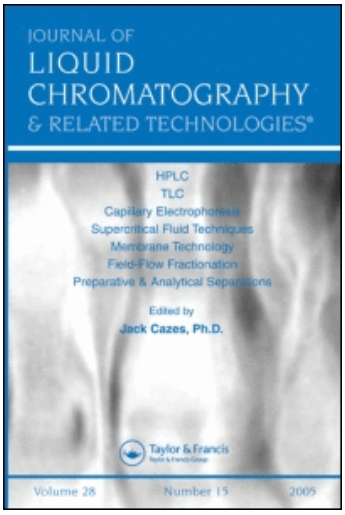

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LIQUID CHROMATOGRAPHIC DETERMINATION OF CYCLOSPORINE-A IN BLOOD, WITH CHROMOSORB P COLUMNS USED FOR SAMPLE PURIFICATION

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ABSTRACT

A fast liquid-chromatographic determination of whole-blood cyclosporine A concenration is described. A sample preparation consisting of diethyl ether extraction and Chromosorb P column purification of extract, requires 1 mL of whole blood and takes 10 min of technical effort per sample. A reversed-phase C_1 column (5-µm particles) is used, with acetonitrile/methanol/water (20/45/35 by vol) as mobile phase. Cyclosporine A is quantified by absorbance at 214 nm, with cyclosporine D as internal standard. Chromatographic development is complete in 8 min. Linearity is verified by a five-point calibration curve in the range 50-900 µg/L, correlation coefficient $r>0.998$, $y = 0.001x - 0.053$. Lower limit of sensitivity is 25 µg/L. Extraction efficiency was over 70%, accuracy varied between -7.1% and +3.3%, CVs were <5.8% within run, <7.5% between runs. No interference was observed from both endogenous compounds, and 33 drugs eventually co-administered during immunosuppression. Over 1,000 patient samples have been analysed by this method in our laboratory in about one year, without any sign of column deterioration.

INTRODUCTION

Cyclosporine A (CyA), a unique cyclic undecapeptide of fungal origin, with potent immunosuppressive properties but low myelotoxicity, has had a major impact on organ transplantation, and recently, on the treatment of some autoimmune diseases. The importance of CyA therapeutic monitoring is well recognized and an understanding of effective blood concentration is developed (1). In response to the clinical need for a better standardization of CyA therapy, the members of the Task Force on CyA Monitoring proposed several impotant recommendations. Their special report contains valuable review on each aspect of CyA monitoring (2). Two procedures are relevant nowadays for the reliable estimation of CyA concentrations: monoclonal radioimmunoassay (RIA) and liquid chromatography (LC). Polyclonal RIA has been quite popular in the near past, but is not further recommended, because of cross-reactivity of CyA metabolites. Both monoclonal RIA and LC assays, have found a place in the drug monitoring arena for CyA, with respective advantages and shortcomings, which are to be overcome by an evidently everlasting laboratory effort. Improved chromatography of CyA has been introduced (3), but still many LC procedures have the major disadvantage of laborious, multistep and time-consuming sample preparation, based on a classical liquid-liquid extraction (4-7, 8), or applying expensive solid-phase cartridges (3,5,6).

Here we present a simple LC procedure for the determination of CyA in whole blood, with use of a rapid single-step diethyl ether extraction, followed by a Chromosorb P column purification of the extract, and a reversed-phase isocratic separation on a C_1 analytical column.

MATERIALS AND METHODS

Chemicals, Reagents, Standards

CyA and cyclosporine D (CyD) were gifts from Sandoz, Inc. (Bazel, Switzerland). Chromosorb P/NAW, 80-100 mesh (diatomite support), and all organic LC-grade solvents were purchased from Merck (D-6100, Darmstadt, F.R.G.). All other chemicals were analytical grade from standard chemical suppliers. Water was de-ionized by Milli-Q System (Millipore, U.S.A.). The stock standards of CyA and CyD, prepared as 10 mg/L solutions in methanol, were kept at +2 to +8°C. Standard concentrations of CyA in whole blood were prepared fresh each month over the range 50-900 µg/L. The mobile phase consisted of acetonitrile/methanol/water (20/45/35 by vol), filtered and degassed through a 0.45 µm (pore size) filter.

Equipment

The LC system consisted of a Series M-45 pump, a U6K manual injector, an absorbance detector M-441, and a single pen recorder (all from Millipore - Waters, Milford, MA 01757, U.S.A.), and a 50 mm x 4.6 mm (i.d.) reversed-phase column, packed with Supelcosil-LC-1, 5 µm particle size (Supelco SA, 1196 Gland, Switzerland), maintained at 60°C in a water bath during analysis.

Microcolumn for Sample Purification

The Chromosorb P microcolumn has been previously described(9). Briefly, plug a 5-mL plastic pipette tip with a small ammount of glass wool or cotton, fill the low part of the tip with about 300

mg of Chromosorb P/NAW, and place the so-prepared column on a stand, above an appropriate-size glass collection tube. Apply 100 µL of NaOH (0.1 M) to the column prior to extraction.

Procedure

Pipette 1.0 mL of whole blood to a chemically clean screw-capped glass tube, add 1.0 mL HCl (0.18 M) and 50 µL of internal standard (CyD) stock solution. Vortex mix for 10 s, add 6.0 mL of diethyl ether, vortex mix for 90 s, and transfer the ether layer to the microcolumn for sample purification. Rinse the column additionally with 2.0 mL of diethyl ether, collect the eluate, and evaporate it under nitrogen in a water bath at 40°C. Reconstitute the residue with 100 µL of acetonitrile/methanol/water (1/1/3 by vol) solution, inject 50 µL onto the LC column, and elute with mobile phase at a flow-rate of 1.0 mL/min (pressure 3-4 MPa), at 60°C. Monitor the column effluent at 214 nm with a sensitivity of 0.01 A full scale. Construct the calibration curves in "internal standard" mode and calculate unknowns from peak height ratios.

RESULTS AND DISCUSSION

Figure 1 presents chromatograms from three specimens of blood derived according to our procedure. CyA and CyD peaks are gaussian and fully base-line separated, which facilitates quantification. No interfering peaks were found at retention times corresponing to those of drug and internal standard at the chromatographic conditions used. The last peak in each chromatogram is an endogenous substance, found in all blood specimens, and is often observed by other authors (3,6). The presence of this peak should be considered a relative disadvantage of the procedure described here, but

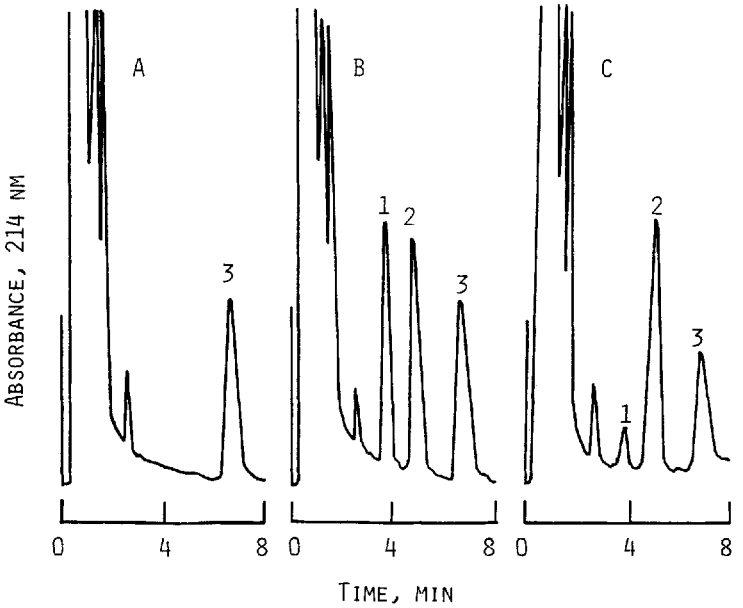

FIGURE 1. Continuous tracing of the detector output during three consecutive specimen extract injections: A, drug free blood; B, donor blood, spiked with 300 µg/L CyA; C, blood of a patient on CyA therapy (CyA concentration, 52 µg/L). Peak 1 is CyA, which has a retention time of 3.84 min; peak 2 is the internal standard, CyD, which has a retention time of 5.00 min; peak 3 is the late eluting endogenous substance, which has a retention time of 6.89 min.

still the output remains sufficiently high: the run time of 8 min allows for analysis of 7-8 specimens per hour.

Other drugs tested, but not detected of found not to interfere, were: acetaminophen, amikacin, amitriptyline, caffeine, carbamazepine, clonazepam, chloramphenicol, desipramine, diazepam, digoxin, disopyramide, ethosuximide, gentamicin, imipramine, lidocaine, methotrexate, N-acetylprocainamide, N-acetylsulfamethoxazole, netilmicin, nortriptyline, phenobarbital, phenytoin, primidone, procainamide, propranolol, quinidine, salicylate, sulfameth-

oxazole, theophylline, tobramycin, trimethoprim, valproic acid, and vancomycin.

CyA gave linear responses with the concentrations studied: 50, 150, 300, 600, and 900 µg/L. The correlation coefficient for plot of peak-height ratios of drug to internal standard vs spiked concentrations was >0,998; the slope of this plot was 0.001, and the intersept was -0.053. The instrument was calibrated at a CyA concentration of 300 µg/L, and the linearity was validated daily by analysis of three blood standards: 50, 150, and 600 µg/L.

Performance characteristics of this method were tested at two different levels - 100 and 500 µg/L. The extraction efficiency was over 70%, and was calculated as follows: peak height of CyA from blood-derived chromatogram was devided by the peak height of the same concentration of the drug in mobile phase, directly injected for chromatography. The accuracy of CyA determination was calculated according to the formula: |(expected concentration - found concentration)/expected concentration|.100, and found to be between -7.1% and +3.3%. The within-run coefficients of variation (n=12) were <5.8%, the between-run coefficients of variation (n=22) were <7.5%. The lower limit of detection was about 25 µg/L, with a signal/noise ratio of 5/1.

The method described, overcomes some limitations of previously reported LC methods. Use of a single-step extraction in combination with a Chromosorb P column purification of extract, has resulted in several advantages and benefits - the procedure is fast, simple and cost-effective. No centrifugation is required for phase separation; back extraction and organic wash of haemolysat and/or reconstituted residue, described by others (4-8), is avoided; Chromosorb P column is less expencive than the commercial solid-phase cartridges. Pretreatment of a single specimen takes about 10 min, a batch of 20 samples can be prepared for chromatography in 45 min. Chromatographic separation is a slight modification of that, developed by T.P.Moyer et al.(3). According to the

suggestions of C.E.Pippenger (10), we used a greater proportion of methanol in the mobile phase, which improved the resolution.

Over a 1,000 patient samples have been analysed by this method in our laboratory in about one year, without any sign of column deterioration. At least 50 specimens can be analysed and reported in a normal day, and the overall performance characteristics well meet the requirements for the routine therapeutic monitoring of CyA.

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