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Note

High-performance liquid chromatographic determination of cyclosporin A in human plasma and urine

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Cyclosporin A (CyA; OL 27-400) is a new fungal cyclic polypeptide with great potential as an immunosuppressive agent in man [1, 2]. The compound has a molecular weight of 1202 and the structural formula is given in Fig. 1.

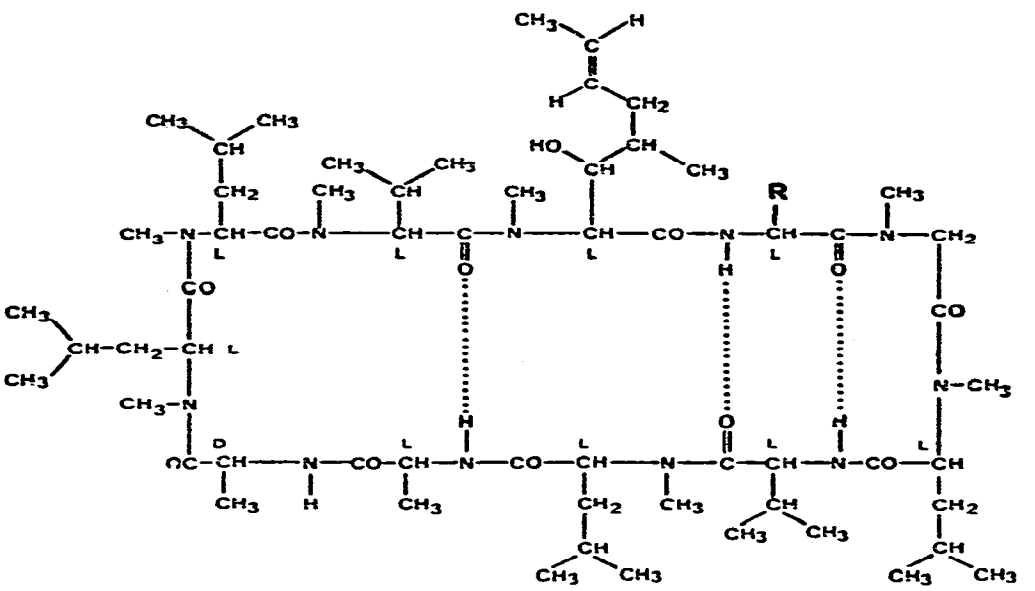

Fig. 1. Structural formula of cyclosporin A (R = CH_2—CH_3) and cyclosporin D [R = $CH(CH_3)_2$].

In this paper, a method for the determination of cyclosporin A in human plasma and urine is described, using a high-performance liquid chromatographic (HPLC) separation in a reversed-phase mode at 72°C and a UV absorption detector at 210 nm. At this wavelength, the compound has a molar extinction ϵ of 45,000 (ϵ_{max} = 66,000 at 195 nm). As an internal standard, cyclosporin D (CyD; 33-804) is used, because it differs only slightly from CyA in structure (Fig. 1) and chromatographic properties.

EXPERIMENTAL

Reagents

For the mobile phases in chromatography, methanol (Lichrosolv, Merck, Darmstadt, G.F.R.), acetonitrile (HPLC grade S, 50% minimal transmission at 205 nm, Rathburn Chemicals, Walkerburn, Great Britain), and water (demineralized and filtered on a Milli-Q-filter) were used. For the extraction, diethyl ether of analytical grade was used. For the development of the method, outdated heparinized blood bank plasma was used.

Standard solutions of cyclosporin A and cyclosporin D (internal standard) at a concentration of 2500 ng/ml each in methanol—water (1:49) were stored at 3°C throughout the study. Both compounds were supplied by Sandoz (Basle, Switzerland).

Extraction procedure

Conical glass centrifuge tubes (25 ml) were rinsed with diethyl ether and dried. One ml of human plasma (or urine) was added and spiked with a standard solution of CyA and of CyD (200 μl, containing 500 ng CyD). Water was added to a total volume of 2 ml and mixed with a vortex mixer during 5 sec. For the extraction, 10 ml of diethyl ether were added. The tubes were shaken mechanically for 20 min on a horizontal shaker (Model Sm 2, Bühler, Tübingen, G.F.R.) at 160 rpm and centrifuged for 40 min at 800 *g*. An 8-ml aliquot of the separated diethyl ether layer was then transferred to another conical centrifuge tube, which had been rinsed previously with diethyl ether. The diethyl ether extract was evaporated to dryness under vacuum for 45 min with a vortex evaporator (Buchler, Fort Lee, NJ, U.S.A.), starting at room temperature and ending at 50°C. The plasma residue, concentrated now at the bottom of the conical tube, was either stored at 3°C or dissolved in 120 μl of the mobile phase B (see under Chromatographic conditions) and mixed on a vortex mixer. An aliquot of 100 μl was injected into the chromatograph.

Chromatographic conditions

The liquid chromatograph consisted of two pumps (Altex Scientific, Berkeley, CA, U.S.A., Model 110A), which were operated with a gradient programmer (Altex, Model 420). A pressure filter (Altex, Model 110-40) reduced the pulsation and a dynamic mixer (Altex, Model 400) mixed the mobile phases of both pumps. Injection was made with a 100-μl syringe (Hamilton, Bonaduz, Switzerland) via a sample injector (Rheodyne, Berkeley, CA, U.S.A., Model 7120 with a 100-μl loop). The absorption at 210 nm was determined at a sensitivity of 0.02 a.u.f.s. with a UV detector (Uvicon LCD 725, Kontron,

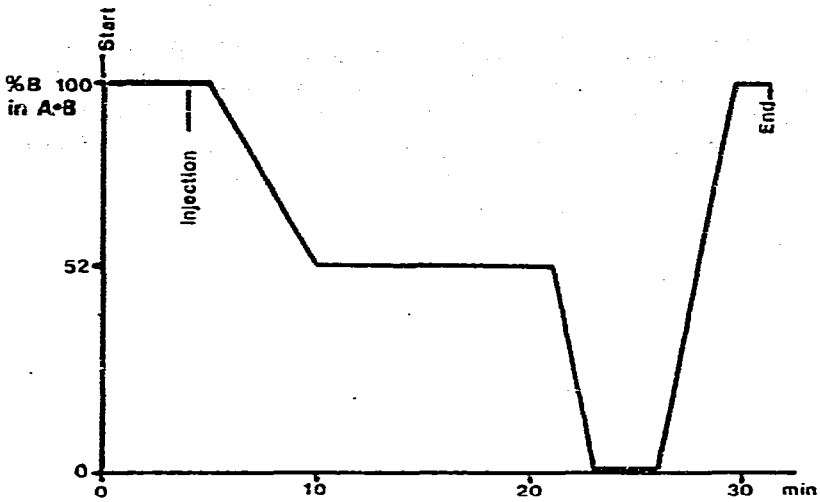

Fig. 2. Gradient profile of the mobile phases water—acetonitrile—methanol (50:750:200) (pump A) and (600:200:200) (pump B).

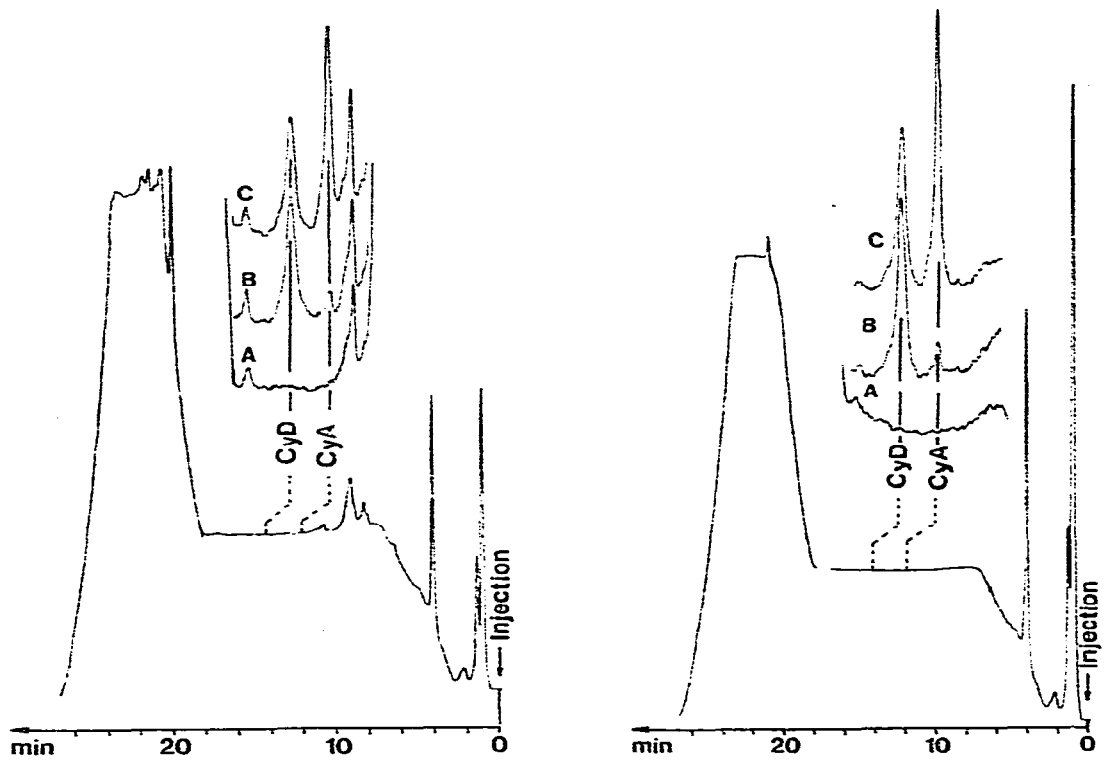

Fig. 3. Liquid chromatograms of (A) blank human plasma and (B) spiked with 50 ng/ml and (C) 500 ng/ml of cyclosporin A. The internal standard cyclosporin D was added at a concentration of 500 ng/ml. The lowest curve is trace A, 10 times attenuated.

Fig. 4. Liquid chromatograms of (A) blank human urine and (B) spiked with 50 ng/ml and (C) 500 ng/ml of cyclosporin A. The internal standard cyclosporin D was added at a concentration of 500 ng/ml. The lowest curve is trace A, 10 times attenuated.

Zürich, Switzerland), which was connected to a two-channel recorder (W + W, Tarkan 600, Kontron).

A stainless-steel column (12.5 cm× 0.3 cm I.D.) was slurry packed (Sandoz) with 5-μm particle size LiChrosorb RP-8 (Merck). It was operated at 72°C [3] inside a Plexiglas jacket (Knauer, Oberursel, G.F.R.), connected to a water circulating thermostat (Haake, Karlsruhe, G.F.R.). To ensure temperature equilibration of the mobile phase, a capillary of 1 m length was placed in front of the column (inside the jacket) [4, 5]. Chromatography was performed using a gradient of the profile given in Fig. 2. The mobile phases consisted of water—acetonitrile—methanol with a composition of 50:750:200 for pump A and of 600:200:200 for pump B. They were degassed with helium before use. With a flow-rate of 1.5 ml/min, the retention times are 12.2 min for cyclosporin A and 14.6 min for cyclosporin D (Figs. 3 and 4). The peak heights were determined graphically.

Human studies

Blood samples were withdrawn into heparinised tubes from two volunteer male human subjects who had received single oral doses of 600 mg cyclosporin A as an olive oil solution (24 ml). The samples were centrifuged, the plasma pipetted off and deep frozen until analysis.

RESULTS AND DISCUSSION

Concentrations of cyclosporin A in plasma and urine were determined from calibration curves for the peak height ratio of the drug CyA to the internal standard CyD (500 ng/ml) over the concentration range 0—2000 ng/ml. The plasma and urine samples, taken in triplicate throughout the extraction procedure described, gave the chromatograms shown in Figs. 3 and 4. For the ratio of the peak heights for CyA to CyD, the coefficients of variation (for 3 determinations) were ± 10, ± 6, ± 9, ± 7, ± 4, ± 1.5 and ± 2.5% for plasma and ± 16, ± 17, ± 7, ± 1, ± 5, ± 1 and ± 2% for urine at 25, 50, 100, 250, 500, 1000 and 2000 ng/ml, respectively. The correlation coefficients of 0.99993 for plasma and 0.99976 for urine indicate a good linearity of the calibration curve. The recovery of CyA was found to be 76 ± 5% in plasma and 104 ± 5% in urine at a concentration of 500 ng/ml.

As no interfering peaks were present in blank plasma and urine (Figs. 3 and 4), the detection limit is determined by the detector noise, which arises mainly from the pulsation of the pumps. Assuming double determinations for later applications, a concentration of 20 ng/ml in plasma or urine differed significantly from the blank according to the *t*-test.

The use of the internal standard CyD is essential for the good precision and linearity of the method. A considerably higher coefficient of variation was found in the upper concentration range taking the peak height of CyA alone compared to the ratio of CyA and CyD. In addition, the correlation coefficients were lower without the internal standard.

The analytical method was applied to plasma and urine samples from the two human subjects dosed with cyclosporin A. The results in Table I show that

TABLE I

PLASMA CONCENTRATIONS OF CYCLOSPORIN A AFTER A SINGLE ORAL DOSE OF 600 mg TO 2 HUMAN SUBJECTS

Time	Plasma concentration* (ng/ml)	
(h)	Subject A	Subject B
0	0	0
1	109 ± 6	0
2	554 ± 17	193 ± 6
4	335 ± 17	642 ± 29
6	176 ± 20	176 ± 8
8	119 ± 6	121 ± 5
24	<20	37 ± 7

*Mean ± standard deviation for $n = 2$.

plasma levels reach a maximum of about 600 ng/ml after 2 h and 4 h for subjects A and B, respectively. The plasma concentrations then decline with an apparent half-life of 5 h (subject A) and 8 h (subject B) for the terminal slope. From urine data, a cumulative excretion of 0.21 and 0.27% of the dose for unchanged drug was found between 0 and 24 h. Peaks from other compounds, known from a radiotracer study [6], were well separated from the peak of the parent compound, thus indicating a good specificity of the method.

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REFERENCES

1 A. Rüegger, M. Kuhn, H. Lichti, H.R. Loosli, R. Huguenin, C. Quiquerez and A. von Wartburg, Helv. Chim. Acta, 59 (1976) 1075.
2 R.Y. Calne, D.J.G. White, S. Thiru, D.B. Evans, P. McMaster, D.C. Dunn, G.N. Craddock, B.D. Pentlow and K. Rolles, Lancet, ii (1978) 1323.
3 F. Erni, personal communication.
4 R. Freiburghaus, personal communication.
5 R.J. Perchalski and B.J. Wilder, Anal. Chem., 51 (1979) 774.
6 G. Maurer, personal communication.