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IMPROVED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR ANALYSIS OF CYCLOSPORIN A USING AN AUTOMATED SAMPLE PROCESSOR

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SUMMARY

Transplant patients receiving the immunosuppressive drug cyclosporin A require regular monitoring to maintain levels within a narrow therapeutic range. A stable, accurate and reproducible high-performance liquid chromatographic method for analysis of cyclosporin A in whole blood has been developed using the Varian Advanced Automated Sample Processor. Starting with 200 μl of blood, absolute recovery of both cyclosporin A and the internal standard was 81% with a detection limit of 12.5 ng/ml. The assay is perfectly linear over the range 0–1000 ng/ml ($r^2 = 1.0$). At a concentration of 250 ng/ml, the coefficient of variation, both between samples and between assays, is 1.87%. Chromatographic cycle time is 10.2 min per sample. Up to eighty samples can be processed by one person in a working day, with final results within 16 h.

INTRODUCTION

The immunosuppressive drug cyclosporin A (Sandimmun, Sandoz, Basle) is used extensively in the field of organ transplantation to prevent graft rejection. Although the use of cyclosporin A has substantially increased the chances of survival of transplant recipients since its introduction in 1978 [1], a number of dose-related side-effects, in particular nephrotoxicity, have been identified [2]. In addition, wide variability has been observed in the pharmacokinetics of cyclosporin A related to age of the patient, their disease state, the type of organ transplanted and to interactions with other drugs [3,4]. Very careful monitoring of cyclosporin A in every patient is therefore necessary to maintain levels within a narrow therapeutic range.

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Two techniques for cyclosporin A assay predominate, namely high-performance liquid chromatography (HPLC) and radioimmunoassay (RIA), although others, such as the fluorescence polarisation immunoassay (Abbot TDx) [5], are being developed. Until the introduction of a monoclonal antibody kit by Sandoz in 1987, specific for cyclosporin A parent compound [6,7], RIA (based on a non-specific polyclonal antibody) lead to substantial, variable overestimation of the drug's concentration through cross-reaction with metabolites [8]. HPLC remains the most sensitive and accurate assay available for cyclosporin A over a large linear range (0–4 μg/ml) and the reference technique where unequivocal values are required.

Analysis of cyclosporin A by HPLC has had a number of operational drawbacks. Laborious manual purification procedures, high column temperatures, long analysis times and blood or plasma volumes between 0.5 and 2 ml have all placed HPLC at a disadvantage compared with RIA. The evolution of cyclosporin A analysis by HPLC through attempts made to address these problems has been summarised by Wallemacq and Lesne [9].

Kabra and co-workers [10,11] and Wallemacq and Lesne [9] have published methods for cyclosporin A analysis based on an Advanced Automated Sample Processor (the Varian AASP). This device utilises solid-phase extraction technology combining sample purification with automatic injection. The present report describes the development of an improved analytical method for cyclosporin A analysis based on the Varian AASP. With this method up to one hundred samples can be processed and analysed in a single automated run. Precise, reproducible results in the range 0–1 μg/ml are obtained starting from 200 μl of whole blood.

EXPERIMENTAL

Reagents and standards

HPLC-grade solvents, acetonitrile (S-grade), hexane, ethanol, methanol, isopropanol and water were all obtained from Rathburn (Walkerburn, U.K.). Cyclosporins A and D were kind gifts from Sandoz (Basle, Switzerland). Separate 500 μg/ml stock solutions of cyclosporins A and D were prepared by dissolving 10 mg of each in 20 ml of methanol. These stock solutions are stable for at least six months at 4°C.

Calibration standards were prepared using a 1 μg/ml solution of cyclosporin A in methanol diluted from the concentrated stock. Appropriate volumes of this solution were dispensed into 10 ml plain-glass blood collection tubes using a microsyringe. The carrier solvent was evaporated under reduced pressure before addition of 200 μl of heparinised whole blood. Thereafter standards were treated in the same way as patient samples.

Sample preparation

Aliquots (200 μl) of whole blood were dispensed into 10 ml plain-glass blood collection tubes. An internal standard of 100 ng of cyclosporin D in 20 μl of methanol was added to each sample or blood standard using a microsyringe. A 1.5-ml volume of acetonitrile–water (2:3, v/v) [12] was then added. Tubes were then vortex-mixed for 10 s and left to stand for 20 min at room temperature to allow complete haemolysis and extraction of cyclosporin A. Finally, extracts were centrifuged at 1300 *g* for 10 min at room temperature.

Preparation of AASP cassettes

AASP cassettes packed with a C_8 bonded-phase silica gel sorbent (Analytichem International) were obtained from Jones Chromatography (Hengoed, U.K.) and processed using an AASP Prep-Station (Varian Instruments). This device operates by passing solvent from a reservoir through each of the ten cartridges in a cassette simultaneously under nitrogen top pressure. Wash solution volumes are in part dictated by the reservoir capacity (approximately 2 ml per cartridge).

Cassettes were first washed by passing 1.5 ml of isopropanol through each cartridge, then conditioned by washing with 1.5 ml of acetonitrile–water (2:3, v/v) and re-wetted with a few drops of the same solution. Sample extract supernatants were transferred to each cartridge reservoir by Pasteur pipette, then passed through the sorbent. Each cartridge was then washed with 1.5 ml of acetonitrile–water (2:3, v/v) and left to purge with nitrogen for 5 min at 1.4 bar. The cassette was then placed in a vacuum desiccator for 10 min before a final wash with 1 ml of hexane followed by a 5-min nitrogen purge. Processed cassettes were then loaded on to the AASP for analysis.

The total extraction and preparation time for each cassette of ten samples is 1 h. However, in practice, where more than one cassette is being prepared, some steps can be performed simultaneously. Fifty to eighty samples can be prepared by one person in a working day.

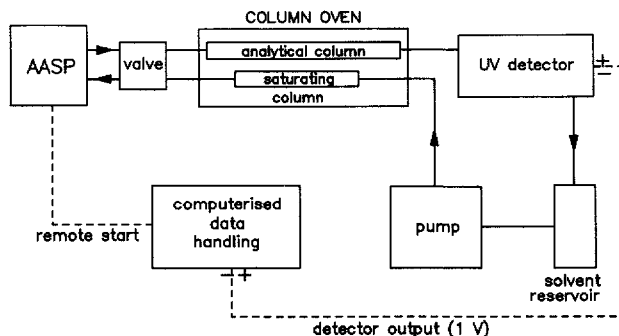

Fig. 1. Arrangement of chromatograph components for analysis of cyclosporin.

Instrumentation

The arrangement of the apparatus is shown in Fig. 1. The chromatographic system consisted of an AASP (Varian Instruments, U.K., Warrington, U.K.), a Merck-Hitachi LiChrograph 6200 pump (BDH Instruments, London, U.K.) and a Merck-Hitachi LiChrograph L-4000 variable-wavelength UV detector (BDH). The analytical column (150 mm×4.6 mm I.D.) was packed with 5-μm CPS Hypersil (Hichrom, Reading, U.K.) which has a cyano-propyl functionality. A 100 mm×4.6 mm I.D. saturating column packed with 37–53 μm silica gel (Pre Column Gel, Whatman, Maidstone, U.K.) was connected between the pump and the AASP[12]. Both columns were housed in an LKB 2155 forced-air column oven (Pharmacia-LKB, Milton Keynes, U.K.). The AASP was interfaced to the chromatograph via a pneumatically operated ten-port valve (Valco Instruments). Sample peaks were integrated and quantified using a Turbochrom chromatography data system (Perkin-Elmer Nelson, Warrington, U.K.) operating on an IBM PS/2 Model 80 microcomputer. Data collection for each sample run was initiated through a contact closure on the AASP.

Chromatographic conditions

The mobile phase was a 9% solution of ethanol in hexane, recycled at a flow-rate of 0.7 ml/min in a closed system. Analytical and saturating columns were maintained at 53°C. The saturating column acts additionally as a heat exchanger, reducing the temperature gradient between affluent and effluent ends of the analytical column. Sample peaks were monitored at 210 nm with full-scale output from the detector set at 1 V.

Run time and cycle time on the AASP were set at 7.2 min. Valve reset was timed at 0.6 min. Purge wait was set at 3 min. Each cartridge was purged with nitrogen for 3 min immediately before injection. Chromatographic run time

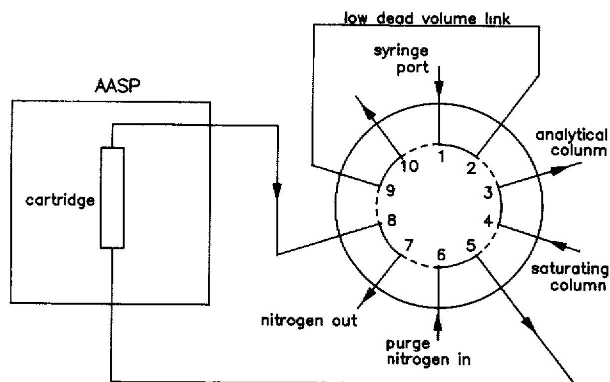

Fig. 2. Nitrogen purging system and modified AASP fluidics.

was 10 min. Purging of the next cartridge with nitrogen commenced before completion of the current run which resulted in a true cycle time of 10.2 min between injections. Purge nitrogen was left on throughout an automated sample sequence.

Nitrogen purging system

Fig. 2 shows the arrangement of the nitrogen purging system. Nitrogen (oxygen-free) at 1.4 bar is fed into the AASP via port No. 6 of the injection valve. In the load position gas exits the valve through port No. 5, purges the AASP cartridge, returns to the valve through port No. 8 and is vented to waste through port No. 7. In the inject position the gas is isolated from the AASP and is vented directly through port No. 7.

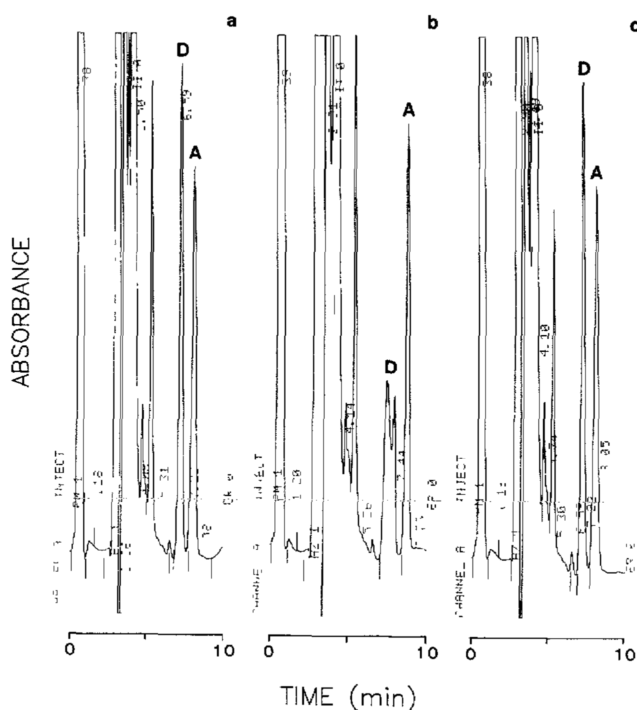

Fig. 3. Effects of pre-injection nitrogen purging on the chromatography of 200-μl blood extracts containing 100 ng each of cyclosporins A and D. (a) Cartridge from a freshly prepared cassette without nitrogen purging. (b) Cartridge from the same cassette after a 90-min delay without nitrogen purging. (c) Cartridge from the same cassette after a 100-min delay, injected immediately after a 3-min nitrogen purge.

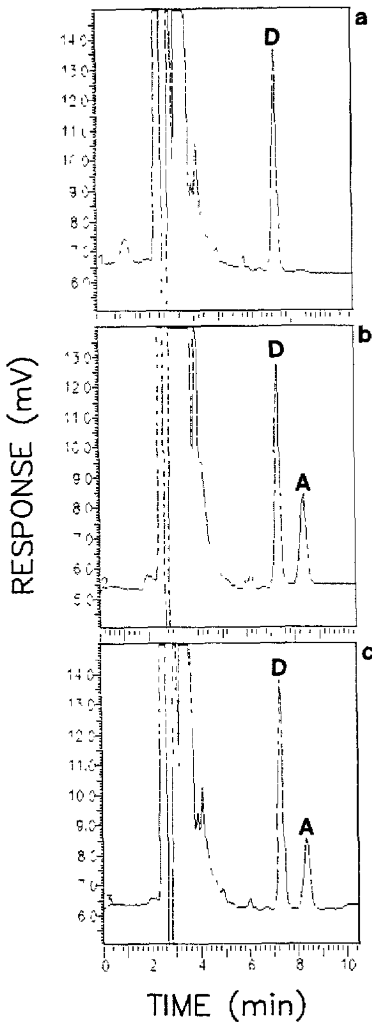

Fig. 4. Chromatograms of 200-μl whole blood extracts. Retention times of cyclosporins D and A were 7.3 and 8.4 min, respectively. (a) Spiked with 100 ng of cyclosporin D standard. (b) Spiked with 100 ng of cyclosporin D and 50 ng of cyclosporin A standards. (c) From a heart transplant recipient containing 196 ng/ml cyclosporin A and 100 ng of cyclosporin D, internal standard.

RESULTS

Nitrogen purging

The effects of cartridge purging with nitrogen before injection are shown in Fig. 3. A chromatogram produced by injecting a cartridge from a freshly prepared cassette of whole blood samples spiked with 100 ng of cyclosporins A and D is shown in Fig. 3a. The next chromatogram (Fig. 3b) is a cartridge from the

TABLE I

COEFFICIENTS OF VARIATION (C.V.) FOR REPEATED ANALYSES OF THREE BLOOD POOLS CONTAINING CYCLOSPORIN A AT DIFFERENT NOMINAL CONCENTRATIONS ($n=10$)

Nominal concentration (ng/ml)	Intra-assay		Inter-assay	
	Observed mean concentration (ng/ml)	C.V. (%)	Observed mean concentration (ng/ml)	C.V. (%)
25	27	7.04	29	12.5
250	244	1.87	245	1.87
1000	975	0.78	972	1.32

same cassette injected after a 90-min wait on the AASP but without any nitrogen purging. The cyclosporin D has split into two peaks (although the total area remains the same) and the retention times of both cyclosporins has been increased. Fig. 3c is the next cartridge in the cassette injected immediately after a 3-min nitrogen purge, that is, approximately 100 min after the sample represented by Fig. 3a. The change in the peak shapes is dramatic and retention times are reduced again to those in the first chromatogram.

Chromatograms

Fig. 4 shows typical chromatograms from (a) a blood extract spiked with 500 ng/ml cyclosporin D, (b) blood spiked with 500 ng/ml cyclosporin D and 250 ng/ml cyclosporin A and (c) from a transplant patient containing 196 ng/ml cyclosporin A. No interfering peaks are present in the blank sample, nor are any differences apparent between spiked blood and patient blood with respect to recovery of internal standard or to quality of chromatography. Occasionally a late-eluting peak (probably a metabolite of cyclosporin A) is seen in some patient's blood but elutes before the frontal peaks of the next sample and does not affect the analysis.

Linearity, precision and reproducibility

The relationship between detector response and the amount of cyclosporin A in whole blood is described by the equation $y=mx+c$ where y is the detector response (mV), x is the concentration of cyclosporin A (ng/ml), m is the gradient (0.002) and c is the y-intercept (0.018). This translates to a perfect linear response over the range of 0–1000 ng/ml of whole blood, with a first-order correlation coefficient (r^2) of 1.0. The y-intercept results from the lack of purity of the cyclosporin D internal standard, two lots of which were found to be contaminated with approximately 1% cyclosporin A.

Coefficients of variation determined over a fourteen-day period using blood

pools spiked with cyclosporin A at three nominal concentrations are reported in Table I. At the 250 ng/ml level, which is approximately the centre of the therapeutic range, coefficients of variation between samples and between assays were both 1.87% ($n=10$).

The overall recovery of both cyclosporins A and D from 200-μl whole blood aliquots was found to be 81% by comparison of peak areas with absolute values obtained by direct injection of 100 ng standards.

The detector baseline noise under the conditions described was less than 5 μV. Taking the detection limit as five times this value, a figure of 2.5 ng of cyclosporin A, on-column, is obtained. For a 200-μl aliquot of blood, this represents a detection limit if 12.5 ng/ml.

DISCUSSION

The preliminary extraction and cassette preparation stages used in this study were adapted and scaled down from a method developed for use with Bond-Elut C_{18} extraction cartridges using 500-μl blood samples [13]. The smaller mass of sorbent (40 mg) contained in the AASP cassettes compared with Bond-Elut cartridges (100 mg) allowed reduction of blood volume to 200 μl without any change in the solvent-to-sorbent ratio. Good results have since been obtained with 100- and 50-μl blood samples, but delivery of such small volumes requires a positive displacement pipette to ensure accuracy. Moreover, limits of detection are obviously raised accordingly.

Initially, the reversed-phase AASP method of Kabra and Wall [10] was investigated with 65% acetonitrile as the mobile phase. Large impurity peaks eluted from the AASP under these conditions which immediately stopped on return of the injection valve to the load position. These peaks did not emanate from the injection valve, nor did pre-washing of the cassettes in similar solvents make any appreciable difference. This problem could not be attributed to sample preparation or to the C_8 sorbent as both blank cassettes and CN cartridges produced similar peaks. The efficiency with which acetonitrile removes plasticisers from a number of laboratory plastics is well known. From the outset care had been taken to avoid all contact with these materials and to use only glass-distilled solvents.

The present method, in common with Wallemacq and Lesne [9], employs reversed-phase sample purification coupled with normal-phase analytical chromatography. With a compound such as cyclosporin that is lipophilic but readily soluble in polar water-miscible solvents, this reversal of separation mechanisms may confer greater peak purity at final analysis. Certainly with the CPS Hypersil analytical column used for this study, no interfering peaks and few extraneous peaks were present on chromatograms (Fig. 4). Pre-washing of cassettes with isopropanol was necessary though to remove impurities from the sorbent, probably residual silanes from the manufacturing process. It

is, therefore, noteworthy that both of the reversed-phase procedures reported by Kabra and co-workers [10,11] were troubled by interference from plasticisers and endogenous contaminants, resulting in the provision of a trapping and backflushing system to remove dioctyl phthalate [11].

The Hichrom CPS Hypersil (CN) analytical column used in this study was found to be extremely stable in use at 53 °C. Retention times of cyclosporins A and D with the present column have scarcely changed during analysis of over 1500 samples. Between analytical sessions the column is allowed to return to room temperature. No guard column or in-line filter are used in this system but the back-pressure has remained absolutely stable at 7 bar for six months. The extraction cartridges themselves act as excellent sample filters. In contrast, Wallemacq and Lesne [9] found the use of an in-line filter essential to maintain low system back-pressure.

In the early development of this method a mobile phase of 15% isopropanol in hexane was used[9]. Whilst the resolution of the cyclosporins was good, some tailing of cyclosporin A was noticeable. Substituting the isopropanol for 12% ethanol produced a substantial improvement in peak shape and a reduction in retention times. A final ethanol concentration of 9% was chosen because greater separation of the internal standard from the solvent front was achieved, without any apparent increase in retention of cyclosporin A by the C_8 extraction cartridge. The mobile phase should always be sufficiently strong to elute the sample from the extraction cartridge within the solvent front in order to prevent peak broadening on the analytical column. Recycling of the mobile phase prolongs column life, gives greater chromatographic stability and reduces costs. A 1-l volume of mobile phase is sufficient for at least 500 samples.

An initial problem with this method was the observation that the internal standard peak shape deteriorated rapidly with time after cassette preparation, such that the last samples in an injection sequence of ten cartridges produced split peaks (Fig. 3) and retention times of both cyclosporins increased. A 10-min period in a vacuum desiccator or 5 min of nitrogen purging on the PrepStation completely restored the peak shapes and retention times to their former state, irrespective of the time elapsed from initial preparation. Purging of cartridges with nitrogen on the AASP immediately prior to sample injection was found to completely overcome this difficulty. Presumably reversible absorption of atmospheric moisture by the sorbent or sample caused this phenomenon. It is possible that cyclosporin D partially hydrates in the presence of water leading to the separation of hydrated and non-hydrated species on-column. A hydrated species would be more polar than the non-hydrated form and have a longer retention time under normal-phase conditions.

The choice of whole blood or plasma as the preferred matrix for cyclosporin A determination has already been the subject of much clinical debate [3,12,14–16]. Although the data presented here relate to whole blood, this method has been tried successfully with 200-μl plasma samples.

In conclusion, normal-phase chromatography allied to the automation provided by the Varian AASP overcomes most of the inherent weaknesses found in other HPLC procedures, allowing sensitive, reliable and reproducible determinations of cyclosporin A to be made in large numbers of blood samples. Presently, this method is being expanded to include analysis of the major metabolites of cyclosporin A found in heart and heart/lung transplant recipients at Harefield Hospital.

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