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DETERMINATION OF 3,7-DIMETHYL-1-(5-OXOHEXYL)-XANTHINE (PENTOXIFYLLINE) AND ITS 3,7-DIMETHYL-1-(5-HYDROXYHEXYL)-XANTHINE METABOLITE IN THE PLASMA OF PATIENTS WITH MULTIPLE DISEASES USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY*

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SUMMARY

Pharmacokinetic studies of pentoxifylline in elderly patients with multiple diseases are described. Because of the low plasma levels of pentoxifylline and its hydroxy metabolite a new high-performance liquid chromatographic method was established. Under our chromatographic conditions, piracetam, a nootropic drug, can be easily separated from simultaneously applied pentoxifylline and its metabolite. Piracetam shows no appreciable ultraviolet absorption above 230 nm and cannot be detected at 268 nm.

INTRODUCTION

The drug Trental® (pentoxifylline) is often used to eliminate or reduce disturbances in the peripheral blood circulation. So far, however, pharmacokinetic parameters of pentoxifylline and its metabolite have been established only for young and healthy persons [1, 2]. Since Trental is also used in the therapy of older patients, a pharmacokinetic study with patients over 60 years old (average age 77.1, S.D. 8.4) with multiple diseases was considered to be necessary. To avoid possible acute side-effects caused by intravenous application of the drug to elderly patients, the drug was applied by infusion (100 mg of Trental during 90 min).

Because of the expected lower plasma levels of pentoxifylline and its hydroxy metabolite, and also because these patients are treated with other

*Dedicated to the 60th birthday of Prof. Dr E. Buddecke, University of Münster.

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drugs, it was necessary to establish a new high-performance liquid chromatographic (HPLC) method instead of using published procedures [2, 3].

Our new method is particularly flexible in analysing various volumes of plasma (1—4 ml), and it is sensitive and selective.

Preliminary results of the parmacokinetic study based on our method will be given.

EXPERIMENTAL

Reagents

Pentoxifylline and its hydroxymetabolite were kindly provided by Albert-Roussel Pharma (Wiesbaden, F.R.G.); HPLC-grade water and methanol were supplied by Promochem (Wesel, F.R.G.).

Extrelut® columns from Merck (Darmstadt, F.R.G.) and Sep-Pak® silica cartridges from Waters (Königstein, F.R.G.) were used for sample clean-up.

The stock solution was prepared by dissolving 100 mg of pentoxifylline and 100 mg of metabolite in 1000 ml of water. A standard solution was then made by diluting 300 ml of the stock solution with water to a final volume of 1000 ml.

Instrumentation and chromatographic conditions

The HPLC system consists of two Kontron LC pumps (Model 410), a Kontron mixing chamber, a Rheodyne Model 7125 sampling valve, a Kontron autosampler (Model MSI 660), a Kontron ultraviolet (UV) detector (Model Uvicon 720 LC), a Kontron programmer (Model 200) and a Spectra-Physics integrator (Model SP 4100).

A LiChrosorb RP 18 column (particle size 10 μm, 25 cm × 4.9 mm I.D.), and an RP 18 guard column (particle size 10 μm, 3 cm × 4.6 mm I.D.) manufactured by Kontron were used.

The analyses were carried out isocratically using a methanol—water mixture (48:52, v/v) as eluent.

The flow-rate of the mobile phase (saturated with helium) was 2.0 ml/min. Always 20 μl of sample solution were injected either manually in a 20-μl loop of the Rheodyne sampling valve or by means of the autosampler. Pentoxifylline and the hydroxy metabolite were detected at a wavelength of 268 nm. The external standard method was chosen for calibration.

UV spectra were run on a Beckman Instruments spectrometer (Model 24).

Preparation of standard plasma samples

Venous blood (containing no pentoxifylline or metabolite) was centrifuged in a heparinized test tube for 10 min at 3000 *g* and the plasma was separated. Then 500, 200, 100, 50, 20, 10 and 5 μl of the standard solution were added to 1-ml aliquots of the plasma.

Method A

The spiked plasma samples prepared as above were first diluted with water to a volume of 16 ml in a calibrated cylinder, and by rinsing the cylinder twice with 2-ml portions of water were transferred directly onto an Extrelut column.

For 30 min the sample was allowed to soak before elution with 40 ml of methylene chloride was started. The solvent was removed from the eluate on a rotatory evaporator at 30°C maximum water bath temperature, and 300 μl of methanol were added to the residue. After shaking for 1 min, the solution was transferred into an Eppendorf micro test tube and centrifuged (Eppendorf centrifuge, Model 3200) for 2 min at 17,000 *g*; 20 μl of the upper layer were injected into the chromatograph via the autosampler. Thus, reference samples for calibrations of chromatographic peak areas were obtained corresponding to the concentrations: 15, 6, 3, 1.5, 0.6, 0.3 and 0.15 μg/ml.

Method B

The spiked plasma samples were chromatographed as described by method A on the Extrelut column. The residue was then dissolved in 3 ml of methylene chloride and transferred to a Sep-Pak cartridge preconditioned with 20 ml of methylenechloride. First, 75 ml of pure methylene chloride were run through the cartridge, then the components were eluted with 20 ml of methylene chloride—methanol mixture (20:1, v/v).

The solvent was removed from this eluate under vacuum and the residue was extracted four times with 500-μl portions of methylene chloride. After transferring 1-ml portions to an Eppendorf micro test tube and evaporation of the solvent by a stream of nitrogen, 300 μl of methanol were added (in some cases only 60 μl). After vortexing for about 1 min and centrifugation for 2 min at 17,000 *g*, 20 μl of the upper layer were injected via the autosampler or manually by the Rheodyne sampling valve.

Patients' plasma samples

The plasma was isolated as described for the standard plasma samples. It was stored at −20°C and thawed at room temperature before use. Plasma volumes of 1—3 ml were analysed by method A; 1—4 ml of plasma were analysed if method B was used.

RESULTS AND DISCUSSION

Detector study

In the literature [3, 4] the absorption maximum of pentoxifylline is reported to be at a wavelength of 274 nm. There is, however, a low-wavelength absorption maximum at 210 nm (Fig. 1) with a more than two-fold higher extinction coefficient. For reasons of better sensitivity, this wavelength was chosen alternatively for detection. The proper specifications of the UV system at that low-wavelength range were tested. The better signal-to-noise ratio at 268 nm (absorption maximum in the mobile phase), on the other hand, results in a more accurate determination of peak areas for quantitative measurements.

Calibration curve

The calibration curve of pentoxifylline and its metabolite from plasma was linear in the range 0.15–6.0 μg/ml with both method A and method B. Linear regression analysis of these data gave correlation coefficients (r) for pentoxifylline and the metabolite consistently greater than 0.999.

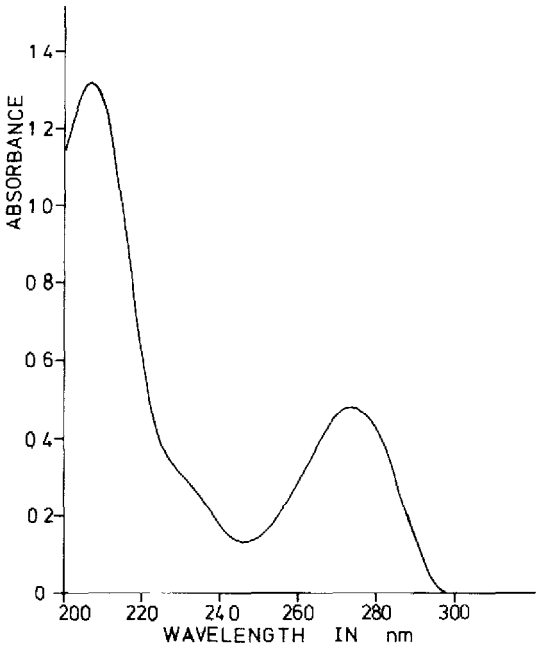

Fig. 1. UV spectrum of pentoxifylline (c = 12.5 μg/ml) in water Reference: water.

Recovery rate, precision, accuracy and sensitivity of the assay

The recovery rate in the concentration range 0.15—15.0 μg/ml using method A is 98.5% (relative S.D. = 7.3%, n = 32) for pentoxifylline and 106.3% (relative S.D. = 11.1%, n = 32) for the metabolite. Using method B, recoveries of 95.7% (relative S.D. = 7.7%, n = 23) for pentoxifylline and 105.4% (relative S.D. = 5.2%, n = 23) for the metabolite are found.

TABLE I

PRECISION AND ACCURACY OF ASSAY METHOD A

Actual plasma concentration (μg/ml)	Measured plasma concentration (μg/ml, mean ± S.D.)		Relative S.D. (%)		Relative error (%) ($\frac{\text{conc. found}}{\text{conc. actual}} \times 100$)		No. of determinations
P* M**	P	M	P	M	P	M	
6.000	5 989 ± 0.408	5.996 ± 0.393	6.817	6.548	99.8	99.9	11
4.500	4.664 ± 0 268	4.502 ± 0.295	5.752	6.561	103.6	100.0	4
2.100	1.997 ± 0.108	2.050 ± 0.079	5.193	3.868	95.1	97.6	4
0.900	0.896 ± 0.055	0.829 ± 0.082	6.095	9.920	99.6	92.1	5
0.300	0.285 ± 0 024	0.278 ± 0.022	8.547	7.914	95.0	92.7	4

*P = pentoxifylline.
**M = metabolite.

TABLE II

PRECISION AND ACCURACY OF ASSAY B

Analysis end-volume = 300 μl

Actual plasma concentration (μg/ml)	Measured plasma concentration (μg/ml, mean ± S.D.)		Relative S.D. (%)		Relative error (%) ($\frac{\text{conc. found}}{\text{conc. actual}} \times 100$)		No. of determinations
P* M**	P	M	P	M	P	M	
6.000	5.869 ± 0.095	5.987 ± 0.179	1.617	2.990	97.8	99.8	4
4.500	4.668 ± 0.125	4.696 ± 0.113	2.687	2.402	103.7	104.4	4
2.100	2.130 ±0.069	2.111 ± 0.035	3.262	1 678	101.4	100.5	4
0.900	0.923 ± 0.043	0.851 ± 0.073	4.621	8.550	102.6	94.6	5
0.300	0.283 ± 0.022	0.276 ± 0.025	7.614	8.897	94.3	92 0	3

*P = pentoxifylline.
**M = metabolite.

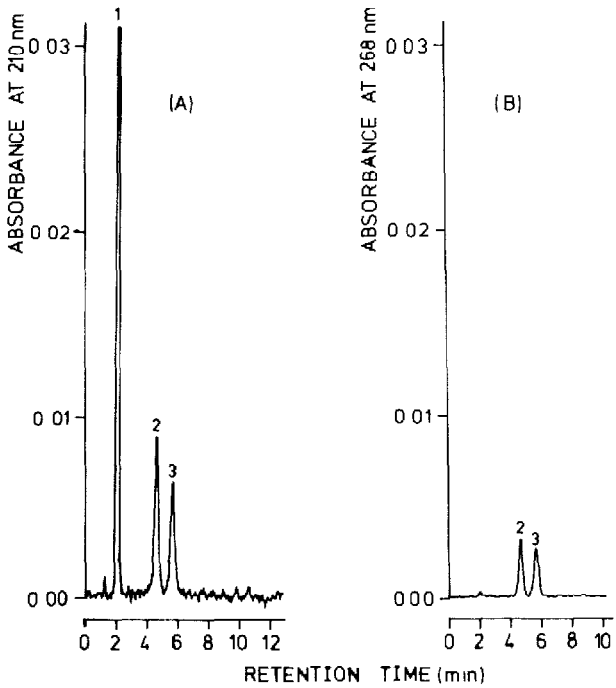

Fig. 2. Chromatograms of a standard solution of piracetam, pentoxifylline and metabolite. (A) absorbance at 210 nm, (B) absorbance at 268 nm. Peaks: 1 = piracetam (2 μg per injection; time 1.9 min), 2 = pentoxifylline (100 ng per injection; time 4 5 min), 3 = metabolite (100 ng per injection. time 5.5 min). Injection volume: 20 μl. For chromatographic conditions see text.

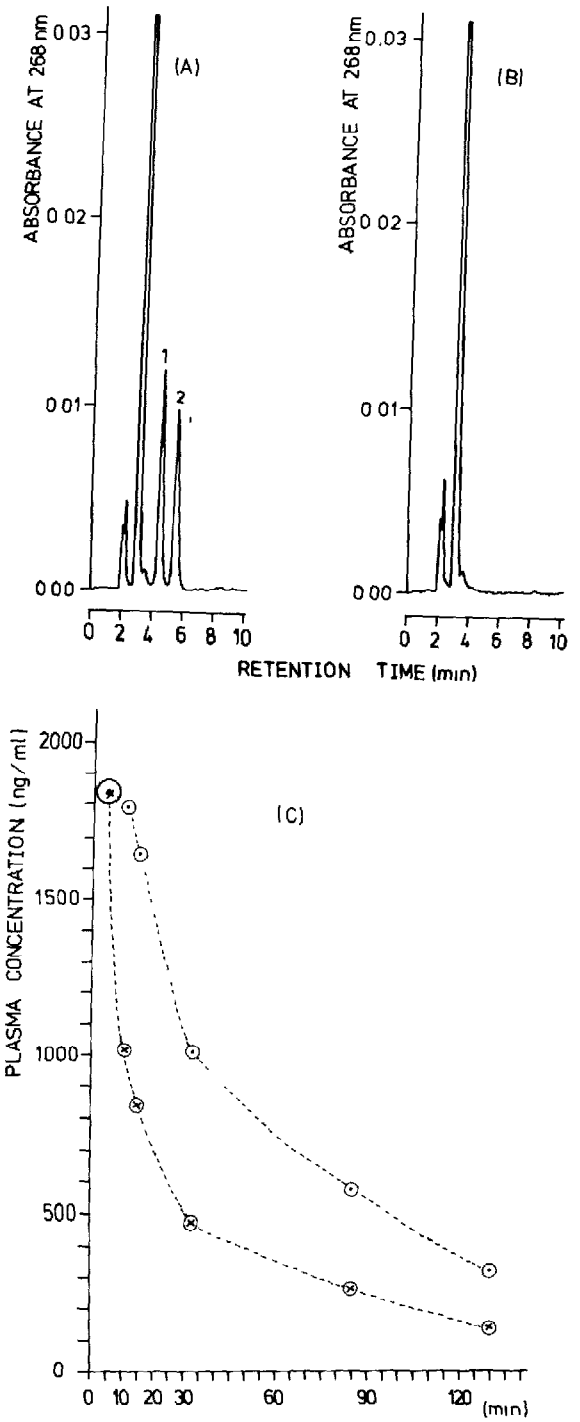

Fig. 3. Chromatograms of a patient's (TR-12) plasma samples: (A) after administration of pentoxifylline; (B) before administration of pentoxifylline. Samples prepared by method A. Peaks: 1 = pentoxifylline (time 4.4 min), 2 = metabolite (time 5.4 min). (C) Profiles of pentoxifylline (×) and metabolite (•) plasma concentrations versus time after an infusion of 100 mg of pentoxifylline (Trental) during 90 min.

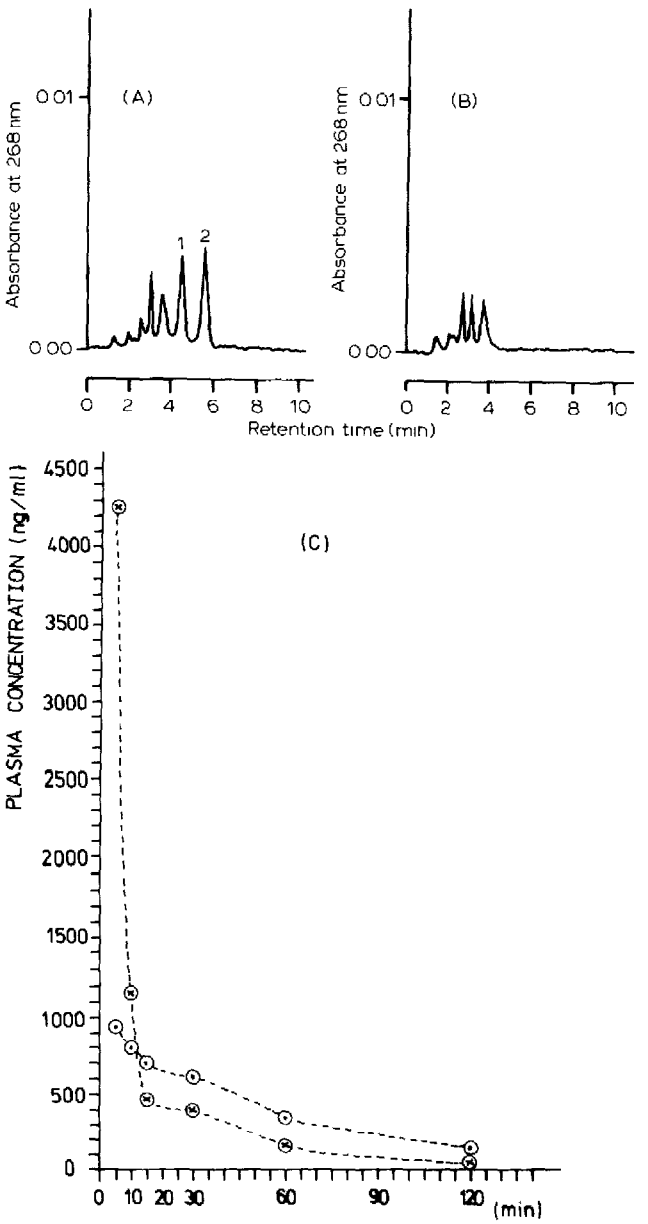

Fig. 4. Chromatograms of a patient's (TR-15) plasma samples: (A) after administration of pentoxifylline; (B) before administration of pentoxifylline. Sample preparation by method B (3 ml of plasma, final analysis volume 300 μl). Peaks: 1 = pentoxifylline (time 4.4 min), 2 = metabolite (time 5.4 min). (C) Profiles of pentoxifylline (×) and metabolite (•) plasma concentrations versus time after an infusion of 100 mg of pentoxifylline (Trental) during 90 min.

The recovery rates are calculated by comparing the peak areas for pentoxifylline and metabolite extracted from spiked plasma samples to those of an equal amount injected directly into the chromatograph. The efficiency of each method is shown by the precision data (Tables I and II).

Following method B and using 3 ml of spiked plasma, the minimum measurable concentration for pentoxifylline and the metabolite is 10 ng/ml, allowing a signal-to-noise ratio of approximately 3. In comparison, method A (3 ml of spiked plasma) gives a minimum measurable concentration for both components of 50 ng/ml with a signal-to-noise level of approximately 3.

Two different procedures for the analysis of the plasma samples (referred to as methods A and B) are essential because with method A, with an analysis end-volume of less than 300 μl recognition of the metabolite peak (retention time 5.4 min) is difficult due to interference from an unidentified component (eluted at 5.9 min). Method B eliminates the problem of peak interference in the concentration range 10—50 ng/ml. Method A, on the other hand, is faster and more economic.

Practical application of the method

The patients examined in our pharmacokinetic study very often, besides a disturbed peripheral blood circulation, showed defective cerebral circulation. This factor, of course, effects the analytical results because such diseases are normally treated simultaneously with high dosages (3—9 g) of Nootrop® (piracetam). Under our chromatographic conditions, piracetam can be easily separated from pentoxifylline and its metabolite. Furthermore, piracetam shows no significant UV absorption above 230 nm and cannot be detected at 268 nm (Fig. 2).

During the study, there were no signs of interference in the analysis of pentoxifylline and its metabolite from the other prescribed drugs.

In figs. 3 and 4 typical chromatograms for two patients are shown, together with the individual kinetic curves. Table III shows the medications administered to these patients.

TABLE III

MEDICATION ADMINISTERED TO TWO PATIENTS WITH MULTIPLE DISEASES

Patient	Drugs	Patient	Drugs
TR-15	Trental	TR-12	Trental
	Nootrop		Digimerck
	Novodigal		Coffein
	Bisolvon		Kalinor-Br. Tabl.
	Sultanol		
	Minipress		
	Sanasthmyl		
	Euphyllin		
	Laevulose		
	Fructose		
	Bactrim		
	Tutofusin K 10		
	Liquemin		

CONCLUSION

In summary, it has been demonstrated that, by using our HPLC method, pharmacokinetic studies with older patients who are treated especially with high doses of piracetam, and even a low dose of Trental, can be established.

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