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Note

Determination of 1-(3′-carboxypropyl)-3,7-dimethylxanthine and 1-(4′-carboxybutyl)-3,7-dimethylxanthine, two major metabolites of oxpentifylline, in urine by high-performance liquid chromatography

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Oxpentifylline (Trental®, pentoxifylline, I in Fig. 1) is a drug often used in the treatment of peripheral vascular disease [1—5]. Determination of oxpentifylline and some of its metabolites in plasma has been previously reported [6—10] but, because of extensive metabolism [11], no unchanged drug can be detected in urine.

The major metabolite of oxpentifylline, in man, is 1-(3′-carboxypropyl)-3,7-dimethylxanthine, CP-DMX (II in Fig. 1) and greater than 50% of administered drug can be excreted in urine as this species. Additionally, up to 10% of oxpentifylline is excreted in urine as the metabolite 1-(4′-carboxybutyl)-3,7-dimethylxanthine, CB-DMX (III in Fig. 1). Information on the bioavailability and pharmacokinetics of various formulations of oxpentifylline can be

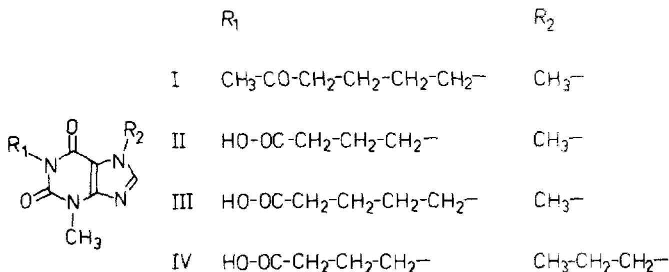

Fig. 1. Structural formulae of oxpentifylline (I), CP-DMX (II), CB-DMX (III) and internal standard (IV).

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obtained from the measurement of these metabolites in urine. Reversed-phase high-performance liquid chromatography (HPLC) provides a fast assay requiring little sample preparation making the assay suitable for both quantitative and compliance studies.

EXPERIMENTAL

Reagents

All chemicals, other than the analytes, were of analytical-reagent grade (Fisons, Loughborough, U.K.) and were used without further purification. Dichloromethane was routinely redistilled before use.

Standard solutions

Standard solutions (1 mg/ml) of CP-DMX, CB-DMX and the internal standard, 1-(3′-carboxypropyl)-3-methyl-7-propylxanthine (IV in Fig. 1) were prepared by dissolving the solid material in 0.01 *M* sodium hydroxide. These solutions were stored at 0—5°C when not in use.

A solution containing both CP-DMX (1 mg/ml) and CB-DMX (0.1 mg/ml) was prepared by dissolving the solid materials in the minimum volume of 1 *M* sodium hydroxide and diluting with drug-free human urine. Portions of this solution were diluted with drug-free urine to produce a series of samples with CP-DMX concentrations over the range 1—1000 μg/ml.

Extraction from urine

A solution of the internal standard (100 μg in 100 μl of 0.01 *M* sodium hydroxide) is pipetted into a 10-ml test tube fitted with a screw cap and a PTFE-faced rubber liner (Sovirel, France) and thoroughly mixed with a sample of urine (1 ml). Four calibration samples are similarly prepared by adding blank urine (1 ml) to tubes containing both the solution of the internal standard and a solution of CP-DMX and CB-DMX (100 μg of each in 100 μl of 0.01 *M* sodium hydroxide). Dichloromethane (6 ml) and 1 *M* hydrochloric acid (1 ml) are added and the urine is extracted for 15 min using a mechanical rotary inversion mixer operating at a fixed speed of 20 rpm (Heto Rotamix, V.A. Howe, London, U.K.). The phases are separated by centrifugation at 2000 *g* for 5 min and the upper aqueous phase is aspirated and discarded. Emulsions, if present, are broken by briefly shaking the tubes. The phases are again separated by centrifugation before the dichloromethane is transferred to a tapered test tube. The solvent is removed under a gentle stream of nitrogen with the tubes immersed in a water bath at 40°C. The residues are taken up in the HPLC mobile phase (2 ml). This solution is transferred to an autosampler vial which is fitted with a PTFE-lined screw cap and aliquots are analysed on the HPLC system described below.

Chromatography

A Model M45 pump (Waters Assoc., Northwich, U.K.) is coupled via a WISP autosampler (Waters Assoc.) to a stainless-steel column (15 cm × 3 mm I.D.) packed with Spherisorb-ODS 1 (5 μm particle size) reversed-phase material. The mobile phase is methanol—0.02 *M* orthophosphoric acid (1:2.5) adjusted to pH 4 with 6 *M* sodium hydroxide. The flow-rate is 1 ml/min.

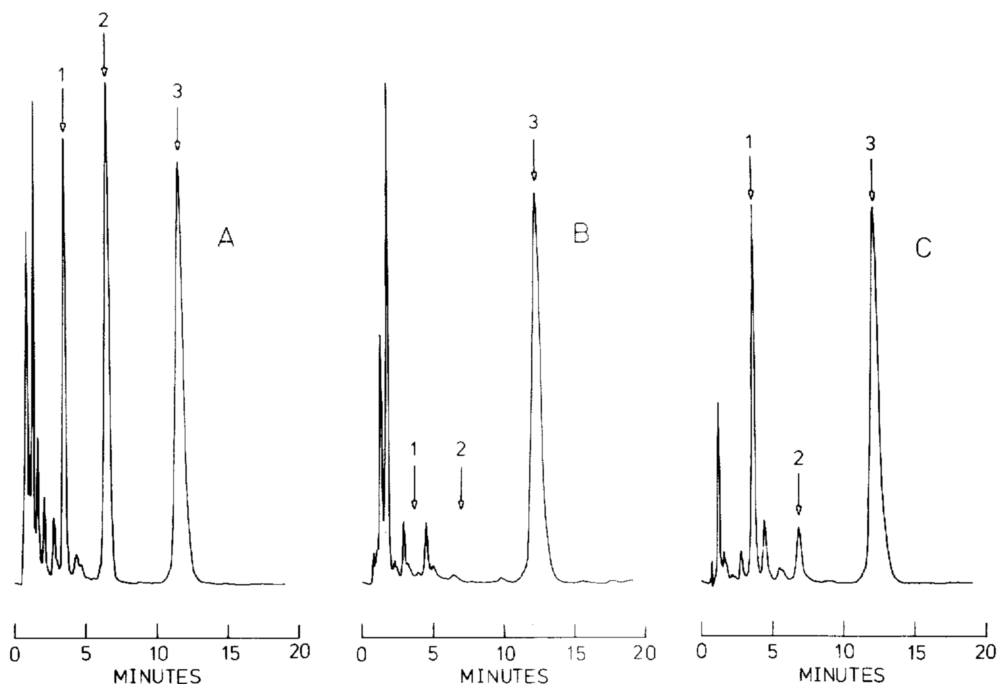

Fig. 2. Examples of chromatograms of extracts of urine containing the internal standard (98 μg/ml). (A) Blank urine to which had been added CP-DMX (97 μg/ml) and CB-DMX (101 μg/ml); (B) sample taken prior to administration of Trental; (C) sample collected 2—4 h after administration of Trental (100 mg). The arrows 1, 2 and 3 indicate the retention times of CP-DMX, CB-DMX and the internal standard, respectively.

The absorbance of the column eluent is measured at 274 nm with a LC/UV detector (Pye Unicam, Cambridge, U.K.) at a sensitivity setting of 0.32 a.u.f.s. Quantification is performed with a SP 4100 integrator (Spectra-Physics, St. Albans, U.K.) using an internal standard procedure.

Typical retention times of CP-DMX, CB-DMX, internal standard and oxpentifylline under the above conditions, are 3.4, 6.4, 11.6 and 18 min, respectively. Chromatograms from urine extracts are shown in Fig. 2.

RESULTS AND DISCUSSION

Extraction procedure

Peak levels of CP-DMX in urine can exceed 0.5 mg/ml and initially, sample preparation consisted only of dilution of the urine with HPLC mobile phase. However, separation of the xanthines from endogenous material was not always achieved because of the wide variation in individual urine samples. Therefore, an extraction step was included to reduce the interference from endogenous material.

Accuracy, precision and detection limit

The accuracy and precision of the assay were assessed by analysis of the urine samples containing known concentrations of CP-DMX and CB-DMX on six separate occasions. The results of these determinations (Tables I and II) show that the precision of the assay is dependent upon the urine concentration of the xanthines. Over the range 2—10 μg/ml the standard deviation (S.D.) of the determined concentrations of CP-DMX is found to be ± 1.5 μg/ml, whereas above 100 μg/ml the standard deviation of the determined values becomes proportional to the concentration and averages ± 5% of the determined values. Thus the overall precision can be defined in terms of the standard deviations of the CP-DMX determinations as S.D. = 1.5 μg/ml + 5% of the measured value. Similarly, the precision for CB-DMX is found to be S.D. = 0.3 μg/ml + 6% of the measured value.

The accuracy of the CP-DMX measurements is 104 ± 6% of the amount added over the concentration range 3—1000 μg/ml. However, the determined values of the minor metabolite, CB-DMX, are consistently underestimated by 1—3 μg/ml over the concentration range 1—100 μg/ml possibly due to incomplete integration of the peak.

TABLE I

DETERMINATION OF CP-DMX ADDED TO BLANK URINE (n = 6)

Added (μg/ml)	Found (mean ± S.D.) (μg/ml)	Relative standard deviation	Mean recovery (%)
0	0		
2.6	2.6 ± 1.9	0.73	100
5.2	6.0 ± 1.2	0.20	115
10.5	9.8 ± 1.4	0.14	93
26.1	28.1 ± 2.5	0.09	108
52.3	55.9 ± 4.8	0.09	107
105	110 ± 6	0.05	105
261	270 ± 17	0.06	103
523	538 ± 36	0.07	103
1045	1092 ± 34	0.03	104

TABLE II

DETERMINATION OF CB-DMX ADDED TO BLANK URINE (n = 6)

Added (μg/ml)	Found (mean ± S.D.) (μg/ml)	Relative standard deviation	Mean recovery (%)
0			
1.0	0.2 ± 0.3	1.5	20
2.5	1.6 ± 0.3	0.19	64
5.0	3.6 ± 0.4	0.11	72
10.0	8.1 ± 1.4	0.17	81
25.0	22.2 ± 2.3	0.10	89
50.0	47.6 ± 3.3	0.07	95
100	98 ± 2	0.02	98

The detection limit (DL) may be defined as the concentration which with a stated degree of confidence is significantly greater than the blank value. This may be expressed in terms of the standard deviation of the determined values close to the detection limit: DL = t S.D. where t is the one-tailed critical value of the t-distribution at the 95% confidence limit and, for six determinations, is 2.0. Thus the detection limit calculated using the data in Tables I and II is 3 μg/ml for CP-DMX and 1 μg/ml for CB-DMX.

Application of the method

The assay has been applied to urine samples from volunteers after oral administration of various oxpentifylline formulations. For example, in a cross-over experiment volunteers were given either the standard enteric-coated Trental tablet (100 mg oxpentifylline) or the slow release Trental 400 formulation (400 mg oxpentifylline) and urine was collected over the following 48 h.

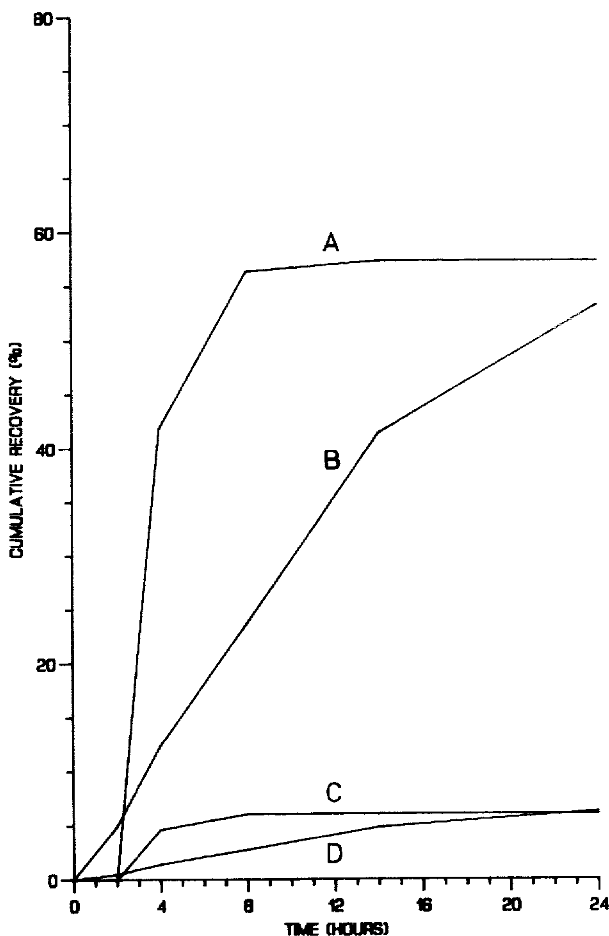

Fig. 3. Cumulative recovery in urine of CP-DMX (A and B) and CB-DMX (C and D) over 24 h, expressed as a percentage of the original dose. The volunteer received either enteric coated Trental (A and C) or Trental 400 (B and D).

The results from the analyses of urine from one volunteer (Fig. 3) clearly demonstrate the slow release properties of Trental 400. In the first 8 h following administration of the standard formulation Trental (100 mg), 65% of the oxpentifylline dose is recovered in the urine as the two carboxylic acid metabolites, whereas after administration of Trental 400, 28% of the dose is excreted over the same period and 34% is excreted over the next 16 h giving a 24-h total of 62%.

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