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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF PRIFINIUM QUATERNARY AMMONIUM ION IN HUMAN SERUM AND URINE

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SUMMARY

A simple, sensitive method for the determination of the prifinium ion, a quaternary ammonium ion, in human serum and urine is described. The method is based on extraction of the test solution with chloroform in the presence of saturated potassium bromide solution and normal-phase high-performance liquid chromatography using aqueous methanol as the mobile phase at pH 10. To prevent the dissolution of silica from the analytical column, the mobile phase is pre-saturated with silica by using a silica saturation column. Quantitation is possible down to 0.5 ng/ml of prifinium ion using 2 ml of serum and down to 5 ng/ml using a 1 ml of urine. The coefficients of variation of the method are less than 1.3% in both serum and urine. Serum levels and urinary excretion data obtained with this method are given for three healthy volunteers who had received a 60-mg oral dose of prifinium bromide.

INTRODUCTION

Prifinium bromide is a quaternary ammonium compound possessing anticholinergic properties [1], which has been used for some years in the treatment of gastro-enteritis, gastro-duodenal ulcer, irritable colon syndrome, etc. Although the pharmacokinetics of ^{14}C-labelled prifinium bromide in experimental animals have been reported [2], little is known about its pharmacokinetics in man. Generally, anticholinergic ammonium compounds are not well absorbed when given by the oral route [3—9]. Consequently, it was necessary to develop a sensitive assay for measuring prifinium ion in serum and urine after oral administration of the usual clinical dose in man.

Gas—liquid chromatography (GLC) and gas chromatography—mass spectrometry (GC—MS) are commonly used to determine quaternary ammonium ions in biological fluids [10—14]. Because these methods usually include a dealkyla-

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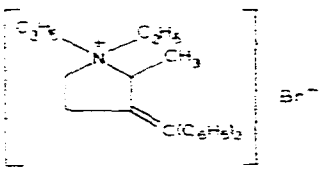

Prifinium bromide

tion or oxidation procedure to produce volatile derivatives, they are time consuming and tedious. Recently, the use of high-performance liquid chromatography (HPLC) for the quantitative determination of quaternary ammonium ions in biological fluds has been shown to have advantages over GLC and GC—MS with respect to speed, simplicity and/or reliability [15, 16]. De Ruyter et al. [15] have reported the use of reversed-phase high-performance ion-pair liquid chromatography for the determination of pyridostigmine, neostigmine and edrophonium in the biological fluids. Assay of ORG NC45 (a myoneural blocking agent) in human plasma using normal-phase HPLC has been reported by Paanakker and Van de Laar [16].

This paper describes a highly sensitive and simple method for the determination of prifinium ion in human serum and urine. The method is based on extraction of the test solution with chloroform in the presence of saturated potassium bromide solution and normal-phase HPLC using aqueous methanol as the mobile phase at pH 10.

EXPERIMENTAL

Reagents and materials

Prifinium bromide (Riabal) was prepared by Fujisawa Pharmaceutical Co. (Osaka, Japan). Methanol and chloroform of UV grade were used. Purified deionized water used for all solutions and mobile phases was prepared with a Millipore Milli-Q water purification system. All other solvents and reagents were of analytical-reagent grade. Sodium hydroxide solution saturated with potassium bromide was prepared from the supernatant after vigorously mixing 100 ml of 0.05 *M* sodium hydroxide with about 70 g of potassium bromide. Blank human serum was obtained from fresh blood of healthy male volunteers. Healthy male volunteers provided blank human urine. Serum and urine samples were stored at —20° C until taken for analysis.

Preparation of standard solution

Diphemanyl methylsulphate was used as an internal standard for the assay. It was dissolved in water and diluted to 1, 2 and 30 μg/ml. Standard solutions of prifinium ion were prepared by dissolving prifinium bromide in water and diluting to appropriate concentrations.

Apparatus

Analysis in serum was made on Waters Assoc. liquid chromatograph equipped with a Model 440 absorbance detector (254 nm fixed wavelength), a Model 6000A pump, a U6K universal injector and a 10-mV recorder. Analysis in urine was made using a Waters Intelligent Sample Processor (WISP) and a Data Module in place of the U6K universal injector and the 10-mV recorder, respectively.

Chromatographic conditions

A pre-packed LiChrosorb Si 60 (particle size 5 μm) analytical column (Umetani Seiki, Osaka, Japan) of 25 cm × 4 mm I.D. and a home-packed LiChrosorb Si 60 (30 μm) guard column of 1 cm × 4 mm I.D. were used. A home-packed LiChrosorb Si 60 (30 μm) pre-column of 5 cm × 2 mm I.D. was placed between the pump and injector (Fig. 1) to prevent the dissolution of silica from the analytical and guard columns at high pH. The mobile phase of 10% 1 *M* ammonium acetate adjusted to pH 10 with 28% ammonia solution in methanol was prepared freshly each day of analysis. The mobile phase was de-aerated under vacuum before use. The operating temperature was ambient and the flow-rate was 1.0 ml/min. Retention times with this system were about 8 min for prifinium ion and 11 min for the internal standard.

Fig. 1. Pre-column and guard column arrangement.

Extraction from biological fluids

To a glass-stoppered 10-ml centrifuge tube containing 2 ml of a serum sample were added 0.1 ml of water, 0.1 ml of internal standard solution (containing 0.1 μg of diphemanil methylsulphate), 3 ml of sodium hydroxide solution saturated with potassium bromide and 6 ml of chloroform. The mixture was shaken for 10 min and centrifuged at 1900 *g* for 10 min. The aqueous phase was carefully aspirated. A 4-ml volume of the chloroform phase was transferred into a glass-stoppered 10-ml centrifuge tube. The solvent was removed by evaporation in a stream of nitrogen. The residue was dissolved in 100 μl of the HPLC mobile phase and an 80-μl aliquot was injected on to the liquid chromatograph.

To a glass-stoppered 10-ml centrifuge tube containing 1 ml of urine sample were added 0.1 ml of water, 0.1 ml of the appropriate internal standard solution (containing 0.2 or 3 μg of diphemanil methylsulphate), 3 ml of sodium hydroxide solution saturated with potassium bromide and 5 ml of chloroform. The other extraction procedure was carried out in the same way as described for serum. An 8- or 40-μl aliquot of the extract dissolved in the HPLC mobile phase was injected on to the liquid chromatograph.

Quantitation

The procedure was standardized by analysing the blank serum or urine samples to which had been added 0.1 ml of prifinium standard solution instead of 0.1 ml of water as in the extraction procedure. Peak-height ratios and peak-area ratios of prifinium ion to internal standard were used to establish the calibration graph for serum and urine samples, respectively. The calibration graph was fitted to a $y = ax + b$ equation by the least-squares method. The concentrations in the unkown samples were subsequently calculated using the calibration graph.

Clinical study

A clinical study was performed in which three healthy volunteers received an

oral dose of 60 mg of prifinium bromide (Riabal tablet). Serum samples were obtained from blood collected by venipuncture at designated time intervals, and stored at −20°C until taken for analysis. The total urine output was collected at intervals of 0—2, 2—4, 4—6, 6—8 and 8—24 h. The urine volumes were measured, and the aliquots were kept at −20°C prior to analysis.

RESULTS

Separation

Typical chromatograms obtained from the human serum and urine samples are shown in Figs. 2 and 3, respectively. As shown in Figs. 2A and 3A, the background peaks of blank human serum and urine have short retention times and are almost completely separated from those of prifinium ion and internal standard. Figs. 2C and 3C show typical chromatograms of serum and urine samples from a healthy volunteer after oral administration of 60 mg of prifinium bromide. In these chromatograms there are no interferences at the retention times of prifinium ion and the internal standard.

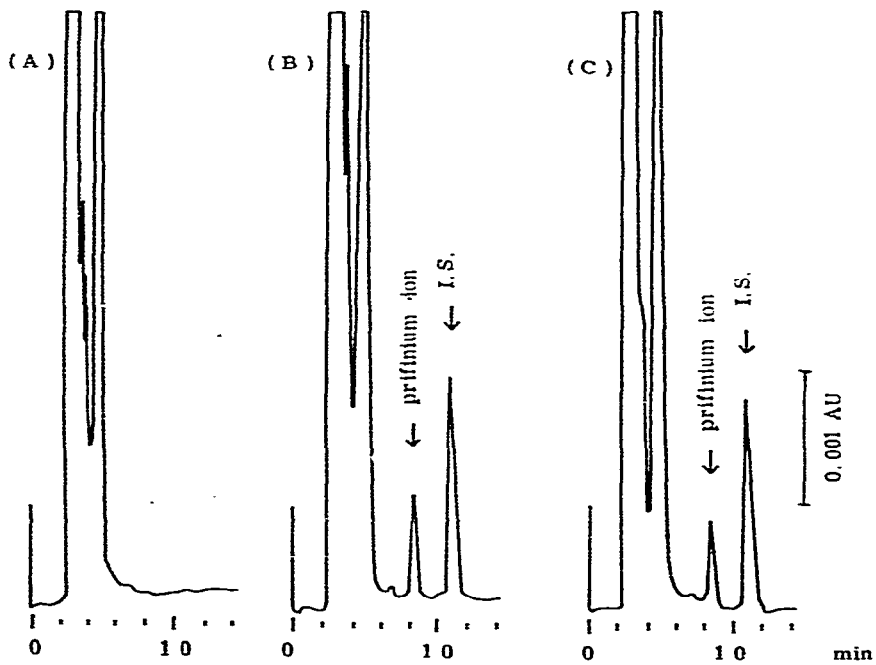

Fig. 2. Chromatograms of (A) blank human serum, (B) serum containing 10 ng/ml of prifinium ion and 50 ng/ml of internal standard and (C) serum from a healthy volunteer after oral administration of 60 mg of prifinium bromide. Conditions: column, 25 cm × 4 mm I.D. LiChrosorb Si 60 (5 μm); mobile phase, 10% 1 *M* ammonium acetate adjusted to pH 10 with ammonium solution in methanol; flow-rate, 1.0 ml/min; detection, UV at 254 nm; injection volume, 80 μl.

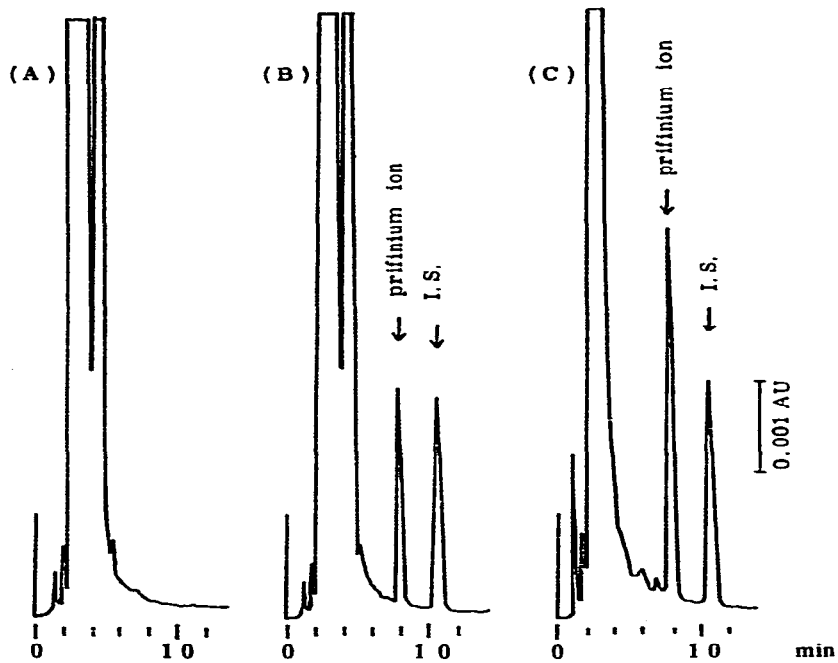

Fig. 3. Chromatograms of (A) blank human urine, (B) urine containing 100 ng/ml of prifinium ion and 200 ng/ml of internal standard and (C) urine from a healthy volunteer after oral administration of 60 mg of prifinium bromide. Conditions as in Fig. 2 except injection volume, 40 μl.

Recovery

Absolute overall recoveries from the samples were estimated by comparing peak heights or peak areas obtained from the injection of known amounts of prifinium ion with peak heights or peak areas obtained from the injection of extracts of samples spiked with prifnium ion. The values obtained from the serum and urine samples were respectively 57.0 ± 1.2% (mean ± S.D.) for prifinium ion concentrations in the range 2.5—50 ng/ml and 72.8 ± 1.5% in the range 25—100 ng/ml. These values, corrected for the ratio of the added solvent volume to the transferred volume, were 85.5% and 91.0% in serum and urine, respectively.

Calibration graph

Typical calibration graphs in human serum and urine are indicated in Table I. All calibration graphs show good linearity in each range. The lower limit of sensi-

TABLE I

TYPICAL CALIBRATION GRAPHS FOR HUMAN SERUM AND URINE

Sample	Concentration range (ng/ml)	Slope	Intercept	Correlation coefficient
Serum	0.5—100	0.04893	0.00169	0.9999
Urine	5 —200	0.007579	—0.00711	0.9998
Urine	50 —2500	0.0005384	—0.00156	0.9996

tivity for serum was 0.5 ng/ml, with a signal-to-noise ratio of 4, when 2 ml of serum was used. In urine, although peaks were obtained at lower concentrations, 5 ng/ml was the lower limit of sensitivity using 1 ml of urine and taking into account the variation of the background peaks in urine.

Reproducibility

The reproducibility was obtained by performing five replicate analyses of spiked serum and urine samples. The results are given in Table II. The coefficients of variation were less than 1.3% in both serum and urine. The actual prifinium ion content measured by HPLC ranged from 97 to 101% in the 15 serum and urine samples analysed. This HPLC method for the analysis of prifinium ion in human serum and urine thus provides good accuracy and precision.

TABLE II

REPRODUCIBILITY OF THE DETERMINATION OF PRIFINIUM ION IN HUMAN SERUM AND URINE

Parameter	Serum	Urine	Urine
Actual prifinium ion concentration (ng/ml)	5.00	50.0	500
Number of analyses	5	5	5
Mean analysed concentration (ng/ml)	4.96	49.7	490
% of actual concentration	99.2	99.4	98.0
Range (ng/ml)	4.91—4.99	48.7—50.3	484—499
S.D. (ng/ml)	0.03	0.62	6.2
Coefficient of variation (%)	0.61	1.2	1.3
Concentration range of calibration graph (ng/ml)	0.5 —100	5 —200	50—2500

Serum levels and urinary excretion in clinical studies

Serum levels of prifinium ion after oral administration of 60 mg of prifinium bromide to healthy volunteers are shown in Fig. 4. The drug reached maximum levels (4.1—14.7 ng/ml) within 3 h after administration of tablets; 0.6—2.1% of the administered dose was excreted as prifinium ion in the 0—24-h urine.

DISCUSSION

Early studies on methods for determining prifinium ion in the biological fluids were carried out by reversed-phase high-performance ion-pair liquid chromatography [15, 17] using heptanesulphonate or laurylsulphate as a counter ion. However, these methods did not separate the background peaks of blank biological fluids from those of prifinium ion. In later studies, normal-phase HPLC with aqueous methanol mobile phases [16, 18] was tested using various salts and pH of the mobile phases. These tests showed that mobile phases at high pH containing ammonium acetate to provide the best conditions for separating the background peaks of blank biological fluids from those of

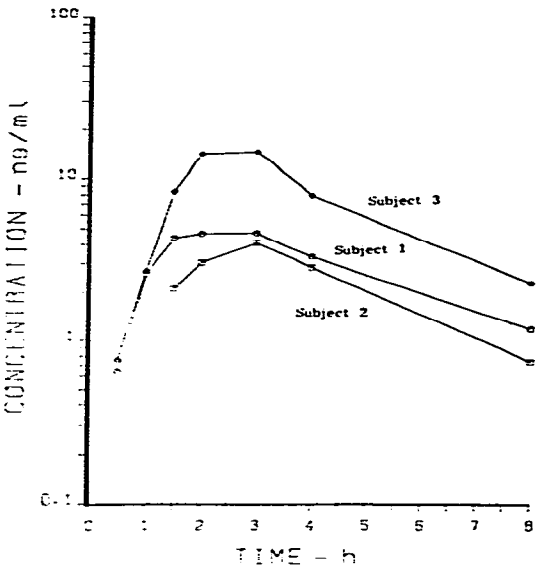

Fig. 4. Serum levels of prifinium ion in healthy volunteers after an oral dose of 60 mg of prifinium bromide.

prifinium ion and the internal standard (Figs. 2 and 3). In this study, a silica gel pre-column was placed between the pump and injector (Fig. 1) to prevent dissolution of silica from the analytical and guard columns at high pH [19—21]. The use of this silica pre-column technique increased the lifetime of the analytical and guard columns. Approximately 200 samples were analysed on one analytical column before a significant decrease in efficiency was observed.

The assay was shown to be sufficiently sensitive to quantify prifinium ion in human serum and urine after oral administration of the usual clinical dose (Fig. 4). A cross-over pharmacokinetic study of prifinium bromide in six healthy volunteers after intravenous and oral administration is in progress. The detailed results and a discussion of the pharmacokinetics of prifinium bromide in man will be published elsewhere.

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