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DETERMINATION OF OXPENTIFYLLINE AND FOUR METABOLITES IN PLASMA AND URINE BY AUTOMATED CAPILLARY GAS CHROMATOGRAPHY USING NITROGEN-SELECTIVE DETECTION

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SUMMARY

A capillary gas chromatographic assay for the determination of oxpentifylline and four hydroxylated metabolites is described. Oxpentifylline, metabolites and internal standards are extracted from basified plasma or urine with chloroform and the metabolites are converted to their O-trifluoroacetates before analysis by capillary gas chromatography using nitrogen-selective detection. A high throughput of samples is achieved by use of an autosampler and a split injection technique which allows the samples to be analysed isothermally. The detection limits of the compounds are in the range 2—10 ng/ml for plasma and 0.1—0.2 μg/ml for urine. The assay has been applied to the analysis of plasma and urine samples after the oral administration of oxpentifylline.

INTRODUCTION

Oxpentifylline (Trental®, pentoxifylline, I in Fig. 1) is a xanthine derivative which is widely used in the treatment of peripheral vascular diseases as it decreases blood viscosity by increasing red cell deformability [1—6]. The metabolism of this drug is extensive and eight metabolites have been reported [7]. One group of metabolites differs from oxpentifylline in that the oxohexyl side-chain of the parent compound is converted into a mono- or dihydroxy-substituted hexyl group. These four neutral metabolites (II—V in Fig. 1), together with oxpentifylline, can be separated from the other acidic metabolites by preferential extraction from basified plasma, and it is these five compounds which are the analytes of the assay described.

Oxpentifylline and its metabolites have been studied by thin-layer chromato-

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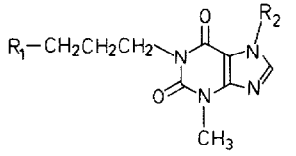

	R_1	R_2
I	CH_3COCH_2-	CH_3-
II	$CH_3CH(OH)CH_2-$	CH_3-
III	$HOCH_2CH(OH)CH_2-$	CH_3-
IV	$CH_3CH(OH)CH(OH)-$	CH_3-
V	$CH_3CH(OH)CH(OH)-$	CH_3-
VI	$HOCH_2CH_2CH_2-$	$CH_3CH_2CH_2-$
VII	$CH_3COCH_2CH_2-$	CH_3-

Fig. 1. Structural formulae of oxpentifylline (I), metabolites (II—V), and internal standards (VI, VII); II = 1-(5′-hydroxyhexyl)-3,7-dimethylxanthine; III = 1-(5′,6′-dihydroxyhexyl)-3,7-dimethylxanthine; IV = *erythro*-1-(4′,5′-dihydroxyhexyl)-3,7-dimethylxanthine; V = *threo*-1-(4′,5′-dihydroxyhexyl)-3,7-dimethylxanthine; VI = 1-(6′-hydroxyhexyl)-3-methyl-7-propylxanthine; and VII = 1-(6′-oxoheptyl)-3,7-dimethylxanthine.

graphy [8, 9] and some of these compounds have also been determined by high-performance liquid chromatography [10—12]. A gas—liquid chromatographic assay, using packed columns, has been previously reported [13]. This paper reports the further development of this assay using capillary chromatography which enables three additional metabolites to be determined whilst reducing the analysis time but maintaining the accuracy and precision of the assay.

The metabolites are converted to their O-trifluoroacetates (O-TFA) prior to chromatographic analysis to eliminate peak tailing by removal of the free hydroxyl groups and also to improve the separation of the xanthines from each other. The use of two internal standards was investigated. The first, 1-(6′-hydroxyhexyl)-3-methyl-7-propylxanthine (VI in Fig. 1), formed an O-TFA derivative like the metabolites whereas the other, 1-(6′-oxoheptyl)-3,7-dimethylxanthine (VII in Fig. 1), like oxpentifylline remained unchanged.

EXPERIMENTAL

Reagents

Ethyl acetate, chloroform, hexane fraction, toluene and sodium hydroxide were of analytical-reagent grade (Fisons, Loughborough, U.K.). Trifluoroacetic anhydride (TFAA; Aldrich, Gillingham, U.K.) was diluted with hexane to form a 5% (v/v) solution which was freshly prepared for each batch of samples. Hexane and chloroform were redistilled before use.

Standard samples of oxpentifylline, metabolites (compounds II—V) and

internal standard VI were supplied by Hoechst Werk Albert (Wiesbaden, F.R.G.). Internal standard VII was synthesised in the authors' laboratory.

Standard solutions

Standard solutions of oxpentifylline, the metabolites (II–V) and the internal standards (VI and VII) were prepared by dissolving the solid materials (25 ± 1 mg) in distilled water (500 ml). Portions of drug and metabolite solutions were mixed and diluted with water to produce a solution containing 5 μg/ml oxpentifylline and its metabolites. The internal standard solutions were similarly diluted to 5 μg/ml. These solutions were used to calibrate the plasma assay. For the urine assay, portions of the metabolite solutions were mixed and diluted to 10 μg/ml of each compound. The internal standard solution (VI) was similarly diluted.

Preparation of the plasma extracts

A solution of the internal standards VI and VII (0.5 μg of each) in water (100 μl) is added to each 8-ml glass test-tube fitted with a PTFE-faced liner (Sovirel, Paris, France). Oxpentifylline and the metabolites II–V (0.5 μg of each) in water (100 μl) are added to six calibration samples and then plasma (1 ml) is added to each tube and thoroughly mixed with the aqueous xanthine solutions. Chloroform (4 ml) and 0.1 *M* sodium hydroxide (0.1 ml) are added and the plasma extracted for 5 min by placing the tubes on a rotary inversion mixer (Heto Rotamix, V.A. Howe, London, U.K.) operating at 20 rpm. The phases are separated by centrifugation at 2000 *g* for 5 min and the upper plasma phase is aspirated and discarded. Occasionally the remaining chloroform phase is emulsified and in these cases the tubes are shaken briefly and centrifuged again. The chloroform is transferred to a tapered glass tube (Quickfit, Fisons) and evaporated under a gentle stream of nitrogen with the tubes held in a heating block (Techne, Fisons) at 60°C.

Freshly prepared 5% (v/v) TFAA in hexane (1 ml) is added and the tubes stoppered. The stoppers are secured with spring clips (HWS, Labap, Huddersfield, U.K.). A vortex mixer is used to aid dissolution of the residues and esterification is completed by placing the tubes in the heating block for 10 min at 60°C. Excess reagent is removed by evaporation under a stream of nitrogen while the tubes are held in the heating block at 60°C. Toluene (50 μl) is added to the residues and a vortex mixer is used to aid dissolution. The samples are transferred to 100-μl autosampler vials (Hewlett-Packard, Winnersh, U.K.). Aliquots (5 μl) are analysed by gas chromatography (GC).

Preparation of urine extracts

Urine samples are prepared in the same way as plasma except for the following changes. The sample volume is reduced to 100 μl and the amount of the metabolites added to the calibration samples and the internal standard (VI) added to all the samples is increased to 1 μg. The volume of sample injected into the gas chromatograph is reduced to 2 μl.

Gas–liquid chromatography

The samples were analysed on the following equipment supplied by Hewlett-

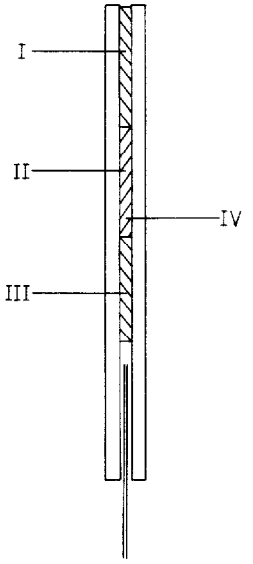

Fig. 2. Silica split liner showing the relative positions of the silanised glass wool (I), 3% OV-1 on Chromosorb W HP (II), silanised quartz wool (III) and the point of injection (IV).

Packard. The gas chromatograph was a Model 5710A equipped with a Model 18789A nitrogen-specific detector and a Model 18740B capillary inlet system. The column was a 25 m × 0.31 mm I.D. fused-silica capillary coated with a 0.17-μm film of cross-linked methyl silicone (Part No. 19091A Opt. 012). The outlet of the column was inserted directly into the jet of the detector. The capillary inlet was operated in the split mode with a silica liner (Part No. 18740-80220) packed with 3% OV-1 on Chromosorb W HP, 100—120 mesh (see Fig. 2). This packing was replaced with fresh material and conditioned at the operating temperature before the analysis of each batch of samples. Quartz wool was used to hold the packing in place and was treated, in situ, with a 5% solution of dichlorodimethylsilane in hexane, followed by a methanol rinse, to eliminate the poor peak shapes obtained with the untreated material.

Samples were injected with a Model 7671A autosampler using a Model 701RN 10-μl syringe (Hamilton, V.A. Howe). The autosampler was operated in the alternate wash mode using ethyl acetate as the wash solvent and was controlled via a Model 3390A integrator and a Model 19400A sampler control module.

Helium was used as the carrier gas (inlet pressure 1 kg/cm^2 giving rise to a mean linear velocity of 43 cm/sec), the septum purge (1—2 ml/min) and the detector make-up gas (16 ml/min). The split flow-rate was normally 20 ml/min for plasma samples and 40 ml/min for urine samples. The detector gases (hydrogen and air) were at flow-rates of 3 and 60 ml/min, respectively, and the detector bead voltage set at 14.5 V. The oven was operated isothermally at 230°C, the injection port at 300°C and the detector at 250°C.

The output of the chromatograph amplifier was connected to the integrator using range 1 for plasma samples and range 10 for urine samples. Peak width

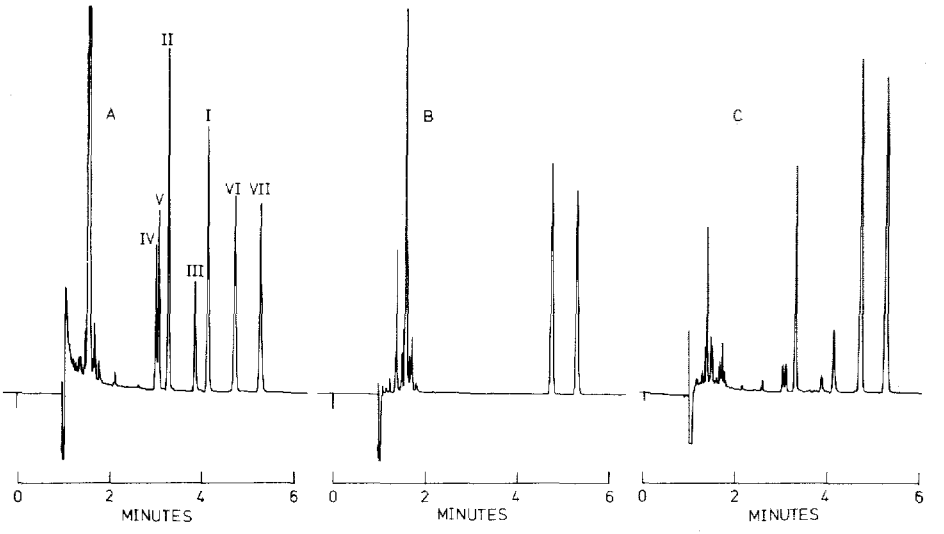

Fig. 3. Chromatograms of plasma extracts. (A) Calibration sample containing 0.5 μg/ml of each compound; (B) sample from a volunteer prior to administration; (C) sample from a volunteer 3 h after administration of oxpentifylline in a retard formulation. The numbered peaks in the calibration sample are as in Fig. 1.

measurements, for determination of column efficiency, were made with the external input of a 5880A gas chromatograph. Chromatograms were also recorded on a 1-mV Model 056 recorder (Perkin-Elmer, Beaconsfield, U.K.) and examples from plasma extracts are shown in Fig. 3.

RESULTS

Validation of the assay

Aqueous solutions of oxpentifylline and the metabolites II—V, in the approximate ratio of 5:5:1:1:1, respectively, were added to pooled human plasma to produce a series of samples of known concentration. Each plasma was analysed on six separate days over a period of five weeks by the assay described and the concentrations were quantified using each of the internal standards. Some of these results using VII as internal standard are shown in Table I. Comparable results were obtained using VI as the internal standard. A series of urine samples containing approximately equal amounts of the metabolites was similarly prepared and analysed (Table II).

Precision

The standard deviations (S.D.) of the results obtained for each validation sample were considered to be a measure of the precision of the assay. Because of the wide concentration range these values are not constant but increase with increase in concentration. For example, considering the measurement of oxpentifylline in plasma using VII as the internal standard, the standard deviations over the range 100—1000 ng/ml average 1.4% of the measured values. However, over the range 1—10 ng/ml the standard deviations are relatively constant and average 0.6 ng/ml. Thus the overall standard deviation

TABLE I

DETERMINATION OF OXPENTIFYLLINE (I) AND METABOLITES (II—V) ADDED TO BLANK PLASMA

In all cases n = 6 using VII as internal standard.

Approximate concentration added (ng/ml)	Concentration found (mean ± S.D., ng/ml)				
	I	II	III	IV	V
0	0 ± 0	0.4 ± 0.5	23.5 ± 3.3	0 ± 0	1.4 ± 1.6
1	1.5 ± 1.0	1.5 ± 1.4	27.8 ± 5.5	0.9 ± 0.8	1.5 ± 1.7
5	5.9 ± 0.7	6.3 ± 0.8	32.3 ± 5.9	4.8 ± 0.3	6.2 ± 0.8
10	9.7 ± 0.2	11.1 ± 0.6	34.3 ± 1.8	10.4 ± 0.8	11.2 ± 1.0
50	49.3 ± 0.9	52.0 ± 3.2	70.1 ± 8.2	50.1 ± 2.8	50.8 ± 4.5
100	99.7 ± 1.6	103 ± 7	123 ± 5	107 ± 4	106 ± 7
500	522 ± 5	538 ± 21	527 ± 28	523 ± 24	516 ± 26
1000	1037 ± 9	1072 ± 51	1007 ± 26	1045 ± 26	1056 ± 34
2500	2570 ± 59	2582 ± 201*			

*Five determinations only.

TABLE II

DETERMINATION OF METABOLITES (II—V) ADDED TO BLANK URINE

In all cases n = 6 using VI as internal standard.

Approximate concentration added (μg/ml)	Concentration found (mean ± S.D., ng/ml)			
	II	III	IV	V
0	0 ± 0	0.02 ± 0.02	0.01 ± 0.02	0 ± 0
0.1	0.10 ± 0.01	0.20 ± 0.16	0.11 ± 0.07	0.12 ± 0.01
0.5	0.49 ± 0.05	0.55 ± 0.04	0.50 ± 0.04	0.44 ± 0.06
1	1.00 ± 0.04	1.16 ± 0.12	0.98 ± 0.07	0.89 ± 0.12
5	5.01 ± 0.15	5.54 ± 0.18	5.06 ± 0.14	4.57 ± 0.21
10	10.2 ± 0.1	11.3 ± 0.1	10.4 ± 0.2	9.83 ± 0.26
50	52.4 ± 3.1	57.2 ± 5.2	53.6 ± 4.5	51.0 ± 9.9

may be described as S.D. = 0.6 ng/ml + 1.4% of the measured value. A summary of the overall precision for each compound is shown in Table III.

Limit of detection

The limit of detection (D.L.) may be defined as the concentration at which the measured value is significantly greater than zero. This may be expressed in terms of the limiting standard deviation as the concentration tends to zero ($S.D._{c\to 0}$) as follows: D.L. = $t_{(n,95\%)} \cdot S.D._{c\to 0}$ where $t_{(n,95\%)}$ is the one-tailed critical value of the t-distribution for n determinations at the 95% confidence limit and, for n = 6, $t_{(n,95\%)}$ = 2.0.

Thus the detection limits of the compounds in plasma are approximately 2 ng/ml (Table III) except for III (10 ng/ml). This compound was subject to interference from a background peak [believed to be due to the plasticiser tris(2-butoxyethyl) phosphate] of similar retention time which originated from the pooled plasma used for the validation. This peak was equivalent to 20—25

TABLE III

PRECISION OF THE DETERMINED VALUES AND THE LIMITS OF DETECTION OF OXPENTIFYLLINE AND ITS HYDROXY METABOLITES IN PLASMA AND URINE

Matrix	Compound	Overall precision	Limit of detection
Plasma	I	± (0.7 ng/ml + 6.5%)	2 ng/ml
	I*	± (0.6 ng/ml + 1.4%)	2 ng/ml
	II	± (1.0 ng/ml + 2.9%)	2 ng/ml
	II*	± (0.9 ng/ml + 5.1%)	2 ng/ml
	III	± (4.5 ng/ml + 6.4%)	9 ng/ml
	III*	± (4.7 ng/ml + 3.8%)	10 ng/ml
	IV	± (0.7 ng/ml + 4.9%)	2 ng/ml
	IV*	± (0.7 ng/ml + 3.8%)	2 ng/ml
	V	± (1.2 ng/ml + 7.7%)	3 ng/ml
	V*	± (1.3 ng/ml + 5.4%)	3 ng/ml
Urine	II	± (0.05 μg/ml + 3.9%)	0.1 μg/ml
	III	± (0.07 μg/ml + 8.1%)	0.2 μg/ml
	IV	± (0.03 μg/ml + 3.1%)	0.1 μg/ml
	V	± (0.10 μg/ml + 4.4%)	0.2 μg/ml

*Compound VII used as internal standard. All other values based upon VI as internal standard.

TABLE IV

ACCURACY OF THE MEANS OF THE DETERMINED VALUES OF OXPENTIFYLLINE AND ITS HYDROXY METABOLITES IN PLASMA AND URINE

Matrix	Compound	Overall accuracy
Plasma	I	± (0.5 ng/ml + 2.6%)
	I*	± (0.5 ng/ml + 1.4%)
	II	± (1.2 ng/ml + 4.8%)
	II*	± (1.1 ng/ml + 3.6%)
	III	± (2.5 ng/ml + 1.3%)**
	III*	± (2.1 ng/ml + 1.7%)**
	IV	± (0.3 ng/ml + 7.1%)
	IV*	± (0.3 ng/ml + 6.6%)
	V	± (1.1 ng/ml + 6.9%)
	V*	± (1.1 ng/ml + 5.7%)
Urine	II	± (0.02 μg/ml + 2.8%)
	III	± (0.09 μg/ml + 13%)
	IV	± (0.04 μg/ml + 2.4%)
	V	± (0.06 μg/ml + 4.9%)

*Compound VII used as internal standard. All other values based upon VI as internal standard.
**After compensation for background measurement.

ng/ml III but was not found in any of the volunteer samples which have been analysed. The detection limits in urine (Table III) were somewhat higher (0.1—0.2 μg/ml).

Accuracy

Accuracy can be considered as being the modulus of the difference or bias

between the mean of the measured values and the amount added. As with the precision, this value increases with increase in concentration and can be described in terms of a minimum value plus a concentration-dependent term. The accuracy of the determined values is summarised in Table IV.

Performance of the splitter

A comparison was made between the manufacturers' recommended split insert and the one shown in Fig. 2. Calibration samples were repeatedly analysed and the split flow-rate varied. The retention times and peak widths were determined by an integrator and the effective plates (N_{eff}) of the column calculated. The performance of the splitters was equivalent for components with a high capacity factor (K) but as the retention times of the xanthines fell, a difference between the two splitters became noticeable. This is illustrated in Fig. 4 where a sharp drop in N_{eff} was found for the O-TFA derivative of II (K = 2.38) using the commercial splitter whereas the performance of the 2-mm I.D. liner is virtually independent of flow-rate.

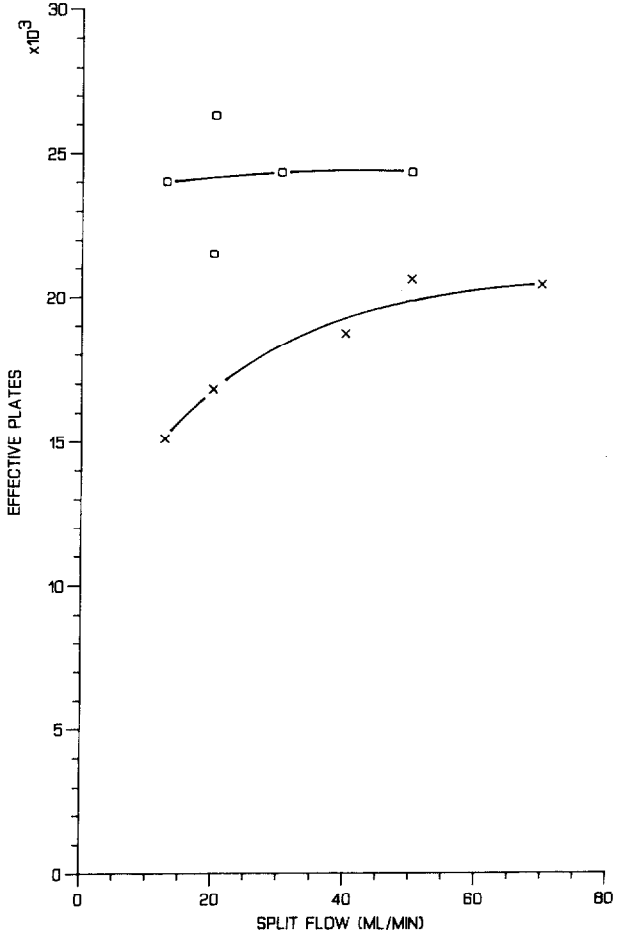

Fig. 4. Comparison of the effective plates obtained from II using the splitter shown in Fig. 2 (○) and a commercial split liner (×).

DISCUSSION

Capillary GC is being increasingly used in the analysis of drugs in biological fluids. The splitless injection technique, developed by Grob and Grob [14—17], is probably the most popular of the automated techniques for trace analysis, but in our experience suffers from two major drawbacks in drug analysis. Firstly, the need to cold-trap high-boiling compounds to prevent band spreading, plus the associated temperature programming, results in long analysis cycles reducing the rate of sample throughput. Secondly, high-boiling material in plasma extracts deposits on the capillary inlet as droplets unless the extracts have undergone additional clean-up steps. This causes a rapid loss in column efficiency and limits the number of samples which can be analysed before these deposits have to be removed.

Split injection techniques have not been popular in trace analysis because they are inherently less sensitive as only a minor fraction of the sample enters the analytical column. However, if the analytes have a relatively narrow range of retention indices, such as the xanthines in this assay (see Table V), the analysis can be performed isothermally resulting in a high rate of sample throughput. None of the high-boiling endogenous material in the samples, e.g. cholesterol (known to be present in relatively large amounts in the extracts), produced a response on the nitrogen detector. Commercially available splitters are usually designed to take a representative sample from a mixture having components over a wide boiling range and often have large internal dimensions requiring high flow-rates. A splitter for use in drug analysis has a different requirement in that only a few components in the sample are of interest and, because of their low concentration, the transfer of these to the capillary column should be maximised. With this in mind the capillary system was modified as follows. Sensitivity could be increased by simply increasing the column flow-rate by 50% above its optimum whilst reducing the column efficiency only slightly. Replacement of the commercial split liner (4 mm I.D.) with the silica tube (2 mm I.D.) allows a reduction in the flow-rate through the splitter by a factor of 4 while maintaining the same linear gas velocity past the

TABLE V

RETENTION INDICES OF XANTHINES ON METHYL SILICONE CAPILLARY AT 230°C

Compound	Retention index
I	2389
II (O-TFA)	2291
III (O-TFA)	2362
IV (O-TFA)	2248
V (O-TFA)	2259
VI (O-TFA)	2445
VII	2489
Caffeine*	1810
Theobromine*	1840
Theophylline*	1485

*Values taken from ref. 18.

column inlet. The liner is partially filled with chromatographic packing to provide a heat sink for rapid volatilisation of the sample and to filter out non-volatile particles in the extracts which might degrade the column. The sample is injected near the base of the packing so that back-diffusion is minimised by the pressure drop along the packing material. The pneumatics are designed to maintain a constant pressure at the column inlet and this, together with the buffering effect of the exit tubing, also reduces back-diffusion of the sample. Injection of 5 μl of a toluene-based sample produces an estimated 1 ml of vapour under the conditions in the injection port resulting in a momentary increase in flow-rate through the splitter and an increase in the split ratio. Some advantageous enrichment of the high-boiling compounds of interest may therefore be expected due to both fractionation on the locally cooled OV-1-coated packing and the flow-pulse produced upon injection of the sample, though this has not been demonstrated.

Operation of the splitter with a split ratio of less than 10:1 resulted in a build-up of high-boiling material in the column, similar to that experienced

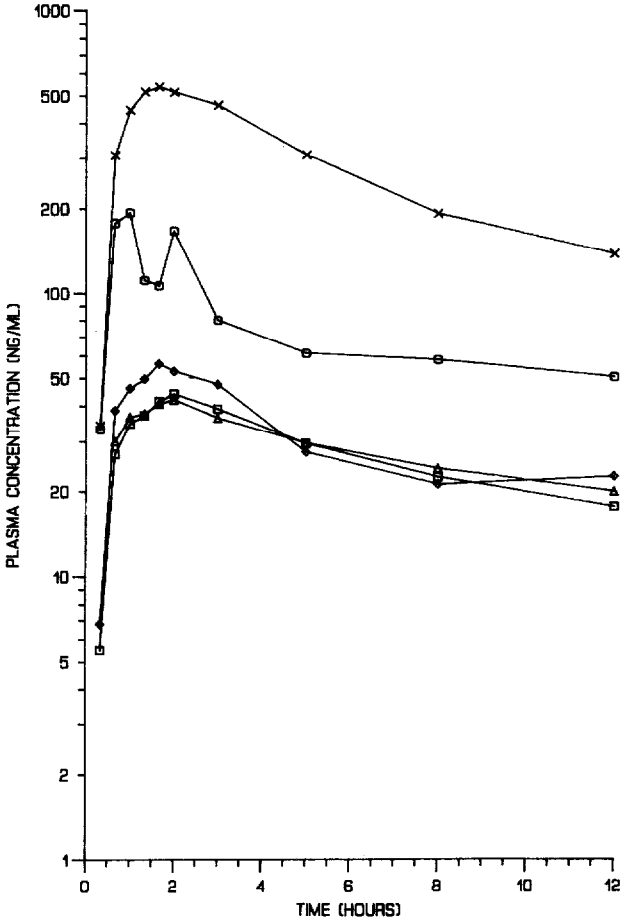

Fig. 5. Plasma levels of oxpentifylline (○), II (×), III (△), IV (□) and metabolite IIIb (◇) after oral administration of a retard formulation of oxpentifylline.

using the splitless technique, causing both loss in column efficiency and increase in retention times of the xanthines. The packing material was supported in the liner with quartz rather than glass wool as the latter material has been found to catalyse the decomposition of similar compounds.

Shoulders appeared on the front of early peaks when the Model 19400A module was used to control the autosampler. This was caused by the delay between insertion of the needle in the injection port and the discharge of the sample by depressing the syringe plunger resulting in a double injection. Subsequent use of the Model 18672A sampler control unit eliminated this effect.

Compound VI was used as the internal standard in our original paper [13] and it was thought that this would be the best candidate as the internal standard for the hydroxy metabolites, whereas VII would be suitable for oxpentifylline. However, the measurements of overall accuracy and precision from the validation samples (Tables III and IV) shows that, in general, VII gave marginally better results, obviating the need for dual internal standards. The extracts were stable for over one week if stored in autosampler vials in a refrigerator

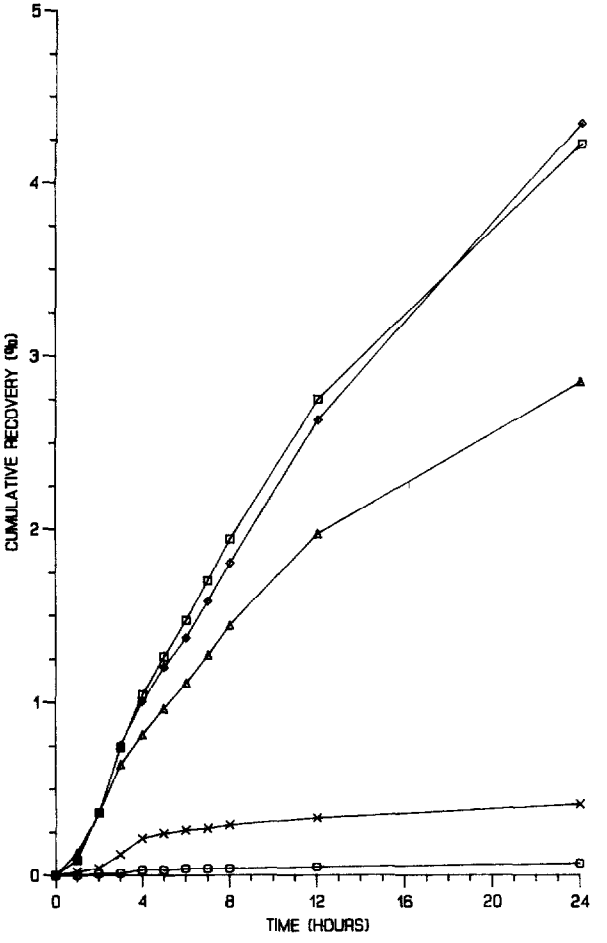

Fig. 6. Recovery of oxpentifylline (○), II (×), III (△), IV (□) and V (◇) in urine after oral administration of a retard formulation of oxpentifylline.

except that an additional peak appeared which was unresolved from VI, again showing VII to be a better choice of internal standard.

The commonly encountered xanthine species caffeine, theobromine and theophylline do not interfere with the assay as their retention indices (Table V) are much less than oxpentifylline and its metabolites. A study of possible interferences from other drugs has not been undertaken as the assay has only been applied to samples from volunteer trials in which Trental was administered alone. However, many neutral and basic nitrogen-containing drugs have been reported as having retention indices in the range 2200—2500 [18]. It is these plus metabolites and TFA derivatives of other drugs and metabolites with similar retention indices which provide a large source of potentially interfering compounds.

Application

The method has been applied to the analysis of plasma from volunteers after the oral administration of various oxpentifylline formulations. In a typical study a volunteer was given oxpentifylline in a retard formulation and blood and urine samples were collected over the following 24 h. Plasma was separated from the red cells by centrifugation immediately after withdrawal of the blood samples to prevent further metabolism of oxpentifylline to compound II, and then stored deep frozen until analysed. A plasma profile obtained from one such experiment is shown in Fig. 5 and the corresponding recovery of the xanthines in urine is shown in Fig. 6.

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