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MEASUREMENT OF CYCLOSPORIN A AND OF FOUR METABOLITES IN WHOLE BLOOD BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-performance liquid chromatographic procedure using cyclosporin D as internal standard for the routine measurement of cyclosporin A and four of its metabolites is described. Whole-blood samples were purified on refillable solid-phase glass extraction columns. The chromatographic method includes a gradient elution using acetonitrile and water (pH 3.0) as eluents and an RP-8 analytical column. More than 1000 samples have already been analysed without any loss. The inter-assay variation was 6.3% and the intra-assay variation 4.9%. A linear correlation was found over a range of 0–3000 ng cyclosporin A per ml whole blood. The detection limit was 20 ng and the recovery was found to be 80–90%. Metabolites 1, 17, 18 and 21 could be characterized.

INTRODUCTION

Cyclosporin A (Cy A) is a lipophilic neutral and cyclic undecapeptide including a C_9 amino acid. The use of Cy A as an immunosuppressive agent [1,2] is well established in the field of organ transplantation [3–5] and is under investigation in the treatment of various other diseases of an immunological nature.

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The therapeutic index of Cy A is very narrow and the individual therapeutic response and the bioavailability are both variable. Therefore regular monitoring of Cy A blood levels is required. This is generally done with a radioimmunoassay (RIA), although its usefulness has been questioned [7,8] because it is difficult to evaluate to what extent the antibodies used cross-react with Cy A metabolites [9–11]. Especially for patients with a liver malfunction, and therefore disturbed Cy A metabolism, it is desirable to have a rapid, specific and sensitive quantitative method to measure Cy A in body fluids, in particular in whole blood. Cy A measurement in serum or plasma due to the changing distribution of Cy A between plasma/serum and blood cells is unreliable [12–15].

In almost 2000 samples we have tried to reproduce several previously reported high-performance liquid chromatographic (HPLC) methods [16–19] under routine conditions, but they all suffered from one or more of the following disadvantages: a short lifespan of the chromatography column [16,17] attributable to plugging or other disturbances, low or variable Cy A recovery [16,18], laborious and/or time-consuming extraction and sample preparation [16–18] and a great proportion (up to 10%) of samples that could not be evaluated because of preparative or chromatographic failure [16,17]. Therefore we developed a new HPLC technique that allows a rapid and reproducible quantitative determination of Cy A and the detection of its metabolites 1, 17, 18 and 21 [20].

EXPERIMENTAL

Materials

The experiments were performed on a Hewlett-Packard HPLC system 1090 equipped with a diode-array detector and a Hewlett-Packard 85 as integrator and data processor and on a Hewlett-Packard HPLC system 1084B equipped with a Merck-Hitachi variable-wavelength monitor and a Merck-Hitachi integrator D-2000. The following columns were used: glass extraction columns and PTFE frits (Krannich, Göttingen, F.R.G.) filled with LiChroprep RP-8, 25–40 μm (Merck, Darmstadt, F.R.G.); a Merck RT (250×4 mm I.D.) analytical column with LiChrosorb RP-8, 5 μm, and a Merck manu-fix (25×4 mm I.D.) precolumn with LiChrosorb RP-8, 5 μm.

The following solvents were used: acetonitrile, water, hexane and dichloromethane (all LiChrosolv, Merck). Cy A and Cy D were a gift from Dr. E. Wiskott, and metabolites 1, 17, 18 and 21 were a gift from Dr. G. Maurer (both Sandoz, Basle). Blood samples for standards and blanks were taken from healthy blood donors, and for Cy A analysis from Cy A-treated allograft recipients.

Extraction procedure

The glass columns were filled with 100 mg of LiChroprep RP-8. For priming, 3.2 ml of acetonitrile and 3.2 ml of water (pH 3.0) were sucked through by vacuum. The internal standard contained 1 μg of Cy D per ml of acetonitrile–water (pH 3.0) (50:50, v/v). To 1 ml of whole blood, 250 μl of the internal standard and 2 ml of acetonitrile–water (pH 3.0) (30:70, v/v) were added. The samples were vortexed for 10 s and centrifuged at 2500 *g* for 2 min. The supernatant, 3.2

ml of acetonitrile–water (pH 3.0) (20:80, v/v) and 0.5 ml of hexane were sucked consecutively through the glass extraction columns. The extraction columns were then set into a 10-ml centrifuge tube, and Cy A was eluted by centrifuging 2 ml of dichloromethane through the columns (400 *g*, 5 min). Subsequently the columns were removed and the eluate was evaporated at 50°C under a stream of nitrogen. The resulting residue was taken up into 250 μl of acetonitrile–water (pH 3.0) (50:50, v/v). Then 0.5 ml of hexane was added and the samples were vortexed for 10 s. After phase separation the acetonitrile–water phase containing Cy A was transferred into a vial for the auto-injector, and 75 μl of a sample were injected into the HPLC system.

Chromatographic procedure

Water (pH 3.0) was used as solvent A and acetonitrile was used as solvent B. The solvents were degassed by vacuum and ultrasound or solvent-heater (temperature for solvent A 80°C and for solvent B 65°C). The water pH was adjusted with hydrochloric acid. The detector wavelength was set at 210 nm, the oven temperature at 75°C and the flow-rate at 1.4 ml/min. For elution during routine analysis a linear gradient running for 14 min from 55 to 63% acetonitrile was used followed by a 5-min column clean-up with 100% acetonitrile. The column was reequilibrated within 5 min.

For determination of the metabolites, which were less lipophilic than the mother compound, the gradient was 45–52% acetonitrile for the first 20 min and changed to 63% acetonitrile within a further 12 min. Subsequently the column was cleaned by 100% acetonitrile for 5 min and reequilibrated for 5 min. Peaks with an equal retention time as that of the metabolite standard solutions were isolated by semi-preparative HPLC. Their cross-reactivity was evaluated with the RIA test (Sandoz, Basle) and their spectral properties with the diode-array detector, after reinjection into the HPLC system.

Inter-assay reproducibility for Cy A was determined by extracting and analysing replicate blood samples from blood donors not treated with Cy A spiked with 100, 200, 300, 400 and 600 ng of Cy A and 250 ng of internal standard. Intra-assay reproducibility was evaluated by repeated injections of a pooled extract from twenty blood samples containing 250 ng of Cy A per ml and the internal standard. For precision control during routine analysis 100 ml of whole blood from Cy A-treated blood donors were spiked with 400 ng of Cy A per ml taken from the standard solution of the RIA kit. A control sample was extracted and analysed every ten samples. A maximum deviation of 10% was regarded as tolerable.

RESULTS

With our routine gradient the retention time of the internal standard Cy D is 13.0 min and that of Cy A is 11.0 min (Fig. 1B). Both peaks, as checked by spectral analysis with the diode-array detector, were found to be pure and not superimposed by material of only slightly different spectral properties. The recovery of Cy A was calculated at a concentration of 250 ng of Cy A per ml of whole blood and was found to be between 80 and 90%. The detection limit was

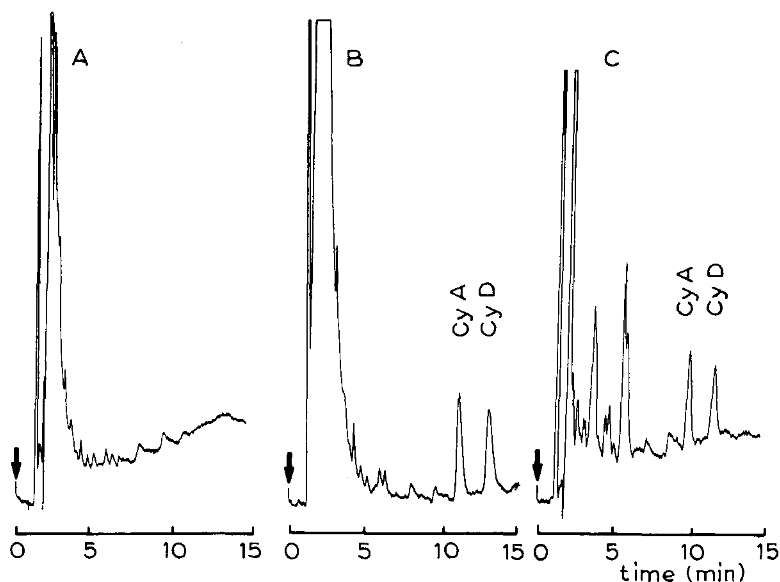

Fig. 1. Chromatograms of whole-blood samples extracted as described and analysed with the routine gradient. (A) Sample from a blood donor not treated with Cy A; (B) sample spiked with 300 ng of Cy A and 250 ng of Cy D; (C) sample from a kidney allograft recipient containing 310 ng of Cy A and the internal standard.

20 ng of Cy A per ml of whole blood. When the amounts of Cy A added to blood samples were plotted versus the amounts calculated from the corresponding chromatograms, a linear correlation was found over the range 0–3000 ng of Cy A per ml of whole blood (Fig. 2). The inter-assay variation was 6.3% ($n=60$) and the intra-assay variation 4.9% ($n=20$). The retention times of the Cy A metabolites, 1, 17, 18 and 21 are shown in Fig. 3 and Table I. In chromatograms of extracted whole-blood samples and spectral analysis of the Cy A, Cy D and metabolite peaks with the diode-array detector no interfering peaks could be found. The fractions collected by semipreparative HPLC showed the same spectral properties as Cy A and the internal standard, and cross-reacted with the RIA test.

DISCUSSION

The results show that the HPLC method described here allows a rapid, sensitive and reliable determination of Cy A in patients' blood, and with a slight modification the determination of its metabolites 1, 17, 18 and 21. The main difficulty in the determination of Cy A stems from its lipophilic nature (the partition coefficient between octanol and water is 120:1). Extraction on prepacked disposable solid-phase extraction columns is associated with plugged columns the more often they are reused [22]. The use of refillable glass extraction columns as described here circumvents this problem because the frits can easily be removed and cleaned. The solid phase is used for only one extraction. The frits can be reused for up to ten extractions. Furthermore, refillable extraction columns are much cheaper to use than prepacked extraction columns. After the addition of acetonitrile–water

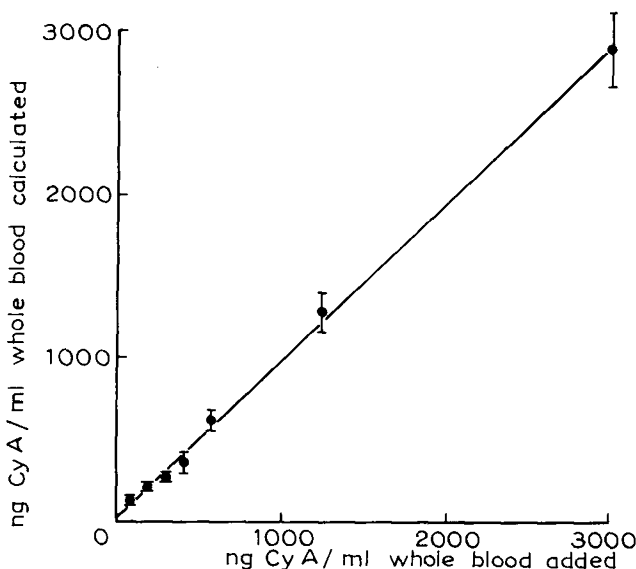

Fig. 2. Values of Cy A (0, 100, 200, 300, 400, 600, 1250 and 3000 ng of Cy A per ml) added to blood samples from blood donors (abscissa) plotted versus values of Cy A calculated from the corresponding chromatograms (ordinate), with $n=6$ for each concentration. The equation for the linear regression is $y=0.975x+8.5$ ($r=0.9997$).

(30:70, v/v) to the whole blood sample and centrifugation, the supernatant must be drawn through the extraction column without great delay. If the sample is left too long in a warm centrifuge or at room temperature, precipitation could occur and the precipitates would plug the column. Poorly anticoagulated samples had the same effect.

Especially after the elution from an external extraction column, there is a danger of contaminating the sample with solubilized plastic materials that are not inert to acetonitrile and dichloromethane [21]. After this step, therefore, glass pipettes and centrifuge tubes rinsed with diethyl ether must be used. In the HPLC system the plastic materials of the inlet and outlet valves of the high-pressure

TABLE I

RETENTION TIMES OF Cy A, Cy D AND Cy A METABOLITES DURING RUNS WITH THE ROUTINE GRADIENT AND THE GRADIENT FOR METABOLITE ANALYSIS

Compound	Retention time (min)	
	Routine gradient	Metabolite gradient
Cy A	11.0	28.1
Cy D	13.0	30.4
Metabolite 17	6.3	16.4
Metabolite 1	6.6	17.2
Metabolite 18	7.0	18.1
Metabolite 21	8.3	22.0

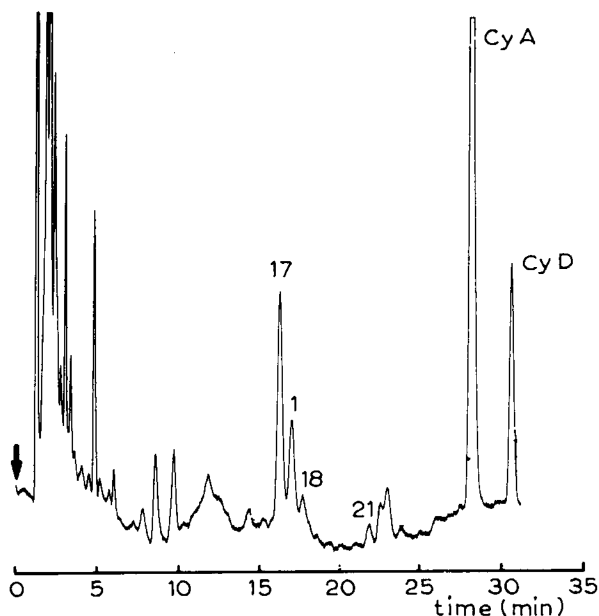

Fig. 3. Chromatogram of a whole-blood sample from a kidney allograft recipient containing 934 ng of Cy A, 315 ng of metabolite 17, 171 ng of metabolite 1, 64 ng of metabolite 18 and 28 ng of metabolite 21 per ml of blood.

pump and of the rotor seals in column switch valves can also cause peaks with a retention time close to the retention time of Cy A. They must be replaced by PTFE parts. Interfering materials from the caps of the autoinjector vials necessitate a thorough cleaning of the injector needle before injection.

Because of its lipophilic properties, its characteristic band broadening and the variable purity of the extracted samples, especially after liquid–liquid extraction, the use of an elution gradient is superior to isocratic elution. With a good inter- and intra-assay precision it could not be confirmed that, as commonly believed, gradient elution is less reproducible and less precise than isocratic elution [23]. Gradient elution decreases the capacity factors (k') of strongly retained bands, such as Cy A and Cy D. The so-called "linear capacity" [24] of the column in isocratic systems is generally less for later-eluting bands and results in band broadening if column overload occurs. A gradient system is less susceptible to poorly purified samples because of its greater linear capacity than an isocratic system. A reasonable separation of the metabolites within an acceptable run time is not possible using an isocratic system.

It has been suggested that rapid degradation of the analytical column has to be attributed to the high oven temperature needed to diminish the characteristic band broadening of Cy A. Like other authors [25], we found that it was not the high oven temperature itself that was the main reason for the short lifetime of the column but too rapid heating and cooling, as well as the injection of poorly purified samples. Thus the analytical column was kept at a temperature of 75 °C, even when no samples had to be analysed, and it was guarded by a precolumn.

During the column clean-up with acetonitrile two peaks were eluted that do not appear after a Cy A standard solution was injected. A rapid overload of the analytical column by materials that were not eluted by the linear gradient could be prevented. Almost 1000 analyses have been performed on our actual analytical column, and no sign of degradation has yet been observed.

To achieve a reasonable peak shape of Cy A and the internal standard the column temperature was set at 75 °C, the water was adjusted to pH 3.0, the injection volume was kept as small as possible and gradient elution was used. We tested RP-2, RP-4, RP-8 and RP-18 columns, and of these the RP-8 columns gave the best results.

We tried to reproduce some previously described methods in almost 2000 samples under routine conditions. After liquid–liquid extraction [16,17] with diethyl ether the number of poorly purified and samples causing column plugging is unacceptably large (up to 10%). Furthermore, these methods are not suitable for urgent samples because they require a time-consuming haemolysis by freezing and thawing. With a haemolysis according to Lensmeyer and Fields [19] measurement of an urgent sample could be performed within 1.5 h. We found liquid–liquid extraction unsuitable for routine extraction of a large number of samples because working with diethyl ether was very uncomfortable and to achieve good and reproducible results a lot of practice is required.

The advantages of our procedure over previously described solid–liquid extraction methods [18,19,26–28] can be summarized as follows:

(1) The use of refillable glass extraction columns with removable frits virtually eliminates the possibility of sample loss caused by plugged extraction columns.

(2) The rate of evaporation from the extraction columns of the dichloromethane used for elution is faster than that of methanol [18] or acetonitrile [19].

(3) The final hexane washing removes remaining interfering materials.

(4) The use of a gradient enables us to measure Cy A and four of its metabolites in the same sample.

We also tested column-switching techniques; most of those described previously were not able to measure Cy A in whole blood [21,29]. Column-switching techniques using whole blood also need a preceding extraction procedure [30]. All in all they proved to be more time-consuming than external column extraction because all the internal extractions had to be done successively and samples for the standard curve must be run intermittently. Because of the large injection volume of the very poorly purified samples our precolumns had a short lifetime and capillaries got plugged by precipitated proteins owing to the high oven temperature.

Especially after liver transplantation, separate measurement of the parent drug and its metabolites could be of value for dose adjustment of Cy A. Until now only Lensmeyer and Fields [19] and Freeman et al. [31] have been able to detect Cy A metabolites in blood using an HPLC system with UV detection. The structures of the four metabolites (1, 17, 18 and 21) were elucidated by Maurer et al. [20]. When the routine linear gradient was used metabolite 17 could be reasonably separated from metabolite 1 and 18. The use of the modified gradient (Fig. 4)

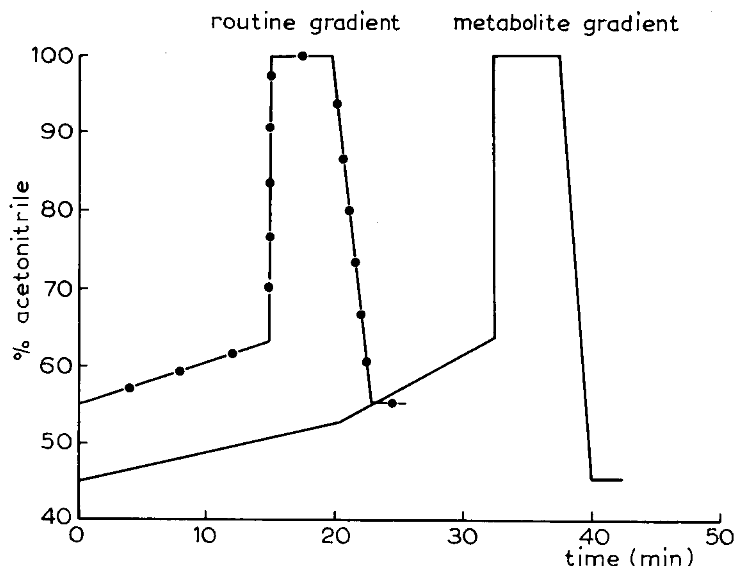

Fig. 4. Curves showing the routine gradient (—●—) and the gradient used for metabolite analysis (—). Water (pH 3.0) was used as solvent A and acetonitrile as solvent B.

considerably improved the separation of metabolites 1, 17 and 18. In most samples metabolites 18 and especially 21 could not be measured because their concentration in 1 ml of whole blood was below the detection limit. With the modified gradient more detailed information about the metabolism of Cy A could be obtained, which would help to improve the use of Cy A.

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