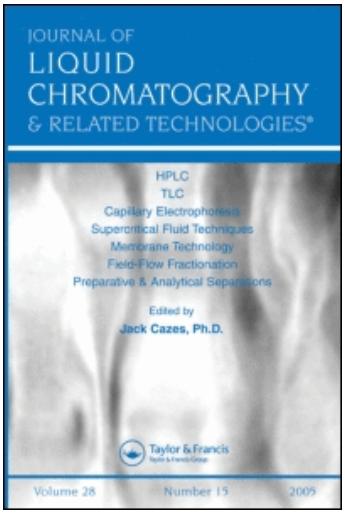

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HPLC DETERMINATION OF CYCLOSPORINE IN WHOLE BLOOD

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ABSTRACT

A rapid sensitive method for measuring cyclosporine concentration in whole blood by HPLC has been developed. The pre-chromatography isolation steps are convenient and rapid and are based on salting out acetonitrile with simultaneous extraction of cyclosporine from the blood. A reversed phase column is used with detection by absorbance at 200 nm.

INTRODUCTION

Cyclosporine (cyclosporin A), whose structural formula is given in Figure 1, is an effective immunosuppressant drug that is used to prevent organ rejection following transplantation surgery. Determination of its blood concentration is important because levels that are too low are therapeutically ineffective while those that are too high are nephrotoxic and have other side effects (1).

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Two techniques currently used for measuring cyclosporine are radioimmunoassay (RIA) and high performance liquid chromatography (HPLC). RIA gives higher results than HPLC because several metabolic products of cyclosporine also react with the test system,(2,3) while the HPLC technique allows more specificity. Whole blood samples are preferred to plasma or serum because cyclosporine is partitioned unevenly between the blood cells and plasma. This relationship varies with time and temperature (4).

We wish to report an HPLC cyclosporine method that is more rapid than those previously described since the pre-chromatography phase is less complex and time-consuming than other procedures (5-10).

MATERIALS AND METHODS

Equipment

The HPLC equipment consisted of: a model 590 pump; a model WISP 710B autoinjector; a model 730 data module for recording and integration (Waters Associates, Inc., Milford, MA 01757); either of two variable wavelength absorbance detectors, a model 773 Spectroflow with a cell path of 8 mm (Kratos Analytical Instruments, Ramsey, NJ, 07446) or a Waters model 481 with a cell path of 10 mm; a 4.6 mm x 25 cm Alltech C_{18} 10 micron analytical column (Alltech Associates, Deerfield, IL 60015), and a 4 mm x 2.4 cm guard column packed with 37-50 micron Waters Bondapak C_{18}/Corasil. The guard and analytical columns were placed in a heater (Fiatron, Milwaukee, WI 53209). Mixing was done with a Vortex Jr. Mixer (American Scientific Products, McGaw Park, IL 60085). The centrifuge was a model GLC-1 (Sorvall).

Materials

HPLC grade acetonitrile (Fisher Scientific, Itasca, IL 60143) and sterile purified water (Travenol Laboratories, Deerfield, IL 60015) were used. HPLC grade n-hexane (Alltech) was purified by washing with concentrated sulfuric acid

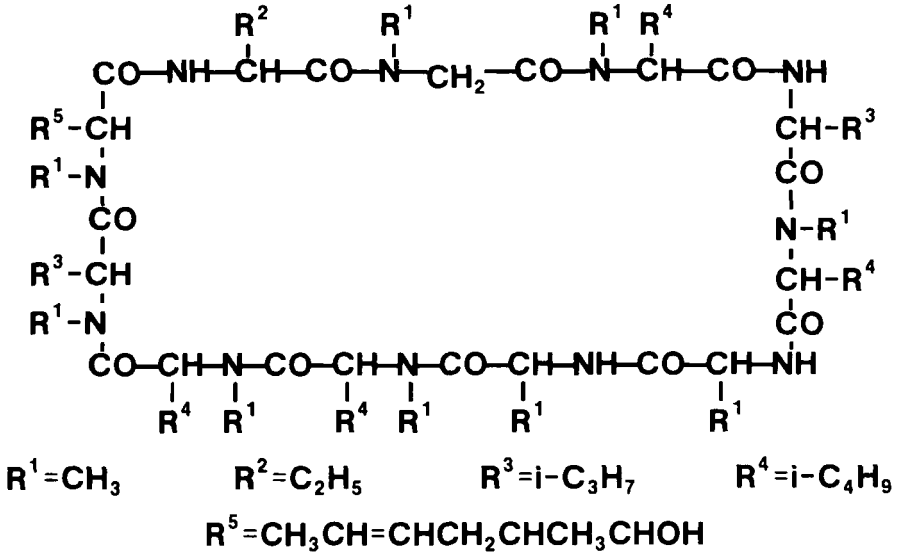

Figure 1. The structure of cyclosporine

until the acid washes lost their yellow color. The hexane was then washed with water to remove traces of acid.

Dowex -50W- hydrogen cation exchange resin, 8% crosslinked, 200-400 mesh (Aldrich Chemical Co., Milwaukee, WI 53233) was stirred with 1 M NaOH for 20 minutes. The supernatant was decanted and the resin was stirred for 20 minutes with water; the supernatant was again decanted and the resin was stirred for 20 minutes with 1 M H_3PO_4. The supernatant was decanted and the resin was washed with water until neutral. Dowex -1- chloride anion exchange resin, 8% crosslinked, 200-400 mesh (Aldrich) was treated in the same way as the cation resin except that the NaOH and H_3PO_4 treatments were exchanged in time. The amounts of Dowex -50W- hydrogen and Dowex -1- chloride were chosen to give equivalent cation and anion exchange capacity. After the resins had been washed to neutrality, they were combined and stirred with water and vacuum filtered.

The resin mixture was stirred twice with acetonitrile. Finally, the resin was vacuum filtered to dryness and stored in a tightly capped container. Even after a period of 24 hours no odor of acetonitrile was detected in the container headspace.

Chromatographic Conditions

The mobile phase was 70:30 acetonitrile to water, and the flow rate was 0.8 mL/min. The detector wavelength was 200 nm, and sensitivity was 0.002 aufs. Injection volume was 40 µL. The column was maintained at 70° C.

Procedure

Whole blood was drawn into a Vacutainer tube (Beckton-Dickinson) containing EDTA and was frozen to lyse the red cells. For analysis the blood was brought to room temperature and vortexed. To 1.00 mL of whole blood was added 1.00 mL of acetonitrile, and the mixture was vortexed for 2 minutes in 1 minute intervals. Protein precipitation occurred. The mixture then was saturated with ammonium sulfate (Aldrich) and vortexed. Two liquid phases were seen, and the acetonitrile phase was cleanly separated by centrifugation for 10 minutes. A 0.70 mL aliquot of the acetonitrile layer was withdrawn and extracted with 1.4 mL of n-hexane. After separating on standing for approximately 2 minutes, the bottom acetonitrile layer was withdrawn carefully. While vortexing the acetonitrile layer, two approximately 50 mg portions of Dowex-ion exchange mixture were added. Following centrifugation the acetonitrile supernatant was used for chromatographic analysis.

Calibration

A stock solution containing approximately 4 ng/µL cyclosporine in acetonitrile was prepared. This solution can be stored without change for at least 8 months at 4° C. Six aqueous solutions were prepared by adding enough stock solution to give concentrations of about 0, 140, 250, 380, 500, 600 ng/mL. An identical calibration curve was obtained if cyclosporine was added to blood instead of

water. If blood samples were encountered that had concentrations much higher than 600 ng/mL, additional higher concentration solutions were used because the calibration curve was linear to at least 1000 ng/mL. Calibration solutions were extracted in exactly the same manner as the whole blood samples. A linear calibration curve of peak height vs. concentration of cyclosporine was calculated and used for subsequent blood determinations.

RESULTS

Figure 2 is a typical chromatogram for a calibration solution. Peaks found in the chromatogram other than cyclosporine are impurities in the reagents used prior to chromatography. Figure 3 is the chromatogram of a blood sample containing no cyclosporine that was carried through the procedure. The time at which cyclosporine elutes is free of interfering peaks. Figure 4 is a chromatogram of the blood of a patient being treated with cyclosporine.

The method has been used to determine the cyclosporine blood content of more than 600 samples. Coefficient of variation of the method as indicated by five determinations was 8% at the 100 ng/mL blood level and 0.3% at the 800 ng/mL level. Recovery of cyclosporine in cases where it was added back to patient's blood was 90% $\pm$ 5% (n=14). Time required for the extraction procedure was less than 1 hour, and the chromatography time was less than 30 minutes.

DISCUSSION

Pre-Chromatography Isolation

Isolation of cyclosporine from blood constituents prior to chromatography in this method required less time than in other methods because it does not employ a step to evaporate a solvent (5-10).

The acetonitrile in this method precipitates proteins and provides an effective solvent for extracting cyclosporine. Ammonium sulfate further precipitates protein amd salts out the acetonitrile to give a second phase. Solutions with ratios of acetonitrile to water in the range of 0.2-2 when salted out give the same volume

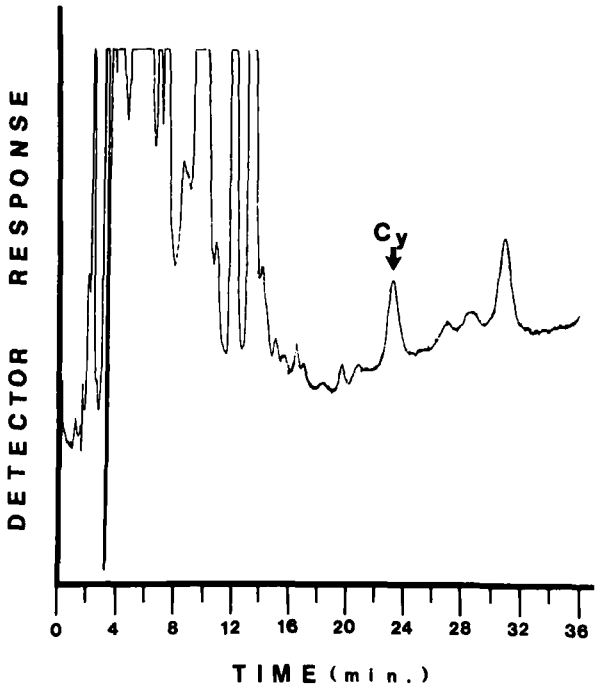

Figure 2. A chromatogram of an extract of an aquous solution used for calibration containing 110 ng/mL cyclosporine (arrow). Detector sensitivity 0.002 aufs.

of acetonitrile layer as the volume of acetonitrile used to prepare the solution. Extraction efficiency studies showed all of the cyclosporine was in the acetonitrile layer. Thus, cyclosporine concentration in the acetonitrile layer after salting out was the same as its concentration in the whole blood sample.

The purpose of ion exchange resin treatment is to remove acidic and basic and ionic constituents which might shorten column life. This treatment also eliminates peaks that otherwise would appear in the early half of the chromatogram. Prior to resin contact, the acetonitrile solution is straw yellow colored, but it becomes colorless after contact.

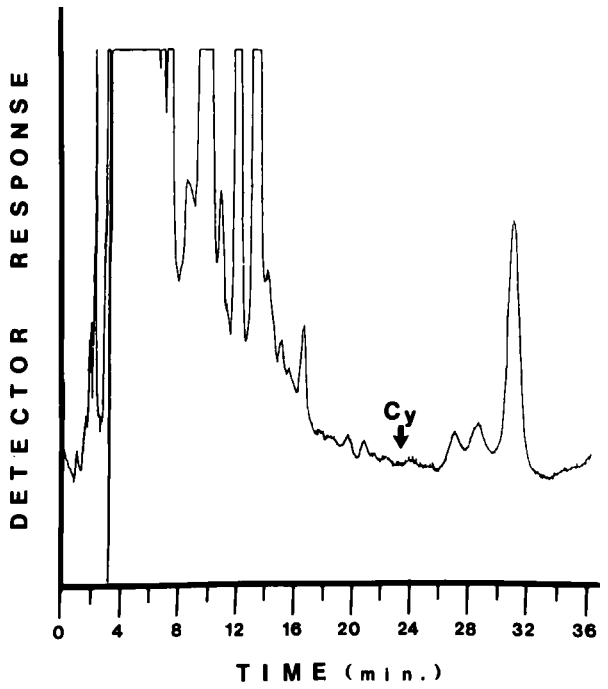

Figure 3. A chromatogram of an extract of whole blood containing no cyclosporine. The arrow indicates where cyclosporine would elute. Detector sensitivity 0.002 aufs.

The n-hexane wash removes some constituents which produce peaks that otherwise elute just prior to the cyclosporine peak and are difficult to resolve from the cyclosporine peak. In the extraction the volume of acetonitrile that dissolves in hexane is exactly the volume of hexane that dissolves in acetonitrile. No cyclosporine is extracted by hexane. The concentration of cyclosporine in acetonitrile is unchanged in this step.

Chromatography

As others have noted in cyclosporine chromatography (7), it is necessary to operate at 70° C or higher when using a reversed phase column. Mobile phase

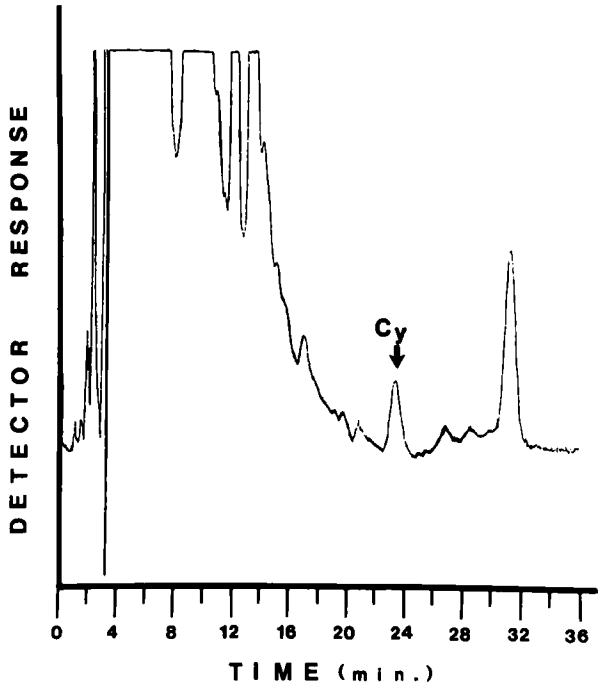

Figure 4. A typical chromatogram of an extract of whole blood from a patient treated with cyclosporine (arrow). Concentration found was 110 ng/mL. Detector sensitivity 0.002 aufs.

temperatures below 70° C gave broader and lower peaks. Likewise, a few percent change in the volume ratio of acetonitrile to water in the mobile phase broadened the cyclosporine peak. The height equivalent to a theoretical plate (HETP) is very sensitive to flow rate, and peaks at 0.8 mL/min are significantly higher than those obtained at 1.0 mL/min.

Injection volumes greater than the volume used in this method (40 µl) gave peak heights less than proportionately greater. Cyclosporine is in a strong solvent, acetonitrile, and it is injected into a weaker mobile phase, 70% acetonitrile -30% water. This contrast in strengths probably accounts for the injection volume limitation.

The 200 nm detector wavelength used in this method was a compromise. At 195 nm a 5% stronger peak was observed for cyclosporine, but the baseline noise was increased. Nearby peaks were also about 5% stronger. At 205 nm a more stable baseline was obtained, but the signal to noise ratio was less than at 200 nm. The injection sample was dilute and, therefore, a high detector sensitivity, 0.002 aufs, was used.

An internal standard was not used in the method. Instead, a linear calibration curve of peak height vs. concentration was employed ($r = 0.990$-0.999). This procedure avoided the risk of internal standard peak interferences from sample constituents. This relationship is linear to at least 1000 ng/mL. The limit of detection is 25 ng/mL in whole blood.

ACKNOWLEDGEMENTS

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