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DETERMINATION OF PENTOXIFYLLINE AND ITS 5-HYDROXY METABOLITE IN HUMAN PLASMA BY SOLID-PHASE EXTRACTION AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ULTRAVIOLET DETECTION

G MUSCH, T HAMOIR and D L MASSART*

Vrije Universiteit Brussel, Farmaceutisch Instituut, Laarbeeklaan 103, 1090 Brussels (Belgium)

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SUMMARY

The simultaneous determination of pentoxifylline and its 5-hydroxy metabolite in human plasma was performed 10 ng/ml pentoxifylline and 15 ng/ml metabolite were determined at a signal-to-noise ratio of 3 The recoveries from plasma at a 100 ng/ml level were 98 0 and 86 9% for pentoxifylline and the metabolite, respectively The intra- and inter-assay coefficients of variation were less than 5% for both pentoxifylline and the metabolite This method shows advantages over many other published extraction procedures prior to high-performance liquid chromatographic analysis in terms of its speed and ease of manipulation

INTRODUCTION

Pentoxifylline (Fig 1) is a xanthine derivative used in the treatment of patients suffering from peripheral and cerebrovascular diseases This drug acts as a haemorrheologic agent and improves the erythrocyte penetration into microcapillaries [1] so that the circulation is ameliorated and ischaemic attacks are prevented. The drug is partly converted into 1-(5′-hydroxyhexyl)-3,7-dimethylxanthine, the 5-hydroxy metabolite in Fig 1, for which it is not yet known whether it possesses pharmacological activity or not [2]

In the literature several methods for the assay of pentoxifylline in biological fluids are described The analytical techniques used are high-performance liquid chromatography [1–7] and gas chromatography [1,8] and liquid–liquid extraction is employed for the sample pretreatment To our knowledge there

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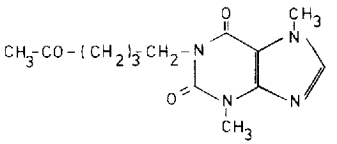
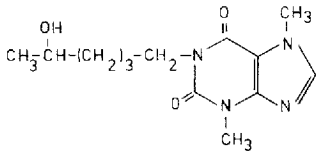
PENTOXIFYLLINE



5-HYDROXY METABOLITE 1-(5-HYDROXYHEXYL)3 7-DI-METHYLXANTHINE



INTERNAL STANDARD 1-(6-OXOHEPTYL)3 7-DIMETHYL-XANTHINE

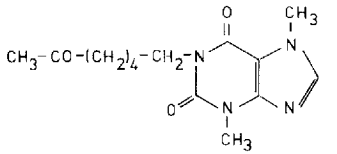


Fig 1 Structures of pentoxifylline, 5-hydroxy metabolite and 1-(6-oxoheptyl)-3,7-dimethylxanthine used as the internal standard

is no report of solid-phase extraction with silica-bonded phases for the isolation of pentoxifylline from plasma. In earlier work [9], a general appraoch for the extraction of basic drugs from plasma was described This work has been extended in an analogous way to the isolation of acidic and neutral drugs from plasma From these unpublished results it can be concluded that neutral drugs possessing a carbon chain length higher than 10 and lower than 25 can be successfully isolated from plasma using a cyanopropyl sorbent

Since pentoxifylline, its 5-hydroxy metabolite and 1-(6-oxoheptyl)-3,7-dimethylxanthine, used as the internal standard (Fig 1), are all neutral drugs possessing 13 or 14 carbon atoms, this approach can be applied here The introduction of solid-phase extraction should simplify the analysis of pentoxifylline and its 5-hydroxy metabolite and make it amenable to automation

EXPERIMENTAL

Apparatus

The liquid chromatograph consisted of a Perkin-Elmer (Uberlingen See, F R G) pump, Series 10, equipped with a Rheodyne Model 7125 injector (injection volume 20 μl), coupled in series with an LC 90 UV detector also from Perkin-Elmer, with variable wavelength set at 280 nm and 0 005 a u f s and optical pathlength of 1 cm An IC-R3A Shimadzu (Kyoto, Japan) integrator was used

For the sample pretreatment an SPE 21 vacuum manifold device was used together with cyanopropyl cartridges containing 100 mg sorbent, both provided by J.T Baker (Deventer, The Netherlands)

Standards and reagents

Pentoxifylline, 5-hydroxypentoxifylline and 1-(6-oxoheptyl)-3,7-dimethylxanthine, used here as the internal standard due to its structural analogy with the parent drug (Fig 1), were supplied by Hoechst (Brussels, Belgium) Stock solutions were prepared by dissolving 10 mg of each drug in 100 ml of methanol and stored at 4 °C. Standard solutions for spiking the drug-free plasma were made fresh daily by diluting the stock solution in Milli Q-deionized water Plasma standards were prepared by adding 100 μl of an appropriate standard solution to 1 ml plasma from healthy plasma donors selected by the blood transfusion unit of the Academic Hospital Jette The recoveries of spiked plasma samples were measured by interpolation on a calibration graph of unextracted standards prepared in the mobile phase when the eluent had to be evaporated and in methanol–0.05 *M* phosphate buffer pH 3 (50 50, v/v) when this solvent was used for the elution. The plasma samples from the human volunteers were measured versus a calibration graph for plasma

The reagents were obtained from Merck (Darmstadt, F R.G). The solvents used were of analytical grade For the chromatography, acetonitrile of HPLC grade was used. Orthophosphoric acid (1 *M*) and sodium dihydrogenphosphate were also provided by Merck Water purified on a Milli Q system (Millipore, Molsheim, France) was used in all the experiments All glass tubes were silanized with Surfasil, diluted 1 10 in acetone Surfasil was obtained from Pierce (Our Beyerland, The Netherlands)

Chromatography

The analytical column was a cyanopropyl LiChrosorb (particle size 5 μm, Merck), 12 5 cm$\times$0.4 cm I D , which was used at ambient temperature (22–25 °C) A precolumn (3 cm$\times$0.4 cm I D) packed with cyanopropyl-bonded LiChrosorb (particle size 10 μm) was used in order to protect the analytical column The mobile phase was acetonitrile–water (1 99, v/v), degassed before use in an ultrasonic bath for 10 min and pumped at a flow-rate of 1 ml/min

Ultraviolet (UV) detection at the absorption maximum of pentoxifylline (280 nm) was used

Sample pretreatment

Drug-free plasma and all the plasma samples were stored at −20°C and allowed to thaw at room temperature (22–25°C) To 1 ml plasma, 100 μl of a 5 μg/ml internal standard solution were added Plasma standards were prepared by loading 1 ml drug-free plasma with 100 μl of a standard solution containing a suitable amount of pentoxifylline and the 5-hydroxy metabolite ranging from 500 ng/ml to 10 μg/ml

The calibration graph consisted of five points (0 ng/ml pentoxifylline, 0 ng/ml 5-hydroxy metabolite, 500 ng/ml internal standard, 50 ng/ml pentoxifylline, 50 ng/ml 5-hydroxy metabolite, 500 ng/ml internal standard, 100 ng/ml pentoxifylline, 100 ng/ml 5-hydroxy metabolite, 500 ng/ml internal standard, 250 ng/ml pentoxifylline, 250 ng/ml 5-hydroxy metabolite, 500 ng/ml internal standard, and 1 μg/ml pentoxifylline, 1 μg/ml 5-hydroxy metabolite, 500 ng/ml internal standard A representative calibration line for the 5-hydroxy metabolite is presented by $y = -0\ 550 + 489\ 719\ x$, $Sb_0 = 6\ 179$, $Sb_1 = 5\ 356$, where Sb_0 = estimated standard error of intercept and Sb_1 = estimated standard error of slope The 95% confidence interval for the intercept is [−17 706, 16 606] and for the slope [474 848, 504 590] For pentoxifylline the calibration line is presented by $y = -26\ 373 + 580\ 935x$, $Sb_0 = 15\ 686$, $Sb_1 = 15\ 573$ The 95% confidence interval for b_0 is [−69 924, 17 177] and for b_1 [537 697, 624 173]

A 1-ml plasma standard or sample was deproteinized by adding 2 ml acetonitrile dropwise under continuous mixing After centrifugation at 600 *g* at ambient temperature (22–25°C) during 20 min, the supernatant was evaporated to dryness under a gentle nitrogen stream at 40°C and reconstituted in 1 ml of water This was introduced onto the cyanopropyl SPE cartridge previously conditioned with 2×1 ml methanol and 2×1 ml water The cartridge was rinsed with 2×100 μl water and the drugs were eluted with 1 ml acetonitrile The eluent was evaporated under a gentle nitrogen stream at 45°C and reconstituted in 1 ml mobile phase From this volume, 20 μl were injected into the liquid chromatograph

Subjects

One healthy male volunteer (23 years, 77 kg) and one healthy female volunteer (28 years, 60 kg) were investigated after an overnight fast They were not on any medication and were requested to abstain from smoking and alcohol during the study At 8 00 h they attended the metabolic unit and a heparin lock was placed in a forearm vein for blood sampling Blood samples were obtained prior to the morning dose (400 mg pentoxifylline per os) and 30 and 40 min and 1, 2, 3, 3,5, 4, 5, 6, 7, 8, 9, 10 and 12 h following administration Each time 10 ml of blood were collected in a heparinized sterile syringe (Sarstedt) Blood

samples were immediately centrifuged at 600 *g* for 30 min at ambient temperature (22–25 °C) and the plasma was separated and stored at −20 °C until analysed The frozen plasma samples were selected at random prior to analysis

RESULTS AND DISCUSSION

For the HPLC system isocratic elution was preferred The composition of the mobile phase where the three compounds were eluted in a k' range of 1–10 was selected by applying the strategy of De Smet et al [10] The percentage of organic modifier indicated, 13% (v/v) methanol, was used as the starting point for optimization The mobile phase eventually selected contained 1% (v/v) acetonitrile The chromatogram obtained using these conditions is presented in Fig 2 and allows quantitation of the 5-hydroxy metabolite, pentoxifylline and the internal standard within an acceptable time Since this paper is meant to illustrate our analytical strategy for the assay of neutral compounds in plasma [1] and this differs importantly from the methodology for the isolation of acidic drugs from plasma, the dosage of the acidic metabolites 1-(3′-carboxypropyl)-3,7-dimethylxanthine and 1-(4′-carboxybutyl)-3,7-dimethylxanthine was considered as less important in this paper However, the

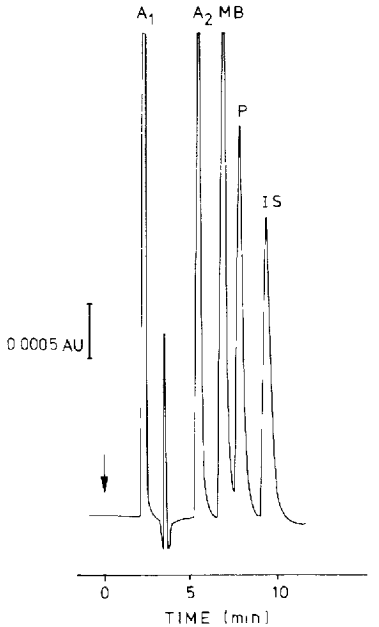


Fig 2 Chromatogram obtained with a cyanopropyl stationary phase in combination with 1% (v/v) acetonitrile in water and monitored at 280 nm, 0 005 a u f s Peaks A_1 = 1-(3′-carboxypropyl)-3,7-dimethylxanthine, A_2 = 1-(4 -carboxybutyl)-3,7-dimethylxanthine, MB = 5-hydroxy metabolite, P = pentoxifylline, IS = internal standard

chromatographic system used allowed separation of the acidic metabolites from the neutral compounds of interest so that possible interferences are eliminated (Fig 2) In the same way it was checked whether other xanthine derivatives, such as caffeine, theophylline and theobromine, and other drugs interfere (Table I). Since the amount of organic modified is very low and the mobile phase did not contain propylamine (masking the silanol functions), the possible interferences expected were rather the acidic and neutral drugs than the basic compounds. Five basic substances, tiapride, amiloride, atenolol, salbutamol and mexiletine, were injected for verification. Indeed, they were not eluted in this chromatographic system

The extraction procedure for the isolation of neutral drugs from plasma used a cyanopropyl-bonded phase (capacity 1 ml). The wash step was performed by using 1 ml of water and the recommended solvent for the elution step was 1 ml methanol or methanol–0 05 *M* phosphate buffer pH 3.

This general approach was first applied for the extraction of 1 μg/ml pentoxifylline from water The elution profiles are presented in Fig. 3 and confirm the use of 1 ml water and 1 ml methanol as wash and eluting solvent, respectively However, acetonitrile (1 ml) was preferred as the eluent since the evaporation step is quicker than when 1 ml methanol is used. The residue was dissolved in 1 ml of mobile phase or in a lower volume when concentration was necessary

This extraction scheme was applied for the extraction of pentoxifylline from plasma A deproteinization step was included. acetonitrile (2 ml) was added and after centrifugation the supernatant was evaporated and reconstituted in 1 ml of water. This was necessary since the presence of acetonitrile in the

TABLE I

RELATIVE RETENTION OF OTHER DRUGS

Drug	Relative retention time	
	Pentoxifylline	5-Hydroxy metabolite
Pentoxifylline	1 00	1 14
5-Hydroxy metabolite	0 88	1 00
Triamterene	0 22	0 25
Phenobarbital	0 69	0 79
Estriol	0 91	1 04
Caffeine	0 69	0 79
Chlortalidone	1 34	1 55
Diclofenac	0 31	0 36
Phenacetine	0 87	0 99
Furosemide	0 25	0 29
Theobromine	0 63	0 71
Theophylline	0 65	0 74

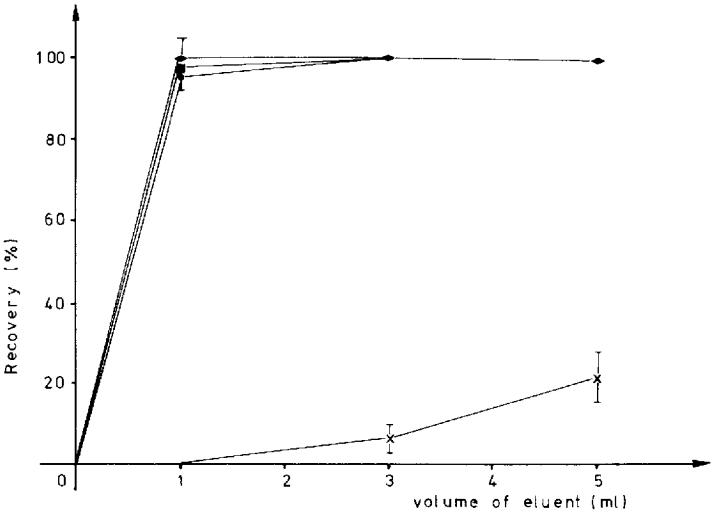


Fig 3 Elution profile for the extraction of pentoxifylline from water (×) Water, (●) methanol, (■) acetonitrile, (♦) methanol-0 05 *M* phosphate buffer pH 3 (1 1)

supernatant may cause a loss of the drug because of insufficient retention For 100 ng pentoxifylline added to 1 ml plasma, 94 7±3 1 ng/ml (mean ±S D , $n=6$) was measured The recoveries obtained at 100 ng/ml for the 5-hydroxy metabolite and the internal standard were 83 and 93 6% ($n=2$), respectively

During the development of this method two additional issues were investigated First, we studied whether the same cartridge can be used several times with reproducible recovery (ca 95%) This was investigated for the extraction of pentoxifylline from water and plasma. On the same cartridge, 100 ng pentoxifylline in water or in plasma were extracted repeatedly After each extraction the cartridge was rinsed with 4 ml methanol before the next extraction cycle was started The results are given in Table II It is seen that, where the extraction yields are relatively high, the same cartridge can be used several (at least three) times for the extraction from plasma thus reducing the unit cost of the analysis Secondly we explored whether the incorporation of a deproteinization step is necessary This was studied for spiked plasma standards as well as for plasma samples obtained from an human volunteer who received 400 mg pentoxifylline per os The extraction yields obtained with or without deproteinization are the same for spiked plasma standards However this was not the case for plasma samples of humans who received one oral formulation containing 400 mg pentoxifylline, when losses of up to 68% occurred without a former deproteinization step This might be explained by the fact that the time that the drug is in contact with the plasma proteins is small for the plasma standards and that the chemical state of pentoxifylline is not the same as in plasma samples obtained from humans who received 400 mg pentoxifylline per

TABLE II

REPEATED USE OF THE SAME SOLID-PHASE EXTRACTION CARTRIDGE FOR THE EXTRACTION OF PENTOXIFYLLINE FROM WATER AND PLASMA

Number of extractions	Recovery from water (%)	Recovery from plasma (%)
1	90	90
2	84	89
3	101	95
4	102	—[a]
5	100	—[a]
6	91	—[a]

[a]Not investigated further

os When the plasma samples from these human volunteers are extracted after deproteinization the extraction yields are significantly higher (1 3–2 8 times for the samples investigated) than those obtained without deproteinization This indicates that a deproteinization step was necessary before applying the sample onto the cartridge The drug–sorbent interaction is not able to break the drug–protein binding Probably, the drug–protein complex cannot reach the active sites of the sorbent since it is excluded from the $60 \cdot 10^{-7}$ mm pores, resulting in an important loss of the drug

The extraction procedure developed for the parent drug, pentoxifylline, was applied to the simultaneous extraction of pentoxifylline, the 5-hydroxy metabolite and the internal standard When the three compounds were extracted together from water the extraction yields for pentoxifylline and its 5-hydroxy metabolite decreased drastically (64 and 56%, respectively, at a 500 ng/ml level) whereas the recovery obtained for the internal standard was acceptable at 92%

We studied whether this competitive effect was due to incomplete retention This was not the case since when 1 ml of an aqueous standard containing 1 μg of each compound was introduced onto the conditioned cartridge the three drugs were totally retained. Secondly the wash fraction (2×500 μl water) was analysed and found to contain 22% of the 5-hydroxy metabolite and 24% of the pentoxifylline. This was not the case when pentoxifylline or the 5-hydroxy metabolite were extracted separately Probably the more apolar drug, here the internal standard, occupies an important part of the more active sites of the sorbent so that the more polar drugs, 5-hydroxy metabolite and pentoxifylline, are more easily eluted by water Therefore we investigated whether it was possible to eliminate the wash step

However, the drug-free plasma obtained in this way were not suitable (Fig 4). It was studied whether a loss of the two drugs of interest appeared when the cartridge was rinsed with 2×100 μl water At a level of 100 ng/ml no loss

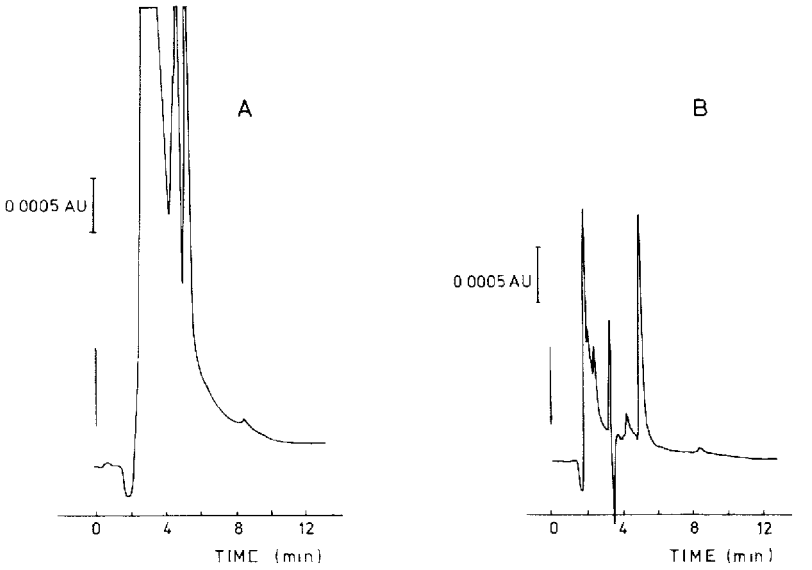


Fig 4 Chromatogram of a drug-free human plasma extract (A) without wash and (B) with 2 × 100 µl water as washing solvent

of pentoxifylline and 6 5% loss for the more polar metabolite was found At a level of 1 µg/ml, 9 6% of the 5-hydroxy metabolite and 6 2% pentoxifylline were found in the wash fraction Since the plasma blanks do not interfere in the chromatography of pentoxifylline, the 5-hydroxy metabolite and the internal standard using this modified wash step (Fig 4), this method was adopted.

This procedure was applied to the extraction from plasma The absolute recoveries obtained at a 100 ng/ml level were 83% for the 5-hydroxy metabolite, 98% for pentoxifylline and 94% for the internal standard (see Fig 5) At a level of 1 µg/ml of plasma the extraction yields were also acceptable 80% for the 5-hydroxy metabolite, 86% for pentoxifylline and 92% for the internal standard The intra-assay reproducibility at a level of 100 ng/ml ($n=6$) was 2 8% (coefficient of variation, C V) for pentoxifylline and 2 3% (C V) for the 5-hydroxy metabolite The inter-assay reproducibility at a 100 ng/ml level ($n=6$) was 3 3% (C V) for pentoxifylline and 4 3% (C V) for the 5-hydroxy metabolite

Application

Finally, this method was applied to the assay of pentoxifylline and its 5-hydroxy metabolite in plasma of two healthy human volunteers who received an oral formulation containing 400 mg pentoxifylline A drug-free plasma from one subject (time = 0 h) is shown in Fig 6A Fig. 6B represents the chroma-

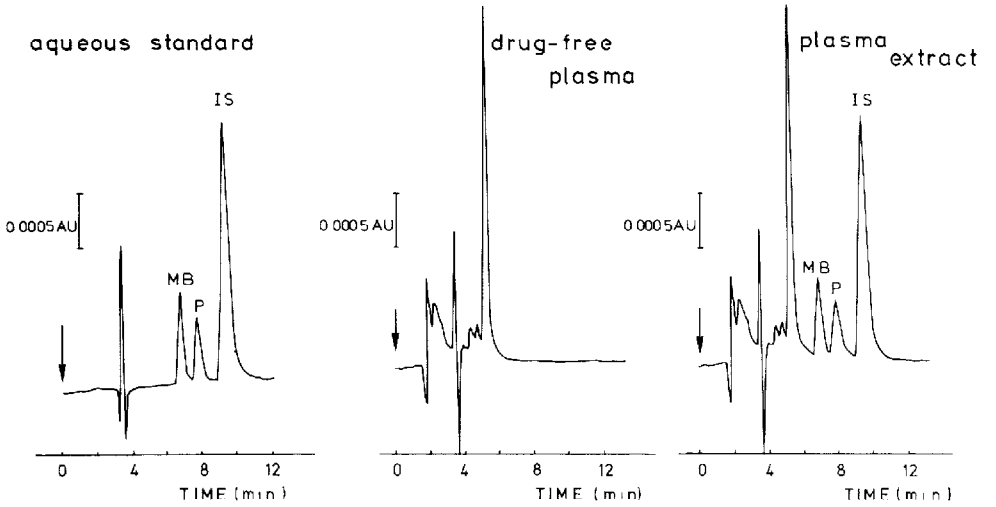


Fig 5 Elution profiles of an aqueous standard, drug-free plasma and a plasma extract Peaks MB = 5-hydroxy metabolite, P = pentoxifylline, IS = internal standard

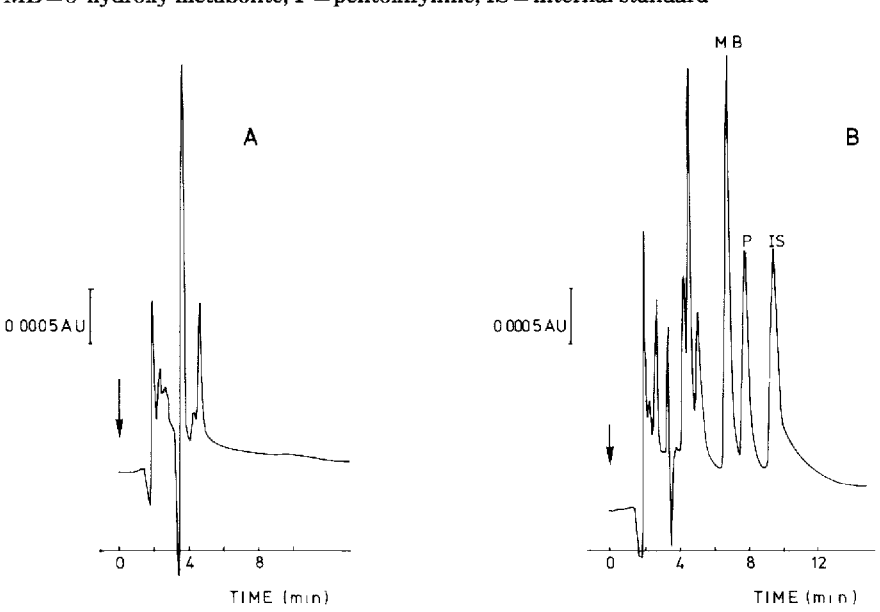


Fig 6 Chromatogram of a plasma extract from a healthy male volunteer, 23 years, 77 kg (A) time = 0 h, (B) 1 h after oral intake of 400 mg pentoxifylline in tablet formulation Peaks MB = 5-hydroxy metabolite, P = pentoxifylline, IS = internal standard

togram of pentoxifylline and its 5-hydroxy metabolite in a sample obtained 1 h after the oral intake of 400 mg pentoxifylline

The plasma concentration as a function of time for one volunteer is given in

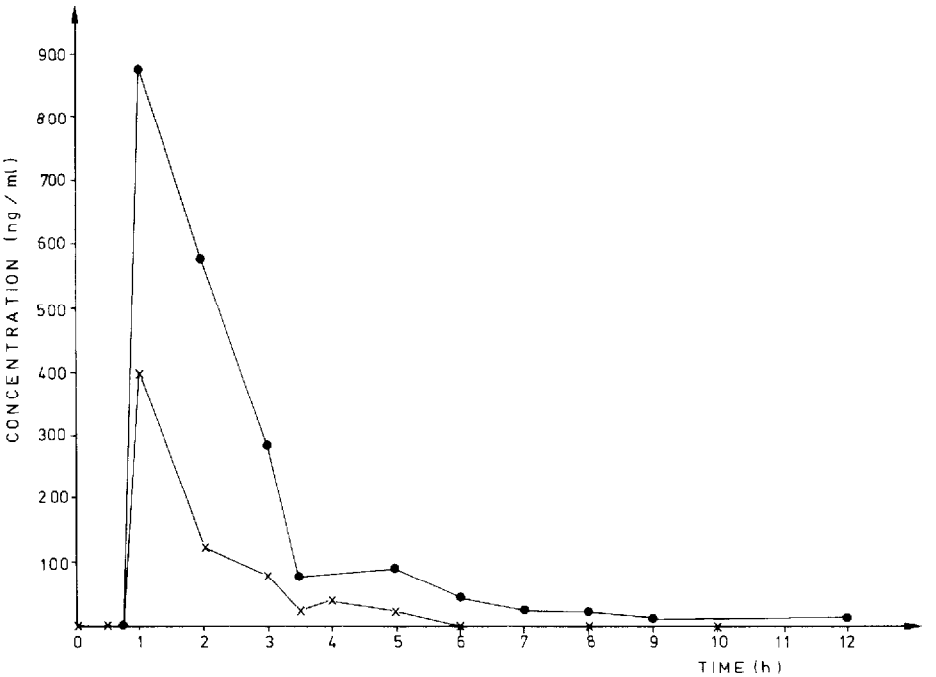


Fig 7 Plasma concentrations as a function of time, after oral administration of one tablet containing 400 mg pentoxifylline (●) 5-hydroxy metabolite, (×) pentoxifylline

Fig 7 The concentration of the 5-hydroxy metabolite was always significantly higher than that of pentoxifylline, which was also reported by Smith et al [1] The peak maximum (T_{max}) for pentoxifylline and for the 5-hydroxy metabolite was found 1 h after administration of 400 mg pentoxifylline per os This differs from the results obtained by Smith et al [1] who found 0 29 h for pentoxifylline For the 5-hydroxy metabolite the peak maximum found by Smith et al [1] was 0 83 h Also the concentration maximum (C_{max}) values were different we found 400 ng/ml of plasma for pentoxifylline versus 1607 ng/ml [7] and 890 ng/ml for the 5-hydroxy metabolite versus 2 μg/ml [7] The subjects included in the study of Smith et al [1] received 400 mg pentoxifylline as an aqueous solution whereas in our study a tablet was administered The different results may be explained by the different dosage forms, or by the small sample size examined ($n=2$)

CONCLUSIONS

The general approach for the isolation of neutral drugs with a carbon chain length higher than 10 and lower than 25 [1] from plasma using solid-phase extraction can be applied after small modifications to the assay of pentoxifyl-

line and its 5-hydroxy metabolite in plasma of healthy humans The first modification concerns the wash step When the two compounds together with the internal standard were extracted from water, the use of two 500-μl volumes of water caused an important loss of the drugs Therefore only 2×100 μl water were subsequently used in the wash step The eluent recommended in general, namely 1 ml methanol [1], was replaced by 1 ml acetonitrile to speed up the evaporation step

When the general procedure was adapted in this way acceptable results were obtained for the determination of pentoxifylline in human plasma The incorporation of a deproteinization step into our strategy [1,10] was found to be necessary Further it was possible to use the same cartridge for at least three extractions from plasma if the sorbent was rinsed with 4 ml methanol after each extraction In this way the costs of the method are reduced In contrast with the liquid–liquid extractions used in the literature, this method is simpler and more amenable to automation

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