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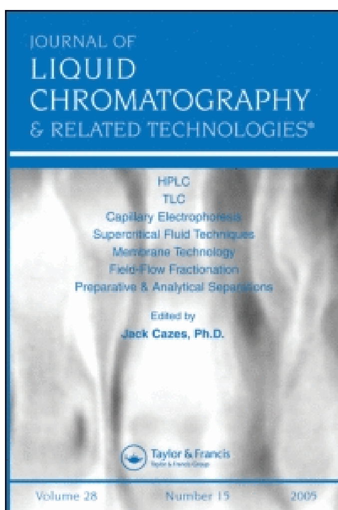

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
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HPLC Measurement of Cyclosporine in Blood Plasma and Urine and Simultaneous Measurement of its Four Metabolites in Blood

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To cite this Article Gupta, Suneel K. and Benet, Leslie Z.(1989) 'HPLC Measurement of Cyclosporine in Blood Plasma and Urine and Simultaneous Measurement of its Four Metabolites in Blood', Journal of Liquid Chromatography & Related Technologies, 12: 8, 1451 — 1462
To link to this Article: DOI: 10.1080/01483918908049516
URL: http://dx.doi.org/10.1080/01483918908049516

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JOURNAL OF LIQUID CHROMATOGRAPHY, 12(8), 1451-1462 (1989)

HPLC MEASUREMENT OF CYCLOSPORINE IN BLOOD PLASMA AND URINE AND SIMULTANEOUS MEASURMENT OF ITS FOUR METABOLITES IN BLOOD

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ABSTRACT

A high performance liquid chromatographic assay has been developed for the estimation of cyclosporine and its four major metabolites in blood and for cyclosporine alone in plasma and urine samples. This assay employs a rapid and very reproducible solid-liquid extraction system. Isocratic chromatographic conditions allow the simultaneous measurement of cyclosporine and its four major metabolites in blood. The method is linear up to 2500 ng/ml and the minimum quantifiable limit for cyclosporine is 30 ng/ml, when 1 ml of sample is analyzed.

INTRODUCTION

Cyclosporine (CYA) is a potent immunnosuppressant widely used in organ transplantation[1]. Chemically, CYA is a lipophilic, neutral, cyclic undecapeptide. Due to the drug's narrow therapeutic index, monitoring of CYA has been of considerable importance. Earlier CYA was estimated

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using a radioimmunoassay employing a polyclonal antibody which cross reacts with the metabolites of CYA. Since certain of the CYA metabolites appear to have immunosupressive activity[2], there is considerable interest in estimating these metabolites separately, especially for immunopharmacokinetic studies. Several HPLC assay methods have been reported in the literature[3-6] for the quantification of CYA. Most of the methods require a tedious extraction step and/or are only suitable for CYA estimation. All the reported procedures use either liquid-liquid or solid phase extraction methods. Most of the reported liquid-liquid extraction procedures are time consuming and not applicable for metabolite estimation. With solid phase extraction methods, a major problem is poor reproducibility. Recently, Christian et al.[6] described a method using self packed glass extraction columns and a gradient system. Kabra et al.[5] reported an extraction procedure yielding high recoveries, using commercially available solid phase systems, for estimation of CYA in blood only. We report here a method which overcomes the problem of variability due to self packed columns and utilizes commercially available sample preparation columns with high and reproducible recoveries. The present technique is applicable for determination of CYA and its four major metabolites in blood.

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EXPERIMENTAL

Apparatus

The chromatographic system consisted of a CM 4000 solvent delivery pump (Milton Roy, Florida), a Waters WISP 710B automatic sample injector, a column heater (Temperature control module, Waters) and a UV detector (Lamda Max model 481, Waters Associates, Milford, MA). Peak integration was recorded with a Hewlett-Packard integrator (Model 3392A). Sample preparation 1 ml columns, (Bond eluet LRC from Analytichem International, Harbor City, CA) were used together with a Adsorbex Sample Preparation Unit with a vacuum pump (Richard Scientific, San Francisco, CA). Lichrosorb RP_8 (Fisher Scientific, San

Francisco, CA) and Ultrasphere ODS (Alltech Associates, Deerfield, IL) analytical columns (250 X 4.6 mm, 5 μ particle size) were used.

Reagents

Acetonitrile, methanol and ethanol were HPLC grade (Fisher Scientific, San Francisco, CA). Zinc sulphate was purchased from Sigma Chemical (St. Louis, MO). Water used for making the mobile phase was de-ionized through a "Nanopure" water filtration system (Millipore, Milford, MA). Cyclosporines (A & D) and metabolites 1, 17, 18 and 21 were obtained as a gift from Sandoz, Basle. Purities of the drug and metabolites were not tested and were used as received. Blood samples were obtained from healthy volunteers following administration of both oral and intravenous CYA.

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Preparation of calibration standards

Stock solutions of CYA and the internal standard cyclosporine D (CYD) were prepared by dissolving 5 mg of the species in 50 ml of methanol and stored at room temperature. The stock solutions were diluted 1 in 10 with methanol and further used in preparation of calibration curves. Stock solutions of metabolites were prepared by dissolving 20 μg in 1 ml of methanol. Calculated volumes of a standard solution of CYA and its four metabolites, equivalent to 100, 300, 600, 900, 1200 and 2500 ng, were added to 10 ml capacity glass tubes and solvent was evaporated. To all the tubes 1 ml of either hemolyzed blood, plasma or urine was added. Tubes were allowed to stand at 37° C for at least 30 minutes and later processed as described below for biological samples.

Extraction procedure

Before extraction, blood samples from subjects were frozen and thawed to hemolyze the red blood cells. Samples or controls (1 ml) containing CYA were spiked with the internal standard (CYD, 500 ng)

and to this 3 ml of protein precipitating reagent (25 % acetonitrile, 25 % methanol and 50 % water containing 10 % Zn SO_4) was added. The mixture was vortexed for 30 seconds and centrifuged at 4000 g for 5 min. Sample preparation columns were arranged on the sample preparation unit and washed with 4 ml of ethanol followed by 3 ml of de-ionized water. Clear supernatant from the samples or controls was eluted through the sample preparation columns. Depending upon the species to be analyzed, irrespective of the sample matrix, the columns were washed with either 2 ml (for CYA and metabolites) or 4.5 ml (for CYA alone) of 50 % acetonitrile solution. After which the columns were eluted with 1 ml of ethanol. Eluent was evaporated to dryness under nitrogen and the residue was reconstituted into 200 µl of mobile phase, from which 150 µl was injected onto the HPLC system. Washing and elution of the sample preparation columns was done using a vacuum of 10 p.s.i.

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Chromatographic conditions

Acetonitrile was mixed with 0.001 M HCl (pH 3.0) in the ratio of 45:55 and the mixture was degassed by vacuum and ultrasound. Solvent was delivered at the rate of 1.5 and 1.0 ml/min for estimation of CYA and CYA plus metabolites, respectively. For estimation of CYA and its metabolites in blood, an Ultrasphere ODS (250 X 4.6 mm, 5 µ) column was used, while for estimation of CYA only in plasma and urine, a Lichrosorb RP_8 (250 X 4.6 mm, 5 µ) column was used. Both columns were maintained at 70° C in a column heater. The detector wavelength was set at 214 nm and 0.002 AUFS. The mobile phase was recirculated and changed after every 100 sample injections.

RESULTS

As shown in Fig.1, when CYA free blood, plasma or urine was extracted and chromatographed no peak was observed at the retention times of CYA and its metabolites. When samples are analyzed for CYA only, the CYA peak eluets at 17 min and the internal standard CYD elutes at 23 min (Fig 2). The peaks are very sharp and well separated at

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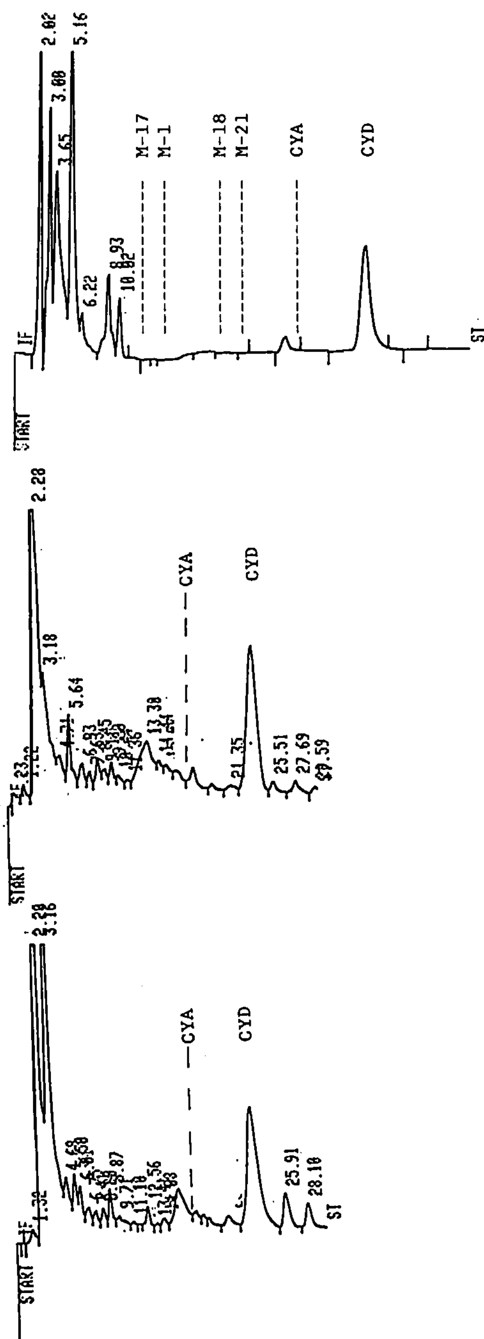

Figure 1. Chromatograms of CYA free (i.e. blank) whole blood (a), plasma (b) and urine (c) samples obtained from a healthy volunteer to which the internal standard CYD (500 ng/ml) had been added.

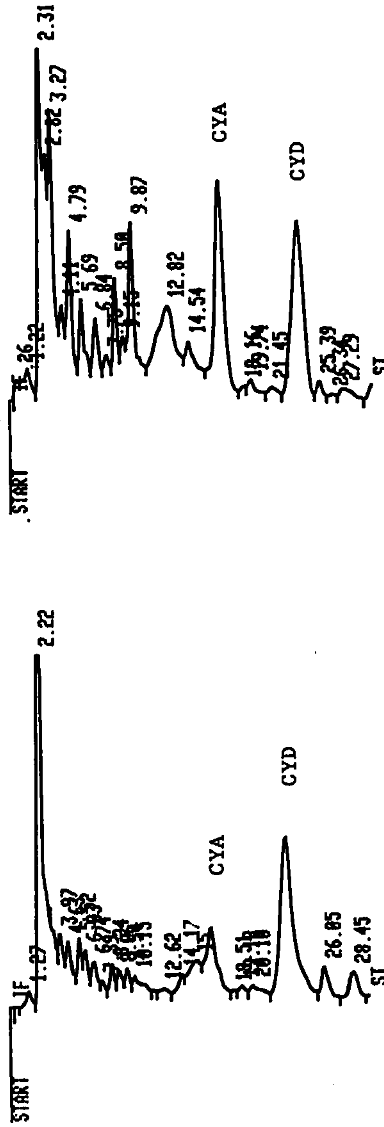

Figure 2. Typical chromatograms of plasma (a) and urine (b) samples containing CYA and internal standard CYD.

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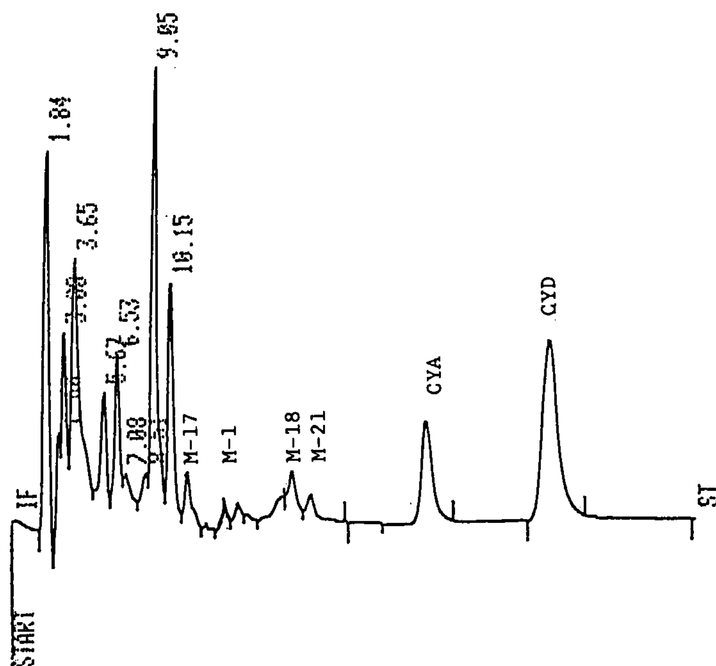

Figure 3. Chromatogram of a blood sample obtained 3 hours following an oral (10 mg/kg) dosing of cyclosporine to a healthy volunteer. The sample contains CYA, metabolites M-17, M-1, M-18, M-21 and the internal standard, CYD.

the base line. No ghost peaks were seen during the analyses, thus samples could be injected every 25 min. When blood samples were analyzed for CYA and its major metabolites, the retention times were 11, 12, 13, 18, 33 and 45 minutes for M-17, M-1, M-18, M-21, CYA and CYD respectively (Fig. 3). The samples were injected every 60 min.

The relationship of peak height ratio (CYA/CYD or Metabolite/CYD) was found to be linear up to 3000 ng/ml for CYA concentrations in blood, plasma and urine and to 2000 ng/ml for metabolites concentrations in blood. Slopes and intercepts obtained following the linear regression of peak height ratios versus spiked concentrations are given in Table 1. The

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Table 1. Linear regression parameters obtained following regression of peak height ratio with concentrations of cyclosporine and its four metabolites spiked into blood from normal healthy volunteer.

Species	Slope	Intercept	r^2
CYA	0.00160	0.0334	0.999
M-1	0.00062	0.0092	0.975
M-17	0.00186	-0.0294	0.985
M-18	0.00144	-0.0976	0.977
M-21	0.00233	-0.0801	0.995

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minimum quantifiable limits for estimation of CYA were 25, 30 and 30 ng/ml for plasma, blood and urine samples respectively. Intra-day and inter-day coefficients of variation were estimated at three CYA concentrations (100, 300 and 1500 ng/ml) and found to be less than 5 % (n=11, for each concentration) for spiked blood and plasma samples. Due to the limited supply of metabolites, the variability for the metabolites was not determined. The intra-day variability for CYA estimation in a blood sample, obtained from a volunteer, was 4.1 % (n=5).

To estimate the absolute recovery of CYA, three different concentrations were added to blood, plasma and urine samples of a healthy volunteer and extracted as described above. The CYA peak height was compared with the peak height obtained for an equal amount of CYA injected directly onto the column. Mean (n=5) recoveries of CYA from blood, plasma and urine samples ranged between 68 and 77 percent (Table 2). Following analyses of several blood samples, obtained from hospitalized patients receiving prednisolone, azathioprine, furosemide, methyldopa and minoxidil, drugs commonly used in post-transplant treatments, no interfering peak at any retention time of interest was observed.

Table 2. Mean percentages (n=5) of cyclosporine recovered following extraction from various biological fluids at three different concentrations .

Concentrations (ng/ml)	% ± S.D. Recovered in : Blood	Plasma	Urine
100 (low)	68±3	70±2	75±3
600 (medium)	70±2	75±2	77±2
1200 (high)	72±2	77±2	77±2

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DISCUSSION

The principal advantages of this extraction procedure are simplicity, high recoveries and reproducibility. With a small modification, the same basic process can be used for CYA measurement in blood, plasma and urine or for simultaneous analysis of CYA and its metabolites in blood. In order to get better separation longer analytical columns (25 cm) were used. The sample extraction columns could be reused for 4 plasma or 3 blood samples and are recharged with elution by ethanol. After repeated use, solvent flow through the columns tends to be slow and longer elution times are required. Inclusion of zinc sulfate in the precipitating reagent results in more complete protein precipitation and yields clearer supernatants. Precipitation using iso-propanol or copper sulfate was found to be equally good in yielding clear supernatants but recovery of CYA was found to be lower than obtained that using zinc sulfate. Zinc sulfate probably has a salting out effect on the recovery of CYA. The present procedure does not require a hexane wash to remove late elueting materials, as proposed in the recently reported procedure of Christian et al.[6]

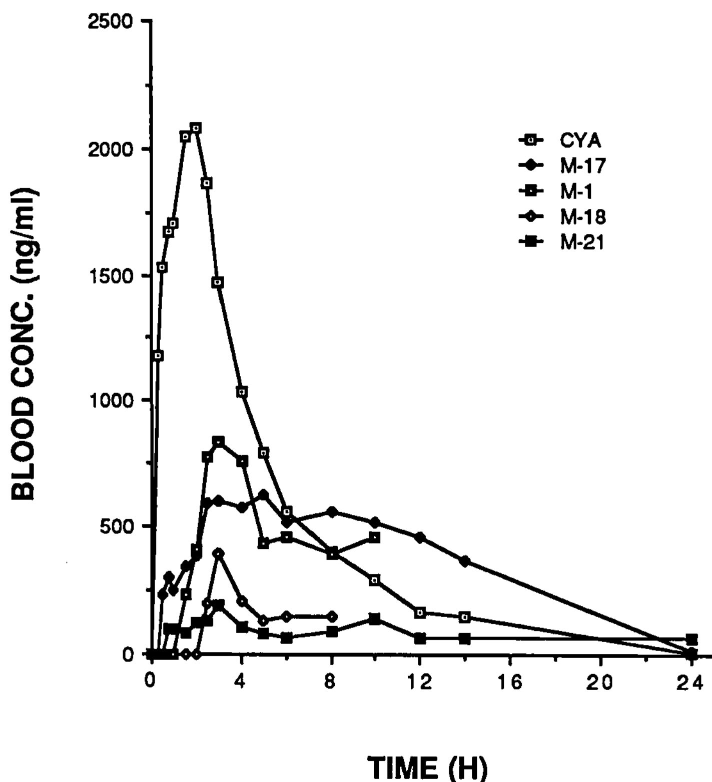

Figure 4. Blood concentration-time profiles for CYA and its four metabolites obtained following oral administration of CYA (10 mg/kg) to a healthy volunteer.

A run time of 45 min for analysis of CYA and its major metabolites is the same as that reported by others[6] using a gradient method for the analysis of metabolites. An isocratic system for the analysis of metabolites was preferred over a gradient method since better peak reproducibility is obtained with isocratic versus gradient elution[7]. We found that employing two different columns allowed us to use the same mobile phase and similar conditions for the assay of CYA and CYA and its four major metabolites. In our experience, flow rates higher than 1.5 ml/min result in high column pressure and shorter column life. Standard

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curves were constructed for each metabolite separately, as indicated by the different slopes in Table 1. Use of the CYA regression parameters for metabolites would have significantly under or overestimated concentrations of metabolites M-1 and M-21, respectively.

The method described here was used to analyze samples obtained from a pharmacokinetic study carried out in healthy volunteers following oral and intravenous administration. Figure 4 depicts blood CYA and metabolites M-17, M-1, M-18 and M-21 concentrations following a 10 mg/kg oral dose to a healthy volunteer. Using this method or any other reported method it is not possible to measure metabolite concentrations in plasma (as opposed to blood) samples with great confidence. Thus we still need assay procedures which are even more sensitive, since the concentrations of metabolites in the plasma are much lower than those found in blood.

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ACKNOWLEDGEMENT

We thank Dr. Pokar M. Kabra of the Department of Laboratory Medicine, University of California, San Francisco, for his help and guidance in the development of this assay. This work was supported by NIH grant GM 26691.

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