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DETERMINATION OF OXPENTIFYLLINE AND A METABOLITE, 1-(5'-HYDROXYHEXYL)-3,7-DIMETHYLXANTHINE, BY GAS—LIQUID CHROMATOGRAPHY USING A NITROGEN-SELECTIVE DETECTOR

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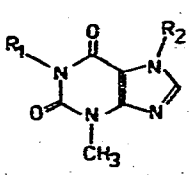
SUMMARY

A gas chromatographic method for the determination of oxpentifylline and a metabolite, 1-(5'-hydroxyhexyl)-3,7-dimethylxanthine is described. Oxpentifylline, metabolite and internal standard are extracted from basified plasma into dichloromethane, then the metabolite and internal standard are converted to their O-trifluoroacetates. Analysis by gas—liquid chromatography using a nitrogen-selective detector allows quantification of oxpentifylline and 1-(5'-hydroxyhexyl)-3,7-dimethylxanthine down to levels of 3 ng/ml and 3—10 ng/ml, respectively. The assay had been applied to plasma samples from volunteers after both intravenous and oral administration of oxpentifylline. The need to separate plasma from erythrocytes immediately after venipuncture sampling to prevent further metabolism of oxpentifylline is emphasized.

INTRODUCTION

Oxpentifylline (Trental®, pentoxifylline, I in Fig. 1) is widely used in the treatment of peripheral vascular disease. It increases blood flow by acting as a vasodilator and by reducing blood viscosity [1—5]. Metabolism of oxpentifyl-



	R_1	R_2
I	$CH_3COCH_2CH_2CH_2CH_2^-$	CH_3^-
II	$CH_3CH(OH)CH_2CH_2CH_2CH_2^-$	CH_3^-
III	$CH_2(OH)CH_2CH_2CH_2CH_2CH_2^-$	$CH_3CH_2CH_2^-$
IV	$CH_3CH_2CH_2^-$	$CH_3COCH_2CH_2CH_2CH_2^-$

Fig. 1. Structural formulae of oxpentifylline (I), metabolite I (II) and internal standards (III and IV).

line is extensive and a major metabolite found in blood is metabolite I, 1-(5'-hydroxyhexyl)-3,7-dimethylxanthine (II in Fig. 1) [6].

A sensitive assay was required to determine plasma levels of oxpentifylline in the presence of metabolites so that the bioavailability and pharmacokinetics in man could be studied. Previously reported assays have used both thin-layer chromatography [7, 8] and high-performance liquid chromatography [9]. This paper describes an assay using gas–liquid chromatography (GLC) which enables plasma levels to be determined from at least 3 μg/ml down to 3 ng/ml for oxpentifylline and 3–10 ng/ml for metabolite I. Whole blood freshly obtained by venipuncture (to which oxpentifylline had been added) metabolises oxpentifylline to metabolite I. No formation of this metabolite was observed in plasma. Thus erythrocytes should be separated from plasma immediately after sampling to prevent further formation of metabolite I.

MATERIALS AND METHODS

Reagents

All chemicals were of analytical grade and were used without further purification, unless otherwise stated. Dichloromethane (Fisons, Loughborough, Great Britain) was redistilled before use. A hexane solution of 5% (v/v) trifluoroacetic anhydride (TFAA; Aldrich, Gillingham, Great Britain) was freshly prepared for each batch of samples.

Standard solutions

A standard solution of oxpentifylline (100 μg/ml) in 0.01 *M* HCl was prepared by dissolving the solid material in 1 *M* HCl and making up to the required volume with distilled water. This solution was diluted with 0.01 *M* HCl to provide a calibration standard containing 10 μg/ml oxpentifylline. Standard solutions containing 10 μg/ml of metabolite I and the internal standard, 1-(6'-hydroxyhexyl)-3-methyl-7-propylxanthine (III in Fig. 1) were prepared in the same way. These solutions were stored at 0–5° and were stable for at least one month.

Extraction from plasma and derivatisation

Internal standard (1 μg in 100 μl of 0.01 *M* HCl) and plasma (2 ml) are pipetted into a 10-ml conical tube fitted with a screw cap having a PTFE-faced rubber liner (Sovirel, Paris, France), and thoroughly mixed. Dichloromethane (5 ml) and 1 *M* NaOH (0.5 ml) are added and the plasma extracted for 15 min using a mechanical rotary inversion mixer operating at a fixed speed of 20 rpm (Heto Rotamix, V.A. Howe, London, Great Britain). The phases are separated by centrifugation at 2000 *g* for 5 min then the upper plasma phase is aspirated and discarded. The remaining dichloromethane emulsion is broken by briefly shaking the tubes and centrifuging again. After careful aspiration of the lipid plug, the dichloromethane is transferred to a 10-ml tapered test tube (Quickfit, Fisons, Great Britain) and evaporated under a gentle stream of nitrogen with the tubes in a water bath at 40°.

A freshly prepared solution of 5% (v/v) TFAA in hexane (1 ml) is added and the stoppers secured in place by means of spring clips (HWS, Labap, Hudders-

field, Great Britain). The residues are taken up in solution with the aid of a vortex mixer and esterification is completed by heating the tubes in a water bath at 60° for 10 min. Excess reagent is removed by evaporation under a stream of nitrogen with the tubes immersed in a water bath at 60°. The residues are taken up in toluene (50 μl) and aliquots (5 μl) of this solution are analysed by GLC.

Gas—liquid chromatography

Analyses were performed on a Perkin-Elmer F17 gas chromatograph equipped with a nitrogen—phosphorus detector. The inside of the glass column (2 m × 1.75 mm I.D.) was treated with a 5% (v/v) solution of dimethyldichlorosilane in hexane, then rinsed with methanol and acetone, and dried before packing with 3% OV-25 on Chromosorb W HP (100—120 mesh). The column inlet was repacked with fresh material after the analysis of each batch of samples and conditioned overnight before re-use. The polarising voltage on the nitrogen detector control box was set to position 3, and the bead current potentiometer set between 500 and 700 depending on the age of the bead. Helium was used as the carrier gas at a flow-rate of 25 ml/min and the air and hydrogen flow-rates to the detector were 80 and 3 ml/min, respectively. The oven temperature was 235°, and the injector/detector block was maintained at 250°.

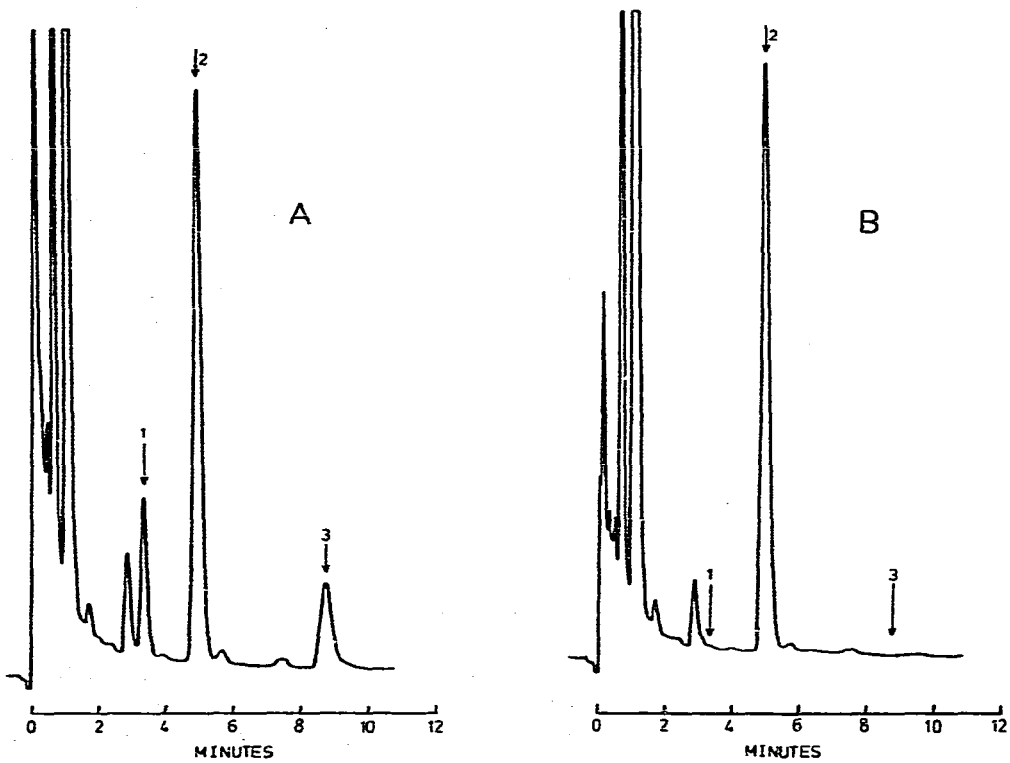


Fig. 2. Examples of chromatograms: A, extract of plasma (2 ml) to which had been added oxpentifylline (106 ng/ml), metabolite I (99.6 ng/ml) and internal standard (497 ng/ml); B, extract of blank plasma (2 ml) to which had been added internal standard (497 ng/ml) alone. The arrows 1, 2 and 3 indicate the retention times of metabolite I (O-trifluoroacetate), internal standard (O-trifluoroacetate) and oxpentifylline, respectively.

Under these conditions typical retention times of the O-trifluoroacetate of metabolite I, the O-trifluoroacetate of the internal standard, and oxpentifylline were 3.4, 5.0 and 8.8 min, respectively.

Chromatograms obtained from plasma extracts are shown in Fig. 2.

Quantification

Plasma levels of oxpentifylline and metabolite I were calculated from peak height measurements using response factors obtained by analysing, in parallel with the unknowns, blank plasma to which had been added oxpentifylline (1 μg in 100 μl of 0.01 *M* HCl) and metabolite I (1 μg in 100 μl of 0.01 *M* HCl).

Extraction efficiency

Oxpentifylline or metabolite I (1 μg) in 0.01 *M* HCl (100 μl) and human plasma (2 ml) were pipetted into screw-top test tubes and thoroughly mixed. 1 *M* NaOH (0.5 ml) and dichloromethane (5 ml) were added and the plasma was extracted for various times between 2 and 20 min on a rotary inversion mixer operating at 20 rpm. The phases were separated by centrifugation, the plasma aspirated, and a portion (4 ml) of the dichloromethane phase transferred to a tapered test tube containing a dichloromethane solution of internal standard (1 μg). The solvent was evaporated, the residue taken up in toluene (50 μl) and analysed by GLC. The recovery of oxpentifylline and metabolite I was estimated by comparison with non-extracted standards.

Metabolism of oxpentifylline by whole blood

Whole blood was obtained from a human volunteer by venipuncture and placed in a heparinised container to prevent coagulation. Plasma was obtained from a sample of the whole blood by centrifugation, and, within 30 min of sampling, portions (2 ml) of whole blood or plasma were added to a solution of oxpentifylline (0.8 μg) in 0.01 *M* HCl (100 μl), thoroughly mixed, and incubated for up to 2 h at 37°. Internal standard (1 μg) was added and the levels of oxpentifylline and metabolite I were then determined by the standard extraction and analysis procedure, except that the volume of 1 *M* NaOH added was increased to 1 ml to reduce the viscosity of the whole blood samples.

RESULTS AND DISCUSSION

Oxpentifylline and metabolite I have similar retention indices when chromatographed on OV-1, OV-17 and OV-25 stationary phases (table I). The use of more selective stationary phases other than the siloxanes was prevented because of the relatively high analysis temperature. Therefore it was necessary to derivatise one of the compounds to achieve separation by GLC. Metabolite I forms a trimethylsilyl ether and a trifluoroacetate, both of which are easily separated from oxpentifylline on siloxane stationary phases (Table I). The latter derivative was chosen as it was formed quantitatively and the excess reagent was easily removed.

The esterification of metabolite I and the internal standard was complete after 5 min, and no difference was observed between chromatograms of plasma extracts which had been reacted with 5% TFAA in hexane for 5 or 60 min.

TABLE I

RETENTION INDICES OF XANTHINE DERIVATIVES ON OV-1, OV-17 AND OV-25

Xanthine as shown in Fig. 1	Retention index on OV-1 at 240°	Retention index on OV-17 at 250°	Retention index on OV-25 at 250°
I	2420	3000	3230
II	2430	2995	3215
II O-trifluoroacetate	2300	2720	2875
II O-trimethylsilyl ether	2485	2915	—
III	2575	3125	3345
III O-trifluoroacetate	2450	2850	3000
III O-trimethylsilyl ether	2635	3045	—
IV	2475	3000	3200

TABLE II

IN VITRO METABOLISM OF OXPENTIFYLLINE

Oxpentifylline (402 ng/ml) was added to each sample, and each result is the mean of two determinations.

Sample	Incubation time (min)	Oxpentifylline found (ng/ml)	Metabolite I found (ng/ml)
Plasma	120	421	Not detected
Whole blood	2	434	<1
Whole blood	120	337	75

Dilution of TFAA with hexane prevented the formation of side-reaction products.

Two compounds were available as possible internal standards; namely, 1-(6'-hydroxyhexyl)-3-methyl-7-propylxanthine (III in Fig. 1) and 1-propyl-3-methyl-7-(5'-oxohexyl)xanthine (IV in Fig. 1). The latter is the most suitable for the analysis of oxpentifylline, but it was not fully resolved from oxpentifylline on methylsiloxane or methylphenylsiloxane stationary phases. Thus, 1-(6'-hydroxyhexyl)-3-methyl-7-propylxanthine, analysed as its O-trifluoroacetate, was chosen as the internal standard, although it is more closely related to metabolite I than to oxpentifylline.

Human plasma containing either oxpentifylline or metabolite I (0.5 μg/ml) was basified and extracted with dichloromethane for various times. The results from this experiment, which are uncorrected for any volume change of the dichloromethane phase which may have occurred during extraction, show that an extraction time of only 2 min is required to extract oxpentifylline and metabolite I with efficiencies of 99% and 96%, respectively.

Incubation of oxpentifylline with either whole blood or plasma shows that the formation of metabolite I occurs only in the erythrocytes (Table II). It is therefore necessary to separate and remove the plasma from whole blood immediately after sampling to prevent further metabolism of oxpentifylline.

Accuracy and precision

The accuracy and precision of the method was determined by analysing blank human plasma to which had been added known amounts of oxpentifyl-

line and metabolite I. The results of six separate determinations at each plasma level are summarised in Tables III and IV. They show that plasma levels can be accurately determined from approximately 3 μg/ml down to 3 ng/ml for oxpentifylline, and from 3 μg/ml down to 3—10 ng/ml for metabolite I. The actual limit of the assay is determined by background interference which has been found to vary between batches of samples.

TABLE III

DETERMINATION OF OXPENTIFYLLINE ADDED TO BLANK PLASMA

Each result is the mean of six determinations.

Oxpentifylline added (ng/ml)	Oxpentifylline found (mean ± S.D., ng/ml)	Relative standard deviation	Mean recovery (%)
0	0.8 ± 0.6	0.8	
1.0	1.0* ± 0.4	0.4	100
3.1	3.0* ± 0.8	0.3	97
10.3	9.3* ± 0.4	0.4	90
30.6	27.6* ± 0.3	0.1	90
106	100 ± 3	0.03	94
309	304 ± 9	0.03	98
1050	1070 ± 40	0.04	102
3017	3063 ± 66	0.02	102

*These values have been corrected for a background level of 0.8 ng/ml.

TABLE IV

DETERMINATION OF METABOLITE I ADDED TO BLANK PLASMA

Each result is the mean of six determinations.

Metabolite I added (ng/ml)	Metabolite I found (mean ± S.D., ng/ml)	Relative standard deviation	Mean recovery (%)
0	Not detected		
2.9	3.4 ± 2.9	0.9	117
9.7	11.0 ± 1.1	0.1	113
28.9	30.5 ± 1.0	0.03	106
99.6	101 ± 1	0.01	101
291	300 ± 3	0.01	103
989	1022 ± 21	0.02	103
2843	2863 ± 85	0.03	101

Application of the method

The assay has been applied to the analysis of plasma from volunteers and patients after intravenous infusion of oxpentifylline and after single or multiple oral administration of various oxpentifylline preparations. In a typical experiment a volunteer was given oxpentifylline (200 mg) in a capsule and blood samples were collected over the following 24 h. Plasma was separated from the red blood cells by centrifugation immediately after withdrawal and stored deep frozen until analysed. A plasma profile obtained from one such experiment is shown in Fig. 3.

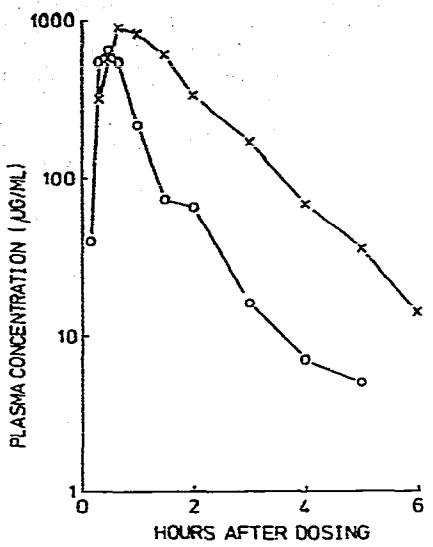


Fig. 3. Plasma levels of oxpentifylline (O) and metabolite I (X) in a volunteer after an oral dose of 200 mg of oxpentifylline.

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