 min) and, after noting the hematocrit, the plasma was removed. Both the plasma and the whole blood samples were divided into several aliquots, a third of which were analyzed immediately, another third after one week, and the rest after four weeks of storage at −18°C. The acid supernatants of the 3- and 5-h plasma samples were rechromatographed after incubation for various times at room temperature and 4°C.

Quantitation

All measurements were done by peak heights. Recovery was estimated by comparing the peak heights of the standard curve with that of the stock α-methyldopa solution. The slope and intercept of the standard curve were obtained by linear regression of peak height on concentration ($Y = AX + B$).

RESULTS AND DISCUSSION

Chromatography

The chromatographic conditions presented here permit the resolution of α-methyldopa from the endogenous catecholamine neurotransmitters (Fig. 1A). The plasma of a human volunteer at 0 h, as well as outdated plasma obtained commercially were found to give essentially clean blanks (Fig. 1B). Fig. 1C shows a chromatogram of plasma extract of the volunteer 5 h after a 250-mg oral dose of α-methyldopa (estimated concentration: 437 ng/ml), and Fig. 1E of blank plasma to which 100 ng α-methyldopa were added.

After several weeks of repeated use of the same column there was a marked deterioration in the resolution between an endogenous substance and the drug; however, by increasing the octanesulfonate concentration in the mobile phase from 0.10 to 0.11% the original resolution was re-established (Fig. 1D and E).

Standard curve

Using the method described above standard curves were constructed for both plasma and whole blood at the concentrations of 0, 0.05, 0.1, 0.5 and 1 μg/ml. The linearity for the standard curve for plasma, with four determinations at each concentration, was excellent ($r^2 > 0.99$, $n = 16$) and a least-squares linear

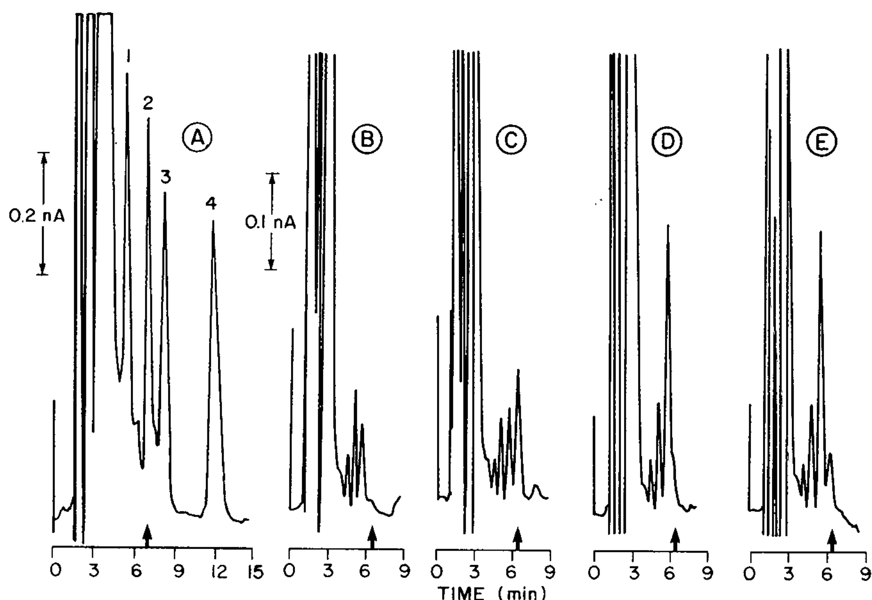

Fig. 1. Chromatography of α-methyldopa and catecholamines. (A) Chromatograms of aqueous standards. Peaks: 1, norepinephrine; 2, α-methyldopa (arrow); 3, epinephrine; 4, dopamine. (B) Plasma blank (volunteer, 0 h). (C) Volunteer 5-h plasma sample following ingestion of 250 mg α-methyldopa (estimated concentration 0.437 μg/ml). (D) Blank plasma spiked with 0.1 μg/ml drug (old column). (E) Same as (D) but with 0.11% sodium octanesulfonate in the mobile phase. Conditions: alkyl phenyl column; mobile phase, 0.02 *M* citrate phosphate buffer, pH 3.05, containing 0.055 *M* disodium EDTA and 0.1% (A—D) or 0.11% (E) sodium octanesulfonate; flow-rate, 2 ml/min; detector, + 0.75 V vs. the Ag/AgCl reference electrode.

regression of peak height on concentration gave a slope (± 95% confidence interval) of 0.429 (± 0.015) nA/ng, a negligible intercept of 0.006 (± 0.008) nA and a mean coefficient of variation (C.V.) of 6% (n = 4 at each concentration). The mean recovery was 93.7% (n = 16). Because of good recovery and the use of constant-volume injection loop the use of an internal standard, as recommended by Cooper et al. [6] was found unnecessary.

Results on whole blood appeared to be much more variable, with a mean C.V. of 17%, possibly due to interference from some components of the erythrocytes. However, as shown below (cf. Fig. 2), α-methyldopa appears to be at least partially excluded from the erythrocytes, reducing the need for an assay method for this drug in whole blood.

Stability

Preliminary experiments using the blood and plasma of three human volunteers to whom α-methyldopa had been administered and whose blood was collected in the presence of heparin only (Vacutainers, Green Stoppers, Becton-Dickinson, Canlab, Ottawa, Canada) gave variable and unreproducible results (personal observations): fresh plasma from volunteers given α-methyldopa, precipitated and kept at room temperature until chromatography, appeared to give bimodal plasma concentration vs. time profiles; repeat analyses of plasma

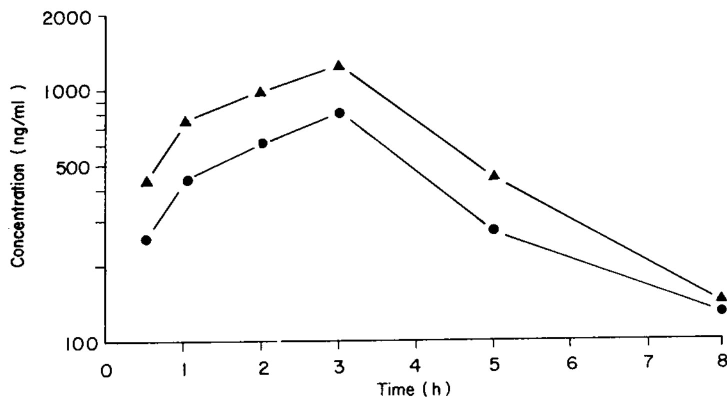

Fig. 2. α-Methyldopa concentrations in the plasma (▲) and whole blood (●) of a volunteer after 250 mg of the drug taken orally. Assay as described in the text using aliquots stored at −18°C for 1 week; for chromatographic conditions, see Fig. 1.

samples which had been kept frozen for several weeks showed a sharp decline in concentrations.

In the final method, as described in the experimental section, the stability of the sample was greatly enhanced by collecting the blood in the presence of EDTA and sodium metabisulfite (to prevent oxidation of the catechol) and reducing the time of contact with the acid precipitant to 30 min at 0°C (to prevent hydrolysis of the conjugated metabolites). Fig. 2 illustrates the plasma and whole blood concentrations in a volunteer during the 8 h following a single oral dose of 250 mg α-methyldopa. From the plasma values it appears that the drug is readily absorbed, absorption half-life being less than 1 h, and rapidly eliminated, with an approximate elimination half-life of less than 2 h. Exact pharmacokinetic modeling was not possible because of the paucity of values, especially between 4 and 8 h. The values for whole blood were consistently lower, indicating a partial, though not complete, exclusion from the red cells.

Plasma α-methyldopa concentrations following 1 and 4 weeks of storage at −18°C are shown in Table I. There was a slight decline in plasma concentra-

TABLE I

THE EFFECT OF STORAGE ON THE CONCENTRATION OF α-METHYLDOPA IN THE PLASMA

The 1- and 8-h samples were not available for immediate analysis.

Time (h) after dose	Concentration (ng/ml) assayed immediately	Percentage remaining after storage at −18°C for	
		1 week	4 weeks
0	0	—	—
0.5	440	98.9	92.3
2	1016	95.6	95.9
3	1193	102.6	97.6
5	475	92.0	82.7
Mean	—	97.3	92.1

tions after 1 week, as compared to the aliquots assayed immediately after blood collection. The mean loss amounted to 2.7%. A somewhat greater loss (mean 7.9%) was observed after 4 weeks at −18°C.

The effect of perchloric acid on the precision of the assay, attributable to possible hydrolysis of the conjugated metabolites, was investigated by chromatographing the acid supernatant of the 3-h and 5-h plasma samples after incubating for 2, 6 and 24 h at room temperature or 24 h at 4°C. The results in Fig. 3 show that the apparent concentration of α-methyldopa increases steadily, this increase being greater at room temperature, and especially with the 5-h sample (more than double by 24 h), presumably due to the greater proportion of metabolites.

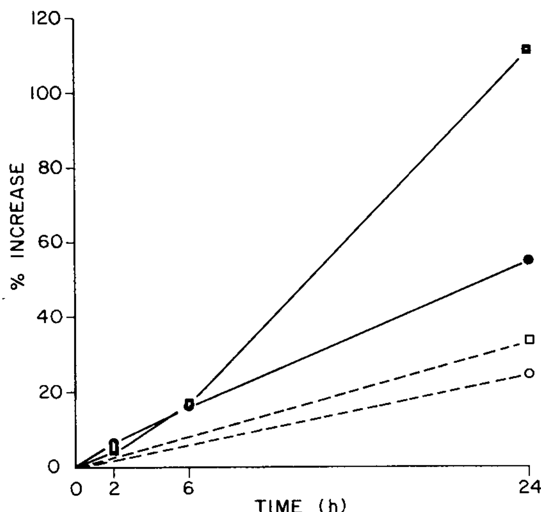

Fig. 3. Release of α-methyldopa from its conjugates in the presence of perchloric acid. Plasma taken 3 h after 250-mg oral dose, incubated at 4°C (○) and at room temperature (●); plasma samples 5 h after the dose, incubated at 4°C (□) and at room temperature (■).

In conclusion, difficulties due to instability associated with blood collection, plasma storage and sample work-up have been reduced to manageable proportions. By using an inhibitor in the collection tubes, avoiding prolonged storage of the plasma samples and keeping the time of contact between the acid precipitant and supernatant to a minimum, a rapid, simple and sensitive assay for this drug in the plasma has been developed which can be used for clinical analyses, as well as for pharmacokinetic studies. Similar precautions may well prove necessary for other, naturally occurring catechols, because of their similar redox and metabolic behavior.

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Note

Separation and quantitation of urinary porphyrins by high-performance liquid chromatography

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(First received November 6th, 1981; revised manuscript received December 8th, 1981)

Quantitative determination of porphyrins in urine has been tedious, inaccurate and time-consuming [1—4]. A variety of analytical procedures has been proposed for the diagnosis and differentiation of the porphyrias, including thin-layer chromatography [5] and high-performance liquid chromatography (HPLC) [6—8]. Evans et al. [7] reported the HPLC separation of porphyrins as free carboxylic acids on an ion-exchange resin. Bonnett et al. [9] and Meyer et al. [10] used reversed-phase ion-pair HPLC for the separation of the urinary porphyrin carboxylic acids.

We present a method for the identification and quantitation of the urinary porphyrins, uro- (8-carboxyl) and copro- (4-carboxyl) porphyrin, as free carboxylic acids, together with some decarboxylation intermediates, by reversed-phase chromatography using a step gradient system with lithium citrate buffer and increasing methanol concentration. An internal standard, deuteroporphyrin, was used to correct for losses of porphyrins during extraction.

EXPERIMENTAL

Apparatus

The HPLC instrument consisted of a Waters Model 6000 pump (Waters Assoc., Milford, MA, U.S.A.) interfaced to a simple three-way solenoid valve (Lee Company, Westbrook, CT, U.S.A.), a U6K loop injector and a Waters Model 440 absorbance detector. The magnetic valve was used for step gradient elution and was controlled by a homemade two-periodic timer. The absorbance of the porphyrins was monitored at 400 nm. A reversed-phase column (300 mm × 4 mm I.D.), slurry-packed with 10-μm LiChrosorb RP-18 (E. Merck, Darmstadt, G.F.R.) was used for the analytical separation. Before

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use, all solvents were carefully vacuum filtered (Millipore Type GS 0.22-μm filters, (Millipore, Bedford, MA, U.S.A.). Air bubbles were removed by bubbling nitrogen through the solution.

Chemicals

Water was deionized and Milli-Q-filtered (Millipore) before use. Methanol, hydrochloric acid, calcium chloride, sodium hydroxide (analytical grade) and hemin were purchased from Merck. Trilithium citrate, analytical grade, was obtained from BDH Chemicals (Poole, Great Britain). The porphyrin standard, consisting of mesoporphyrin, coproporphyrin, penta-, hexa- and hepta-carboxylic acid substituted porphyrin, and uroporphyrin was obtained from Pierce Chemical Company (Rockford, IL, U.S.A.). Deuteroporphyrin was synthesized according to the procedure of Dinello [11]. The compound was dissolved in 0.1 *M* hydrochloric acid and stored at −70°C (when not in use).

Sample preparation

Urine samples were collected over 24 h in dark plastic bottles containing 5 g of sodium carbonate. A 2.5-ml aliquot of urine was mixed in a 10-ml screw-capped glass tube with 40 μl of deuteroporphyrin solution (48 nmol/l). The porphyrins were adsorbed on calcium hydroxide by adding 2.0 ml of 3% (w/v) calcium chloride and 4.0 ml of 1.0 *M* sodium hydroxide solution. After centrifugation and washing of the precipitate with 1 ml of 0.1 *M* sodium hydroxide, 100 μl of concentrated hydrochloric acid were added to each tube. It is important to check the pH (<2.0) of the solution to ensure that the porphyrins are completely protonated and dissolved. Urine specimens known to contain a high concentration of porphyrins were diluted 1:5 with normal urine, i.e. urine with non-detectable amounts of uro- and coproporphyrin.

The porphyrin standard solution containing porphyrin carboxylic acids (0.4 nmol/l each) was prepared by dissolving the freeze-dried standard substances in 100 μl of 0.1 *M* hydrochloric acid and 2.4 ml of normal urine. The solution was treated as above.

Chromatographic conditions

The column was first equilibrated with a buffer containing 0.05 *M* lithium citrate (pH 2.5)—methanol (30:70, v/v) (buffer 1). Fourteen minutes after sample injection, a buffer containing 0.05 *M* lithium citrate (pH 2.5)—methanol (5:95, v/v) (buffer 2) was introduced into the pump for 10 min. The solvent flow-rate was 1 ml/min and the detector sensitivity 0.05 absorbance units full scale (a.u.f.s.). A 25-μl volume of the sample or standard solution was injected to the column.

RESULTS AND DISCUSSION

Fig. 1 shows the separation of a standard mixture of uroporphyrin, hepta-, hexa- and pentacarboxylic acid porphyrin, and copro-, deutero- and meso-porphyrin. The acids are eluted in order of decreasing polarity. The retention time for the least-polar mesoporphyrin is 29 min. Buffer change from 1 to 2 is made after 14 min. A switch back to the equilibration buffer (buffer 1) is

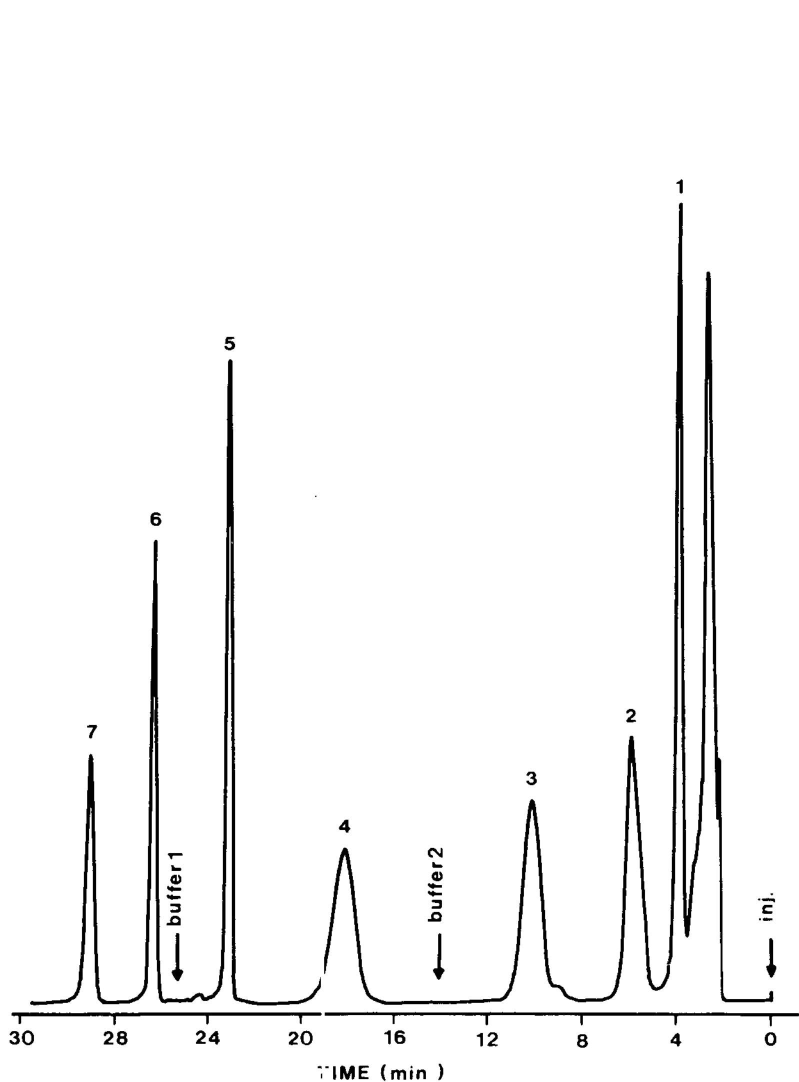

Fig. 1. HPLC separation of a porphyrin carboxylic acid standard mixture, containing uroporphyrin (1), hepta- (2), hexa- (3), and pentacarboxylic acid (4), and copro- (5), deutero- (6) and mesoporphyrin (7). A 10-μm LiChrosorb RP-18 column (300 mm × 4 mm I.D.) was used. Elution system: 0.05 *M* lithium citrate (pH 2.5)—methanol (30 : 70, buffer 1; and 5 : 95, buffer 2). Flow-rate 1 ml/min; absorbance monitored at 400 nm.

made after 25 min. Analysis of new samples can be started every half an hour. The retention times for the biologically most interesting porphyrins, uro- and coproporphyrin, are 4 and 23 min, respectively. It was found necessary to add an internal standard to obtain reproducible results if the analysis was not performed on the same day. A sample analysed one day, three days and one week after sampling gave a coefficient of variation (C.V.) of 1.2% for uroporphyrin and 5.5% for coproporphyrin using deuteroporphyrin as internal standard. Previous reports on HPLC analysis of porphyrins were based on the use of external standards.

Fig. 2 shows a chromatogram from the urine of a patient with porphyria cutanea tarda. Apart from high levels of uro- and coproporphyrin, trace levels of hepta-, hexa- and pentacarboxylic porphyrins were also detected but not quantitated. A linear correlation (r = 0.998, four points) was found between the area (height × width at half height) ratios of pure standard substances and internal standard vs. the uro- and coproporphyrin concentrations in the range 0.05—0.8 μmol/l (1.2 μmol/l deuteroporphyrin added). The standard preparations were treated in the same way as the urine samples. The mean recoveries of uro- and coproporphyrin are 99% and 97%, respectively.

Some of the samples were also analysed by Askevold's method [4]. Plots

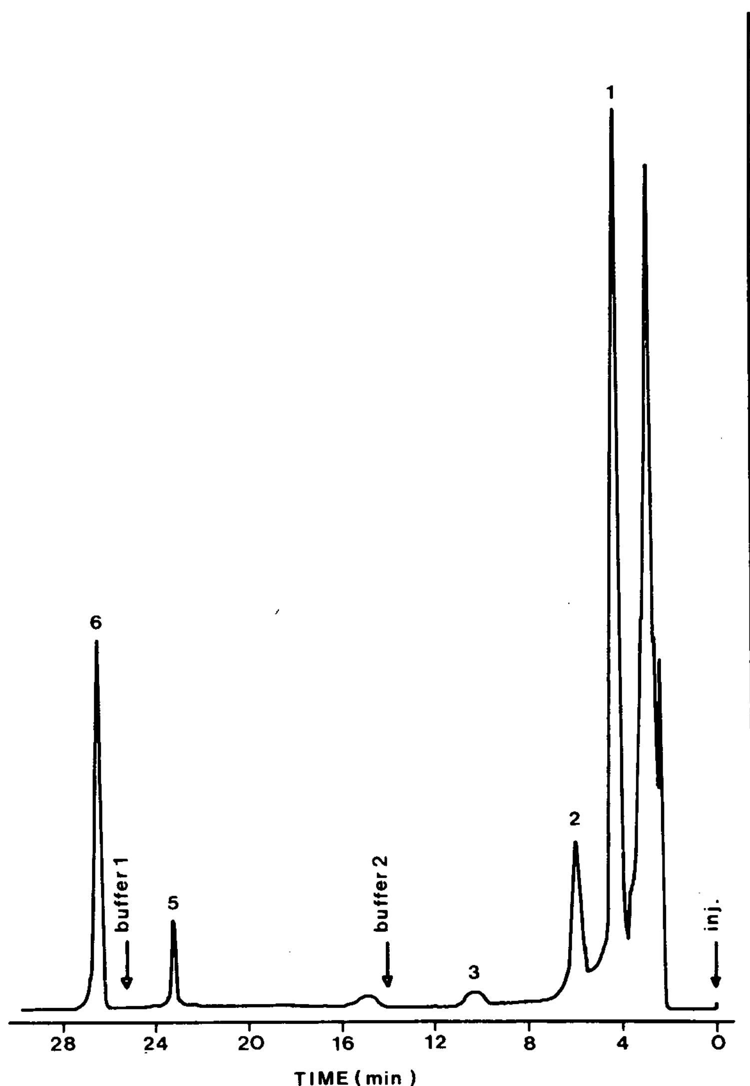

Fig. 2. HPLC separation of porphyrin carboxylic acids obtained from the urine of a patient with porphyria cutanea tarda. Column and elution system as in Fig. 1.

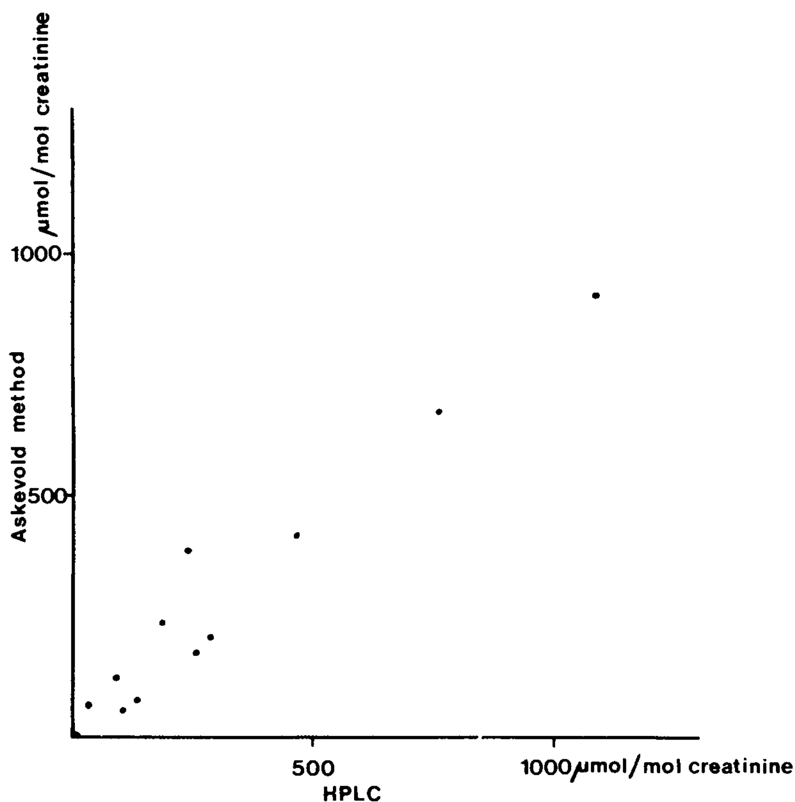

Fig. 3. Correlation of uroporphyrin concentration values, analysed by HPLC and by the method of Askevold.

between data obtained by our HPLC technique and Askevold's method are presented in Figs. 3 (uro-) and 4 (copro-). The molar values are related to creatinine excretion because some of the urines were not collected for 24 h. The correlations are good if the two points (marked with arrows) in the coproporphyrin plot are neglected. For this reason no correlation coefficients are presented. The deviations in µmol values for these latter coproporphyrin points are almost 100%. The HPLC traces of the corresponding urine samples revealed, apart from high uro- and coproporphyrin levels, also detectable levels of hepta- and hexacarboxylated products (see Fig. 2). It is most plausible that these compounds are co-determined in the Askevold method, giving rise to high concentration values and a large deviation in the correlation plot.

CONCLUSION

The described HPLC method offers a good alternative to the classical Askevold method as it is faster and more accurate, especially in cases where decarboxylation products appear.

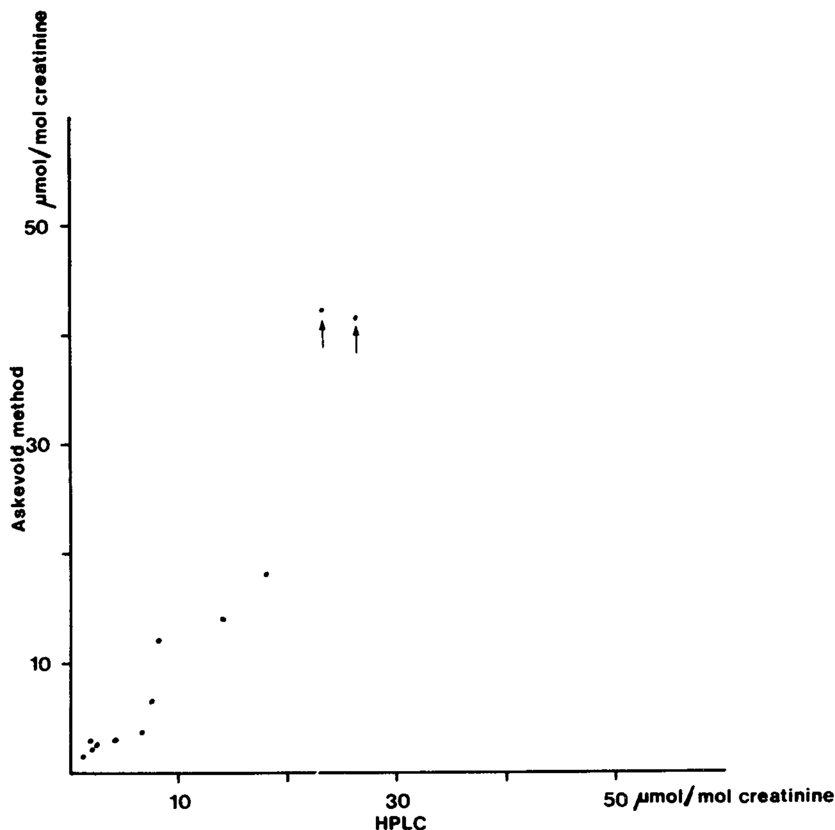

Fig. 4. Correlation of coproporphyrin concentration values, analysed by HPLC and by the method of Askevold.

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CHROMBIO. 1217

Note

Hydrolysis of riboflavin nucleotides in plasma monitored by high-performance liquid chromatography

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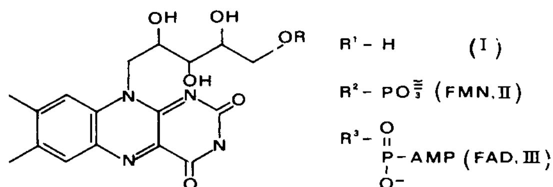
Riboflavin (I) carries out its functions in body in the form of one or other of two coenzymes, riboflavin phosphate (FMN, II) and flavin adenine dinucleotide (FAD, III). It was found that both FAD and FMN are hydrolyzed and resynthesized during the incorporation process [1]. Okumura and Yagi [2] reported that FAD and FMN are dephosphorylated very rapidly in whole blood. These authors observed that plasma is responsible for the hydrolysis of both FAD and FMN, the former being hydrolyzed at a faster rate.



However, these results are not completely consistent with the report of Jusko and Levy [3] that FMN is not decomposed by human blood plasma, and this difference might be ascribed to the analytical methods used for flavins. For this reason, in the present study the hydrolysis of FAD and FMN in blood plasma was monitored by high-performance liquid chromatography (HPLC).

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This technique has recently provided a rapid, specific and reproducible method of analyzing nucleotides using normal or reversed-phase columns [4—7].

The method described in this paper is a reversed-phase HPLC procedure for the direct determination of FAD, FMN and riboflavin in blood plasma using a fixed-wavelength detector (254 nm) and ammonium phosphate buffer—acetonitrile as eluent.

EXPERIMENTAL

Reagents and materials

FAD, FMN, riboflavin and nicotinamide were purchased from Sigma (St. Louis, MO, U.S.A.) and diammonium hydrogen phosphate (analytical-reagent grade) from Merck (Darmstadt, G.F.R.). Acetonitrile was of HPLC grade (Chromasolv; Riedel-De Haën, Hannover, G.F.R.). Water was distilled in glass and then passed through a 0.45-μm membrane filter (Type HA, Millipore, Bedford, MA, U.S.A.).

Standard stock solutions were prepared by dissolving riboflavin (10 μg/ml), FAD, FMN (150 μg/ml) and the internal standard, nicotinamide (200 μg/ml), in 0.9% sodium chloride solution. These stock solutions were prepared in amber glass and used within 12 h.

Sample preparation

A 1-ml volume of blood plasma was mixed with 100 μl of FAD or FMN stock solution and fortified with 50 μl of internal standard. Incubation was carried out in the dark at 37°C, and the hydrolysis was stopped by injecting 5-μl aliquots into the liquid chromatograph every 10 min during 1 h. Control blanks were performed under the same conditions using the above solutions containing everything but blood plasma.

Chromatography

A Waters Assoc. (Milford, MA, U.S.A.) analytical liquid chromatograph equipped with a 30 cm × 3.9 mm I.D. μBondapak C_{18} (10 μm) column was fitted with a Waters Model 440 UV detector using low-dead-volume hardware. A pre-column (Waters Assoc., part No. 84550) consisting of a short stainless-steel column (2 cm × 4 mm I.D.) packed with Bondapak—Corasil (37—50 μm) was used to prolong the life of the analytical column. A mobile phase of 10 m*M* diammonium hydrogen phosphate (adjusted to pH 5.5 with 20% phosphoric acid)—acetonitrile (1000:120) was filtered, degassed and used at a flow-rate of 2.0 ml/min (130 bar). The effluent stream was monitored using a 254-nm filter on the detector. The range setting was fixed at 0.02 a.u.f.s., with the signal monitored by a 10-mV strip-chart recorder (1.0 cm/min) interfaced with a Model 730 Data Module (Waters Assoc.).

RESULTS AND DISCUSSION

Some difficulty was encountered in finding an isocratic eluent to separate rapidly FAD, FMN, riboflavin and the internal standard from each other and from plasma components. The most satisfactory solvent was ammonium

phosphate buffer—acetonitrile; the retention times were strongly dependent upon the acetonitrile concentration and the pH. A mixture of aqueous buffer—acetonitrile (1000:120) and a pH value of 5.5 were selected. Capacity factors in the system as described were: nicotinamide (internal standard), 1.4 min; FAD, 2.3 min; FMN, 3.9 min; riboflavin, 8.1 min.

The chromatograms in Fig. 1 demonstrate the lack of interference and the specificity of the assay procedure for the measurement of FAD, FMN and riboflavin in plasma. Calibration curves were constructed by determining the response from known amounts of FAD, FMN and riboflavin and the internal standard added to 0.9% sodium chloride solution. Assay linearity was demonstrated over the range 0.6—20 μg/ml; the amount of the internal standard was held constant at 10 μg/sample. Typical calibration curves are shown in Fig. 2.

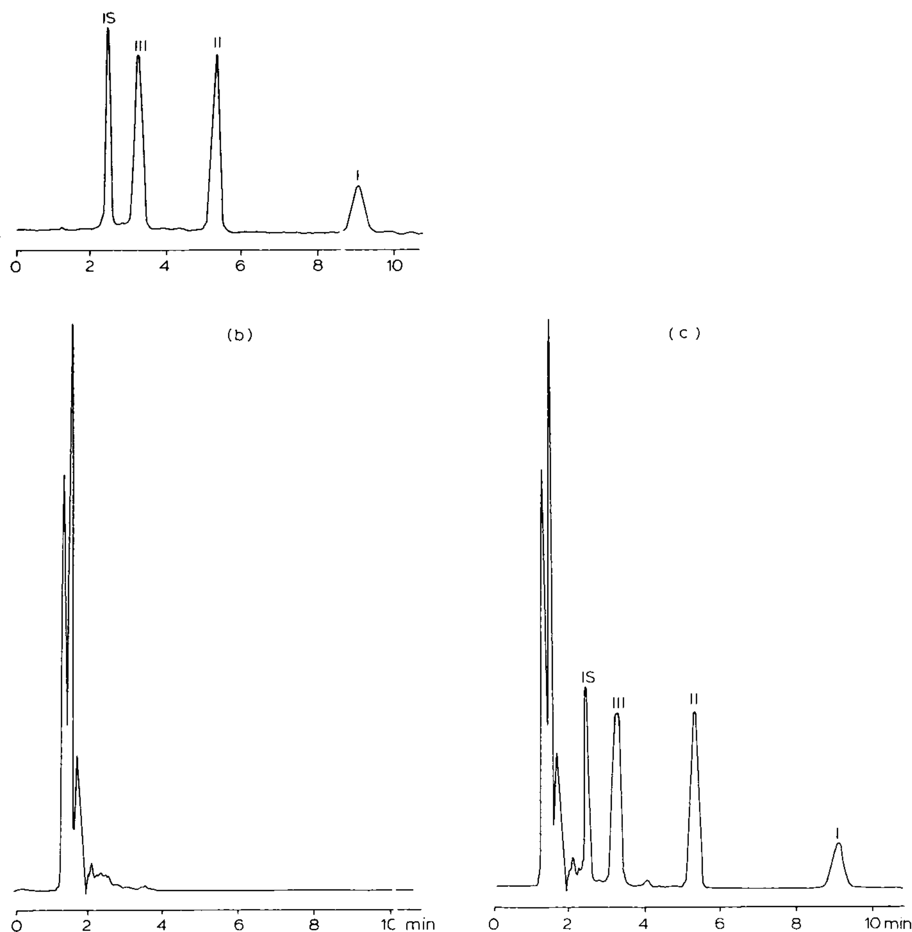

Fig. 1. Chromatograms of: (a) a standard mixture of internal standard (IS, 45 ng), FAD (III, 65 ng), FMN (II, 65 ng) and riboflavin (I, 25 ng); (b) control plasma; (c) plasma fortified with internal standard (IS, 45 ng), FAD (III, 65 ng), FMN (II, 65 ng) and friboflavin (I, 25 ng).

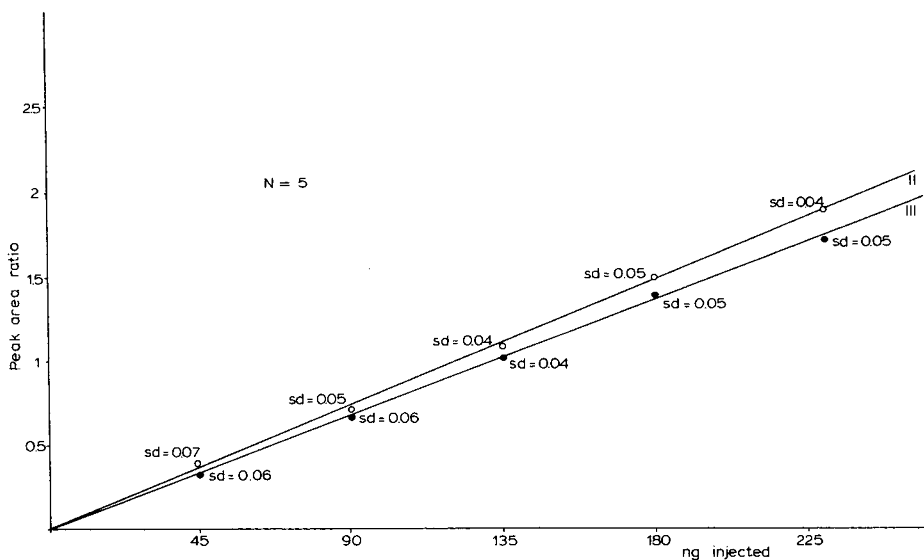

Fig. 2. Calibration graph of the peak area ratio (II or III peak area/internal standard peak area) versus the amount of II and III injected.

TABLE I

HYDROLYSIS OF FAD AND FMN BY BLOOD PLASMA AT 37° C

The reaction mixture consisted of 1 ml of blood plasma, 0.1 ml of FAD or FMN stock solution and 0.05 ml of internal standard. Incubation was at 37° C. Values given express the amount of FAD or FMN remaining as a percentage of the value at time 0.

	Incubation time (min)					
	10	20	30	40	50	60
FAD	68.5	43.4	25.3	15.2	5.1	3.4
FMN	99.4	98.9	98.5	98.3	97.9	97.5

The sensitivity of this method was estimated to be 0.5 μg/ml and was adequate for following the hydrolysis.

The time—course for hydrolysis of FAD and FMN in blood plasma is shown in Table I. FAD added to blood plasma was hydrolyzed to FMN within 60 min; on the other hand, FMN added to blood plasma was hydrolyzed only to a very small extent, comparable to that of the blank. These results indicate that the rapid loss of plasma FAD is due to enzymatic catalysis, while the slow hydrolysis of FMN should not be ascribed to enzymatic activity.

In conclusion, this HPLC method is a simple, reproducible and specific procedure, which allows the monitoring of FAD and FMN hydrolysis in blood plasma. The extension of the procedure to whole blood as well as to red cells has been demonstrated in preliminary experiments.

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Note

High-performance liquid chromatographic analysis of nitrate in urine and feces

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Nitrate is important to human health because of methemoglobin formation in infants [1] and because carcinogenic N-nitroso compounds can form in vivo, either in the stomach [2] or in an infected urinary bladder [3], after ingestion of nitrate and the corresponding amines. Several methods, which have been described for the analysis of nitrate, are either subject to interferences by other compounds present in the samples or they are time-consuming because of lengthy clean-up steps [4,5] and/or conversion to nitro compounds [6], nitrite [7,8], or ammonia [9]. Reduction to nitrite requires subsequent diazotization [4] or additional chemical reactions [10,11]. furthermore, cadmium used for nitrate reduction and azo dyes that are subsequently produced are themselves toxic [12,13]. Two direct high-performance liquid chromatographic (HPLC) methods for nitrate and nitrite have been reported, by Thayer and Huffacker [14] for plant extracts, and by Gerritse [15] for sewage. In preliminary experiments, we found that the former [14] did not provide clean separations from other compounds present in urine and feces, while the inferior peak resolution is obtained in the latter [15]. We therefore set out to find conditions for assay of nitrate in complex biological samples.

MATERIALS AND METHODS

Reagents

Analytical-grade reagents and degassed, glass distilled, deionized water (specific resistance 16—18 MΩ/cm) were used.

Biological samples

Urine and feces from Sprague-Dawley rats or humans (authors) were pre-

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pared as previously described [16]. A nitrate-free 0.5-μm or 0.2-μm filter (Millipore, Bedford, MA, U.S.A.) was used before HPLC analysis.

High-performance liquid chromatography

The following equipment was used: a Perkin-Elmer Series 2 pump (Perkin-Elmer, Norwalk, CT, U.S.A.); a Whatman Partisil 10 SAX (Whatman, Clifton, NY, U.S.A.) or Ultrasil AX (Altex, Berkley, CA, U.S.A.) 25-cm anion-exchange column; a 6-cm guard column packed with Whatman HC Pellosil or Whatman Partisil 10 SAX; a Rheodyne 7010 sample injector (Rheodyne, Cotati, CA, U.S.A.); and a Gilson Holochrome HM/HPLC spectrophotometer (Gilson Medical Electronics, Middleton, WI, U.S.A.). After digitization, the output was interfaced to a Microflop Descope computer (Charles River Data Systems, Natick, MA, U.S.A.).

Columns were routinely eluted with 22.5 m*M* aqueous phosphate buffer, pH 2.35. For a given type of sample the pH was altered to a value where nitrate eluted alone.

Reduction of nitrate to ammonia

Sample (5 ml of urine or intestinal contents), 5 ml of 0.4 *N* sodium hydroxide and 1 g of Devarda's Alloy (J.T. Baker, Phillipsburg, NJ, U.S.A.) were combined and heated at 100°C for 15 min. Phosphoric acid (0.1 ml) was then added and the precipitate was removed by centrifugation at 10,000 *g*.

Reduction of nitrate to nitrite

Samples were passed through a column (10 × 0.5 cm) of coarse mesh cadmium (E. Merck, Darmstadt, G.F.R.). Spongy cadmium [16] increased analysis time. Nitrite was then determined by a modified Griess test [16].

RESULTS

Comparison of nitrate analysis by HPLC and the cadmium reduction method

Unlike the HPLC method the cadmium reduction system was linear to only 60 nmol/ml of nitrate (Fig. 1). Furthermore, nitrate recovery from urine samples by the cadmium reduction method was less consistent than for HPLC analysis (Table I).

Identification of the nitrate peak

Three methods were used to verify the nitrate peaks. First, known amounts of nitrate were added to samples. Fig. 2 represents an example of this with rat urine. Figs. 2 and 3 have been drawn from computer displays; the blank areas immediately following injection represent void volume peaks not included in the figures. Fig. 2C shows a typical elution profile of nitrate (16.1 nmole) in distilled deionized water. Figs. 2A and B are elution profiles of the same urine sample except that 2A has been spiked with 16.1 nmole of nitrate. The sum of the nitrate peak areas in Fig. 2B and C equals the area (amount of nitrate) in the peak of 2A indicating the efficiency (98.6%) of nitrate recovery by this HPLC method. In similar experiments, with over 40 different urine and fecal samples, the mean difference between added and recovered nitrate was 2.83% (standard deviation: 2.61).

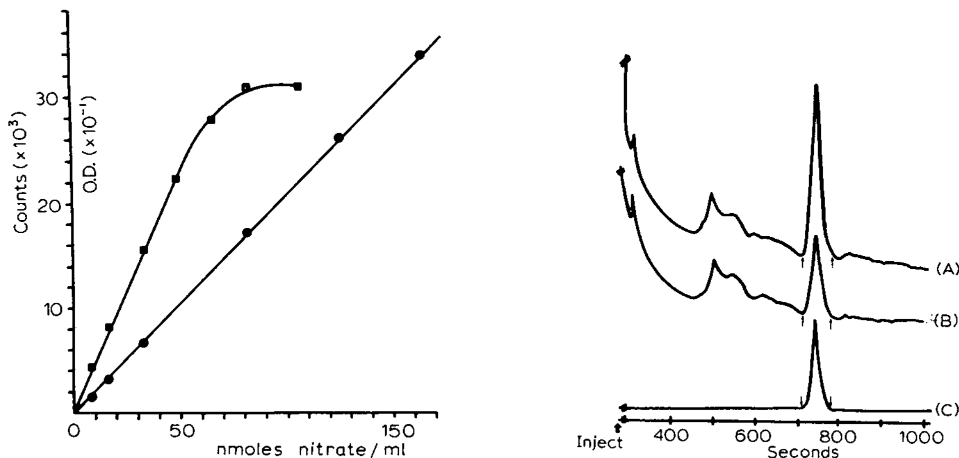

Fig. 1. Comparison of analysis of nitrate in distilled deionized water by the cadmium reduction method and HPLC. Circles represent HPLC (counts) and squares represent cadmium (O.D.) analysis of nitrate.

Fig. 2. Representative example of a rat urine (A) after and (B) before addition of a standard nitrate spike (16 nmol/ml). The same standard spike is shown in (C) in distilled deionized water. These three separate analyses have been stacked to allow better comparison of the results. The first part of each trace has been omitted. Arrows indicate computer marks used for nitrate peak area integration; counts in A = 7324, B = 3490, C = 3943. Flow-rate = 1.5 ml/min.

TABLE I

ACCURACY OF THE CADMIUM REDUCTION METHOD AND HPLC FOR THE ANALYSIS OF NITRATE IN RAT URINE

	Cadmium			HPLC		
	O.D. Observed	O.D. Expected	Recovery (%)	Counts Observed	Counts Expected	Recovery (%)
Sample (S)	0.023	N.A.*	N.A.	1299	N.A.	N.A.
S + 1.6 nmol/ml of nitrate	0.100	0.112	89.3	1569	1594	98.4
S + 8.0 nmol/ml of nitrate	0.395	0.417	94.7	3000	2922	102.6
S + 16.1 nmol/ml of nitrate	0.675	0.831	81.3	4396	4405	99.8

*N.A. = not applicable.

Secondly, nitrate peaks were identified by cadmium reduction of effluent fractions followed by azo dye determination of nitrite [16]. Assignments agreed with those based on spiking experiments (data not shown).

The third method was conversion of nitrate to ammonia with Devarda's alloy. The peak bounded by arrows in Fig. 3A was not present after alloy treatment (Fig. 3B) indicating it was nitrate.

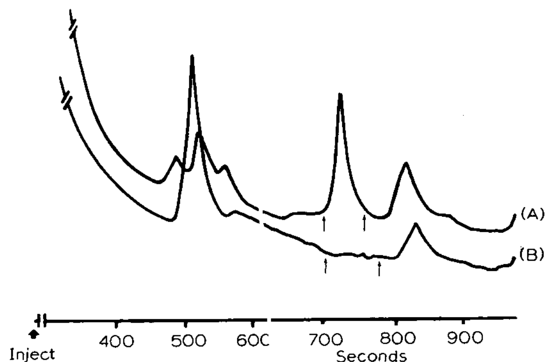

Fig. 3. Representative example of HPLC effluents of a rat urine containing nitrate (A) and the removal of nitrate (B) by Devarda's alloy. The first part (left) of each trace represents the void volume and has not been reproduced. Arrows (at 700—800 sec) indicate computer marks used for nitrate peak area integration. Flow-rate = 1.5 ml/min.

Precision of the HPLC method

Precision was estimated by analyses of 52 urine and fecal samples in triplicate. The mean coefficient of variation was 1.91% (standard deviation 1.33).

DISCUSSION

HPLC analysis is superior to the cadmium reduction method for assay of nitrate in biological samples. Previously reported HPLC techniques [14,15] proved to be inadequate for quantitating nitrate in urine, feces and animal tissues. The modification described in this report allowed such quantitation. Rat blood, kidney and liver extracts as well as rat and human urine and feces and several foodstuffs were successfully analyzed for nitrate. Furthermore, this HPLC technique allowed the demonstration of nitrate synthesis by mammalian tissues in vivo [17].

Several practical points can be made for this HPLC method. Nitrate analysis times were 15—25 min depending on buffer pH (and to a lesser extent on ionic strength) and on the presence of compounds eluting after nitrate. More acidic buffers gave shorter retention times. Chloride, but not sulfate, adversely affected resolution of the nitrate peak. There were only minor differences in the nitrate peaks obtained with Partisil SAX or Ultrasil AX columns. Both columns gave good service for over a year. Guard columns packed with HC Pellosil were superior to those packed with Partisil. Comparison of raw samples with those treated with Devarda's alloy greatly helped in correct assignment of nitrate peaks.

In conclusion, this report describes a procedure for nitrate analysis of complex biological samples which is safe, rapid and direct. It can detect 0.8 nmol/ml with a precision of better than ±5%.

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Note

Lipids in human neutrophils determined by a microanalytical method

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Lipids in neutrophils have been investigated in de novo synthesis using precursors labelled by radioisotopes [1]. However, the lipid composition of neutrophils has not been reported except in one case [2]. Lipids in blood cells are usually analysed by thin-layer chromatography and each spot is quantitated either by densitometry [3] or by colorimetry of lipid eluted from the silica gel [4]. These methods are quite useful if sufficient sample can be obtained.

We established the microanalytical quantitation of lipids in platelets [5] and lymphocytes [6] using a Chromarod with thin-layer chromatography connected to a flame ionization detector. In this present paper, lipids in neutrophils separated from 20 ml of human peripheral blood were quantitated using this microanalytical technique.

MATERIALS AND METHODS

Subjects

Five females and six males, whose physical and laboratory examinations were determined to be within normal limits, were examined. Their ages, serum total cholesterol, triglyceride and phospholipid were, respectively, 25 ± 2 years, 162 ± 25 mg/dl, 75 ± 6 mg/dl and 169 ± 18 mg/dl.

Preparation of neutrophils

A 20-ml sample of heparinized venous blood was drawn from each subject after overnight fasting. Leukocyte-rich plasma obtained by the dextran (molecular weight 25,000, Nakarai Chemicals, Tokyo, Japan) sedimentation method was placed on Conray—Ficoll solution (density 1.077 at 25°C) and centrifuged at 400 *g* for 40 min at 20°C to remove mononuclear cells [7].

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Residual red blood cells in amongst the neutrophils were lysed by hypotonic saline [8]. Erythrocyte ghosts and hemoglobin were removed by centrifugation at 400 *g* for 10 min with 0.15 *M* sodium chloride solution containing 1 mg/dl EDTA (Wako Chemicals, Osaka, Japan) and the number of neutrophils was adjusted to $1 \cdot 10^6$ per ml. More than 95% of the cells collected were neutrophils. The number of neutrophils obtained from 20 ml of peripheral blood ranged between $1.5 \cdot 10^7$ and $3.0 \cdot 10^7$. The neutrophils were filled with nitrogen and stored at —70° C until the lipid analysis.

Lipid extraction from neutrophils and lipid analysis by thin-layer chromatography in combination with flame ionization detection

Lipid extraction from neutrophils and lipid analysis by thin-layer chromatography in combination with flame ionization detection were performed by the method reported previously for lymphocytes [6].

Statistical evaluation

Student's *t*-test was used for the statistical evaluation.

RESULTS

Quantitation of neutral lipids and phospholipids

Phospholipid, 54.4% and 18.1 μg per 10^6 neutrophils, was the major lipid component of neutrophils. Triglyceride, 21.9% and 4.5 μg per 10^6 neutrophils, was the major component of the neutral lipids. Cholesteryl ester, 10.9% and 3.3 μg per 10^6 cells, and free cholesterol, 13.4% and 3.4 μg per 10^6 cells, were nearly equal in the lipids of neutrophils. The molar ratio of free cholesterol to phospholipid was 0.47. No sex difference existed for cholesteryl ester, free cholesterol, phospholipid and the molar ratio of free cholesterol to phospholipid except for triglyceride, which was slightly higher in male than in female neutrophils ($0.05 < P < 0.1$) (Table I).

Quantitation of phospholipid fractions

Phosphatidylethanolamine (40.4%) and phosphatidylcholine (36.8%) were two major components of the phospholipid fractions. The third component was sphingomyelin (15.2%); phosphatidylinositol plus phosphatidylserine (7.4%) was a minor component. There was no sex difference in any of these phospholipid fractions (Table II).

Quantitation of free fatty acid

$C_{16:0}$, $C_{18:0}$ and $C_{18:1}$ were each determined as approximately 20% of the free fatty acids using gas chromatography; $C_{20:4}$ was 13.5%; $C_{14:0}$, $C_{16:1}$ and $C_{18:2}$ ranged between 7.4 and 8.9%. No sex difference was observed (Table III).

DISCUSSION

Freshly isolated human neutrophils can be used to estimate the activities of the insulin receptor and of the low density lipoprotein receptor which regulate

TABLE I

LIPID COMPOSITION AND THE MOLAR RATIO OF FREE CHOLESTEROL TO PHOSPHOLIPID IN MALE AND FEMALE NEUTROPHILS

Lipids were qualitated and quantitated by thin-layer chromatography with a flame ionization detector and expressed as weight per cent and μg per 10^6 neutrophils. Figures in parentheses are the number of experiments. All values are expressed as mean ± S.D.

	Total (11)	Female (5)	Male (6)
Cholesteryl ester (weight per cent)	10.9 ± 5.1	10.1 ± 4.9	11.7 ± 5.4
(μg per 10^6 cells)	3.3 ± 2.1	2.9 ± 2.3	3.8 ± 1.9
Triglyceride (weight per cent)	21.9 ± 14.8	16.6 ± 11.4	30.4 ± 14.3
(μg per 10^6 cells)	4.5 ± 2.1	3.6 ± 2.1	6.2 ± 0.9
Free cholesterol (weight per cent)	13.4 ± 3.4	14.7 ± 2.3	12.2 ± 4.0
(μg per 10^6 cells)	3.4 ± 1.7	2.8 ± 1.5	3.9 ± 1.9
Phospholipid (weight per cent)	54.4 ± 14.7	58.6 ± 13.7	50.7 ± 15.6
(μg per 10^6 cells)	13.1 ± 9.8	13.5 ± 5.2	21.4 ± 11.1
Molar ratio of free cholesterol to phospholipid	0.47 ± 0.08	0.48 ± 0.03	0.47 ± 0.11

TABLE II

PHOSPHOLIPID DISTRIBITION IN MALE AND FEMALE NEUTROPHILS

The phospholipid distribution was analysed by thin-layer chromatography with a flame ionization detector and expressed as weight per cent. Figures in parentheses are the number of experiments. All values are expressed as mean ± S.D.

	Total (11)	Female (5)	Male (6)
Phosphatidylethanolamine	40.0 ± 6.2	43.2 ± 4.7	37.9 ± 7.3
Phosphatidylinositol plus phosphatidylserine	7.4 ± 1.9	7.8 ± 2.7	7.6 ± 1.9
Phosphatidylcholine	36.8 ± 6.9	36.3 ± 8.3	37.2 ± 6.4
Sphingomyelin	15.2 ± 4.6	14.0 ± 3.1	18.4 ± 6.8

TABLE III

FATTY ACID COMPOSITION OF MALE AND FEMALE NEUTROPHILS

Fatty acid composition was analysed by gas chromatography and expressed as weight per cent. Figures in parentheses are the number of experiments. All values are expressed as mean ± S.D.

	Total (11)	Female (5)	Male (6)
$C_{14:0}$	8.9 ± 3.3	7.5 ± 1.9	10.4 ± 4.1
$C_{16:0}$	20.9 ± 3.5	21.6 ± 3.9	20.2 ± 3.3
$C_{16:1}$	8.5 ± 1.7	9.2 ± 1.0	7.6 ± 1.9
$C_{18:0}$	18.9 ± 2.9	19.5 ± 2.7	18.4 ± 3.4
$C_{18:1}$	22.1 ± 5.1	23.1 ± 4.2	21.2 ± 6.3
$C_{18:2}$	7.4 ± 2.0	7.8 ± 1.7	7.0 ± 2.4
$C_{20:4}$	13.5 ± 3.5	12.0 ± 3.5	14.9 ± 3.2

the intracytoplasmic lipid metabolism of cells [9, 10]. The relationship between these receptor activities and lipid metabolism needs to be clarified beyond analysis of adenylate cyclase and the cyclic AMP system. Studies on the lipids of neutrophils have been performed using a radioisotopic method [1]. The lipid composition of neutrophils has been reported in only one paper,

which was published in 1967 [2]. Gottfried separated his neutrophils on glass-bead columns from 250 ml of peripheral blood. However, it is now known that both glass-bead and nylon-wool columns cause adherence of not only neutrophils but also monocytes, B lymphocytes, some T lymphocytes and platelets [11]. We have prepared much purer neutrophils using gradient sedimentation methods [7]. This combination of dextran, Ficoll—Conray, and hypotonic lysis yielded over 95% pure neutrophils.

Extraction of lipids from the nuetrophils was performed by the Folch method [12]. However, the solvent system for chromatographic development on thin-layer plates was hexane—diethyl ether—acetic acid (80:20:1) for neutral lipids [2], while we used *n*-hexane—diethyl ether (9:1) with the Chromarod. Gottfried's [2] results were for cholesteryl ester — triglyceride could not be located — and he reported that cholesterol was $5.33 \pm 0.70 \times 10^{-15}$ moles/cell and 10% of the total lipids. This agrees well with our determination. Furthermore, cholesteryl ester, triglyceride and free cholesterol were clearly identified in our system, indicating that thin-layer chromatography with a Chromarod connected to an ionization detector is superior to classical thin-layer chromatography with silica gel plates.

The mobile phase for the phospholipids was chloroform—methanol—water (60:30:3.5) for our thin-layer chromatography with flame ionization detection, while classical two-dimensional thin-layer chromatography depended on chloroform—methanol—acetic acid—water (25:15:4:2) as solvent for the first direction and diisobutyl ketone—acetic acid—water (40:25:5) as solvent for the second dimension. We examined which of these and several other solvent systems would provide a clear fraction of phospholipids on the Chromarods. The best separation of phosphatidylinositol and phosphatidylserine, which were very difficult to identify as two spots even by two-dimensional thin-layer chromatography [1], was obtained with a chloroform—methanol—water (60:30:3.5) system. Even so, clear separation of these two phospholipids was obtained by chance. This is the reason why they are expressed as phosphatidylinositol plus phosphatidylserine.

We used cholesterol acetate as an internal standard for calibration of relative amounts of lipid fractions and also for absolute amounts in combination with calibration curves for cholesteryl ester, triglyceride and phosphatidylcholine. For the determination of phospholipid, this method using phosphatidylcholine as a standard presents something of a problem because of the difference in molecular sizes of total phospholipids and phosphatidylcholine; however, a good correlation was obtained between this method and the colorimetric method reported previously [6].

Calibrations of weight percentages of the fractionated lipids were performed automatically by a densitometer together with a flame ionization detector. It takes only 50 min from development to densitometry.

Thus, this newly developed microanalytical method can be used as a clinical technique to quantitate neutral lipids and fractions of phospholipids in neutrophils as well as in lymphocytes [6] and platelets [5]. Fatty acids in the extracted neutrophil lipids were detected by ordinary gas chromatography. A sex difference among these lipids and fatty acids was noted only for the triglycerides, but it was not statistically significant.

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Note

Separation by thin-layer chromatography of nucleotides from bases and nucleosides in trichloroacetic acid extracts of cells

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Trichloroacetic acid (TCA) is commonly used to precipitate nucleic acids and to extract bases, nucleosides and nucleotides from cells after they have been grown with a radioactive nucleic acid precursor. Analysis of the acid-soluble fraction for radioactivity in the phosphorylated and nonphosphorylated derivatives is sometimes required [1]. Thin-layer chromatography (TLC) has been used but TCA interferes with the chromatographic separation and must be removed; this can lead to the loss of nucleotides [2]. Recently, K_2CO_3 has been used to neutralize TCA extracts prior to TLC [3] and because this approach would appear to overcome the problems associated with TCA removal, we have developed TLC systems that separate common bases and nucleosides from nucleotides when these compounds are in either TCA or TCA neutralized with K_2CO_3.

EXPERIMENTAL

The compounds were dissolved in 5% (w/v) TCA which in some cases was neutralized immediately afterwards with K_2CO_3. The final concentration of K_2CO_3 was between 0.10 and 0.20 M. The radioisotopes, [2,8-^{3}H]adenosine-3′,5′-cyclic monophosphate or [8-^{3}H]guanine, were added to 5% (w/v) TCA to give 5 μCi/ml; some solutions were neutralized immediately with K_2CO_3.

Acid-soluble fractions were prepared from human or Chinese hamster fibroblasts that were labelled in one chamber of a two-chamber LabTek slide (Miles Laboratories, Naperville, IL, U.S.A.) or on a Thermanox (Lux, Newbury Park, CA, U.S.A.) plastic coverslip (15 mm diameter) that was held in a well of a Costar (Cambridge, MA, U.S.A.) 24-well plate. [G-^{3}H]Hypoxanthine or [8-^{3}H]-adenine was added to the cultures to give 5 μCi/ml. Four hours later the

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cultures were rinsed three times with cold phosphate-buffered saline and extracted with 500 μl of ice cold 5% (w/v) TCA for 30 min. Microliter aliquots of 2.5 M K_2CO_3 were added to the extract and on each addition the pH of the solution was tested on wide-range B Accutint indicator paper (Anachemica Chemicals, Champlain, NY, U.S.A.). When the paper turned light green, which indicated a pH of between 7 and 8, the extract was suitable for chromatography. Generally 20 μl of 2.5 M K_2CO_3 neutralized 250 μl of extract.

Precoated TLC sheets (20 × 20 cm) of polyethyleneimine (PEI) cellulose

TABLE I

R_F VALUES OF NUCLEOTIDES, NUCLEOSIDES AND BASES ON PEI SHEETS

Chromatography conditions: in A and B the compounds were in neutralized TCA whereas in C they were in TCA. The solvent was methanol—water (7:3) in A. In B and C the first solvent was methanol and this was followed by development in the same direction with methanol—water (7:3). The mean and standard deviation are given in A where n=3 except for cAMP (n=10), NAD^+ (n=6), $NADP^+$ (n=4) and adenine (n=4). Chromatography was done only once in B and C.

	Chromatography conditions		
	A	B	C
Nucleotides			
cAMP	0.10±0.04	0.02	0.10
AMP	0.02±0.01	0.00	0.08
dAMP	0.02±0.01	0.00	0.05
ADP-ribose	0.05±0.05	0.00	0.00
ADP	0.01±0.01	0.00	0.02
ATP	0.01±0.00	0.00	0.00
NAD^+	0.08±0.06	0.00	0.00
$NADP^+$	0.01±0.01	0.03	0.06
GMP	0.02±0.01	0.00	0.06
dGMP	0.03±0.01	0.00	0.05
GDP	0.01±0.00	0.00	0.00
XMP	0.02±0.01	0.03	0.05
dTMP	0.01±0.00	0.10	0.05
UMP	0.00±0.00	0.05	0.08
Nucleosides			
Adenosine	0.48±0.02	0.60	0.79
Deoxyadenosine	0.49±0.03	0.61	0.76
Guanosine	0.26±0.02	0.23	0.63
Deoxyguanosine	0.50±0.03	0.58	0.70
Inosine	0.47±0.01	0.50	0.62
Xanthosine	0.29±0.01	0.29	0.60
Thymidine	0.76±0.01	0.78	0.95
Uridine	0.66±0.01	0.69	0.83
Bases			
Adenine	0.46±0.02	0.63	0.66
Hypoxanthine	0.46±0.01	0.57	0.59
Guanine	0.36±0.01	0.29	0.70
Xanthine	0.23±0.03	0.33	0.53
Thymine	0.70±0.02	0.72	0.88
Uracil	0.63±0.02	0.67	0.84

cultures were rinsed three times with cold phosphate-buffered saline and extracted with 500 μl of ice cold 5% (w/v) TCA for 30 min. Microliter aliquots of 2.5 M K_2CO_3 were added to the extract and on each addition the pH of the solution was tested on wide-range B Accutint indicator paper (Anachemica Chemicals, Champlain, NY, U.S.A.). When the paper turned light green, which indicated a pH of between 7 and 8, the extract was suitable for chromatography. Generally 20 μl of 2.5 M K_2CO_3 neutralized 250 μl of extract.

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were run in distilled water and dried prior to use. Aliquots of 10 and 20 μl of marker compound (1 mg/ml) and 20 and 40 μl of neutralized extract were spotted 2.5 cm from the bottom of the chromatogram. The chromatograms were developed in either distilled water, methanol, methanol—water (1 : 1) or methanol—water (7 : 3). The solvents were run approximately 12 cm above the origin and this generally took less than 2 h. Detection of the compounds has been described previously [4].

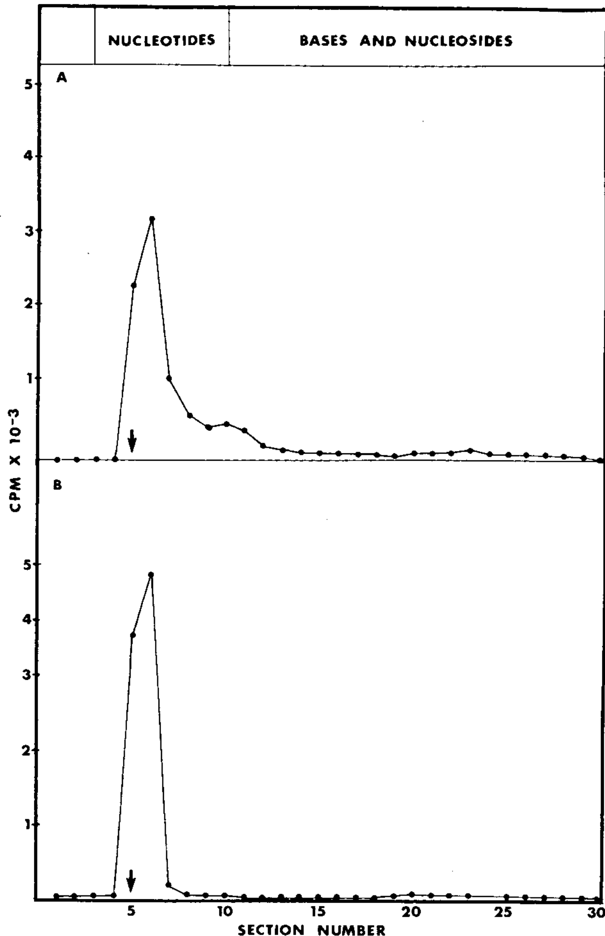

Fig. 1. Chromatography of TCA cell extracts. Human fibroblasts were grown with [^{3}H]-hypoxanthine for 4 h and then extracted with 5% TCA for 30 min at 4°C. In A a PEI strip (2.5 × 20 cm) was spotted with 20 μl of extract whereas in B the extract first was brought to a pH of between 7 and 8 with 2.5 M K_2CO_3 prior to the application of 20 μl to a PEI strip. The chromatograms were run in methanol, dried, developed again in the same direction with methanol—water (7 : 3), and then cut into 0.5 × 2.5 cm segments. These were counted in a liquid scintillation counter. The regions where known nucleotides (segments 4—10) and known bases and nucleosides (segments 11 to solvent front) migrated are outlined at the top of the figure. The arrows indicate the origins.

RESULTS AND DISCUSSION

Solvents were tested for their ability to separate nucleotides from bases and nucleosides when these compounds were in neutralized TCA or in neutralized TCA cell extracts. Only methanol—water (7 : 3) gave the desired separation. The nucleotides remained at the origin while the bases and nucleosides migrated well away (Table I). The success of this solvent was established with radioactive standards and cell extracts. When [^{3}H]cAMP that was in neutralized TCA was chromatographed, 96.3% of the radioactivity recovered on the chromatogram was found in the nucleotide region, whereas when [^{3}H]guanine was chromatographed, 99.5% of the radioactivity was found in the base and nucleoside region. Fibroblasts were labelled with [^{3}H]adenine and extracted under conditions that would preserve the nucleotides and under conditions that would hydrolyze them. These extracts were compared by chromatography. When the cells were extracted with 5% TCA for 30 min at 4°C, 93.1% of the radioactivity was found in the nucleotide region, whereas when the cells were extracted with 5% TCA for 6 h at 70°C, 88% was found in the base and nucleoside region. If the pH of an extract was above 8 due to an excess of K_2CO_3, separation was not achieved but could be achieved in the solvent system described below.

Inasmuch as acetic [5], formic [6], and perchloric [7] acid cell extracts have been chromatographed directly, solvent systems were sought for the separation of nucleotides from bases and nucleosides in TCA or TCA cell extracts. If the chromatograms were run in absolute methanol, dried and developed again in the same direction with methanol—water (7:3), separations were achieved whether the compounds were in TCA or neutralized TCA (Table I). When [^{3}H]cAMP that was dissolved in 5% TCA was chromatographed, 94.7% of the radioactivity was found in the nucleotide region. The chromatographic behaviour of TCA and neutralized TCA cell extracts was compared immediately after preparation (Fig. 1). In the first case the nucleotide region contained 88.3% of the radioactivity found on the chromatogram while in the second instance this region contained 97.8%. This suggests some breakdown of nucleotides in TCA. In contrast, nucleotides appeared quite stable in neutralized TCA cell extracts as a sample stored for 10 months at −20°C showed no change.

In summary TLC methods for separating nucleotides from bases and nucleosides in TCA extracts of cells have been described that unlike previous methods do not require the removal of TCA.

ACKNOWLEDGEMENT

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Note

Determination of two nitrosourea antitumor agents by chemical ionization gas chromatography—mass spectrometry

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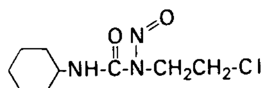
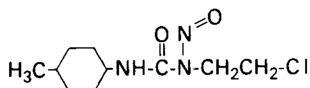
Two nitrosoureas, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) and 1-(2-chloroethyl)-3-(*trans*-4-methylcyclohexyl)-1-nitrosourea (MeCCNU), are clinically useful for the treatment of neoplastic diseases. The quantitative determination of these drugs in biological samples requires high sensitivity combined with selectivity because of their rapid decomposition in aqueous media. Previous assays used for nitrosoureas include high-performance liquid chromatography [1], colorimetry [2—4], radiochemical analysis of labelled compounds [5, 6], differential pulse polarography [7], chemical ionization mass spectrometry with direct probe [8] and gas chromatography—mass spectrometry (GC—MS) [9]. The GC—MS method, using electron ionization (EI) of their trifluoroacetyl derivatives, combines the sensitivity of radiochemical analysis with the selectivity necessary to distinguish the parent drugs from their decomposition products. This assay has been used to study the pharmacologic disposition of MeCCNU [10]. Occasional samples, however, give erroneous results because of interfering components. Modification of this method for chemical ionization (CI) should increase the selectivity for these drugs.



CCNU



MeCCNU

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CI generally imparts less energy than EI to the sample molecule, resulting in less fragmentation and, in general, a greater proportion of ions relating to the intact molecule. Although a careful study indicated the relative sensitivities of EI and CI are essentially equal [11] the reduced fragmentation in the CI process significantly decreases contaminant interference in selected ion chromatograms of biological samples. This increased selectivity should in effect lower the limits of quantitation for these samples. This paper describes the chemical ionization of CCNU and MeCCNU by several reagent gases and its use for improving the assays for these agents.

MATERIALS AND METHODS

CCNU and MeCCNU were provided by the Drug Development Branch, Division of Cancer Treatment, National Cancer Institute. Standard solutions of these drugs were prepared from weighed samples by serial dilutions in methylene chloride. Trifluoroacetic anhydride and acetonitrile were obtained from Pierce (Rockford, IL, U.S.A.).

Plasma samples were extracted and prepared as previously described [9]. Variable volumes of urine (1—30 ml) were extracted twice with one-half volumes of diethyl ether—hexane after adding the internal standard. The extracts were combined, dried over anhydrous magnesium sulfate and the solvents evaporated under a nitrogen stream before derivatizing as described [9].

Instrumentation

Mass spectra and quantitative determinations were obtained from a Finnigan Model 3300F gas chromatograph—mass spectrometer with chemical ionization capability and interfaced with an Incos 2300 data system. The 1.2 m $\times$ 2 mm glass gas chromatographic column was packed with chemically bonded Carbowax 20M (Ultrabond 20M, RFR Corp., Hope, RI, U.S.A.). Chemical ionization mass spectra and selected ion chromatograms were obtained using methane, isobutane and ammonia reagent gases. Isobutane and ammonia were added as a make-up gas to the nitrogen carrier gas while methane also served as the chromatographic carrier gas. EI and CI data were obtained at 70 and 90 eV respectively.

RESULTS AND DISCUSSION

The mass spectra of trifluoroacetylated CCNU under EI, methane CI, isobutane CI, and ammonia CI conditions are compared in Fig. 1. The corresponding spectra of MeCCNU are very similar. As expected the mass spectra show less fragmentation in the order EI > methane CI > isobutane CI > ammonia CI. Surprisingly, the relative intensities of the protonated molecular ions in the methane and isobutane CI spectra are not significantly enhanced over that of the EI molecular ion, precluding their use in selective ion monitoring. However, the base ion in the ammonia CI spectrum is the ammonium adduct ion at m/z 414. Fig. 2 shows proposed decomposition pathways to account for the major ions derived from trifluoroacetylated CCNU under methane CI conditions.

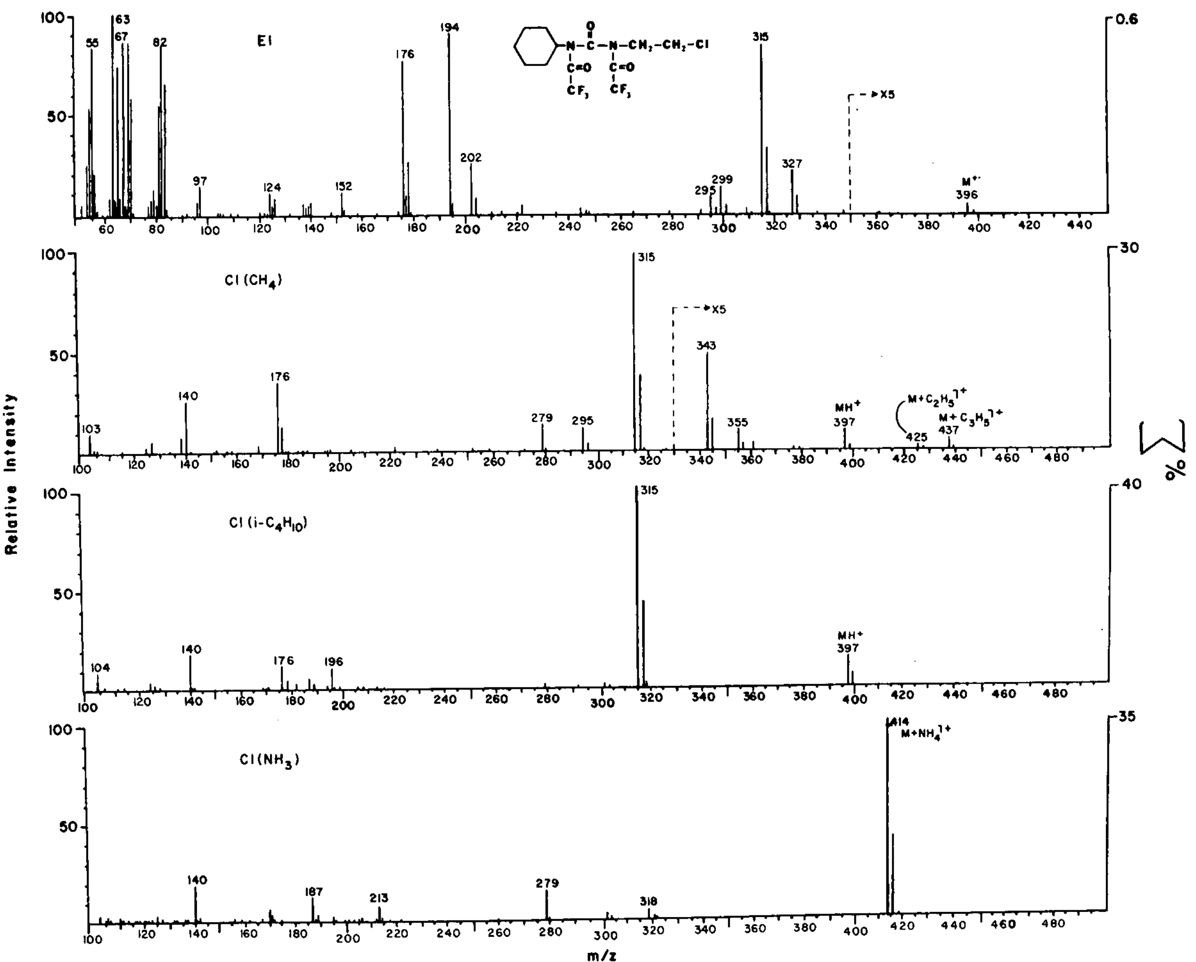

Fig. 1. Mass spectra of CCNU using EI, methane CI, isobutane CI and ammonia CI.

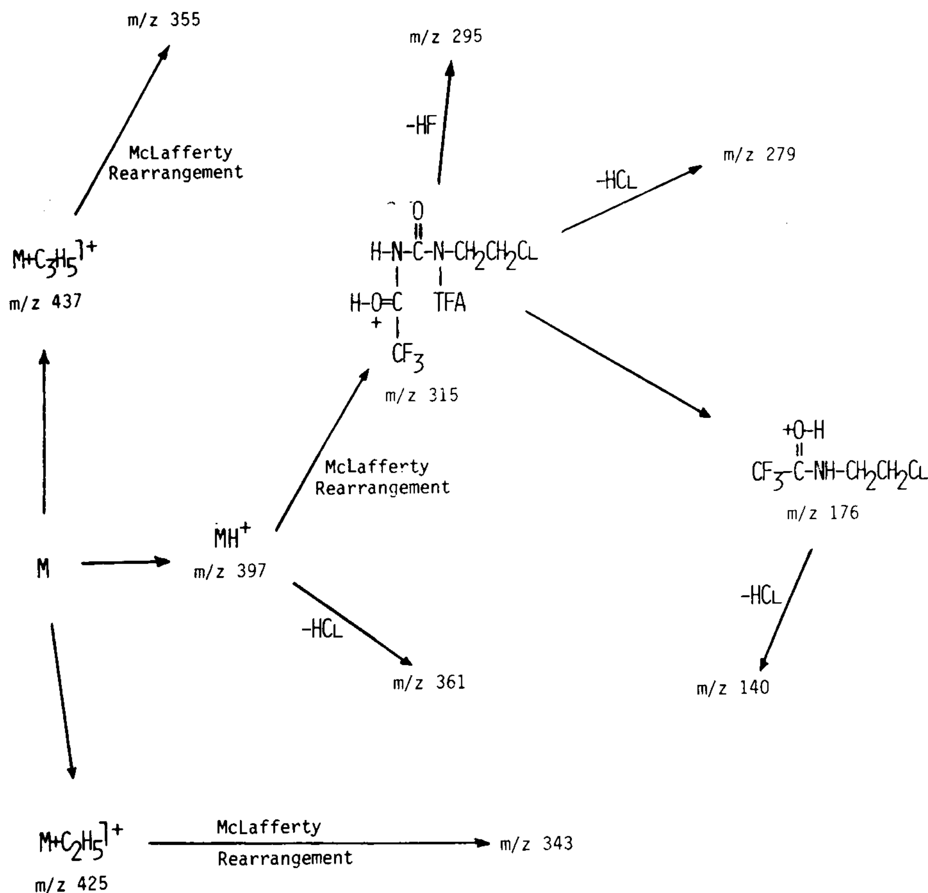

Fig. 2. Major decomposition pathways observed for CCNU after methane CI.

Selective ion monitoring at *m/z* 315 gives adequate sensitivity under EI conditions but interfering components from a few samples have necessitated changing to ions at *m/z* 317 or *m/z* 194. Despite the greatly reduced fragmentation with isobutane and ammonia reagent gases the sensitivity with these reagent gases was unsatisfactory. However, when monitoring *m/z* 315 with methane CI the responses for CCNU and MeCCNU appear slightly increased over those observed from EI. More important, the interference in the ion current chromatograms from extracted plasma is significantly reduced. Fig. 3 shows a selected ion chromatogram (*m/z* 315) of plasma containing 1.0 ng/ml of CCNU and 100 ng/ml of MeCCNU. The injected quantities were approximately 300 pg and 30 ng of CCNU and MeCCNU, respectively. A plasma blank exhibits no significant signal at the retention times of these two components. That the interference from plasma is minimal is also evidenced by a linear response over the drug concentration range of 1.0 ng/ml to 1.0 μg/ml.

Methane CI selective ion monitoring is being applied to pharmacology studies of CCNU and MeCCNU in experimental animals. The urinary excretion of unchanged MeCCNU from a dog after an intravenous administration

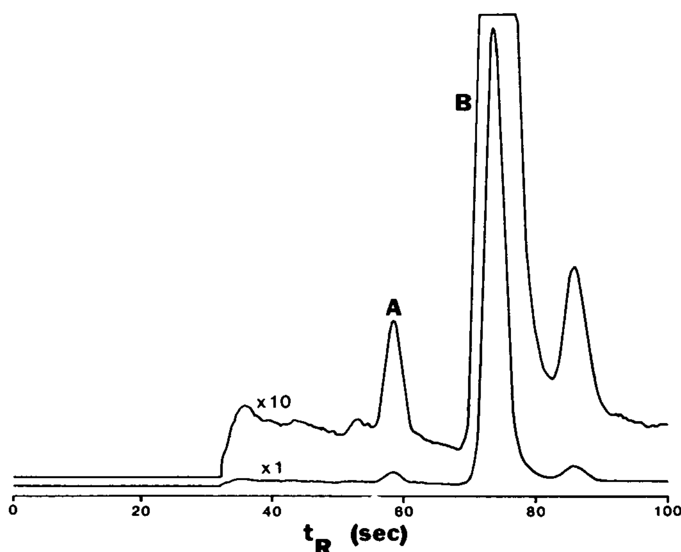

Fig. 3. Selected ion chromatogram (at m/z 315) for a plasma sample containing 1.0 ng/ml of CCNU (A) and 100 ng/ml of MeCCNU (B).

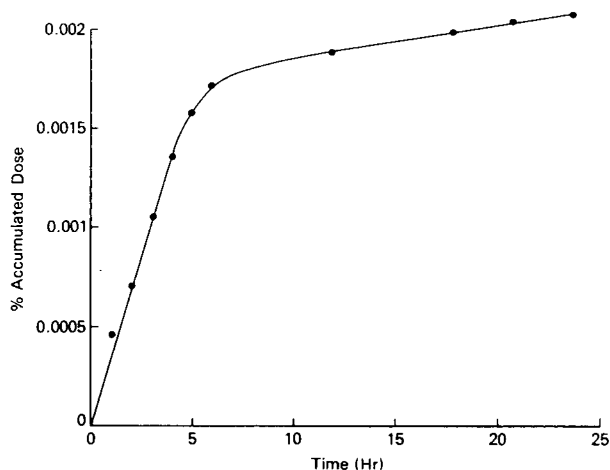

Fig. 4. Urinary excretion of MeCCNU from a dog after an intravenous administration of 15 mg/kg of this drug.

of 15 mg/kg of the drug is shown in Fig. 4. The excretion of the unchanged drug in minor, approximately 0.002% of the administered dose in 24 h. This is a dramatic contrast to the 50—60% of dose excreted by patients as measured by radioactivity [6]. This discrepancy is due to the nonspecific nature of the radiochemical assay and emphasizes the need for more definitive methodology.

CONCLUSION

Modification of the sensitive GC—MS selected ion monitoring assay for

CCNU and MeCCNU to use methane CI provides even greater sensitivity and selectivity; a ten-fold increase in sensitivity is realized by reducing the background signal due to endogenous components. Although this modification has not yet been applied to 1,3-bis(2-chloroethyl)-1-nitrosourea, it is reasonable to expect a similar improvement.

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Note

High-performance liquid chromatographic method for the determination of diethylamine, a metabolite of disulfiram, in urine*

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(First received October 20th, 1981; revised manuscript received December 21st, 1981)

Disulfiram (tetraethylthiuram disulfide, Antabuse®, Ayerst Labs., New York, NY, U.S.A.) is a drug used in the treatment of alcoholism in man [1]. Methods to measure compliance to a disulfiram regimen are important for two reasons: (1) in clinical practice to know if patients prescribed the drug are actually taking it, and (2) to determine the extent of compliance in clinical trials evaluating the efficacy of disulfiram. Disulfiram, after ingestion and absorption, is rapidly metabolized and excreted in the form of carbon disulfide, diethylamine and esters of diethyldithiocarbamate [2, 3]. The former is excreted in expired air and the latter two are excreted in urine [3, 4]. In previous studies, we found that after ingestion of disulfiram labelled with [1-^{14}C]diethylamine, 87% of the radioactivity was excreted in the urine and that a major portion of this radioactivity was in the form of [^{14}C]diethylamine [2]. We have been interested in the determination of diethylamine as a measure of disulfiram intake because diethylamine is not normally present in urine [5] but appears in increased concentrations after ingestion of disulfiram [2]. A thin-layer chromatographic (TLC) method was developed to measure diethylamine in urine [6] and this method has been used to measure compliance to a disulfiram regimen [7].

Although the TLC screening procedure proved to be a rapid and useful method for testing whether a patient was taking disulfiram regularly, we had some urine specimens which gave faint or questionable positive values for diethyl-

*This work was presented in part at the Medical-Scientific Conference of the National Alcoholism Forum, New Orleans, LA, April 12—15, 1981. An abstract appeared in Alcoholism: Clin. Exp. Res., 5 (1981) 163.

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amine. It was not possible to be certain whether these specimens were from patients who were still taking disulfiram but analyzed at a time when excretion of the drug was at a minimum or were from patients who were no longer taking the drug. We also, on occasion, found a few specimens from hospitalized patients who were known not to be taking disulfiram that gave faintly positive results. This necessitated that a reliable confirmational procedure be developed that could be used in combination with the TLC screening procedure to determine diethylamine in urine. This report presents a new high-performance liquid chromatographic (HPLC) method for the detection and quantitative analysis of diethylamine in urine.

MATERIALS AND METHODS

Derivatization of diethylamine

Diethylamine was purchased from Fisher Scientific (Pittsburgh, PA, U.S.A.) and ethylpropylamine, the internal standard, from The Ames Laboratory (Milford, CT, U.S.A.). The standard 3,5-dinitrobenzamide derivatives were prepared and purified as previously described [6]. Diethylamine and/or ethylpropylamine added to urine were converted to the benzamide derivative in the urine and extracted with diethyl ether [6, 7]. As previously reported, the efficiency of this derivatization is greater than 90% [6].

Processing of urine specimens

For analysis of unknown concentrations of diethylamine, 530 nmol of ethylpropylamine, the internal standard, were added per ml of urine prior to processing. The recovery experiments were performed with urine specimens from volunteers known not to be taking disulfiram. The urine specimens from a volunteer given disulfiram (250 mg) orally were collected and made acid with glacial acetic acid to a pH of less than pH 4.0. They were stored frozen (−10°C) until analysis. After thawing, the specimens were adjusted to pH 7.0 with 10 *N* sodium hydroxide. In all studies, 1 ml of urine was used for preparation of the benzamide derivative.

HPLC procedure

The HPLC separations were carried out on a Waters liquid chromatograph equipped with a Model 440 absorbance detector and a Model 6000A solvent delivery system. The residue from the benzoylation of urine was dissolved in ethanol (usually 0.5 ml) and a 15-μl aliquot was analyzed on a μBondapak C_{18} column (10 μm, 30 cm × 3.9 mm I.D.) with a mobile phase containing methanol—water (55:45, v/v) at a flow-rate of 2 ml/min. All chromatograms were run at ambient temperature. The column effluent was monitored by absorbance at 254 nm at a sensitivity of 0.5 a.u.f.s. Calibration curves and quantitation were determined using peak areas.

TLC procedure

The residue from the benzoylation of urine with no added internal standard was dissolved in chloroform (usually 0.5 ml). A 50-μl aliquot was applied to a TLC plate precoated with silica gel G, and containing a 254-nm phosphor

(Kontes, Vineland, NJ, U.S.A.). The plates were developed with chloroform–ethanol (99:1, v/v) and the resulting spots were visualized under short-wave ultraviolet light [6].

RESULTS

A chromatogram illustrating the separation of the benzamide derivatives of diethylamine and ethylpropylamine, the internal standard, is given in Fig. 1A. The benzamide derivatives of these amines and other secondary amines tested

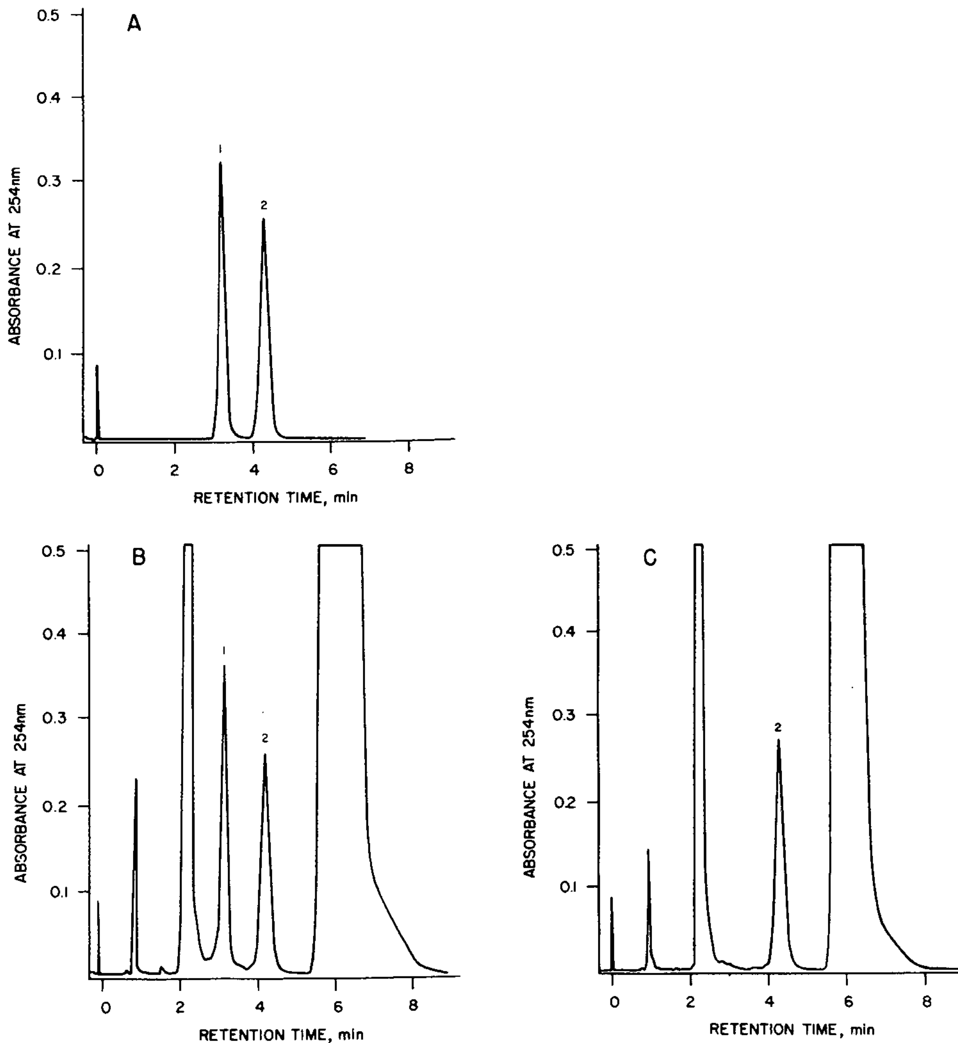

Fig. 1. Chromatograms of (A) standard N,N-diethyl-3,5-dinitrobenzamide (1) and the internal standard, N,N-ethylpropyl-3,5-dinitrobenzamide (2), (B) representative urine from a patient prescribed disulfiram to which the internal standard had been added, and (C) representative urine from a control patient not prescribed disulfiram to which the internal standard had been added.

but not present in the figure had the following retention times: N,N-dimethyl 2.3 min, N,N-diethyl 3.2 min, N,N-ethylpropyl 4.3 min, N,N-dipropyl 5.8 min, and N,N-dibutyl 16.0 min. The benzamide derivative of norpropoxyphene, the metabolite of Darvon which has previously been reported to give a spot by the TLC screening procedure that was near that of diethylamine [7], had a retention time of 2.6 min. Our derivatization is selective for secondary amines such as diethylamine; primary amines are removed by a washing procedure while tertiary amines do not react with the reagent [6].

In Fig. 1B, a representative chromatogram from urine of a patient known to be taking disulfiram is given. The peaks from the point of injection to 2.4 min and the one at 6.4 min are by-products of the benzoylation reaction and are found in all samples. The peak area for diethylamine was equivalent to 620 nmol of diethylamine per ml of urine. Control urine from a patient not taking disulfiram (Fig. 1C) does not give a response at the retention time for the derivative of diethylamine.

The calibration curves for the diethylamine and ethylpropylamine derivatives were determined. Linearity was observed between 1 and 20 nmol (r = 0.998 and 0.999, respectively). This response would be observed for concentrations of diethylamine ranging from 22 to 670 nmol per ml of urine. The limit of detection for diethylamine was 10 nmol/ml urine. We analyzed urine to which known quantities of diethylamine and the internal standard were added and we observed quantitative recovery of the diethylamine as the benzamide derivative (Table I).

TABLE I

THE RECOVERY OF DIETHYLAMINE ADDED TO URINE

Mean ± S.D., n = 4.

Added (nmol/ml)	Measured (nmol/ml)	Recovery from urine (%)
72.3	70.9 ± 7.3	98.0 ± 10.1
144.6	142.3 ± 26.0	98.4 ± 18.0
361.5	356.1 ± 56.5	98.5 ± 15.6
723.1	721.5 ± 9.3	99.8 ± 1.3

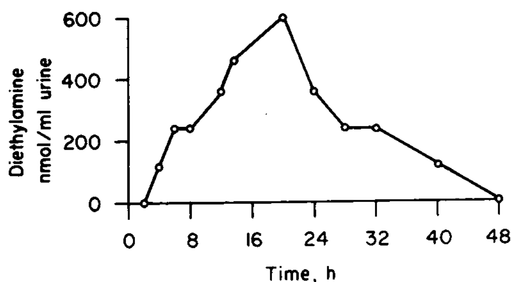

Fig. 2. Time course of urinary excretion of diethylamine in a human subject following oral administration of 250 mg disulfiram.

We also tested urine from a patient given a single dose of 250 mg disulfiram. Urine specimens were collected for 48 h after dosage. The diethylamine content of this urine is given in Fig. 2. No diethylamine was found 2 h after dosage. Values ranging from 40 to 600 nmol/ml urine were found 4 to 32 h after dosage and the urine was again negative at 48 h. When these same specimens were analyzed by our TLC screening procedure, the 6- through 24-h specimens were judged positive, the 4-, 28- and 32-h specimens were faintly positive or questionable, and the 2- and 48-h specimens were negative. When the same patient was given 250 mg disulfiram daily for 4 days and the urine was then analyzed by HPLC for diethylamine after the last dose, the 0- and 2-h specimens were also positive as well as the other specimens up to 96 h after dosage.

DISCUSSION

The present HPLC procedure provides an improved reliable method for the detection of diethylamine, a metabolite of disulfiram, in urine. This method allows both the detection and the quantitation of diethylamine in the urine of patients receiving disulfiram. The urinary concentration of diethylamine depends on the time of collection of the specimen after ingestion of disulfiram. Our method can reliably detect concentrations of diethylamine as low as 22 nmol/ml urine. This enables the detection of diethylamine up to 48 h after a single dose of disulfiram and 96 h after multiple doses. Because specimens that are collected from patients during a period when diethylamine excretion is minimal will be correctly identified as containing diethylamine by HPLC, this method can separate urine specimens which appear questionably or faintly positive by TLC into true positives or true negatives. The procedure when combined with the TLC screening assay provides an accurate method for determining compliance to a disulfiram regimen.

ACKNOWLEDGEMENTS

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CHROMBIO. 1194

Note

Determination of N-methylpyridinium 2-aldoxime methylsulfate (Contrathion®) in rat plasma and urine by high-performance copper(II)—silica ligand-exchange chromatography

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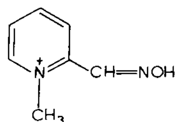
A number of oximes have been found to reactivate the cholinesterases inhibited by organophosphates [1]. A quaternary pyridinium structure containing an oxime function in combination with atropine has proved effective in the treatment of poisoning by some of these toxic chemicals and in reducing their toxicity in various animal species [2].

The chemical structure of the cationic moiety, N-methylpyridinium 2-aldoxime, is



Several salts are used: N-methylpyridinium 2-aldoxime chloride, iodide, methanesulfonate and methylsulfate (Contrathion®). The aim of this paper was to study the determination of Contrathion in rat plasma and urine for use in pharmacokinetic studies.

Several analytical methods have been described for the quantitation of N-methylpyridinium 2-aldoxime in biological fluids, including hydrolysis (with hydroxylamine) of the deproteinized supernatant, and absorptiometric analysis [3] or direct spectrophotometric measurement of absorption of the oximate

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anion at 335 nm [4, 5]. This latter method has been made more rapid and accurate by automatization [6].

Over the past five years, modern liquid chromatography has established itself as the choice method for solving many biochemical and clinical analysis problems. Recently a quantitative determination of another oxime (N,N-trimethylene bis-pyridinium-4-aldoxime, TM B-4) was achieved using ion-pair chromatography coupled with spectrophotometric detection at 254 nm [7, 8]. This chromatographic technique could be used for the quantitative estimation of Contrathion in biological fluids, but we prefer ligand-exchange chromatography on copper(II)-modified silica gel, previously developed in our laboratory [9—13]. A primary benefit of ligand-exchange chromatography on copper(II)-modified silica-gel particles compared with ion-pair chromatography is the improvement of selectivity and a better efficiency compared with ligand-exchange chromatography on classical polystyrene—divinylbenzene resins.

An additional advantage of ligand-exchange chromatography is the use of an alkaline mobile phase, the Contrathion molar absorptivity being higher in basic medium than in acidic medium [14]. Furthermore, the selectivity is better in a basic mobile phase owing to the fact that the wavelength used for detection (340 nm) is higher in an acidic medium (295 nm). This last observation is very important for the analysis of complex mixtures such as biological fluids.

EXPERIMENTAL

Apparatus

This study was performed with a Hewlett-Packard (Waldbronn, G.F.R.) Model 1084B liquid chromatograph equipped with an automatic sampling system (79842A) and a variable-wavelength detector (190—540 nm) (79875A) operating at 340 nm.

Stationary phases

Copper(II)-modified silica-gel columns were prepared as described previously [9]. Stainless-steel columns (15 × 0.48 cm I.D.) were packed, according to the slurry packing technique, with pure Partisil 5 silica gel of 7 μm mean particle size (Whatman, Clifton, NJ, U.S.A.). An aqueous solution of copper(II) sulfate (10^{-2} mol/l) and ammonia (1 mol/l) was then allowed to percolate through the column until equilibrium was reached [when copper(II) ions appeared in the effluent]. The column was then fitted on the chromatograph and equilibrated with the mobile phase.

Chemicals

Acetonitrile was of LiChrosolv grade and was purchased from E. Merck (Darmstadt, G.F.R.). Ammonia was of Rectapur grade and was obtained from Prolabo (Paris, France). Copper(II) sulfate was of Purissimum grade from Fluka (Buchs, Switzerland). The water was bidistilled. Contrathion® and the N-methylpyridinium 4-aldoxime methanesulfonate used as internal standard were supplied by our department of organic synthesis.

Preparation of samples

Plasma. Each 100 μl of plasma with an added 10 μl of internal standard

solution was deproteinized with 500 µl of acetonitrile. After being shaken vigorously the samples were centrifuged. Aliquots of 20 µl were injected into the chromatographic column.

Urine. A suitable volume, usually 1 ml or less, was diluted with distilled water to 10 ml and a protein-free solution was prepared as described for plasma.

RESULTS AND DISCUSSION

Choice of mobile phase composition

In a previous paper [9] we demonstrated that the retention on copper(II)-modified silica gel is governed by both ligand-exchange and normal-phase partition mechanisms. Consequently, we can take it for granted that the solute capacity factor (k') is a function of both ligand and organic modifier concentrations in the mobile phase.

The variations in Contrathion and internal standard capacity factors with ammonia concentration in the mobile phase (acetonitrile—water, 75:25, v/v) are shown in Fig. 1. The higher the ammonia concentration in the mobile phase the smaller the capacity factor. This behaviour is in good agreement with a ligand-exchange mechanism.

The variations in Contrathion and internal standard capacity factors with acetonitrile content in the mobile phase containing 0.5 mol/l ammonia are shown in Fig. 2.

The capacity factor increases as the acetonitrile volume fraction in the

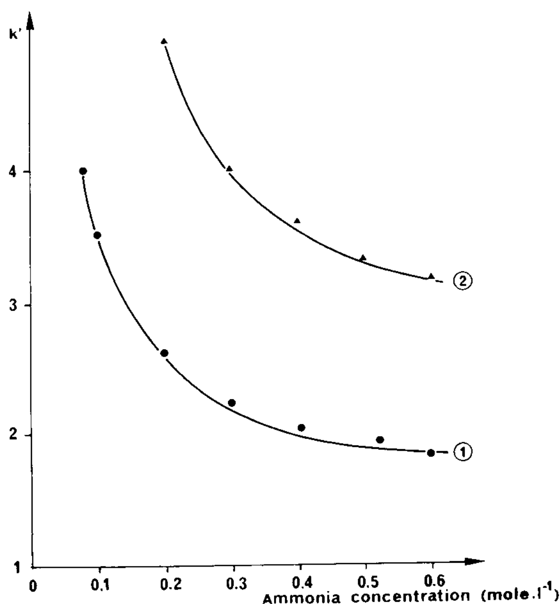

Fig. 1. Variation of capacity factor (k') of Contrathion® and internal standard with ammonia concentration in the binary mixture acetonitrile—water (75:25, v/v). Column: 15 × 0.48 cm I.D. Packing: copper(II)-modified silica gel (Partisil 5), 7 µm. Flow-rate: 1.5 ml/min. 1 = Contrathion; 2 = internal standard.

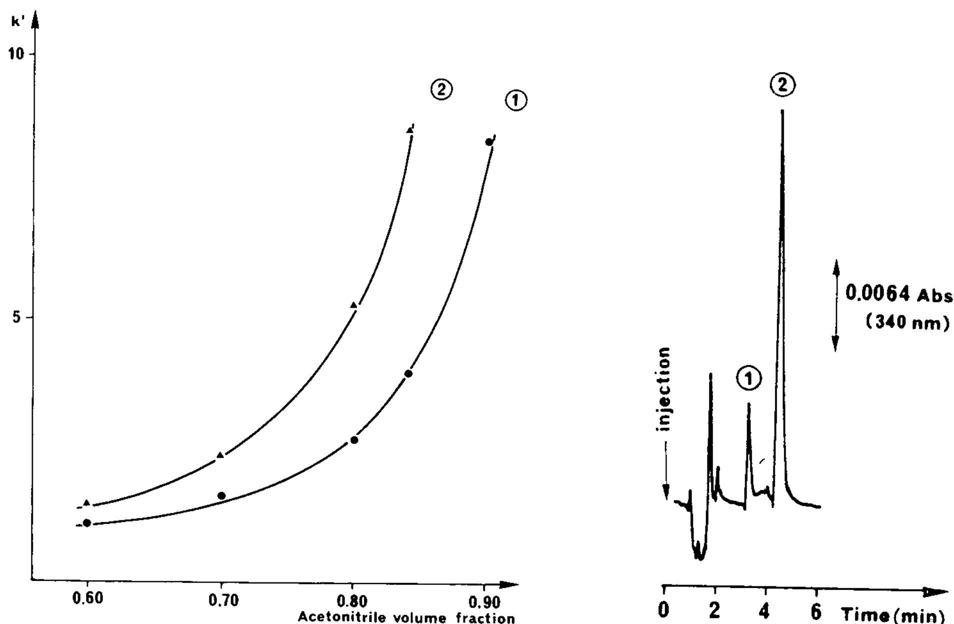

Fig. 2. Variation of capacity factor (k') of Contrathion® and internal standard with volume fraction of acetonirile in the binary mixture acetonitrile—water. Ammonia concentration: 0.5 mol/l. Operating conditions as in Fig. 1. 1 = Contrathion; 2 = internal standard.

Fig. 3. Ligand-exchange chromatographic separation of Contrathion® in rat plasma 2 min after a single muscular injection of 60 mg/kg. Internal standard was injected after prelevement. Column: 15 × 0.48 cm I.D. Packing: copper(II)-modified silica gel (Partisil 5), 7 μm. Mobile phase: acetonitrile—water (75:25, v/v) with ammonia 0.5 mol/l. Flow-rate: 1.5 ml/min. ΔP: 60 atm. Temperature: 30°C. Detection: UV at 340 nm. Injected volume: 20 μl. 1 = Contrathion; 2 = internal standard.

mobile phase increases, that is to say, as it becomes increasingly hydrophobic. In a mobile phase rich in acetonitrile, the solubilities of hydrophilic molecules such as Contrathion and internal standard are smaller than in a mobile phase poor in acetonitrile. Thus, the normal-phase partition mechanism also governs the variations in capacity factor.

Following from these results, a mobile phase having an acetonitrile—water composition of 75:25 (v/v) and an ammonia concentration of 0.5 mol/l was selected for the Contrathion analysis. Fig. 3 illustrates ligand-exchange analysis of Contrathion in rat plasma 2 min after a single intramuscular injection of 60 mg/kg. The plasma level of Contrathion measured is $42 \cdot 10^{-6}$ mol/l (UV absorption at 340 nm). No interference from biological components of plasma was observed.

Quantitative analysis

The calibration plot is linear from 5 pmol to 1 μmol, and passes through the origin, for aqueous solutions as well as for deproteinized biological samples. Unfortunately, some loss of Contrathion is observed during the plasma work-up procedure and the yield is only ca. 90%. In this latter case, the calibration plot was constructed using samples of rat plasma spiked with Contrathion.

The reproducibility is high both for the chromatographic technique itself (relative standard deviation measured for retention time is 0.5%) and for the quantitative analysis. The relative standard deviations are 0.6%, 1.8% and 4.5% for Contrathion quantities close to 0.6, 0.1 and 0.05 nmol injected, respectively.

These results prove the good stability of chromatographic columns filled with copper(II)-modified silica gel eluted with mobile phases containing less than 50% water. For higher water concentrations the silica support is slowly attacked by the hydroxide ions.

Detection limit

Fig. 4 shows the chromatogram (for a rat plasma sample) corresponding to the injection of 20 pmol of Contrathion and internal standard.

The detection limit is generally taken as the solute quantity that will provide a signal-to-noise ratio of two. According to this definition, 5 pmol of Contrathion are detected. In consequence of sample preparation and an injected volume of 20 μl, the detection limit in rat plasma and in urine is $1.5 \cdot 10^{-6}$ mol/l.

With operating conditions (ion-pair chromatography) described in a recent publication [8] a detection limit of 30 pmol has been obtained (at 295 nm) by us. Ligand-exchange chromatography at 340 nm is six times more sensitive.

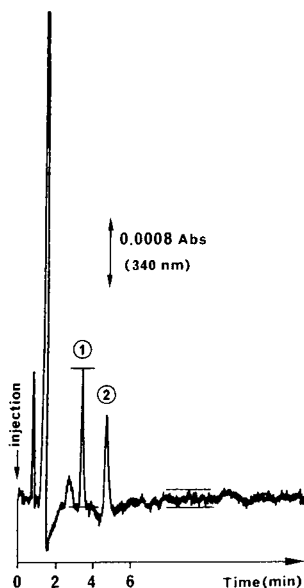

Fig. 4. Separation of 20 pmol of Contrathion® and internal standard in spiked rat plasma. Operating conditions as in Fig. 3. 1 = Contrathion; 2 = internal standard.

CONCLUSION

Ligand-exchange chromatography on copper(II)-modified silica-gel microparticles appears to be the best chromatographic technique for quantitative analysis of Contrathion and its derivatives in biological fluids. Advantages are

high selectivity due to the ligand-exchange mechanism, high efficiency due to silica microparticles and very good mechanical properties of the silica-gel matrix. Also, this new technique for ligand exchange constitutes an interesting alternative to ion-pair chromatography for the separation of numerous hydrophilic compounds which are donors of electronic lone pairs.

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CHROMBIO. 1196

Note

Quantitative determination of 1-(2-chloroethyl)-3-(β-D-glucopyranosyl)-1-nitrosourea in blood and urine of man*

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(First received December 12th, 1980; revised manuscript received December 8th, 1981)

A newly synthesized antitumor drug, 1-(2-chloroethyl)-3-(β-D-glucopyranosyl)-1-nitrosourea (GANU), is a water-soluble nitrosourea derivative. In animal experiments, GANU exhibited high activity against experimental tumors; its action was mainly associated with inhibition of DNA synthesis [1, 2]. In this communication, a method for the estimation of blood levels as well as of the urinary excretion of GANU has been developed using high-performance liquid chromatography (HPLC).

MATERIALS AND METHODS

GANU was thoroughly dissolved in physiological saline solution just before injection. In the case of drop infusion, 250 ml of saline were used, and 20 ml of saline were used in the case of bolus injection.

Blood samples were collected at 15, 30, 55, 65, 75, 90, 120 and 180 min after injection. A 2-ml volume of the blood sample was heparinized with 0.1 ml of heparin and immediately poured into a tube with 8 ml of a cooled mixture of isotonic citrate buffer (pH 4.0) and 0.9% sodium chloride solution (1:3, v/v). The mixture obtained was carefully mixed to avoid hemolysis. The successive procedures are summarized in Fig. 1. The sample thus obtained was dissolved in 0.1 *M* acetate buffer (pH 4.0) and used for HPLC.

The HPLC apparatus was from Waters Co. Ltd. A stainless-steel column (300 × 3.9 mm) was packed with μBondapak C_{18} (8—10 μm). As the mobile

*A preliminary report was presented at the 39th Annual Meeting of the Japanese Cancer Society in November, 1980, in Tokyo.

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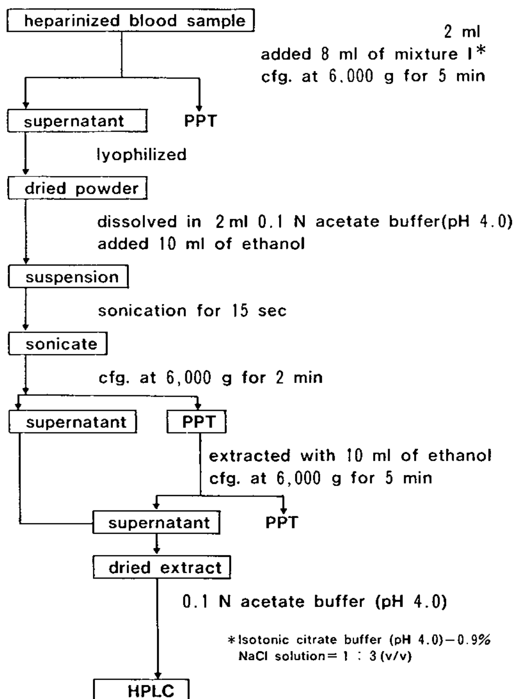

Fig. 1. Procedure for the extraction of GANU from human blood. Whole blood was used for the extraction of GANU. The mixture of isotonic citrate buffer (pH 4.0) and saline solution (1:3, v/v) was used to avoid the decomposition of GANU during the extraction procedures.

phase, a water—methanol mixture (9:1, v/v) was used throughout the experiments. Details of the conditions for the determination of GANU are described in the legends to the figures. The elution pattern was detected at 254 nm using a recorder from National Electric Co. Ltd.

Urine was also collected into bottles containing 0.1 *M* acetate buffer (pH 4.0) at different time intervals, then centrifuged at 6000 *g* for 10 min. The supernatant solution thus obtained was immediately used for HPLC. The HPLC procedure was the same as that for blood samples.

Water used for these experiments was prepared by a water purifier, Milli-R/Q, Millipore Co. Ltd. Methanol was chromatographic grade and was obtained from Wako Chemical Co., Tokyo, Japan. All other chemicals of the highest quality were obtained from commercial sources.

The solution of the authentic sample of GANU was prepared by dissolving 2 mg of GANU in 1 ml of water, and used for HPLC. Also, 2 mg of GANU were dissolved in 10 ml of pooled plasma and extracted by the method described in Fig. 1. Urine samples containing various concentrations of GANU were used for HPLC without further purification.

RESULTS

Separation of authentic samples

When a water—methanol mixture (9:1, v/v) was used as mobile phase, the retention time of GANU was 4.6 min (Fig. 2A), but the peak decreased in size when 0.2 *M* ammonia solution (pH 8.4) was used as mobile phase (Fig. 2B).

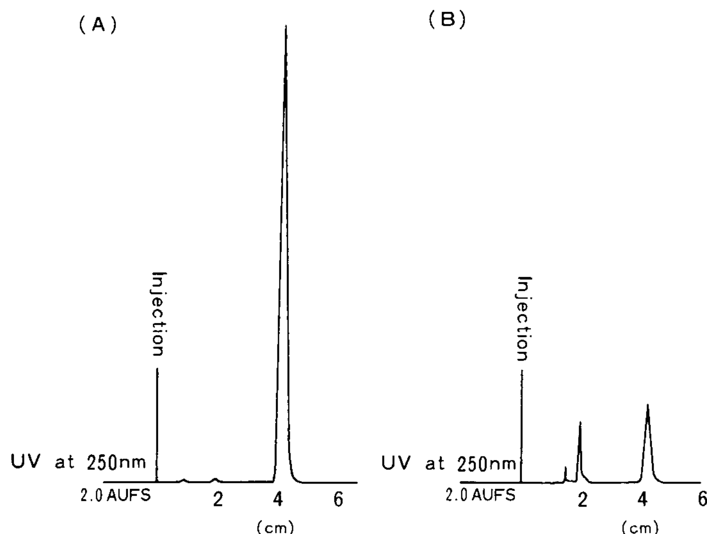

Fig. 2. Elution pattern of GANU and its decomposition product by different eluting solutions. (A) GANU is eluted at 4.6 min without any decomposition product when a water—methanol mixture (9:1, v/v) is used as mobile phase. (B) 0.12 *M* ammonia solution (pH 8.4) has been used as mobile phase. The height of the GANU peak has decreased and a new peak has appeared at 2.1 min.

Recovery of GANU from pooled plasma

The HPLC pattern of GANU extracted from plasma is presented in Fig. 3A. The peak of authentic GANU has a retention time of 4.6 min and no disturbing peak has been observed in the area of the GANU peak (Fig. 3B). The recovery of GANU was calculated to be approximately 68%.

Calibration curve for the estimation of GANU in human blood

The calibration curve is linear until a GANU concentration of 5 μg/ml. The calibration equation has been calculated to be $y = 5.209x - 0.208$ ($r = 0.999$) and the recovery has been estimated as 68.8%. The coefficient of variation has been estimated as 3.8%.

Calibration curve for the estimation of GANU in human urine

The calibration curve is linear until 500 μg/ml GANU. The recovery of GANU from urine is 76.4%, and the r value 0.7754.

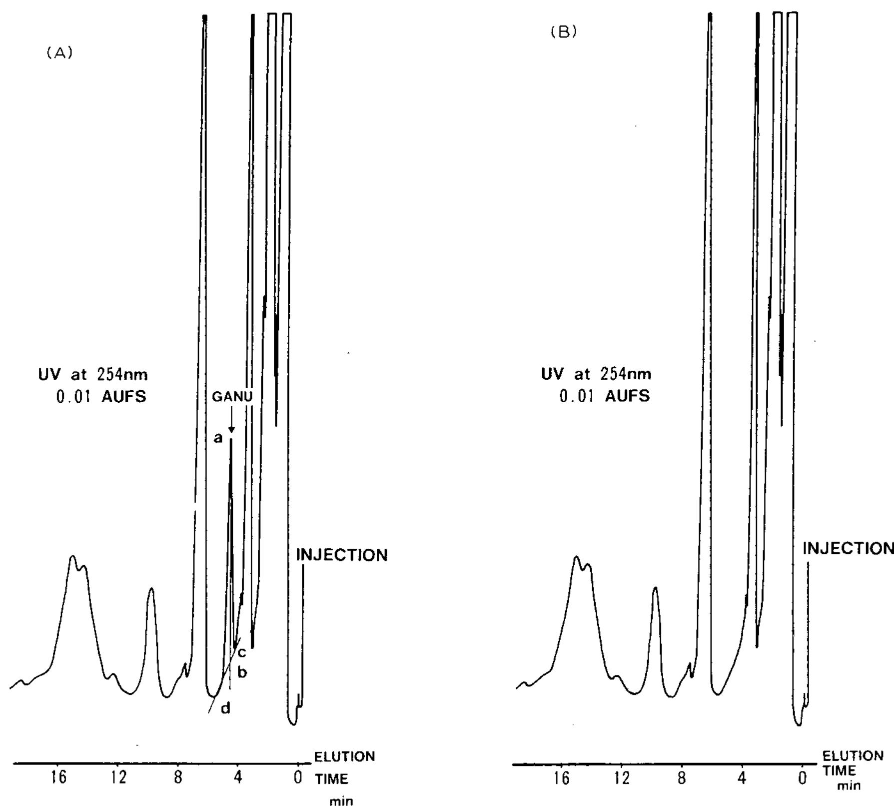

Fig. 3. Chromatograms of GANU extracted from human plasma. HPLC conditions: column 30 × 0.4 cm, μBondapak C_{18}; mobile phase, water—methanol (9:1, v/v); flow-rate 2 ml/min; chart speed 0.5 cm/min. (A) Authentic GANU was mixed with pooled human plasma and extracted by the method described in the text. The concentration was determined using the height between a and b. (B) Blank plasma assayed by the same method. No disturbing peak was observed in the region of the GANU peak.

Concentration of GANU in human blood

The concentration of GANU has been determined in the blood of two patients with cancer for each of the doses 60, 80, 100, 120, 140, and 160 mg/m^2. From the observed concentrations of GANU in the blood the pharmacokinetic data calculated by one-compartment theory have been demonstrated as shown in Table I.

In addition, the same dose of GANU was injected by bolus injection into two patients. The concentration of GANU was assayed, and the pharmacokinetic data are presented as well (Table I).

Excretion of GANU in human urine

As shown in Table II, the excretion of GANU (dose administered 120 mg/m^2) in the urine was 49.5 μg/dl in the initial hour, but after that no GANU was detected in the urine in either case. The excretion rate of GANU was estimated to be 2.11%. Metabolites of GANU such as 1-(2-chloroethyl)-3-(β-D-

TABLE I

COMPARISON OF PHARMACOKINETIC PARAMETERS BETWEEN DROP INFUSION AND INTRAVENOUS BOLUS ADMINISTRATION

Dose administered = 120 mg/m^2, n = 2.

	Drop infusion	Bolus injection
Elimination constant (min^{-1})	0.0873	0.162
Half-time of blood level (min)	7.98	4.63
Distribution volume (l/m^2)	18.0	5.66

TABLE II

URINARY EXCRETION OF GANU IN HUMANS AFTER CONSTANT INTRAVENOUS DROP INFUSION OF GANU

Dose administered = 120 mg/m^2.

Subject	Time after administration (h)	Urine volume (ml)	Concentration of GANU (μg/ml)	Amount recovered (mg)	Percentage of dose
A	0–1	60	34.8	2.09	1.11
	1–2	180	N.D.*	—	—
	2–6	220	N.D.	—	—
	6–24	630	N.D.	—	—
	0–24	1090	—	2.09	1.11
B	0–1	92	64.2	5.90	3.11
	1–2	63	N.D.	—	—
	2–6	1100	N.D.	—	—
	6–24	1980	N.D.	—	—
	0–24	3235	—	5.90	3.11

*N.D. = not detected.

glucopyranosyl)urea (GAU) and β-D-glucopyranosylamine (1-AG) have never been detected by the method described above.

DISCUSSION

The assay method for GANU has been established by HPLC in acidic solution. GANU is freely soluble in water but is unstable in the light, at high temperatures and at alkaline pH [3]. Therefore, to prevent the degradation of GANU in blood and urine of patients, it was essential that the biological materials are immediately mixed with acid solution after collection, as shown in Fig. 1. By adding the acid solution, the extraction and purification of GANU from biological material have been quantitatively developed. However, it was not necessary to add saline solution in the case of urine. The recovery of GANU from pooled plasma has been estimated as approximately 68%, and the recovery from urine has been estimated to be 76%. The limit of sensitivity was 100 ng/ml in blood and 5 μg/ml in urine.

The concentration of GANU in the blood and urine of the patients who have been administered GANU either by drop infusion or bolus injection has been assayed by the method described above. GANU was detected until 90 min in cases of drop infusion and 30 min with bolus injection. On the other hand, GANU was only detected in the urine in the first hour. GANU was not detected after 1 h of drop infusion at bolus injection because of the dilution of GANU in urine. Metabolites of GANU such as GAU and 1-AG have not been detected in either blood and urine; this is probably due to the low concentration of these compounds in biological materials. The quantitative determination of these metabolites should be investigated by a different method. However, the method described in this communication has been sufficient to assay the concentration of GANU in human blood and urine and to work out details of the pharmacokinetics of this compound.

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CHROMBIO. 1198

Note

Reversed-phase high-performance liquid chromatographic method to determine vidarabine and hypoxanthine arabinoside in biological fluids

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Vidarabine is an arabinosyl nucleoside used to treat various herpes virus infections including herpes simplex keratitis, herpes simplex encephalitis, and herpes zoster in immunosuppressed patients.

Humans rapidly deaminate vidarabine to arabinosyl hypoxanthine (ara-Hx) [1]. Ara-Hx exhibits antiviral activity although to a lesser extent than vidarabine. Methods for measuring vidarabine in biological fluids for clinical or pharmacokinetic studies must be able to measure ara-Hx as well as vidarabine, because of the rapid appearance of the deaminated metabolite [2].

Previously described methods to determine vidarabine and ara-Hx concentrations have relied on measurement of total antiviral activity in tissue culture [3] or on ion-exchange high-performance liquid chromatography (HPLC) methods [4]. The biological assays are cumbersome, suffer from considerable random variation inherent with the assay system, and do not distinguish between parent drug and active metabolites. The ion-exchange HPLC method of Schneider and Glazko [4] requires a minimal chromatography time of 30 min and is subject to interference by endogenous and exogenous arabinoside analogues.

We describe here a reversed-phase HPLC method to quantitate vidarabine and ara-Hx. This method requires a small sample volume, provides the required sensitivity and precision, and substantially reduces chromatographic time. Other arabinosides, as well as a number of other drugs, do not interfere with the assay.

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MATERIALS AND METHODS

Reagents

Pentostatin (adenosine deaminase inhibitor) was supplied by Warner-Lambert (Ann Arbor, MI, U.S.A.). Vidarabine and ara-Hx analytical standards and isoamyl alcohol were obtained from Sigma (St. Louis, MO, U.S.A.). HPLC grade sodium pentanesulfonate, chloroform, acetonitrile and acetone were obtained from Fisher Scientific (Fairlawn, NJ, U.S.A.).

Vidarabine and ara-Hx stock standards containing 0.5 mg/ml and 1.0 mg/ml, respectively, were prepared monthly in deionized water alkalinized with two drops of concentrated ammonium hydroxide and stored at 5°C. The mobile phase consisted of 20 ml of acetonitrile in 480 ml of a 5 m*M* sodium pentanesulfonate buffer, pH 7.2 at room temperature.

Chromatography

The chromatography was performed with a Perkin-Elmer Model 601 high-performance liquid chromatograph equipped with an LC 55 UV/VIS variable-wavelength detector interfaced to a Sigma 10 data system (Perkin-Elmer, Norwalk, CT, U.S.A.). The data system provided the integrated areas under the peaks and the retention times for vidarabine and ara-Hx. A 25 × 0.46 cm LiChrosorb RP-8, 5-μm particle size, reversed-phase column (Supelco, Bellefonte, PA, U.S.A.) was used for the chromatography. The oven temperature was maintained at 40°C, the flow-rate was 1.0 ml/min, and the effluent was monitored at 250 nm.

Procedure

Pentostatin was added to all blood samples at the time of venipuncture to inhibit any in vitro deamination of vidarabine to ara-Hx in patient and control sera. Two hundred μl of sample (serum, cerobrospinal fluid, or urine diluted 1:10 with distilled water) were placed into a 1.5-ml Eppendorf tube. Twenty μl of isoamyl alcohol (to prevent emulsion formation) and 50 μl of chloroform were added. The tube was vortexed for 30 sec and centrifuged for 10 min at 20,931 *g* in an Eppendorf 3200 centrifuge. The aqueous layer was transferred to another Eppendorf tube to which was added 1.0 ml cold acetone (0°C). This mixture was vortexed for 10 sec and centrifuged for 5 min. The supernatant was transferred to a clean 10 × 75 mm culture tube and evaporated to dryness at 40°C under nitrogen. The dried sample was reconstituted with 50 μl of deionized water just prior to injection into the chromatograph.

RESULTS

Typical chromatograms of vidarabine and ara-Hx are shown in Fig. 1. Retention times for ara-Hx, pentostatin, and vidarabine were 4.30, 5.82, and 6.65 min, respectively. Fig. 1A shows a chromatogram of extracted blank serum. Fig. 1B illustrates a chromatogram of drug-free serum spiked with 5.0 μg/ml ara-Hx and 2.5 μg/ml vidarabine. Fig. 1C is a chromatogram obtained from a patient serum sample in which the determined concentration of ara-Hx was 3.4 μg/ml. Similar chromatograms were obtained from urine and cerebrospinal fluid.

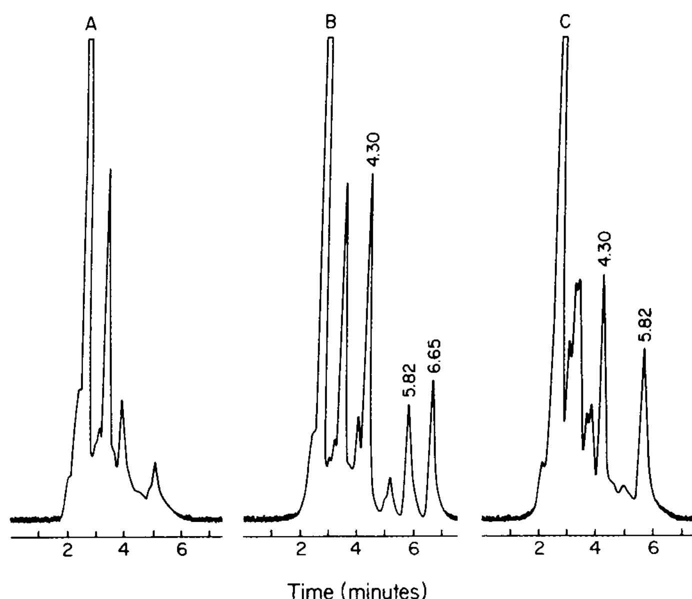

Fig. 1. Chromatograms obtained from: (A) blank serum; (B) serum prepared with 5.0 μg/ml ara-Hx and 2.5 μg/ml vidarabine; (C) patients' serum determined as 3.4 μg/ml ara-Hx. Vidarabine was not detected. The retention times for ara-Hx, pentostatin and vidarabine were 4.30, 5.82, and 6.65 min, respectively.

A standard curve was constructed by adding ara-Hx and vidarabine to five sets of drug-free serum or urine samples to yield concentrations in the range of 1.0—40.0 μg/ml and 0.5—20.0 μg/ml, respectively. Concentrations and peak areas were linearly related over the respective ranges in both serum and urine. The least squares regression for vidarabine was $y = 0.943x + 0.48$ and for ara-Hx was $y = 1.05x - 0.558$. The coefficient of correlation for both lines was 0.998.

Within-run precision was evaluated by assaying five aliquots of each of the following prepared serum standard concentrations: 0.5, 5.0, and 20.0 μg/ml of vidarabine; 1.0, 10.0, and 40.0 μg/ml of ara-Hx (Table I). Between-run

TABLE I

WITHIN-RUN PRECISION IN SERUM

In all cases $n = 5$.

	Mean (μg/ml)	S.D. (μg/ml)	C.V. (%)
Vidarabine	0.6	0.1	16.7
	5.0	0.3	6.0
	21.3	0.9	4.0
Ara-Hx	1.3	0.1	7.6
	10.0	0.6	6.0
	28.0	0.9	3.2

precision was determined by freezing aliquots of a pooled, drug-free serum to which vidarabine and ara-Hx were added and assaying the aliquots over a period of one month (Table II). There was no significant loss of vidarabine or ara-Hx during storage at −20° C for one month.

Five samples at each of the three different concentrations were assayed to determine the analytical recovery of known amounts of vidarabine and ara-Hx. Recovery of vidarabine from serum concentrations of 1.25, 5.0, and 20 μg/ml was 78, 80, and 84%, respectively. Recovery of ara-Hx from serum concentrations of 2.5, 10.0, and 40.0 μg/ml was 76, 80, and 78%, respectively.

The detection limit of this assay for both vidarabine and ara-Hx is 0.5 μg/ml in serum and cerebrospinal fluid and 2.5 μg/ml in urine.

Penicillin G, hydroxyzine, chlorothiazide, phenobarbitol, theophylline, prednisone, cytosine arabinoside, uracil arabinoside, guanine arabinoside, nystatin, kanamycin, sulfamethoxazole, trimethoprim and metaproterenol did not produce interfering peaks. Serum and urine samples obtained from patients receiving cancer chemotherapeutic agents were also free of interfering substances.

TABLE II

BETWEEN-RUN PRECISION

		Vidarabine	Ara-Hx
Serum	Mean (μg/ml)	8.5	7.0
	S.D. (μg/ml)	0.6	0.7
	C.V. (%)	7.0	10.0
	n	8	8
Urine	Mean (μg/ml)	7.6	7.3
	S.D. (μg/ml)	1.1	0.6
	C.V. (%)	14.5	8.2
	n	9	9

DISCUSSION

Previously described methods for measuring vidarabine and ara-Hx in biological fluids have relied on bioassays in tissue culture or ion-exchange HPLC methods. Biological assay systems measure total antiviral activity and do not distinguish between parent drug and active metabolites. In addition, tissue culture assays require prolonged incubation time resulting in excessively long turn-around time for the assay. The ion-exchange HPLC method described by Schneider and Glazko [4] also requires long turn-around time because of the chromatography time of 30 min. The latter method is also susceptible to interference from endogenous and exogenous analogues of vidarabine.

The method described here reduces the chromatography time to approximately 8 min, requires a sample size of only 200 μl and is not subject to interference from a number of concomitantly administered drugs or endogenous nucleosides. The short chromatographic time and small sample size make this

method particularly useful in monitoring vidarabine and ara-Hx levels during therapy in infants and small children.

We have observed serum and urine concentrations of vidarabine and ara-Hx in infants and children during therapy which are comparable to those reported in adult patients. Vidarabine is rarely detected because it is rapidly deaminated in vivo and is present in concentrations less than the minimum level of detection of our method (< 0.5 μg/ml in serum and cerebrospinal fluid and < 2.5 μg/ml in urine). Ara-Hx concentrations typically range from 2.0 to 12.0 μg/ml in serum depending on the dose and the time after the dose at which the sample is obtained.

The reproducibility, precision, and simplicity of the method described herein make it practical for use in any clinical laboratory with HPLC capability.

ACKNOWLEDGEMENT

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Note

Simultaneous determination of acetaminophen, theophylline and salicylate in serum by high-performance liquid chromatography

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Determination of acetaminophen, theophylline and salicylate is commonly requested in our laboratory, for studies of both drug therapy and drug intoxication. A method for the simultaneous determination of the three drugs would therefore be very useful.

Many methods for the separate high-performance liquid chromatographic (HPLC) determination of acetaminophen, theophylline and salicylate have been described [1—26]. The simultaneous determination of acetaminophen and salicylate was reported by Miceli et al. [27]. However, theophylline was not resolved from acetaminophen and could therefore interfere.

Preliminary results using isocratic reversed-phase liquid chromatography with radially compressed columns, have indicated that the composition of the mobile phase is critical to obtain resolution of the peaks. A simple pretreatment of the serum sample, e.g. addition of acetonitrile to precipitate proteins, centrifugation and injection of the clear supernatant liquid, was investigated and found not to be applicable since endogenous compounds interfering with the determination of acetaminophen were detected.

EXPERIMENTAL

Apparatus

A Liquid chromatography pump Model 45 (Waters Assoc., Milford, MA, U.S.A.) connected to a UV detector Model 445 (Waters Assoc.) were used for the determinations. The detector was equipped with a 280-nm interference filter. The liquid chromatograph was connected to a Sigma 10 Chromatography Data Station (Perkin-Elmer, Norwalk, CT, U.S.A.).

The HPLC column was a plastic C_{18} column (10 cm × 5 mm I.D.; 10 μm

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particle size) fitted into a radial compression module RCM 100 (Waters Assoc.).

The mobile phase was 28% (v/v) of methanol in acetate buffer (pH 3.6). (Buffer preparation: to 2.7 g of sodium acetate in 1000 ml of deionized water were added 15.0 ml of concentrated acetic acid.) The mobile phase flow-rate was 1.0 ml/min.

Reagents

Sodium acetate and concentrated acetic acid were of A.R. grade (Merck, Darmstadt, G.F.R.). Methanol, methylene chloride and isopropanol were of HPLC grade (Baker, Phillipsburg, NJ, U.S.A.)

The internal standard solution was prepared by dissolving 15 mg of 8-chlorotheophylline (Sigma, St. Louis, MO, U.S.A.) in 100 ml of ethanol.

Serum was supplied from the hospital's blood bank. Serum drug standards were prepared by adding known amounts of acetaminophen, theophylline and salicylate to drug-free serum.

The extraction solvent was methylene chloride—isopropanol (90:10).

Procedure

To 200 μl of serum were added 20 μl of the internal standard solution and 200 μl of 1.0 *M* HCl. After thorough mixing, 2 ml of the extraction solvent were added. The tubes were vortexed for 5 min in a multi-tube vortexer and then centrifuged for 2 min at 2500 *g*.

The aqueous supernatant layer was aspirated off and discarded. The organic phase was transferred to a new tube with the aid of a pasteur pipette and evaporated to dryness at 50°C with a gentle stream of air.

The residue was dissolved in 100 μl of the mobile phase. The extract was then transferred to a Spinco plastic tube and centrifuged in a hematocrite centrifuge (10,000 *g*) for 3 min. A 20-μl volume of the clear supernatant liquid was then injected into the chromatograph.

RESULTS

Fig. 1 shows chromatograms obtained with the procedure. Acetaminophen, theopylline, salicylate and 8-chlorotheophylline were all resolved under the chromatographic conditions used. However, salicylate showed minor peak tailing, but this did not seriously affect its quantitative determination.

No major interfering peaks of biogenic origin were detected. Blank values as computed by the Sigma 10 Data Station were as follows: acetaminophen 0.2 μg/ml; theophylline 0.7 μg/ml; salicylate 1.1 μg/ml.

Table I gives the precision data obtained by repeated analysis of a serum to which all the drugs had been added. The following practical detection limits (computed as approximately three times the blank values) were set: acetaminophen 1 μg/ml; theophylline 2 μg/ml; salicylate 3 μg/ml.

No interference to acetaminophen, theophylline or salicylate was found from: hexapropymate, meprobamate, methyprylon, methaqualone, glutethimide ("neutral" drugs); oxazepam, chlordiazepoxide, diazepam (benzodiazepines); phenobarbital, metharbital, heptabarbital, butalbital, secobarbital, aprobarbital (barbiturates); gentamicin, tobramycin, cloxacillin (antibiotics; for

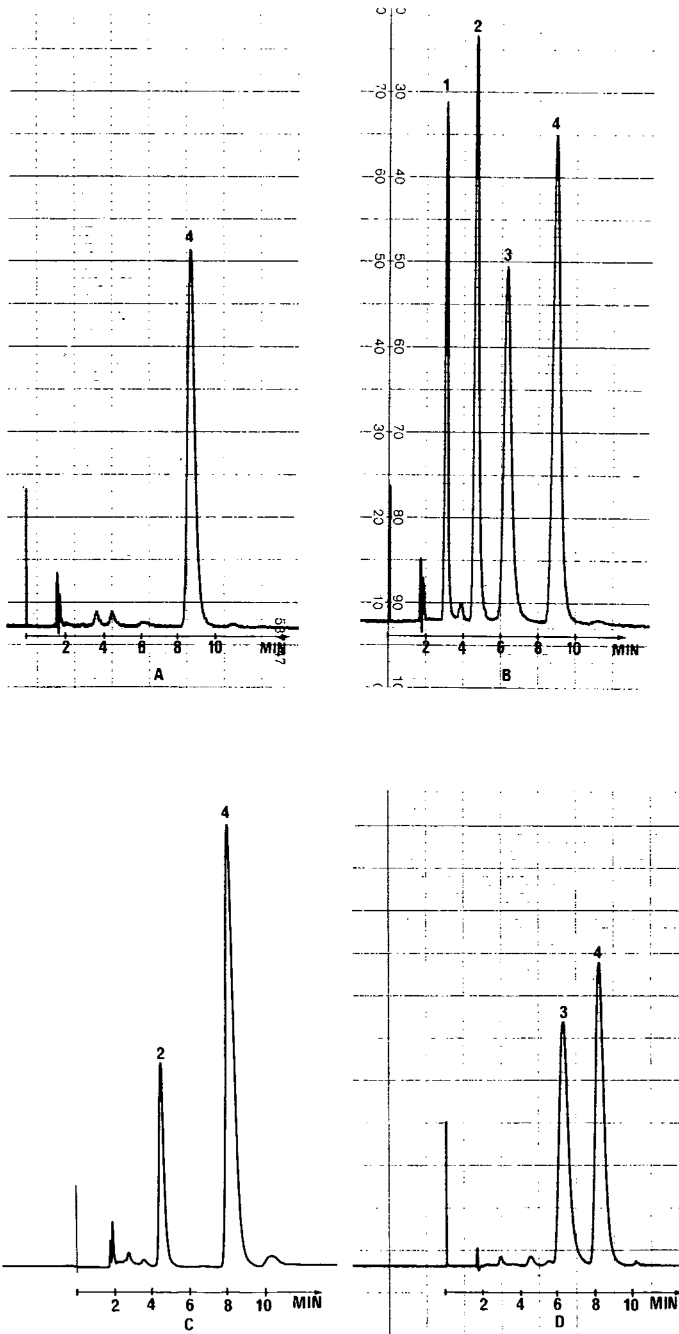

Fig. 1. Chromatogram A: a typical serum blank. Chromatogram B: serum standard. 1 = acetaminophen (100 μg/ml), 2 = theophylline (20 μg/ml), 3 = salicylate (200 μg/ml), and 4 = 8-chlorotheophylline (internal standard). Chromatogram C: sample from a patient on theophylline medication (theophylline concentration = 8.1 μg/ml). Chromatogram D: sample from a patient on salicylate medication (salicylate concentration = 240 μg/ml).

TABLE I

PRECISION DATA

Compound	Mean (μg/ml)	n	C.V. (%)	Added (μg/ml)	Range of linearity* (μg/ml)
Acetaminophen	10.5	8	12.1	12.5	10—200
	24.7	5	3.0	25.0	
	51.0	8	3.4	50.0	
	98.4	8	3.9	100.0	
Theophylline	3.7	8	4.1	3.8	2—60
	7.7	5	3.5	7.5	
	15.2	8	2.3	15.0	
	32.5	8	3.2	30.0	
Salicylate	26.2	8	9.1	25.0	20—500
	97.1	8	5.8	100.0	
	212.4	13	7.2	200.0	

*The calibration graphs of peak area ratios of the drugs and the internal standard versus drug concentration are linear in the given ranges (all correlation coefficients are greater than 0.98). The calibration graphs all pass through the origin at zero drug concentration ("zero intercept").

exception see cefuroxime, Table II); amitriptyline, imipramine, desipramine, nortriptyline, protriptyline, trimipramine (tricyclic antidepressants); phenytoin, ethosuximide, carbamazepine, primidone (antiepileptic drugs); sulfadimethoxine, sulfaisodimidine, sulfaproxylin (sulfonamides; see Table II).

TABLE II

RETENTION TIMES OF SOME COMPOUNDS

Peak No.*	Compound	Retention time (min)
1	Acetaminophen	3.0
	Sulfadiazine	3.0
	Cefuroxime	3.1
	Theobromine	3.4
	β-Hydroxyethyltheophylline	4.2
	1,7-Dimethylxanthine	4.4
2	Theophylline	4.6
	Sulfamethizole	4.9
	Sulfamethoxidiazine	5.0
3	Salicylate	6.2
	Sulfametoxazole	6.4
	Sulfafurazole	7.9
	Salicylamide	8.1
4	8-Chlorotheophylline	8.7
	Persedon	9.1
	Caffeine	10.2
	3-Isobutyl-1-methylxanthine	>15

*The drugs are numbered as the peaks in Fig. 1.

All compounds either eluted later than 20 min after injection or did not elute at all.

Table II shows the retention times of some compounds detected by the procedure. A short comparison between theophylline analyzed by HPLC and enzyme-multiplied immunoassay technique (EMIT) (according to our earlier reported procedure for the Gemsaec centrifugal fast analyzer [28]) is given in Table III.

TABLE III

COMPARISON OF THEOPHYLLINE ANALYZED BY HPLC AND EMIT

Patient code	Theophylline (μg/ml)	
	EMIT	HPLC
1	19.8	18.7
2	5.8	7.6
3	12.7	13.5
4	15.3	15.6
5	12.2	12.5
6	9.6	9.8
7	15.6	15.6
8	12.4	12.9
9	10.5	10.9
10	8.0	8.5

DISCUSSION

As seen in Table II, β-hydroxyethyl-theophylline was not resolved from theophylline and could therefore not be used as an internal standard in our procedure (β-hydroxyethyltheophylline is a commonly used internal standard for the determination of theophylline by HPLC).

In most cases a patient's medication is known and therefore unknown peaks are not likely to be found in the chromatograms. However, in situations of suicidal drug overdose, confirmation of peak identity is of prime interest since the drug "panorama" then may be unknown and complex. UV spectra of eluted peaks may help identify the drugs.

The procedure has now been used in our laboratory for half a year, primarily to determine theophylline in sera from patients undergoing theophylline therapy. Possible interference from compounds like sulfonamides and antibiotics has to be considered. We recommend others to investigate the interference from the latter groups of compounds. The chemical structures of these compounds in medical use may differ from country to country. The use of an extraction technique to recover the drugs from serum is beneficial since the extracts are relatively free from endogenous interfering compounds and suspended particles.

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Note

Fluorometric liquid chromatographic assay of the antiarrhythmic agent flecainide in blood plasma

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(First received September 22nd, 1981; revised manuscript received December 28th, 1981)

Flecainide [R818, the acetate salt of 2,5-di-(2,2,2-trifluoro-ethoxy)-N-(2-piperidylmethyl)benzamide] is a novel antiarrhythmic agent. It suppresses induced arrhythmias in dogs and pigs [1—3]. The antiarrhythmic properties of the drug are currently being tested in patients [4], but a good assay has not been reported. Gas chromatography has been used [2, 4, 5]. However, no details are given in the literature. We combined the resolving power of high-performance liquid chromatography with the sensitivity of fluorescence detection to assay flecainide in deproteinized blood plasma. This paper describes a rapid, sensitive and accurate method which is relatively specific, and is linear over a very wide concentration range.

EXPERIMENTAL

Reagents

Flecainide acetate was obtained from Riker Laboratories (Loughborough, Great Britain), and H_3PO_4, KOH, K_2CO_3, $HClO_4$ and methanol (LichroSolv) were obtained from Merck (Darmstadt, G.F.R.). $NH_4H_2PO_4$ was supplied by Baker Chemical Co. (Phillipsburg, NJ, U.S.A.). Water was purified with the Milli-RO/Milli-Q System (Millipore, Bedford, MA, U.S.A.). The elution buffer consisted of a mixture of 600 ml of 50 m*M* $NH_4H_2PO_4$ (adjusted to pH 3.0 with H_3PO_4) and 400 ml of methanol; before use, the buffer was filtered through a 0.45-μm filter (Millipore).

Apparatus

A Model 5010 liquid chromatograph (Varian, Palo Alto, CA, U.S.A.) equipped with a Valco injector (Valco Instruments Co., Houston, TX, U.S.A.) was

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used. Fluorescence was measured with an Aminco SPF-500 ratio detector (American Instrument Cy., Silver Spring, MD, U.S.A.) with an Aminco J4-9618 Microcell Condensing System Accessory. Some studies were conducted with a Vari-Chrom UV detector (Varian). Fluorescence emission or UV absorbance was monitored with a Servigor S RE543 chart recorder (Goerz Electro AG, Vienna, Austria). A C_{18} μBondapak column (particle size 10 μm; Waters Assoc., Milford, MA, U.S.A.) was cut into half to shorten the analysis time. Final dimensions of the column were 15 $\times$ 0.4 cm.

Procedure

Heparinized blood plasma was stored at -20°C until analyzed. A 1-ml aliquot was deproteinized with 1.0 ml of 0.8 *M* $HClO_4$ at 0°C. The mixture was shaken for 1 min and centrifuged for 4 min at 5500 *g* using an Eppendorf 3200 table centrifuge (Eppendorf, Hamburg, G.F.R.). An aliquot of the supernatant fluid (1.5 ml) was adjusted to pH 5—7 with 140 μl of a 6 *M* KOH—1 *M* K_2CO_3 solution. After centrifugation at 5500 *g* for 4 min, 200 μl of the supernatant fluid were applied to the column.

Chromatographic conditions

The flow-rate was 2.0 ml/min and the column was kept at 30°C. Under these conditions the retention time of flecainide is about 6 min. The fluorescence detector was set at an excitation wavelength of 300 nm and an emission wavelength of 370 nm. The excitation and emission slits on the monochromators were set at 10 and 15 nm bandwidths, respectively. UV absorption was measured at 280 nm. Calibration with standards was done every five determinations.

RESULTS AND DISCUSSION

Chromatograms

Fig. 1 shows chromatograms obtained with a plasma blank and plasma spiked with 200 ng/ml flecainide.

Linearity

Standards added to buffer or plasma gave a linear response up to 10 μg/ml when 200-μl samples were injected (Fig. 2). The sensitivity of the technique permits the detection of 50 ng/ml plasma flecainide, the detection limit being defined as a signal three times the height of the noise level.

Recovery

Plasma was deproteinized with $HClO_4$ and subsequently neutralized as described in the Experimental section. This procedure did not affect flecainide in water or buffer (recovery $>$ 98%). We conclude from the calibration curves of flecainide in buffer and plasma (Fig. 2) that 35% of the drug was precipitated with plasma protein due to the $HClO_4$, but that this was independent of the flecainide concentration. Thus the recovery over the whole concentration range studied was 65.4 $\pm$ 3.2% (mean $\pm$ S.D., n = 17). Deproteinization with HCl, trichloroacetic acid, methanol, ethanol or acetone did not improve this recovery.

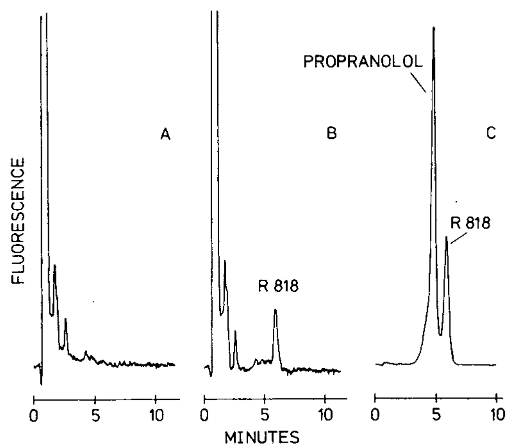

Fig. 1. Chromatograms obtained by high-performance liquid chromatography of (A) plasma blank, (B) plasma spiked with 200 ng/ml flecainide (R818), and (C) propranolol and flecainide in elution buffer (500 ng/ml). For deproteinization of plasma, see Experimental.

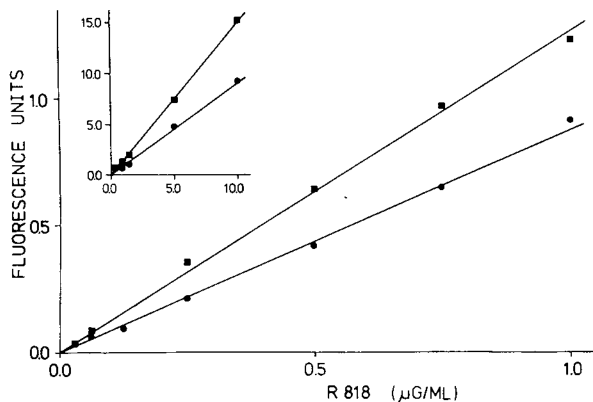

Fig. 2. Calibration curves of flecainide up to 10 μg/ml added to elution buffer (▪) or plasma (•). After the addition of the drug, plasma was deproteinized as described under Experimental.

Interference

Table I lists drugs that have been tested for potential interference with the procedure. All these drugs, including β-blocking agents, heart glycosides, vasodilators and antithrombolytics, could be detected by UV absorption, but only a number of them were fluorescent under the assay conditions. Propranolol showed considerable fluorescence; however, the separation from flecainide is adequate (Fig. 1), as is the case for the other drugs (Table I).

Precision

Inter-assay precision expressed as relative standard deviation (coefficient of variation) was 2.2, 1.2 and 1.2% for the 100, 500 and 1000 ng/ml aqueous standards, respectively (n = 5). For plasma flecainide determinations (concentration range 400—1500 ng/ml) both intra- and inter-assay standard deviation was found to be about 40 ng/ml.

TABLE I

DRUGS TESTED FOR THEIR POTENTIAL INTERFERENCE WITH THE FLECAINIDE ASSAY

Drug	UV detection		Fluorescence detection	
	Concentration tested (μg/ml)	Relative retention time(s)	Concentration tested (μg/ml)	Relative retention time(s)
Flecainide acetate	10	1.00	1	1.00
Acetylsalicylic acid	3600	0.43	50	0.40
Caffeine	1000	0.26	1000	NPD**
Diazepam	10	0.31; 0.49	600	NPD
Digitoxin	20	0.29; 0.40	200	NPD
Digoxin	2.5	0.29; 0.40	250	NPD
Dipyridamole	75	0.21; 0.23	75	NPD
Disopyramide phosphate	10	5.00	1000	NPD
Fenprocoumon	1500	0.09; 0.14	300	0.20
Heparin (sodium salt)	500 IU	0.46	100 IU	1.83
Nifedipine	100	2.57*	25	NPD
Nitroglycerin	5	0.17	5000	0.19
Practolol	500	0.16; 0.37	500	NPD
Procainamide chloride	10,000	0.20	1000	0.25
Propranolol chloride	10	0.86	0.5	0.83
Quinidine sulfate	150	0.30*	1	0.47*
Sulfinpyrazone	10,000	0.11; 2.40	20,000	NPD
Thiazide chloride	10,000	0.14	5000	NPD

*Relative retention time of main peak; other peaks observed with relative retention times < 0.90 and > 1.10.
**NPD = no peak detected.

Plasma concentrations

In patients treated with flecainide we found plasma concentrations of 540 ± 180 ng/ml (mean ± S.D., n = 60). The therapeutic concentrations in human plasma probably range between 180 and 900 ng/ml (cf. refs. 4–6), which means that subtherapeutic concentrations and overdoses can also be determined with the method described.

CONCLUSION

This assay based on high-performance liquid chromatography with fluorescence detection is rapid, sensitive and relatively specific. It seems useful in estimating plasma flecainide levels for clinical management.

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Note

Determination of clofazimine in plasma by high-performance liquid chromatography

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Clofazimine [3-(*p*-chloroanilino)-10-(*p*-chlorophenyl)-2,10-dihydro-2-(isopropylimino)phenazine; B663] is now an important secondary drug for use in patients infected with *Mycobacterium leprae* resistant to the primary drugs, dapsone and rifampin [1–3]. Early methods for the analysis of B663 in biological systems were based on the intense yellow-orange color of B663 in aqueous acid [4] or the production of a fluorescent derivative of B663 by reduction with titanous chloride [5]. Application of these methods by Levy [6] indicated that the practical limits of sensitivity for the colorimetric and fluorometric procedures were about 300 and 200 ng B663 per ml of plasma, respectively. Other workers [7] concluded that the two techniques were of equal sensitivities at about 200 ng/ml of plasma.

In this paper, we report the development of a method for measuring B663 in plasma with a limit sensitivity of about 10 ng/ml. This method involves extraction of B663 into organic solvents, separation of B663 from potential interfering materials by high-performance liquid chromatography (HPLC), and quantitation via the high absorbance of B663 at 285 nm. Following completion of our studies, Gidoh et al. [8] reported a similar HPLC method for B663.

EXPERIMENTAL

Materials

B663 was supplied by Dr. L. Levy (Hebrew University Hadassah Medical School, Jerusalem, Israel). Methanol, chloroform, and hexane were obtained from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.); water was purified through a Super-Q water purification system (Millipore, Bedford, MA, U.S.A.). All solvents were filtered through a type HA filter (0.45 μm, Millipore); other chemicals were reagent grade.

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Screw cap glass culture tubes (50 ml) and 16 × 100 mm disposable culture tubes (Kimble, Toledo, OH, U.S.A.) were vapor-phase silylated [9], rinsed with methanol, and allowed to air-dry. The tubes were closed with foil-lined caps (Arthur H. Thomas Co., Philadelphia, PA, U.S.A.). Injection vials for HPLC were disposable polypropylene microvials with polyurethane locking caps (Vanguard International, Neptune, NJ, U.S.A.).

Dr. R.R. Jacobson (Carville, LA, U.S.A.) kindly supplied us with heparinized plasma from his patients receiving B663 regimens therapeutically. We also used plasma from his patients receiving dapsone provided earlier [10] to test for interference by this drug and its metabolites and for recovery of B663 using the current method. The samples were stored and shipped frozen. We employed drug-free Sprague-Dawley rats (Simonsen Labs., Gilroy, CA, U.S.A.) as donors for heparinized rat plasma.

Extraction

B663 was extracted from 1.0-ml aliquots of rat or human plasma by addition of 1.0 ml of phosphate—citrate buffer, pH 6.0 (12.6 ml of 0.2 *M* Na_2HPO_4 and 7.4 ml of 0.1 *M* citric acid), and 14 ml of chloroform—methanol (4:1, v/v) in a 50-ml culture tube. The tube was closed with a foil-lined cap, and was shaken for 20 min at 80—100 strokes/min on a shaker (Eberbach, Ann Arbor, MI, U.S.A.). After centrifuging for 10 min at 400 *g*, the aqueous layer was aspirated off. Ten ml of the organic layer were transferred to a 16 × 100 mm test tube and evaporated to dryness under a gentle stream of high-purity nitrogen using a Meyer N-Evap (Organomation Assoc., Shrewsbury, MA, U.S.A.). The residue was reconstituted in 150 μl of mobile phase (0.0425 *M* phosphoric acid in 81% methanol) and 0.5 ml of hexane. Following centrifugation to separate the phases, the hexane layer was discarded and the mobile phase was transferred to an injection vial.

High-performance liquid chromatography

Samples were chromatographed using a Hewlett-Packard Model 1084B liquid chromatograph (Hewlett-Packard, Santa Clara, CA, U.S.A.) equipped with a variable-volume injector, automated sampling system, variable-wavelength detector, and automated fraction collector. B663 was separated from interfering plasma material using a reversed-phase Ultrasphere-octyl column (250 × 4.6 mm, 5-μm particle size; Altex, Berkeley, CA, U.S.A.) maintained at 40°C. Sample injection volume was 100 μl. The mobile phase for elution was 0.0425 *M* phosphoric acid in 81% methanol, pH 2.4, at a flow-rate of 1.5 ml/ min. Detection of B663 was accomplished by monitoring the column effluent at 285 nm. Plasma concentrations were quantitated by using integrated peak areas in comparison to standard quantities of B663 (in plasma) carried through the entire procedure.

Mass spectrometry

Fractions at the elution position of B663 were collected using the automatic fraction collector from extracts of plasma samples. B663 was extracted from the collected fractions with chloroform—methanol (4:1) and the organic phase was evaporated to dryness under a stream of nitrogen. The residue was sub-

jected to mass spectrometry using authentic B663 as the reference standard. Spectra were determined by direct introduction into an LKB 9000 mass spectrometer, using a 12-eV ionizing potential.

RESULTS

The elution profile of a plasma extract from a patient receiving B663 is shown in Fig. 1C. Fig. 1A shows the elution profile of an extract of control plasma and Fig. 1B the profile of a control plasma sample spiked with 332 ng/ml of B663. B663 exhibits a retention time of 9.6 min and is well resolved from extraneous UV-absorbing material. The antileprosy drugs, dapsone and ethionamide, eluted at the solvent front and rifampin was retained 3 min longer than B663.

To determine the recovery of B663 from plasma, pooled human plasma from patients receiving dapsone was used. The mean recovery of B663 from duplicate aliquots of plasma containing 33, 50, 330, and 830 ng/ml was 116% (S.D. = 10.4%; range was 97.3—130%). Duplicate determinations agreed within an average of 3.2% of their means. Fig. 2 shows a linear relationship between the peak areas of these samples and the quantity of B663 extracted ($r = 0.9981$, $P < 0.001$). The lower limit of sensitivity was estimated to be 10 ng/ml. Limited studies on the stability of B663 in frozen rat plasma showed that, at a concentration of 248 ng/ml, there was < 2% change in the level of B663 after nine days of storage. The mean recovery of 332 ng/ml of plasma on six different days was 105% (S.D. = 6.4%), thus demonstrating good reproducibility from day to day.

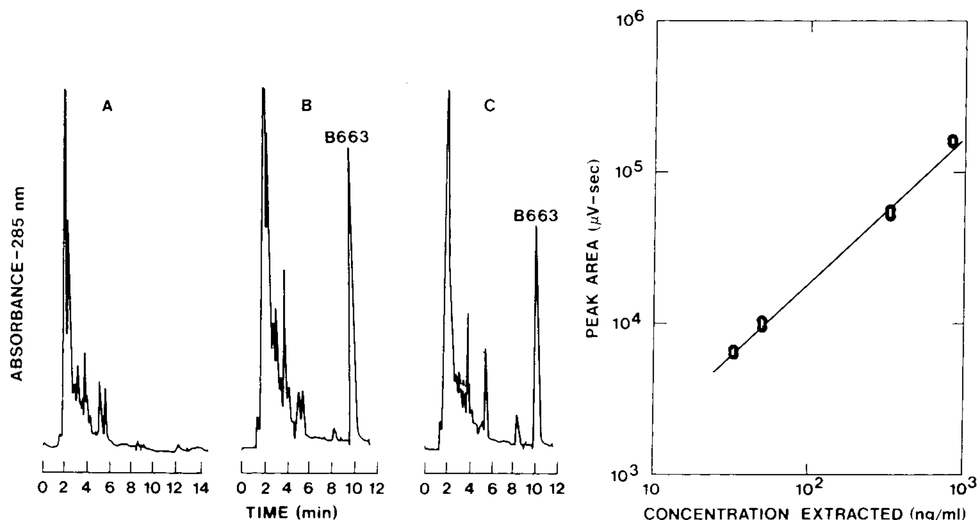

Fig. 1. Elution profiles of (A) B663-free plasma extract; (B) an extract of human plasma containing added B663; (C) an extract of a 2-h plasma sample from a patient receiving 100 mg B663 daily. The estimated level in the latter sample was 520 ng/ml of B663.

Fig. 2. Calibration plot of peak area at 285 nm versus concentration of B663 in plasma extracted.

Plasma levels of B663 in patients receiving B663 therapeutically are shown in Table I. All patients had received a dose of 100 mg B663 at zero time before the plasma samples were collected, regardless of their regimen. Patient 4 also received 250 mg ethionamide at zero time prior to sampling. The four patients (numbers 1, 2, 3, and 6) receiving 100 mg B663 daily exhibited variable times of maximum B663 levels from 2—8 h. These peak levels ranged from 550—1600 ng/ml in this group. Patient 4 receiving 250 mg ethionamide with 100 mg B663 daily was not different from the four receiving only B663 daily. Patient 5, receiving 100 mg B663 three times per week, exhibited the lowest levels of all patients; and patient 7, receiving 100 mg B663 three times per day, exhibited the highest levels at most time periods.

Because variable body weights of the patients could contribute to some of the differences found in the plasma levels of B663 of Table I, we have calculated the dose-adjusted levels shown in Table II wherein each plasma level was expressed in units of ng/ml divided by the mg B663 per kg of body weight of each patient. While these data can now be compared more rigorously, they do not eliminate the extreme variability in levels observed in the different patients. In all patients, a pattern of slow absorption and slow clearance from the body is indicated. At no time were levels found near the lower limit of our method. Also, no other peaks were seen on the chromatograms that indicate B663 metabolites. Finally, fractions collected at the retention time of authentic B663 from extracts of plasma exhibited mass spectra identical to that of authentic B663 (spectra are available from the authors on request).

DISCUSSION

Early methods for B663 depended upon absorption in visible light at about 540 nm [4,6,7] or conversion to a fluorescent derivative [5] but the practical limits of sensitivity of these techniques were 200—300 ng/ml of plasma [6]. In

TABLE I

LEVELS OF B663 IN PLASMA OF LEPROSY PATIENTS

Patient No.	Mean level (ng/ml) of B663 found after administration at				
	1 h	2 h	4 h	8 h	24 h
1*	350	460	610	590	410
2*	480	550	480	440	300
3*	1090	1030	1230	1600	1470
4**	— ***	630	660	680	490
5§	370	370	350	390	370
6*	570	630	640	700	510
7§§	1710	1880	2300	1270	1490

*Dose was 100 mg B663 daily.
**Dose was 100 mg B663 daily plus 250 mg ethionamide twice daily.
***No sample was available.
§Dose was 100 mg B663 three times per week.
§§Dose was 100 mg B663 three times per day.

TABLE II

DOSE-ADJUSTED LEVELS OF B663 IN PLASMA OF CARVILLE PATIENTS

Patient No.	Body weight (kg)	Hours after administration (ng/ml/mg B663/kg)				
		1	2	4	8	24
1*	52.7	184	245	323	309	215
2*	79.5	377	438	381	352	242
3*	63.6	696	657	783	1020	938
4**	92.7	—***	585	613	626	455
5§	70.9	265	261	245	278	260
6*	80.0	454	502	514	558	410
7§§	84.5	481	530	648	359	419

* Dose was 100 mg B663 daily.

** Dose was 100 mg B663 daily plus 250 mg ethionamide twice daily.

*** No sample was available.

§ Dose was 100 mg B663, three times per week.

§§ Dose was 100 mg B663, three times per day. For calculating dose-adjusted levels, a total single dose of 300 mg B663 was used.

our method, we have lowered the limit of sensitivity for measuring B663 to approximately 10 ng/ml of plasma using an original sample volume of 1 ml. Also, we have demonstrated no interference from plasma of patients receiving dapsone and separation of B663 from ethionamide and rifampin. Independently of us, Gidoh et al. [8] developed a similar HPLC method for B663. In their system, B663 exhibited an absorbance maximum at 282 nm (molar absorptivity = $8.8 \cdot 10^4$). In our mobile phase, we found the maximum for B663 and 285 nm (molar absorptivity = $8.4 \cdot 10^4$). Under these conditions, a second UV-absorbing peak for B663 was observed at 496 nm but the absorption was only one-half of that observed at 285 nm. We would expect that the method of Gidoh et al. [8] would be similarly applicable to analysis of plasma samples from patients receiving B663, although these authors reported only results of recoveries of B663 and other drugs added to pooled guinea pig serum. Their goal of development of a single method for measuring B663, dapsone, and rifampin has yet to be attained because the crucial tests of potential mutual interference by parent drug metabolites in human plasma in the assay of the other drugs have yet to be performed.

In the meantime, our current method has sufficient sensitivity and selectivity to measure B663 in patients receiving dapsone in combination with B663. Thus, in conjunction with methods we have already published for the primary antileprosy drugs, dapsone [11] and rifampin [12], and the newer secondary drugs, ethionamide and prothionamide [13], it is now possible to monitor antileprosy drug therapy of patients receiving combinations of these drugs. Recently, we have shown that saliva can be employed instead of plasma for monitoring dapsone therapy [14].

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Errata

J. Chromatogr., 225 (1981) 440–445

Page 443, last line of the legend to Table I should read: "volume, and V_t = total bed volume".

J. Chromatogr., 226 (1981) 325–332

Page 325, first sentence of the Introduction should read: "2-Ketoglutaric acid is a normal metabolite of glutamic acid by transamination".

Page 330, line 16, "Phenyllactic acid" should read: "*p*-Hydroxy-phenyllactic acid".

J. Chromatogr., 227 (1982) 187–192

Page 191, Fig. 4, the tabel of the *y*-axis should read: "DMP (ng/ml)".

Page 191, legend to Fig. 4, "an oral dose of 40 ng of MP" should read: "an oral dose of 40 mg of MP".

Vol. 229, No. 2

JOURNAL OF CHROMATOGRAPHY
BIOMEDICAL APPLICATIONS

NEWS SECTION

MEETING

9th INTERNATIONAL MASS SPECTROMETRY CONFERENCE

The 9th International Mass Spectrometry Conference will be held from August 30 to September 3, 1982, at the Congress Centre, Hofburg, in Vienna, Austria. This Conference is one in a triennial series, the last having been held in Oslo in 1979.

The Conference is organized by the Austrian Mass Spectrometry Group of the Austrian Society for Microchemistry and Analytical Chemistry of the Austrian Chemical Society, and the Institute of Analytical Chemistry of the University of Vienna, in cooperation with an International Scientific Committee.

The scientific programme will cover all aspects of organic and inorganic mass spectrometry: theory, instrumentation, data processing and all fields of applications. An important aim of the Conference is to stimulate the exchange of information between scientists working in different fields of mass spectrometry.

The language of the conference will be English. No simultaneous translation will be provided.

Plenary and keynote lectures will be presented by invited speakers dealing with different important aspects of mass spectrometry. Other parts of the scientific programme will consist of contributed papers (oral or poster presentations) and a panel discussion.

An extensive exhibition will also be organized.

The proceedings will be published by the Elsevier Scientific Publishing Company as special volumes in the *International Journal of Mass Spectrometry and Ion Physics*.

Further information may be obtained from: the Secretariat, 9th International Mass Spectrometry Conference, c.o. Interconvention, P.O. Box 80, A-1107 Vienna, Austria. Telephone: (0222) 57 62 88, 57 63 05; Telex: 11 12 10.

NEW PRODUCTS

N-1669

AUTOMATED TLC APPLICATION

Sample application has been not only the most time-consuming operation in quantitative TLC, but due to human errors also the least dependable. The CAMAG Automatic TLC Sampler I is a microprocessor-controlled device that makes sample application both fully automatic and reliable. A tube rack with up to 16 samples is inserted in the unit, and the sample vials are sealed, if so desired, with a membrane. A fused-silica capillary, which is connected to a dosage syringe operated by a stepping motor, draws an appropriate quantity of sample solution from the vial and delivers the desired volume at a preselected speed to the plate. The sequence in which the samples are applied as well as their positioning on the layer, can be freely selected. Rinsing of the capillary after each sample is also automatic.

N-1668

TLC APPLICATOR AND SOLVENT SELECTER

J.T. Baker offers a time-saving device for the simultaneous application of up to 16 uniform samples to TLC plates. The Automated TLC Spotter is said to be 75% faster in spotting multiple samples and provides more reproducibility than manual methods. Operating with either hydrophobic, or hydrophilic "wicks", this device can accommodate any size TLC plate. It can also be used with the Multi-Chamber Solvent Reservoir to select spotting solvents.

N-1665

MICROPROCESSOR-CONTROLLED GAS CHROMATOGRAPH

The Tracor Model 570 gas chromatograph offers a combination of microprocessor technology and field-proven chromatographic design. Its large integral CRT/keyboard controller uses both hard and soft key annotation to eliminate confusing input codes of language. Simple operating logic makes it easy to use. Up to ten methods can be stored, each with up to five temperature programming rates. Many other capabilities are included such as method linking, auto capillary start, timed events and Hall detector auto vent. Another feature of Model 570 is its permanent memory.

N-1663

IMMUNOELECTROPHORESIS SYSTEM

Pharmacia Fine Chemicals have introduced a system for immunoelectrophoresis. This system is based on the company's flat bed electrophoresis system and is designed for all conventional forms of agarose-gel electrophoresis and immunoelectrophoresis. The electrophoresis cell is equipped with a cooling plate made of epoxy resins and resistant to cooling fluid mixtures, based on methanol, ethanol, and ethylene glycol. The plate has an extended, black surface, imprinted with a white grid for easy observing of the immunoprecipitates. The positioning of plates and placing of samples are said to be simplified by this grid. As an option, the company offers a system for the casting of gels and imprinting of various patterns for immunological techniques.

N-1661

POST-COLUMN REACTOR SYSTEM

Varian Associates has announced a post-column reactor system which provides highly sensitive HPLC detection of primary amines. Compounds of these amines eluting from the HPLC column are mixed with a buffered solution of *o*-phthalaldehyde, and the resultant derivatives are detected fluorimetrically. Varian offers three types of post-column reactor systems. Each type consists of the Varian Fluorichem fluorescence detector, a reagent pump with an attached reaction coil cassette, and a chemicals and buffer kit. The three new systems are said to be optimized for fast *o*-phthalaldehyde reaction at room temperature.

N-1672

FIXED-WAVELENGTH HPLC DETECTOR

Micromeritics introduced a fixed-wavelength HPLC detector at the 1982 Pittsburgh Conference. The Model 791 is designed for maximum reliability and easy operation. A specially engineered electronics system reduces time between lamp changes. The detector detects UV absorbing compounds as they elute from the HPLC column at selectable wavelengths. A push button allows baseline zeroing during automatic analysis.

N-1670

DUAL VARIABLE-WAVELENGTH DETECTOR

Micromeritics introduced the 788 dual variable-wavelength detector at the Pittsburgh Conference, March 8-12, 1982. This detector simultaneously measures the light absorbance at two selected wavelengths, which gives the chromatographer another tool to speed HPLC analysis by allowing him to measure compounds not fully resolved. This detector provides accurate quantitation of unresolved components, including those having identical retention times, through selective elimination of an interfering peak. The Model 788 supplies data for the dual characterization of chromatographic samples. In addition to the information from the absorbance signals displayed on an ordinary dual pen recorder, the two absorbance signals can be arithmetically processed to give the sum, quotient and difference of the two signals. Absorbance ratio data can be used to characterize chemical species as well as to determine the purity of the components in the flow cell.

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PUBLICATION SCHEDULE FOR 1982

Journal of Chromatography (incorporating *Chromatographic Reviews*) and *Journal of Chromatography, Biomedical Applications*

MONTH	J	F	M	A	M	J	J	A	S	O	N	D
Journal of Chromatography	234/1 234/2 235/1 235/2	236/1 236/2	237/1 237/2 237/3	238/1 238/2 239	240/1 240/2 241/1	241/2 242/1 242/2	The publication schedule for further issues will be published later.					
Chromatographic Reviews		251/1		251/2								
Biomedical Applications	227/1	227/2	228	229/1	229/2	230/1						

INFORMATION FOR AUTHORS

(Detailed *Instructions to Authors* were published in Vol. 209, No. 3, pp. 501–504. A free reprint can be obtained by application to the publisher.)

Types of Contributions. The following types of papers are published in the *Journal of Chromatography* and the section on *Biomedical Applications:* Regular research papers (Full-length papers), Short communications and Notes. Short communications are preliminary announcements of important new developments and will, whenever possible, be published with maximum speed. Notes are usually descriptions of short investigations and reflect the same quality of research as Full-length papers, but should preferably not exceed four printed pages. For review articles, see page 2 of cover under Submission of Papers.

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