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

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SUMMARY

A procedure is described for the simultaneous determination of oxpentifylline and three metabolites in plasma by capillary gas chromatography. Plasma samples are acidified and extracted with chloroform. The carboxylic acid compounds are converted to their methyl esters and the hydroxylated metabolites to their O-trifluoroacetates prior to analysis by capillary gas chromatography using nitrogen-selective detection. The detection limits for the compounds are 5 ng/ml and the assay has been applied to the analysis of plasma from volunteer trials after oral administration of oxpentifylline.

INTRODUCTION

Oxpentifylline (Trental®, pentoxifylline, I in Fig. 1) is used in the treatment of peripheral and cerebrovascular diseases. This drug is a xanthine derivative and undergoes extensive metabolism resulting in the formation of eight reported metabolites [1]. Six of these are solely due to changes in the oxohexyl side-chain and the other, minor metabolites, involve loss of the methyl group from the 7 position of the xanthine ring.

Assays have been reported for the determination of oxpentifylline and various hydroxylated metabolites in plasma using either high-performance liquid chromatography (HPLC) [2–4] or gas chromatography (GC) [5,6]. Two carboxylic acid metabolites of oxpentifylline are also found in plasma (III and IV in Fig. 1) and one of these (III) is often the major circulating species. The analysis of these metabolites in urine by HPLC has been reported [7] but the assay has not been applied to plasma samples. This communication describes a GC method for the determination of these two metabolites in plasma together with oxpentifylline and the major hydroxylated metabolite (II in Fig. 1).

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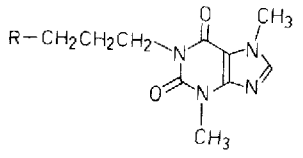

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I CH_3COCH_2-

II $CH_3CH(OH)CH_2-$

III $HOOC-$

IV $HOOCCH_2-$

V $CH_3COCH_2CH_2-$

VI $HOOCCH_2CH_2-$

Fig. 1. Structures of oxpentifylline (I), metabolites (II-IV) and internal standards (V, VI).

Prior to GC analysis, the carboxylic acids were converted to methyl esters with diazomethane, and the hydroxylated metabolites are esterified with trifluoroacetic anhydride. These derivatives not only improve the chromatographic peak shapes of the analytes, but also facilitate their mutual separation. Two internal standards are used in the assay because of the differences in chemical properties of the analytes. The first (V in Fig. 1) is used to quantify oxpentifylline and the hydroxy metabolite (II) and the second (VI in Fig. 1) is used for the acid metabolites (III and IV).

EXPERIMENTAL

Reagents and solvents

Chloroform, dimethyldichlorosilane, diethyl ether, ethyl acetate, hexane fraction, hydrochloric acid and potassium hydroxide were obtained from Fisons (Loughborough, U.K.). Trifluoroacetic anhydride (TFAA) and Diazald® were obtained from Aldrich (Gillingham, U.K.) and absolute ethanol from James Burrough (Witham, U.K.). Chloroform and hexane were re-distilled before use.

Oxpentifylline, the metabolites II, III and IV and the internal standard VI were supplied by Hoechst Werk Albert (Wiesbaden, F.R.G.). The internal standard V was synthesised in the author's laboratory (can be supplied upon request).

An ethereal solution of diazomethane was prepared by a scaled-down version of the method described by De Boer and Backer [8]. All work with diazomethane was performed in a fume cupboard because of its high toxicity. The explosion hazard associated with this reagent was minimised by keeping the diazomethane in dilute ether solution and by use of glassware equipped with Clear-Seal joints (Aldrich). Diazald (2.2 g) was dissolved in diethyl ether (30 ml) and added over a period of 10 min to a solution of potassium hydroxide (0.6 g) in 95% ethanol

(12.5 ml) at 65°C. Diazomethane co-distilled with the ether from the reaction mixture. The resultant yellow distillate of ethereal diazomethane was usable for at least two weeks if stored at 0°C.

TFAA was diluted to a 5% (v/v) solution with hexane and was freshly prepared for each batch of samples.

Standard solutions

Separate solutions of oxpentifylline, metabolites II–IV and internal standards V and VI were prepared by dissolving the solid materials (25 ± 1 mg) in distilled water (250 ml). A solution of oxpentifylline and the metabolites (5 μg/ml each) was prepared by mixing and diluting portions of these. A solution containing 5 μg/ml of each of the internal standards was prepared in the same way.

Preparation of plasma standards

Three batches of calibration samples were prepared by diluting a portion (5 ml) of the solution of oxpentifylline and the metabolites to 50 ml with pooled human plasma. Aliquots (2.5 ml) of these were dispensed into plastic tubes (Sarstedt, Leicester, U.K.), stoppered and stored at −20°C. One plasma sample from each of the three pools was used to provide two 1-ml samples to calibrate the assay.

Preparation of the plasma extracts

Adsorption of the xanthines onto glassware was prevented by pre-treating the 8-ml screw-cap glass test tubes (J. Bibby, Stone, U.K.) with a 5% (v/v) solution of dimethyldichlorosilane in hexane.

A solution of the internal standards V and VI (0.5 μg of each) in water (100 μl) was added to each tube and thoroughly mixed with the plasma sample (1 ml). Chloroform (4 ml) and 1 *M* hydrochloric acid (0.5 ml) were added, and the plasma was 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 were separated by centrifugation at 2000 *g* for 5 min, and the upper plasma phase was aspirated. If the chloroform phase was emulsified, the tubes were briefly placed on a vortex mixer, centrifuged again, and the remaining lipids were aspirated. The chloroform was transferred to a tapered glass test tube (Quickfit, Fisons) and evaporated under a gentle stream of nitrogen while the tubes were held in a heating block (Techne, Fisons) at 60°C.

The tubes were cooled and a vortex mixer used to take up the residues in an ethereal solution of diazomethane (100 μl). After about 1 min, the excess reagent was removed under a gentle stream of nitrogen. A solution of 5% (v/v) TFAA in hexane (1 ml) was added, and the tubes were stoppered using spring clips (HWS, Labap, Huddersfield, U.K.) to secure the stoppers. A vortex mixer was used to aid dissolution of the residues, and esterification was completed by placing the tubes in a heating block for 10 min at 60°C. Excess reagent was removed by evaporation in a stream of nitrogen while the tubes were held in the heating block at 60°C. The residues were taken up in toluene (100 μl) and transferred to 0.3-ml autosampler vials (Chromacol, London, U.K.).

Gas chromatography

The samples were analysed with the following equipment supplied by Hewlett-Packard (Winnersh, U.K.). The gas chromatograph was a Model 5710A equipped with a Model 18789A nitrogen-specific detector and a Model 18740B capillary inlet system. A fused-silica capillary column (25 m×0.31 mm I.D.) coated with a 0.52-μm film of cross-linked 5% phenylmethyl silicone (Part No. 19091B Opt. 112) was used for the analysis, and the column outlet was inserted directly into the detector jet. The capillary inlet was operated in the split mode with a silica liner (Part No. 18740-80220) packed with 3% OV-73 on Chromosorb W HP, 100–120 mesh, as previously described [6]. This packing was replaced with fresh material and conditioned at the analysis temperature before the analysis of each batch of samples.

Samples (5 μl) were injected with a Model 7672A autosampler using a Model 701RN 10-μl syringe (Hamilton, V.A. Howe). The autosampler operated in the alternate wash mode using ethyl acetate as the wash solvent.

Helium was used as the carrier gas with an inlet pressure of 0.9 kg/cm^2 which gave rise to a mean linear velocity of 35 cm/s. The split flow-rate was 25 ml/min, the make-up gas flow-rate 25 ml/min and the septum purge flow-rate 2 ml/min. The injection port and detector temperatures were 300°C and the oven was operated isothermally at 250°C. The nitrogen detector bead voltage was approximately 14.5 V.

The chromatograph amplifier was connected, via range 10, to the external input of a Model 5880A gas chromatograph for integration and data reduction. Concentrations of the analytes were calculated with an internal standard procedure using V to quantify oxpentifylline and metabolite II, and VI to quantify metabolites III and IV. Values of the response factors were determined for each compound from the calibration samples carried through the extraction procedure with each batch of unknowns.

Chromatograms of plasma extracts produced on a 1-mV Model 056 recorder (Perkin-Elmer, Beaconsfield, U.K.) are shown in Fig. 2.

RESULTS

Aqueous solutions of oxpentifylline and the metabolites II, III and IV, in the approximate ratio 10:10:10:1, respectively, were added to pooled human plasma to produce a series of nine samples of known concentrations. The concentration of oxpentifylline in these samples, for example, ranged from 5 to 2500 ng/ml. Three dihydroxy metabolites, whose analysis has been previously reported [6], were also included in the mixture at a concentration of about 10% that of oxpentifylline. Each plasma was analysed on six separate days by the assay described. The results obtained from three of these samples are shown in Table I.

Precision

The results from the six validations were used to assess the precision of the assay. At low plasma concentrations, the precision is best described in terms of a limiting value of the standard deviation which, for the lowest four concentrations

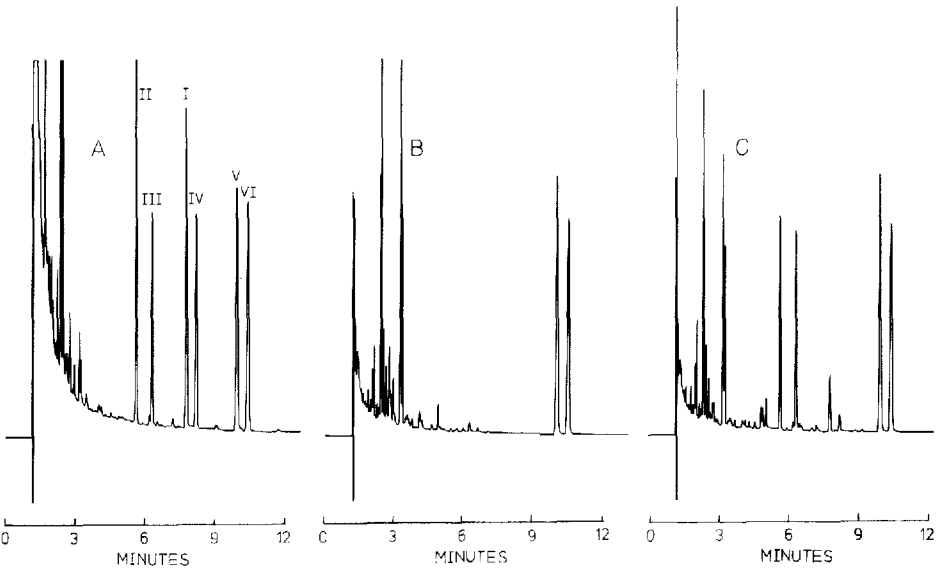

Fig. 2. Chromatograms of plasma extracts. (A) Calibration sample containing 0.5 μg/ml of each compound; (B) pre-dose sample from a volunteer containing the internal standards; (C) sample from a volunteer 2 h after administration of a retard formulation of oxpentifylline (400 mg). The numbered peaks in chromatogram A correspond to the compounds in Fig. 1.

of oxpentifylline (5–50 ng/ml), averages 0.6 ng/ml. As the concentration increases so do the associated values of the standard deviation, whereas the coefficient of variation falls to a relatively constant value. This averages 1.5% of the measured value over the highest four concentrations (250–2500 ng/ml). Thus the overall precision of the measurement of oxpentifylline can be described as ± (0.6 ng/ml + 1.5% of the measured value). A summary of the overall precision for each compound determined in this way is shown in Table II. The within-day precision, measured by the six calibration samples, was similar (0.9–1.9%).

TABLE I

DETERMINATION OF OXPENTIFYLLINE (I) AND METABOLITES (II-IV) ADDED TO BLANK PLASMA

Values in parentheses are coefficients of variation (%).

Approximate concentration added* (ng/ml)	Recovery (%)			
	I	II	III	IV
10	109.5 (5.5)	140 (8)	116 (25)	109.9 (9.5)
100	99.4 (1.2)	104.2 (2.7)	100.4 (2.9)	101.2 (3.3)
1000	101.2 (1.1)	101.4 (3.2)	101.6 (2.0)	

*Individual concentrations lie in the range 100–104% of these values.

TABLE II

PRECISION AND ACCURACY OF THE DETERMINED VALUES OF OXPENTIFYLLINE AND METABOLITES IN PLASMA

x = measured value.

Compound	Precision (ng/ml)	Accuracy (ng/ml)
I	$\pm(0.6+0.015x)$	$0.6+0.013x$
II	$\pm(1.5+0.029x)$	$4.0+0.018x$
III	$\pm(2.1+0.015x)$	$1.6+0.016x$
IV	$\pm(2.0+0.025x)$	$1.4+0.024x$

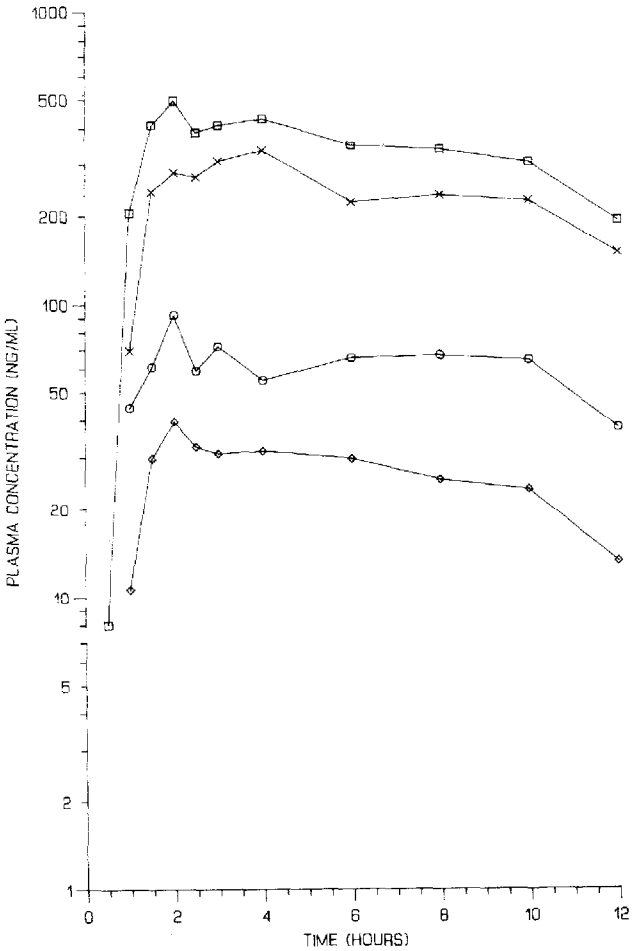

Fig. 3. Plasma levels of oxpentifylline (○), II (×), III (□) and IV (◇) after oral administration of a retard formulation of oxpentifylline (400 mg).

Limit of detection

The detection limit (DL) 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 (S.D.) as the concentration tends to zero ($S.D._{c\to 0}$) by the equation $DL = t_{(n,95\%)} \cdot S.D._{c\to 0}$. The value of $t_{(n,95\%)}$, which is the one-tailed critical value of the t-distribution for n determinations at the 95% confidence limit, is 2.0 for $n = 6$. Values of the minimum precision from Table II show the detection limits to be between 1 and 4 ng/ml for the four compounds.

Accuracy

Accuracy can be considered as being the modulus of the difference or bias between the mean of the measured values of the validation samples and the amount added. As with precision, these differences increase over the range of the assay and can therefore be described in terms of a minimum value and a concentration-dependent term. For example, using the data from the same concentration ranges as for precision, the overall accuracy of the oxpentifylline measurements can be expressed as 0.6 ng/ml + 1.3% of the measured value. A summary of the accuracy of the determined values is given in Table II.

Recovery of the compounds shows the assays to be linear over the expected concentration ranges except for II. This metabolite shows a positive bias of approximately 4 ng/ml due to an unresolved endogenous compound in the plasma extracts of the validation samples. Compensation for this in samples from volunteers was only made if the component was present in the plasma taken prior to administration of oxpentifylline.

DISCUSSION

Previous analytical methods have concentrated on the measurement of oxpentifylline and its hydroxy metabolites but have ignored the acid metabolites, one of which (III) is often the major species in plasma. These acid metabolites have been previously measured in this laboratory using a separate, unpublished, GC analysis and so the possibility of their simultaneous measurement with the major neutral xanthines was investigated in order to eliminate the need for two assays.

Basic extraction conditions were used in the previously reported GC assays [5,6] but to also extract III and IV, acidic conditions must be employed. This results in an increase in the amount of endogenous plasma material which is coextracted and, in order to prevent fouling of the column by a build up of lipid material, a smaller portion of the extract is injected. Studies involving up to a thousand plasma samples with daily batches of 50–60 samples have been analysed by the GC system described without deterioration in column performance.

Ethanol containing 1 *M* hydrogen chloride as a catalyst was initially used to esterify the acids because the ethyl esters gave better separation from the other xanthines than the methyl esters. Use of this reagent led to erratic recoveries of I and V, probably due to ketal formation. Esterification with diazomethane eliminated this problem and so this reagent was used despite its high toxicity.

The three dihydroxy metabolites of oxpentifylline were also included in the validation samples but, because of the higher chromatographic background as-

sociated with extracts from acidified plasma, these could not be determined with sufficient accuracy or precision to be of use pharmacokinetically. Measurement of these, if required, is recommended using the previously reported method [6]. The inclusion of these metabolites in the validation samples did, however, demonstrate that their presence did not affect the quantification of the other xanthines.

Two internal standards have been incorporated into the assay, one (V) for the neutral compounds and the other (VI) for the acidic. Little difference was observed between either the day-to-day or within-day precision of the response ratios calculated from each of these standards. This indicates that either could be used to quantify all of the analytes with equal results. However, the differences in pK_a of the analytes plus their different behaviour in the two derivatisation steps are obvious potential sources of error, and an extra degree of confidence in the results is imparted by the continued use of both internal standards.

Application

The assay has been applied to volunteer studies in which subjects received various formulations of oxpentifylline. Blood samples were taken at various intervals, and the plasma was quickly separated and removed to prevent further metabolism of oxpentifylline to II by the erythrocytes [9]. A plasma profile from one such study in which the subject received a sustained release formulation of oxpentifylline (400 mg orally) is shown in Fig. 3 and it can be seen that the carboxypropyl metabolite (III) is in fact the major xanthine circulating in plasma.

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