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DETERMINATION OF HEPTAMINOL IN HUMAN PLASMA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Heptaminol was measured in plasma and urine following pre-column derivatisation with *o*-phthalaldehyde and reversed-phase high-performance liquid chromatography employing fluorescence detection. The limits of detection were sufficient for pharmacokinetic studies of the drug after clinically-used doses. Plasma concentrations of heptaminol reached peak levels (2.19 μg/ml) at 0.75 h after single oral doses (0.47 g of heptaminol) and declined with a half-life of 2.1 h (± 0.5 S.D.). Heptaminol was well absorbed and excreted rapidly, mainly unchanged in urine, 82% dose (± 10 S.D.).

INTRODUCTION

Heptaminol (6-amino-2-methyl-2-heptanol) hydrochloride (Fig. 1a) has been used for many years as a cardiotonic agent with a positive inotropic action [1]. Heptaminol has been measured in human urine by gas—liquid chromatography [2, 3] and in rat plasma and urine using radiochemical procedures [4]. Two further methods have been reported incorporating derivatisation of heptaminol to form fluorescent adducts [5, 6] but were not applied to biological samples.

The reaction of primary amines with *o*-phthalaldehyde (OPA) in alkaline medium in the presence of 2-mercaptoethanol to form highly fluorescent adducts is well established [7]. Pre-column fluorescence derivatisation of

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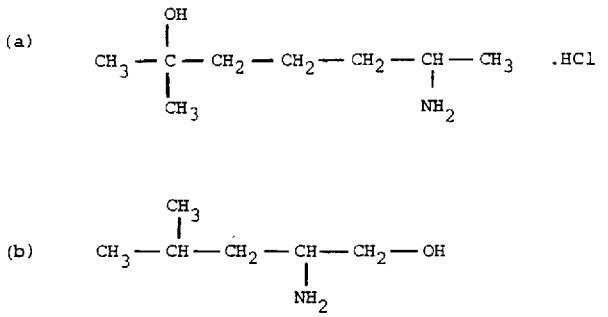

Fig. 1. Chemical structures of (a) heptaminol hydrochloride and (b) leucinol.

amino acids with OPA followed by high-performance liquid chromatography (HPLC) in a reversed-phase mode has been previously used [8].

This paper describes a simple and rapid procedure for the measurement of heptaminol in plasma and urine using pre-column OPA derivatisation and HPLC with fluorescence detection. L-Leucinol (2-amino-4-methyl-1-pentanol) (Fig. 1b) is incorporated as an internal standard.

No sample pre-treatment is required other than the removal of plasma proteins. The method is capable of measuring circulating levels of heptaminol in plasma and urine after administration of clinically-used doses of the drug.

EXPERIMENTAL

Materials and reagents

All reagents were of analytical grade and all inorganic reagents were prepared in freshly glass-distilled water. Acetonitrile was HPLC-far UV grade (Fisons Scientific Apparatus, Loughborough, Great Britain). *o*-Phthalaldehyde (OPA, Fluoropa®) was purchased from Pierce (Gillingham, Great Britain). Heptaminol hydrochloride was supplied by Delalande (Courbevoie, France). OPA derivatising solution (40.3 m*M*) was prepared as previously described [8]. The solution was stored at room temperature and regenerated every alternate day by adding 2-mercaptoethanol (20 μl). The activity of this solution was checked daily by measuring the chromatographic—fluorimetric response of a standard mixture containing heptaminol and leucinol. An aliquot of a standard mixture (40 μl, containing 200 ng of each compound in acetonitrile) was mixed with the derivatising solution (20 μl) using a vortex mixer for 30 sec and an aliquot (10 μl) of this mixture injected into the chromatograph. Response of this standard mixture showed a coefficient of variation of ± 3% during a four-week period.

Standard solutions of heptaminol and leucinol were prepared at concentrations of 1 mg free base per ml and 10 μg free base per ml in acetonitrile and stored at 4°C in the dark.

Plasma derivatisation procedure

Conical centrifuge tubes (10 ml capacity) were spiked with internal standard solution (10 μl, containing 100 ng leucinol in acetonitrile). The total volume of acetonitrile was adjusted to 100 μl and plasma (100 μl) was slowly added so

that precipitation of the proteins occurred. The tubes were vortex mixed for 30 sec and then centrifuged at 2000 *g* for 10 min. Aliquots (50 μl) of the acetonitrile layer were removed to clean conical tubes and derivatising solution (20 μl) was added with mixing using a vortex mixer for 30 sec. Aliquots (40 μl) of this solution were then immediately injected into the liquid chromatograph.

Urine derivatisation procedure

Aliquots (10 μl or 100 μl) of urine samples diluted 1 in 100 with glass-distilled water were transferred into conical tubes, spiked with internal standard solution (10 μl, containing 100 ng leucinol in acetonitrile). The total volume of acetonitrile was adjusted to 100 μl and the tubes mixed for 10 sec using a vortex mixer. Derivatising solution (20 μl) was added to the tubes with mixing using a vortex mixer for 30 sec and aliquots (30 μl) of this solution immediately injected into the liquid chromatograph.

Calibration procedures

Samples of control (drug-free) plasma (100 μl) were spiked with amounts equivalent to 10, 20, 40, 80, 120 and 200 ng of heptaminol free base and taken through the plasma derivatisation procedure. Samples of control urine (10 μl) which had been diluted 1 in 100 with glass-distilled water were spiked with amounts equivalent to 5, 10, 20, 40, 60, 80, 100, 120 and 150 ng of heptaminol free base and taken through the urine derivatisation procedure.

Instrumentation

The liquid chromatograph consisted of a Waters M6000A pump (Waters Assoc., Cheshire, Great Britain) connected to an LC1000 fluorescence detector (Perkin-Elmer, Beaconsfield, Great Britain) using a 338-nm excitation filter and an emission wavelength of 445 nm. Injection was performed manually using a U6K universal injector (Waters Assoc.). Chromatograms and peak area measurements were recorded using a 3380A computing integrator (Hewlett-Packard, Hitchin, Great Britain).

Chromatography

Chromatography was performed in a reversed-phase mode using a mobile phase of 45% (v/v) acetonitrile in aqueous sodium acetate buffer (0.05%, w/v) with a final pH adjustment to 6.1 using glacial acetic acid. The column used for the analysis was constructed of stainless-steel (25 cm × 0.46 cm I.D.) and packed with Zorbax® C_8 (mean particle diameter 6 μm) (Dupont, Stevenage, Great Britain). A pre-column constructed of stainless steel (7 cm × 0.2 cm I.D.) and dry-packed with Co:Pell® ODS (particle diameter 25—37 μm) (Whatman, Maidstone, Great Britain) was installed in series in front of the main analytical column to protect it from contamination and could be changed if the back-pressure in the system increased beyond reasonable limits (> 280 bar). A mobile phase flow-rate of 2.5 ml/min was maintained, and under these conditions the adducts of heptaminol and leucinol had retention times of about 6.5 min and 7 min, respectively (Fig. 2). Samples could be injected every 10 min.

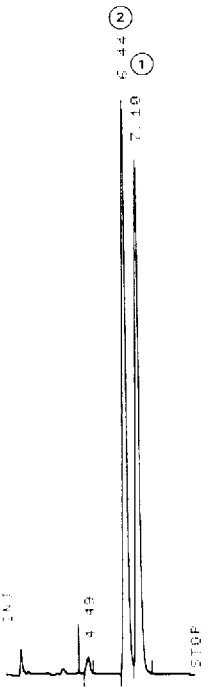

Fig. 2. Chromatogram of OPA-adducts of leucinol (peak 1) and heptaminol (peak 2). Column: 25 cm × 0.46 cm I.D., containing Zorbax C_8; flow-rate: 2.5 ml/min; solvent system: 45% (v/v) acetonitrile in aqueous sodium acetate, pH 6.1; detector: fluorescence, excitation wavelength = 338 nm, emission wavelength = 445 nm, scale expansion × 50.

Studies in human subjects

Plasma and urine samples were obtained from five human volunteer subjects dosed orally with a syrup formulation containing 0.47 g heptaminol and analysed by the foregoing procedures. These volunteer studies were conducted under conditions similar to those described by Brodie et al. [9].

RESULTS AND DISCUSSION

Heptaminol shows negligible UV—visible spectral properties but does possess a primary amine group which can be readily reacted with derivatising agents to form fluorescent adducts. Of the more popular fluorescence derivatising procedures, dansylation required elevated temperature and rather long reaction times, fluorescamine reacted rapidly at room temperature but in common with amino acids resulted in the formation of two fluorescent derivatives which separated during chromatography [10]. OPA gave the most satisfactory results. The derivatisation occurred rapidly at room temperature, was reproducible and the resultant adducts could be chromatographed with short analysis times.

Precision and accuracy of measurement

Derivatisation and measurement were repeated on five occasions at each concentration over the calibration ranges during a three-day period. The

TABLE I

BETWEEN-ASSAY PRECISION MEASUREMENTS OF HEPTAMINOL IN PLASMA

Concentration of heptaminol (μg/ml)	Peak area ratio $\frac{\text{heptaminol}}{\text{internal standard}}$					Mean (± S.D.)	Coefficient of variation (%)
0.1	0.10	0.10	0.12	0.09	0.08	0.10 (0.01)	10
0.2	0.18	0.21	0.18	0.19	0.20	0.19 (0.01)	5
0.4	0.35	0.37	0.38	0.37	0.37	0.37 (0.01)	3
0.8	0.74	0.73	0.74	0.77	0.72	0.74 (0.02)	3
1.2	1.07	1.09	1.11	1.11	1.13	1.10 (0.02)	2
2.0	1.76	1.73	1.83	1.77	1.82	1.78 (0.04)	2

TABLE II

BETWEEN-ASSAY PRECISION MEASUREMENTS OF HEPTAMINOL IN URINE

Concentration of heptaminol (μg/ml)	Peak area ratio $\frac{\text{heptaminol}}{\text{internal standard}}$					Mean (± S.D.)	Coefficient of variation (%)
0.5	0.06	0.03	0.06	0.05	0.05	0.05 (0.01)	20
1	0.09	0.09	0.10	0.09	0.11	0.10 (0.01)	10
2	0.19	0.18	0.20	0.19	0.19	0.19 (0.01)	5
4	0.36	0.36	0.38	0.38	0.39	0.37 (0.01)	3
6	0.54	0.55	0.56	0.59	0.59	0.57 (0.02)	4
8	0.72	0.74	0.73	0.75	0.77	0.74 (0.02)	3
10	0.92	0.93	0.91	0.92	0.95	0.93 (0.02)	2
12	1.06	1.10	1.10	1.10	1.20	1.11 (0.05)	5
15	1.31	1.35	1.32	1.40	1.45	1.37 (0.06)	4

between-day precision of the method for the plasma assay, as indicated by the coefficient of variation of peak area ratio measurements of drug to internal standard ranged from ± 10% at 0.1 μg/ml to ± 2% at 2.0 μg/ml (Table I). The precision of the method for the urine assay was ± 20% at 0.5 μg/ml to ± 4% at 15 μg/ml (Table II).

The calibration line for the determination of heptaminol in plasma constructed from five replicate measurements at six concentrations in the range 0.1—2 μg/ml, was linear ($Y = 0.017 + 0.884X$). The accuracy of the method as indicated by the standard error of the fitted least squares regression line was ± 0.027 μg/ml.

The calibration line for the estimation of heptaminol in urine, constructed from five replicate measurements at nine concentrations over the range 0.5—15 μg/ml of diluted urine was also linear ($Y = 0.009 + 0.0913X$). The standard error of this fitted line was ± 0.3 μg/ml.

Recovery and limit of detection

Heptaminol was completely recovered from plasma and urine. No interfering peaks with the same retention time as heptaminol were present in control plasma and urine samples, although a small peak was sometimes present with a similar retention time to that of the internal standard (Fig. 3). The concentration of internal standard was selected so that the contribution of this occasional component was negligible.

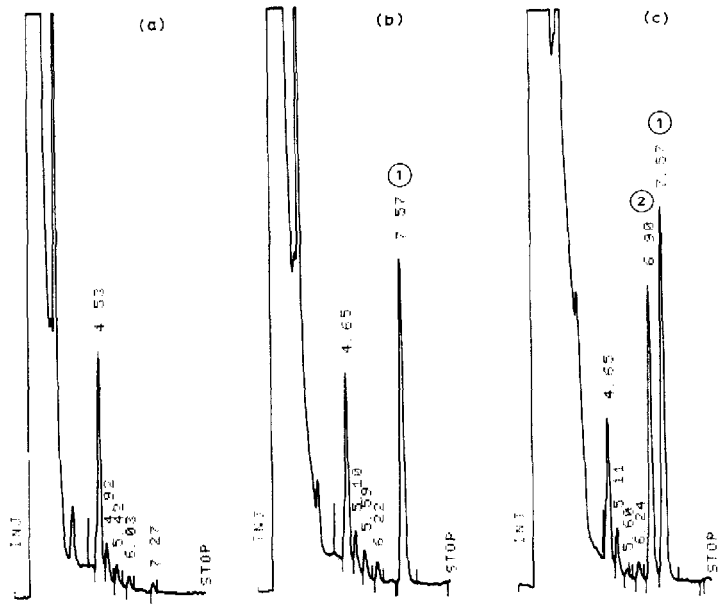

Fig. 3. Chromatograms of (a) control plasma (b) control plasma containing internal standard and (c) plasma containing heptaminol at a concentration of 0.8 μg/ml. Peaks: (1) = internal standard adduct; 2 = heptaminol adduct. Chromatography conditions as for Fig. 2.

Concentrations of heptaminol in plasma could be measured down to 0.1 μg/ml when assaying 100 μl plasma and using a final injection volume of 40 μl. This represents < 2 ng of heptaminol on column. The sensitivity of the method could be improved if necessary by analysing larger volumes of plasma or injecting larger volumes of derivatised solution.

Concentrations of heptaminol in urine could be measured down to 0.05 μg/ml of diluted urine when assaying 100 μl and using a final injection volume

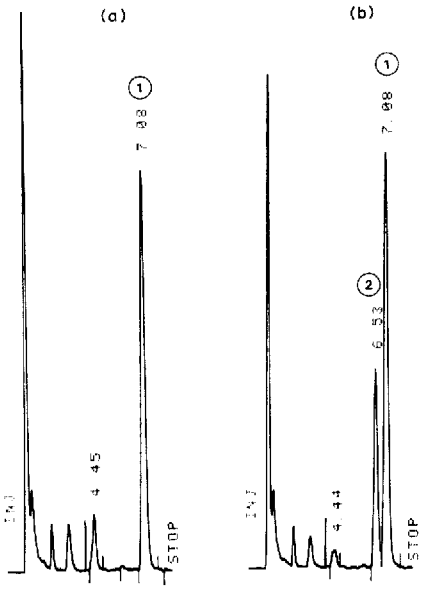

Fig. 4. Chromatograms of (a) pre-dose human urine and (b) 0—12 h diluted human urine containing heptaminol at a concentration of 4.8 μg/ml. Peaks: 1 = internal standard adduct; 2 = heptaminol adduct. Chromatography conditions as for Fig. 2.

of 30 μl. The sensitivity of the method is more than adequate since urinary concentrations of heptaminol are high (Fig. 4). As a highly polar compound, heptaminol is not metabolised to any extent, and between 75 and 95% of an oral dose is eliminated as unchanged drug within 24 h of dosing [2].

Concentrations of heptaminol in human plasma and urine

After single oral doses of a syrup formulation containing 0.47 g heptaminol, a peak of mean plasma heptaminol concentrations of 2.19 μg/ml occurred at 0.75 h and remained at about this level for about 2 h (Table III), indicating that heptaminol was relatively rapidly absorbed from the gastrointestinal tract of humans. Thereafter plasma levels of heptaminol declined monoexponentially with a half-life of 2.1 h (± 0.5 S.D.). Assuming complete absorption of heptaminol, the mean clearance and volume of distribution of heptaminol were 48.5 l/h (± 14.5 S.D.) and 145 l (± 45 S.D.), respectively. This clearance is numerically similar to the renal plasma flow-rate indicating that the renal extraction ratio for heptaminol is close to unity.

Urinary excretion of unchanged heptaminol was almost complete (77 ± 11% S.D.) within 12 h of dosing as might be expected from the plasma drug clearance since heptaminol is eliminated mainly, if not totally, by renal excretion. A mean of 82 ± 10% S.D. (range 68—92%) of the administered dose was excreted in the urine as unchanged drug. These data indicate that heptaminol was well absorbed from the gastrointestinal tract of humans. Similar results have been obtained by Chanoine et al. [3] who also reported a biexponential decline of plasma concentrations (half-lives 1.8 and 6.6 h) in contrast to the monoexponential decline obtained in the present study over the same time-course. Nonetheless, consideration of the urinary excretion data indicates that

TABLE III

PLASMA CONCENTRATIONS (μg/ml) OF HEPTAMINOL AFTER SINGLE ORAL DOSES OF 0.47 g HEPTAMINOL TO HUMAN SUBJECTS

For calculation of means, the concentration is taken as zero if < 0.10 μg/ml.

Time (h)	Subject No.					Mean	S.D.
	1	2	3	4	5		
0.25	0.45	0.28	0.63	2.37	1.48	1.04	0.87
0.50	1.40	2.00	1.20	2.16	2.11	1.77	0.44
0.75	2.68	2.45	1.59	2.37	1.87	2.19	0.45
1.0	2.22	2.33	1.32	2.19	1.80	1.97	0.42
1.5	2.01	2.56	1.41	2.24	1.56	1.96	0.48
2.0	2.63	2.16	1.78	2.51	1.47	2.11	0.49
2.5	2.37	1.94	1.42	2.14	1.49	1.87	0.41
3.0	2.13	1.86	1.31	1.94	1.47	1.74	0.34
4.0	1.83	1.22	0.88	1.33	0.82	1.22	0.41
5.0	1.16	1.03	0.51	1.02	0.61	0.87	0.29
6.0	0.93	0.60	0.40	0.82	0.40	0.63	0.24
7.0	0.60	0.57	0.31	0.66	0.30	0.49	0.17
8.0	0.21	0.44	0.12	0.45	0.19	0.28	0.15
10.0	0.12	0.25	<0.10	0.27	<0.10	0.13	0.13
12.0	<0.10	0.14	<0.10	0.18	<0.10	0.06	0.09

the half-life of about 2 h probably accounts for elimination of most of the administered dose.

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