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Short Communication

Automated high-performance liquid chromatographic assay for Trapidil in human plasma*

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Introduction

Trapidil, 5-methyl-7-diethylamino-s-triazolo<1,5-a>pyrimidine (T), is a drug which was introduced for the therapy of ischemic heart diseases. It shows cardiac and antiarrhythmic efficacy, peripheral vasodilatation, antihypertonic, rheological and antisclerotic effectiveness. The drug is in use in East-European countries and in Japan. Although the compound is not new, only a few laborious assays are available for the determination of T and its metabolites in human plasma based on HPLC [1, 2] and GC [3]. To manage the large number of analyses required in pharmacokinetic studies we have developed an automated HPLC assay on the basis of a column-switching technique with on-line sample enrichment.

The metabolism of T (Fig. 1), where the main pathway for transformation consists of *N*-dealkylation and hydroxylation of the aromatic system with subsequent sulphonation [4], is well-known. The key for this route is the monodesethylated compound, 5-methyl-7-ethylamino-s-triazolo<1,5-a>pyrimidine (M), and so the assay method was developed to include this key metabolite.

Experimental

Chemicals and reagents

All chemicals were of analytical-reagent grade (Merck, Darmstadt, FRG). Trapidil and

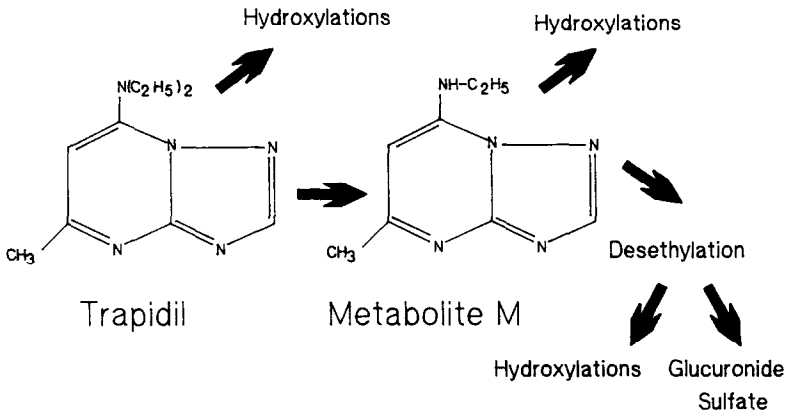

Figure 1
Biotransformation of Trapidil.

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metabolite M were obtained from VEB Deutsches Hydrierwerk (Rodleben, GDR). Other materials were BONDAPAK Corasil C_{18} 37–50 μm (Waters, Eschborn, FRG), blank plasma (Blutzentrale, Ulm, FRG), HPLC-water prepared with MilliQ (Millipore, Eschborn, FRG).

Instruments and conditions

Analytical separation: Pump A VARIAN LC 5000 (Varian, Darmstadt, FRG), flow rate 1.0 ml min^{-1}. Eluent: aqueous 0.01 M Na_2CO_3–acetonitrile–methanol (76:12:12%, v/v/v) adjusted with dilute H_3PO_4 to pH 9.0 and degassed. HPLC-column Hyperchrome 125 × 4.6 mm filled with SHANDON Hypersil ODS 5 μm (home-made). Column heater 35°C. UV-detector Spectroflow 873 G (ABI, Weiterstadt, FRG) set to 295 nm (Attenuation = 0.02 AUFS). Data collected VISTA CDS 402 (Varian, Darmstadt, FRG), external standard method. Sample enrichment: Pump B KONTRON LC Pump 414 (Kontron, Eching, FRG), flow rate 1.0 ml min^{-1}. Eluent:HPLC water, washing period 4 min. Sampler WATERS WISP 712 B (Waters, Eschborn, FRG) sample volume 200 μl. Precolumns Hyperchrome 40 × 4.6 mm dry filled with BONDAPAK Corasil C_{18} 37–50 μm. Switching valve VALCO 10-port-multifunctional valve with air actuator (Chrompack, Frankfurt, FRG). Time and event control: Labtimer (KNAUER, Bad Homburg v.d.H., FRG).

Calibration and control samples

Calibration samples were prepared from Trapidil and metabolite M by adequate dilution steps with water to yield 250 or 2000 ng ml^{-1} T and 125 or 1000 ng ml^{-1} M, respectively. Control samples were prepared from aqueous stock solutions with plasma in the two last dilution steps to yield 50, 500 or 5000 ng ml^{-1} T and 25, 250 or 2500 ng ml^{-1} M.

Sample preparation

The frozen plasma samples were thawed at 37°C and centrifuged at 5000 rpm for 5 min; 0.5 ml of the clear supernatant fluid was diluted with 0.5 ml water in adequate vials. Each sample was analysed in duplicate.

Chromatographic procedure (see Fig. 2)

The sample is injected by the autosampler into the flow from pump B to be concentrated on the first conditioned precolumn with water as solvent. After 4 min the valve is switched to the alternate position and the adsorbed material is flushed back to the analytical column with the stronger eluent from pump A. During the separation time the next precolumn

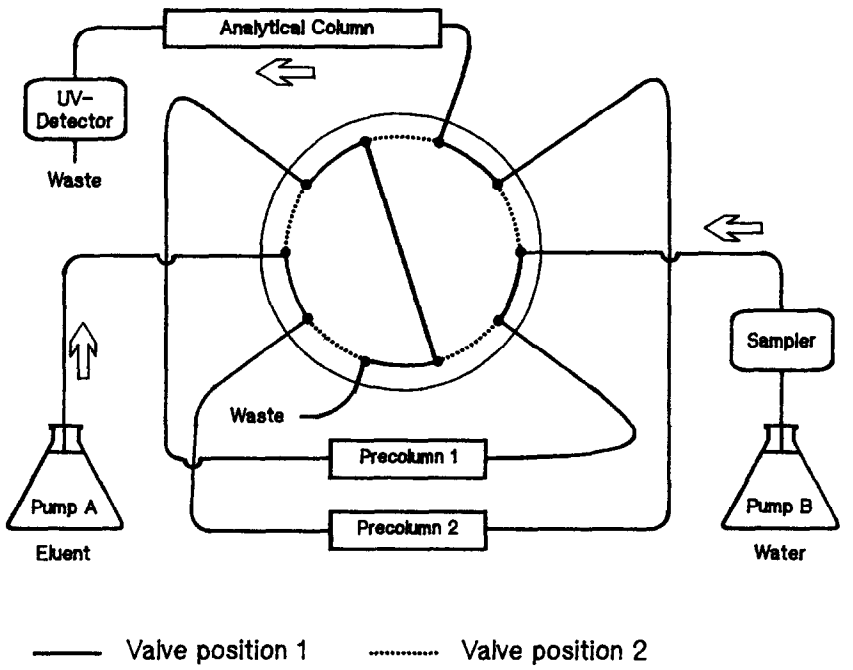

Figure 2
Switching positions of the 10-port valve during preconcentration and analysis of plasma samples.

is conditioned with water and the next sample is injected and preconcentrated. After the washing time this sample is introduced into the flow of pump A for the HPLC separation.

Results and Discussion

Method development

The first step was to develop a good separation between polar plasma components and M as well as M and T. As shown in Fig. 3 a good separation between M and plasma components has been achieved, although the retention time for T seems rather long. Using a gradient system did not save time due to the need for re-equilibration.

The next step was the optimization of the conditions for the on-line sample enrichment with respect to recovery and timing. For column switching we used a 10-port-multifunctional valve for which the connections for both positions is shown in Fig. 2. The time for wash cycles with quantitative recovery was 4 min on CORASIL RP_{18} as stationary phase and pure water as solvent.

The overall analysis time equals the time for the analytical separation.

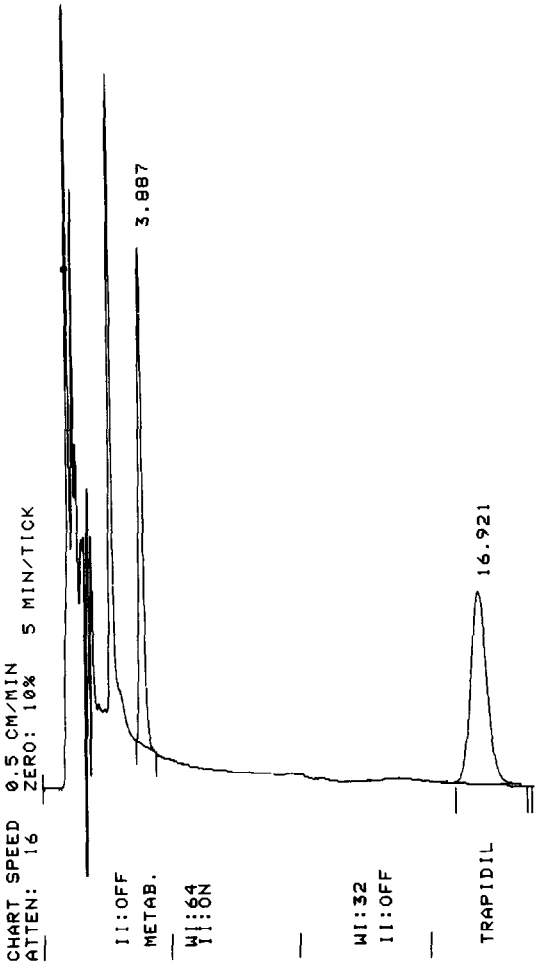

Figure 3
Chromatogram of a plasma sample with about 500 ng ml^{-1} Trapidil and 250 ng ml^{-1} metabolite (M).

Method validation

Linear range. After the oral administration of 100 mg T maximal concentrations up to 5 μg T and 2 μg M in plasma were expected. For higher oral doses the ranges up to 15 μg T and 5 μg M, respectively were examined (Table 1). Higher concentrations could not be operated by the integrator, but if there was a need to measure in a higher range, the injected volume could be diminished.

Limit of determination. The limit of determination was calculated on a statistical basis [5] to be 5.3 ng ml^{-1} for T and 2.6 ng ml^{-1} for M.

Recovery. The recovery over the whole analysis was determined with spiked plasma samples. It was calculated for several concentration ranges by comparison with the same amounts of T and M injected as aqueous solutions directly on to the analytical column (Table 2).

Precision. The precision of the method was determined as *repeatability* in one series and as *reproducibility* from day to day over the measurements of a study lasting several weeks (Table 2).

Table 1
Linear range

	Range (ng ml^{-1})	Results of the linear regression: Slope (ng ml^{-1})	Intercept	Correlation coefficient, r
Trapidil (T)	5–15,000	0.99	0.47	0.9999
Metabolite (M)	5–5000	0.995	−2.04	0.9999

Table 2
Recovery, precision

	Concentration range (ng ml^{-1})	Recovery with respect to direct injection (%)	Repeatability (within a series) Mean (%)	*n*	RSD (%)	Reproducibility (day to day) Mean (%)	*n*	RSD (%)
Trapidil (T)	50	98.7	96.4	10	0.98	97.1	9	3.68
	500	99.0	99.7	9	0.70	99.4	10	2.07
	5000	94.6	100.3	10	0.66	99.3	8	1.03
Metabolite (M)	25	100.7	100.0	10	0.94	95.6	9	4.20
	250	96.9	99.7	9	0.54	97.9	10	2.12
	2500	95.4	100.8	10	1.31	99.3	8	2.20

Application of the method

Stability of samples. For the practical execution of sample measurements the stability of samples under storage conditions of −20°C in glass and plastic tubes was examined. For the simulation of realistic protein binding conditions the spiked samples were heated to 37°C prior to freezing. Over a storage time of 6 months no loss of T or M was observed and no influence of the material of the container on the results was seen. Therefore it was deduced that a storage period of several weeks between the first plasma sampling and the last measurement in a study did not cause any problem.

Pharmacokinetic study. In a pharmacokinetic study with healthy volunteers more than 600 plasma samples were measured. Together with calibration and control samples this study resulted about 750 injections. The analyses were performed without any troubles as, for example clogging of columns or influence of endogenous substances on the chromatographic separation. As a precaution precolumns were changed every 120 injections. An example of a time versus concentration profile following the administration of 100 mg T as a tablet is given in Fig. 4.

Conclusions

A rapid, sensitive and selective HPLC method has been developed for the determination of T and its mono-desethylated meta-

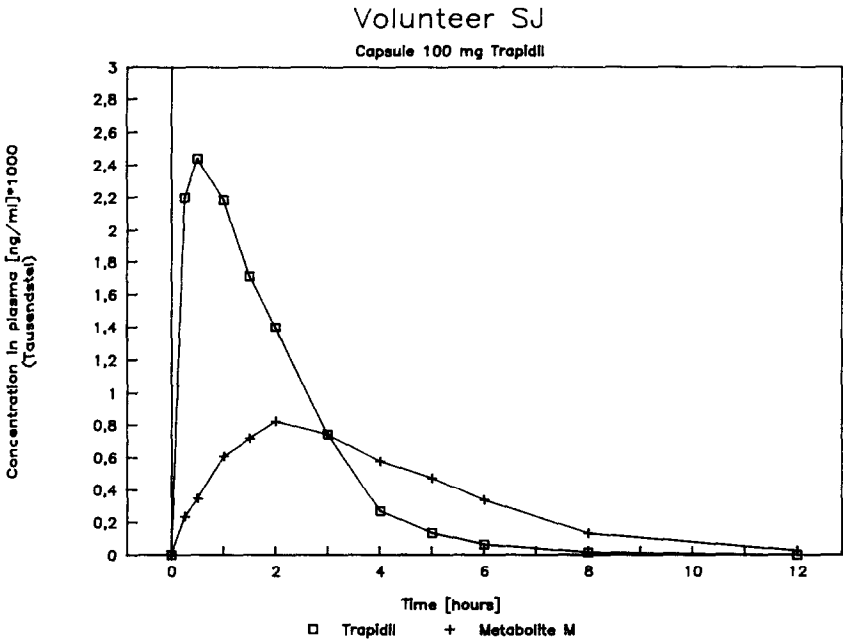

Figure 4
Time–concentration profile of Trapidil and metabolite (M) after administration of a 100-mg Trapidil-capsule peroral.

bolite. After a single dilution step the analysis is performed by an automated system employing a switching technique and sample enrichment on two precolumns. The method shows complete recovery of the analytes and very good precision within a linear range from 5 to 15,000 ng ml^{-1} T and 5 to 5000 ng ml^{-1} M. The method was used for a pharmaco-kinetic study without any problems in a series of more than 750 chromatograms. The method is robust for routine work and only few and inexpensive accessory units and modifications to a conventional HPLC apparatus are required.

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