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Letter to the Editor

Optimized high-performance liquid chromatographic method for the analysis of cyclosporine and three of its metabolites in blood and urine

Sir,

Cyclosporine (cyclosporine A, CsA) is an immunosuppressive drug which has been used extensively in organ transplantation and autoimmune disease [1]. Several high-performance liquid chromatographic (HPLC) methods have been published for the analysis of CsA. Some of these employed liquid–liquid extraction [2–4] and others used solid-phase extraction [5–9].

We report an optimized HPLC method for the determination of CsA in whole blood and urine to achieve a highly sensitive assay with a sensitivity of 5 ng/ml. In addition, an easy method for the determination of CsA metabolites 1, 17 and 21 is also reported. The method employs a previously described solid-phase extraction procedure developed by Moyer et al. [6] for the extraction of CsA and its metabolites. This method has been used extensively in our institution.

EXPERIMENTAL

Instrumentation and chromatographic conditions

A Model 510 solvent delivery system, a Model 710B WISP automatic injector, both from Waters Chromatography (Division of Millipore, Milford, MA, U.S.A.), a Model SPD-6A variable-wavelength UV detector (Shimadzu Scientific Instruments, Columbia, MO, U.S.A.), a Fiatron HPLC column temperature controller and heater (Fiatron Laboratory Systems, Oconomonoc, WI, U.S.A.) and a Model SP4290 Spectra-Physics integrator (Spectra-Physics Autolab Division, San Jose, CA, U.S.A.) were used.

The separation of CsA alone was carried out on a 15 cm×4.6 mm I.D. prepacked, microparticulate (5 μm average particle size) reversed-phase column LC-18 (Supelco, Bellefonte, PA, U.S.A.). The column was heated to a temperature of 70°C. The detector wavelength was set at 202 nm. The mobile phase was acetonitrile–distilled water (68.5:31.5, v/v) with the flow-rate set at 1.4 ml/min. The separation of CsA and metabolites 1, 17 and 21 utilized a 25 cm×4.6 mm prepacked, microparticulate (5 μm average particle size) Zorbax cyano column (Dupont Instruments, Wilmington, DE, U.S.A.). The column temperature was set

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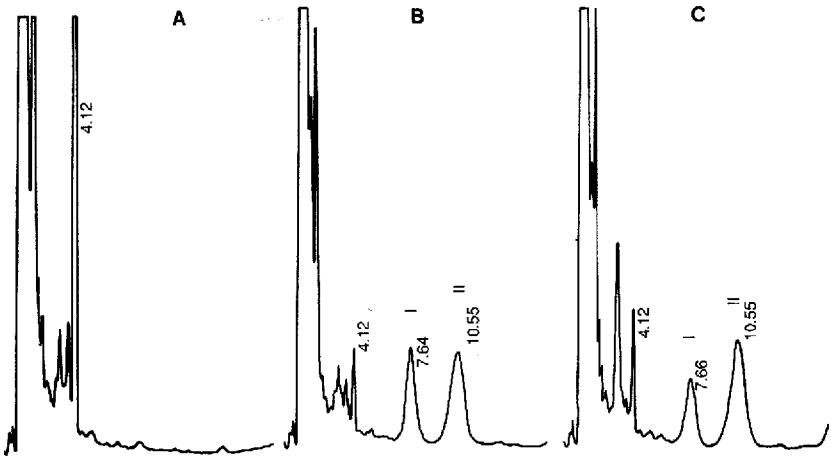

Fig. 1. Typical chromatograms obtained in the analysis of 2 ml of (A) blank blood, (B) standard curve sample containing 205 ng/ml CsA and (C) a patient's blood sample containing 112 ng/ml CsA using the method for measurement of CsA alone. Peaks: I = CsA; II = CsD.

at 60°C. The absorbance was monitored at 214 nm. The mobile phase was distilled water–acetonitrile–methanol (54.5:31:14.5, v/v/v). The flow-rate was set at 0.7 ml/min.

Extraction procedure

The method of Moyer et al. [6] was used for the measurement of CsA alone or CsA and metabolites in blood or urine. Briefly, 10% methanol in acetonitrile is added to each blood or urine sample (2 ml) and the denatured proteins are separated by centrifugation. The entire supernate is then added to a 6-ml C_{18} Bond Elut cartridge and the solvent is discarded. The cartridge is then washed with 70% methanol in water and then 1% acetone in hexane. The final wash uses 25% isopropyl alcohol in ethyl acetate to elute CsA and its metabolites. The effluent is collected and passed through a 3-ml Bond Elut silica cartridge, which had previously been rinsed with the isopropyl alcohol–ethyl acetate solution.

The effluent collected from the silica cartridge is evaporated to dryness and reconstituted in 105 μl of the appropriate mobile phase solution. An aliquot of the reconstituted sample (90 μl) is injected into the HPLC system. The peak-height ratios of CsA and its metabolites to cyclosporine D (CsD; internal standard) were calculated.

RESULTS AND DISCUSSION

In the method used for CsA alone the retention times for CsA and CsD were approximately 7.6 and 10.5 min, respectively (Fig. 1). However, an unknown biological component appears at about 29 min. Therefore, sample injection was set at 15-min intervals.

In the method used for CsA and its metabolites, the retention time for metabolites 17, 1, 21, CsA and CsD were approximately 16, 18, 23, 31 and 37 min, re-

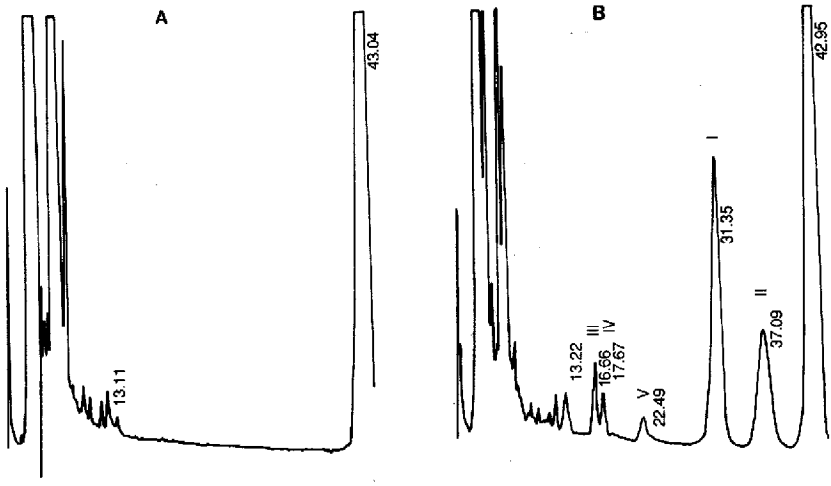

Fig. 2. Typical chromatograms of (A) blank blood and (B) a patient's blood containing CsA and its three metabolites. Peaks: I = CsA; II = CsD; III = metabolite 17; IV = metabolite 1; V = metabolite 21.

spectively (Fig. 2). The samples were injected every 45 min. Because of the very limited quantity of the pure metabolites available to us, the standards were used for identification purposes only.

The day-to-day precision in blood was determined by comparing the peak-height ratio for the standard samples during a two-week period. The coefficient of variation was less than 10% over the entire range of the standard curve. Sample variability for samples run within the same day was calculated by multiple determinations ($n=5$) of a standard containing 205 ng/ml. The coefficient of variation was 10%.

The recovery was determined from the ratio of the calculated to the added concentrations of CsA standards. The recovery ranged from 97.4 to 104.9% with coefficients of variation less than 10% over the range of the standard curve.

The sensitivity of CsA assay was determined by preparing spiked blood samples containing 5, 10 and 20 ng/ml CsA. All three concentrations were measurable and at a signal-to-noise ratio of 5:1, the sensitivity of the assay was 5 ng/ml using 2 ml of whole blood.

This method has been used extensively in the study of CsA pharmacokinetics in renal transplant patients at steady state following oral administration.

In summary, highly sensitive, accurate and reproducible HPLC methods for the determination of CsA alone or CsA and three of its metabolites in whole blood and urine are reported. Cyclosporine concentrations as low as 5 ng/ml can be measured in blood or urine.

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