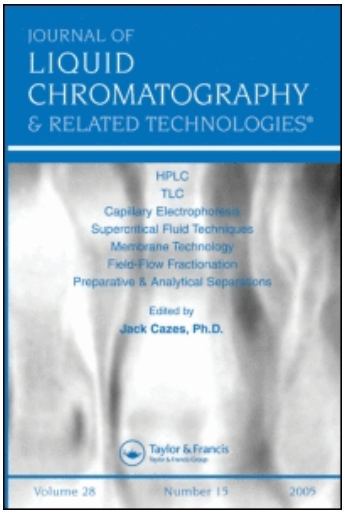

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Supercritical Fluid Chromatography for Therapeutic Drug Monitoring of Immunosuppressants: Selectivity for Cyclosporine A, Fk 506 (Tacrolimus), and Rapamycin

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SUPERCRITICAL FLUID CHROMATOGRAPHY FOR THERAPEUTIC DRUG MONITORING OF IMMUNOSUPPRESSANTS: SELECTIVITY FOR CYCLOSPORINE A, FK 506 (TACROLIMUS), AND RAPAMYCIN

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ABSTRACT

Clinical monitoring of cyclosporine A (CsA) in whole blood is currently performed by either immunoassays or high-performance liquid chromatography. A new immunosuppressant, FK 506 - Tacrolimus, is currently undergoing multi-centers clinical trial for liver transplant, while active research and clinical studies are being performed for another new immunosuppressant, rapamycin. The present study investigated their chromatographic selectivities by supercritical fluid chromatography, in comparison to HPLC. Feasibility studies were performed for the analyses of extracts of whole blood samples, after solid-phase extraction. SFC analyses were performed by using an open tubular SB-biphenyl capillary column, CO_2 as the mobile phase, pressure programming from 100 to 300 atmospheres, separation temperature of 70° C, and FID detection. CsA eluted after the internal standard CsD, while FK 506 tautomer eluted after FK 506. From the " reversal " of

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elution order as compared to reversed-phase LC, the selectivity of the above column with CO_2 as the mobile phase was characterized as " normal-phase HPLC-like ". Analysis of extracts of CsA patient's samples, and FK 506 spiked whole blood samples showed that the current SFC procedure did not achieve sufficient sensitivity limit for clinical therapeutic drug monitoring.

INTRODUCTION

Supercritical fluid chromatography(SFC) was advocated for routine pharmaceutical and clinical drug analyses [1]. However, recent reviews by the author indicated limited acceptance as compared to other chromatographic and analytical techniques [2,3]. For pharmaceutical analysis, high-performance liquid chromatography and gas liquid chromatography have provided satisfactory selectivity, capacity, reproducibility, and flexibility, supplemented by GC/MS for definitive analysis. Other forms of analysis include immunoassay and various chemical techniques. For clinical drug analysis, immunoassays have been the methods of choice for high throughput, ready reagent supply, cost effectiveness, and ease of operation, while chromatography, HPLC in particular, has been complementary. For newly introduced drugs such as sertraline [4], an antidepressant, and felbamate [5], an antiepileptics, HPLC provides a readily available and viable alternative. Thus, the role of chromatography for clinical analysis of newly introduced drugs remains firmly established.

In understanding the metabolism of new drugs, the definitive identification and quantitation of both parent drugs, and active and inactive metabolites may be achieved by chromatography, and if needed, interfacing with mass spectrometer for definitive identification. SFC offers similar range in general, and in certain applications, unique advantages, in operational characteristics and chromatographic selectivity as compared to HPLC and GLC [2]. Thus, its clinical application was previously evaluated for therapeutic drug monitoring (TDM) of phenobarbital.[6]

Further, tremendous research and development demonstrated the feasibility of using supercritical fluid extraction(SFE). Messer, Taylor, et al. [7] recently reviewed SFE for drug analysis. To continue our effort in establishing the clinical efficacy, SFC for monitoring immunosuppressants was undertaken.

Currently, several new immunosuppressants are under active clinical investigation for various organ transplants such as liver and kidney. Clinically, CsA has been successfully used for immunosuppression for a variety of organ transplants, while new immunosuppressants, under active clinical investigation but not yet approved by the Federal Drug Administration, would include FK 506 and rapamycin [8]. The current study re-established the " normal-phase HPLC-like " selectivity of SFC for three immunosuppressants - CsA, FK 506, and rapamycin, and described preliminary feasibility extraction studies.

The origin and principle of SFC were previously reviewed [1]. Wong and DellaFera performed SFC analysis of phenobarbital in serum, showing the " normal-phase HPLC-like " selectivity of SFC using CO_2 as the mobile phase [6]. This was important in the design of extraction protocol. Further, solid-phase extraction was advocated in order to introduce " clean " extracts for analysis by open-tubular capillary columns as their sample loading capacity was limited. Li et al. [9] demonstrated the analysis of panaxadiol and panaxatriol in ginseng and Chinese herbal medicine. Biermanns et al. [10] demonstrated SFC chiral analysis of β-blockers such as propranolol. The above applications showed that SFC for drug analysis are still limited for research studies.

First [11] recently projected the future of transplantation. Among issues such as organ availability, further understanding of transplant immunology including tolerance induction, immunosuppressant - both currently used and new ones, had been and will be a vital therapy in preventing organ rejection. New

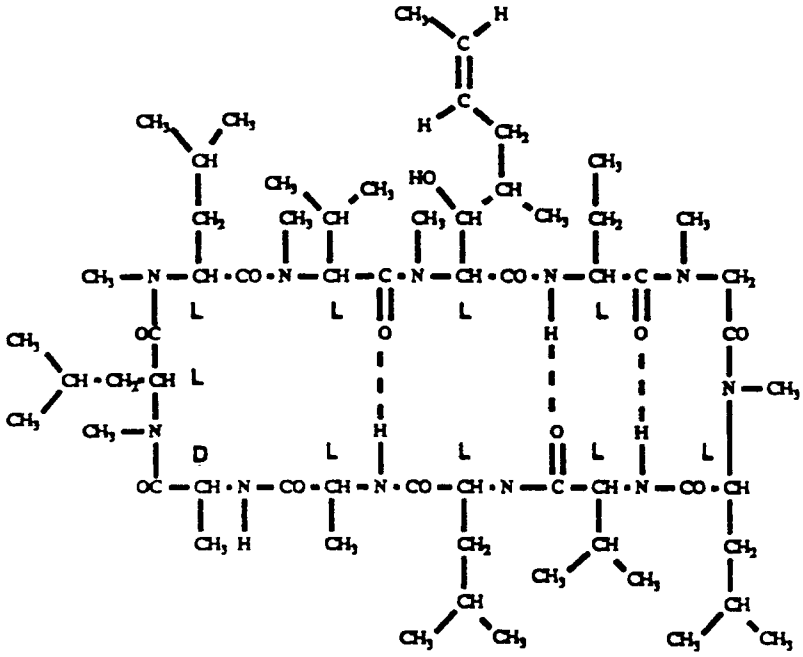
immunosuppressants, according to a recent review by Napoli, supplementing the currently available CsA, include FK 506 (Tacrolimus) which is undergoing final stage of the multi-center clinical trial for liver transplant, and the beginning stage of renal transplant, cyclosporine G (CsG) - an analog of CsA, rapamycin in limited clinical trials, and other newer agents in various stages of research and clinical studies - mizoribine, mycophenolic acid and its morpholinoethyl ester - RS-61443, brequinar sodium, and deoxyspergualin. TDM of some of these new immunosuppressants were recently addressed [12]. Figure 1 shows the structures of CsA, FK506 and rapamycin.

The clinical pharmacology and monitoring of CsA have been extensively reviewed and had been the subject of a Task force, review, and Consensus conference [13-15]. From these reports, the mechanism of action of CsA was due to the inhibition of T-cell mediated responses., reduction in interleukin-2(IL-2) synthesis., and inhibition of γ–interferon synthesis. The major side effect of CsA therapy is nephrotoxicity. Monitoring of whole blood CsA is performed clinically by using immunoassays - RIAs, fluorescence polarization immunoassay(FPIA), and enzyme multiplied immunoassay technique., and high performance liquid chromatography using solid-phase extraction and reversed-phase liquid chromatography. According to the latest Consensus conference report [15], the guidelines included specific methods such as selective immunoassays and HPLC to differentiate between CsA and metabolites , and that some of the proposed therapeutic ranges for liver, kidney, bone marrow, and heart tranplants are:100-150, 80-125, 150-400, and 100-150 μg/L respectively.

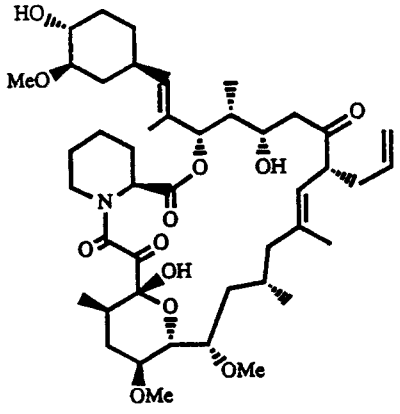
Recently, an analog of CsA, CsG underwent clinical trial for renal transplant[16]. Monitoring of CsG was demonstrated by Annesley et al.[16] by using monoclonal RIA and FPIA, and HPLC., and by Yatscoff et al.[17] using selective RIA and FPIA. Their clinical efficacy were acceptable.

Tacrolimus (FK 506), another new immunosuppressant, has been subject of extensive clinical and basic research, and was recently reviewed by Napoli [8], and Wallemacq and Reding [18]. FK 506 is a macrolide isolated from *Streptomyces tsukubaensis*, originally found in Japan [19]. Clinical pharmacology studies showed that FK 506 binds to a cytosolic protein - FK binding protein(FKBP) immunophilin. It inhibits Ca^{2+} dependent division of both T and possibly B cells., inhibits IL-2 and secretion of cytokines., and inhibits the expression of IL-2 receptor. Monitoring of FK 506 was initially performed by ELISA using serum or whole blood [20-23]. More recently, an automated microparticle enzyme immunoassay(MEIA) was introduced by Abbott Laboratory, capable of monitoring whole blood concentrations from 5 to 60 μg/L [24]. For clinical pharmacological research, HPLC and LC/MS have been used to quantify both parent drug and metabolites [25,26].

Rapamycin is another macrolide, isolated from *Streptomyces hygroscopicus* [27-29]. Morris reviewed that rapamycin inhibits both Ca^{2+} dependent and independent pathways in both T and B cells, and IL-2 cannot obviate the inhibition of rapamycin. It blocks T-cell division at the G1/S phase, different from that of FK 506 at the GO/G1 phase. It lowers internalization of IL-2. Similar to FK 506, it binds to FKBP immunophilin. Napoli and Kahan [30] optimized an HPLC analysis by using two microbore Novapak columns(150 mm x 2.1 mm), water/methanol(1:9), 0.1 mL/min., and 40° C. Retention time was 12 min. Yatscoff, Faraci and Bolingbroke [31] recently described an HPLC assay of rapamycin, using an ether extraction, followed by a reversed-phase analysis using two Spherisorb C-8 columns in tandem, methanol/water as the mobile phase, 0.35 mL/min, 45° C, and detection at 278 nm. Linearity was established for 250 μg/L, with a sensitivity limit of 1 μg/L. From the animal model study using rabbits and

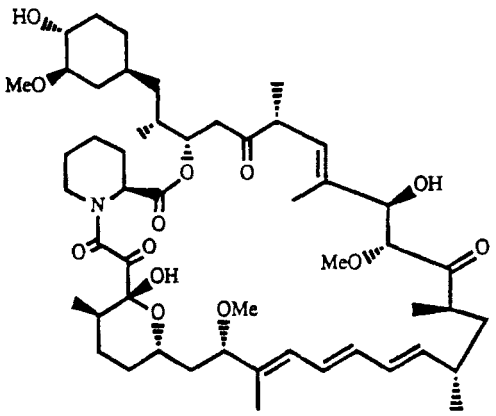


cyclosporine A



FK 506

Figure 1: Structures of cyclosporine A, FK 506 and rapamycin.



rapamycin

FIGURE 1 (continued)

from other pharmacokinetic studies, a trough concentration of about 0.75 to 30 μg/L was proposed.

The current studies investigated the SFC selectivity of CsA, FK 506 and rapamcyin, and some feasibility studies were performed for CsA and FK 506 whole blood extracts [32].

Experimentals

I. Reagents - Hexane, resi-analyzed grade, acetonitrile, methanol, HPLC grade, and zinc sulphate were obtained from J.T. Baker (Phillipsburg, NJ). Bond-Elut C-18 extraction cartridges were obtained from Varian (Walnut Creek, CA). Supercritical grade carbon dioxide was provided by Scott Specialty Gases (Plumsteadville, PA).

II. Drug standard solutions - Cyclosporine A and D were kindly donated by Sandoz Pharmaceuticals(Hanover, NJ). FK 506 (Tacrolimus) was generously supplied by Fujisawa Pharmaceutical Company (Deerfield, IL). Rapamycin was

donated by Wyeth-Ayerst Research (Princeton, NJ). To prepare the primary drug standard solutions of 1 mg/mL, 10 mg of each of the above drugs was dissolved in 10 mL of methanol. In preparing the working stock solutions, 100 μL was further diluted with 10 mL of methanol, yielding a final concentration of 10 ng/μL. Primary stock solutions were injected into both SFC and HPLC for selectivity studies, while the working stock solutions were used for spiking into drug-free whole blood for extraction feasibility studies.

III. Instrumentations - Two SFC were used for the present study. For the selectivity and feasibility studies of CsA, the SFC was consisted of a Model 500 SFC pump from Dionex/Lee Scientific (Salt Lake City, Ut). A model 600 controller was used to execute the pressure programming. Supercritical fluid was delivered to a Valco injector with a 200 nL loop, mounted on top of a model 5880 gas chromatograph equipped with a FID detector from Hewlett Packard (Avondale, PA). An open tubular capillary column was used, SB-biphenyl-100, 10 m in length and 50 μm internal diameter, obtained from Dionex/Lee Scientific Division (Salt Lake City, Ut). A Hewlett Packard monitor was used for recording key chromatographic parameters and the signal from the FID. The SFC used for the analyses of FK 506 and rapamycin was a Model 600 series from Dionex/Lee Scientific. HPLC analysis was performed by a Model 9533 liquid chromatograph, equipped with a Model 9523 variable wavelength detector from IBM instrument(Danbury, CT), and a reversed-phase μBondapak C-18 column(20 cm x 3.6 mm), Waters/Millipore(Milford, MA).

IV. Sample preparation - Drug-free whole blood samples, 1 to 4 mL were spiked with the working stock solutions. Along with quality control and selected patient samples, a solid-phase extraction was performed using a Bond-Elut C-18 cartridge. Aliquots, 1 to 4 mL of the above whole blood samples were vortex-mixed with zinc sulfate/methanol/acetonitrile for protein precipitation. After

centrifugation, the supernatant was transferred to a series of Bond-Elut C-18 extraction cartridges, pre-conditioned by elution with methanol and water. After elution of the supernatant, the column was washed with acetonitrile and water(9:11), followed by water. The cartridges were dried for 30 minutes under vacuum. Then, the extracted drugs were eluted with methanol. Following concentrating the extracts by evaporating the methanol, the residues were re-constituted with methanol for SFC analysis. For HPLC analysis, methanolic extracts were mixed with water, followed by washing with n-hexane. Aliquots of the transferred aqueous layer was injected into the HPLC for analysis.

V. Chromatographic conditions - SFC parameters were: SB-biphenyl column, CO_2 as the mobile phase, 70° C, 100 to 300 atmosphere at 10 atmos/min for the three drugs, and 175 to 250 atmosphere at 2 atmos/min for CsA and CsD, splitted injection of about 200 nL injected, and FID detection. HPLC parameters were: μBondapak C-18, acetonitrile/water at 1 mL/min, 70° C, injection volume of 50 to 100 μL, and detection at 202 nm at 0.002 AUFS.

Results and discussions

Based on the previous study of SFC analysis of phenobarbital in serum [6], a solid phase extraction protocol was used for the whole blood extraction of CsA. Figure 2 shows the SFC chromatogram of an extract of a patient's whole blood with about 228 μg/L of CsA. Pressure programming was used, with initial 100 and final 300 atmosphere as stated previously. CsA eluted at about 18.4 minutes, equivalent to the elution pressure of 270 atmosphere. Unfortunately, CsA co-eluted with the internal standard CsD. Thus, this would not useful for quantitative application. However, this data was useful to guide further optimization study.

In order to achieve the necessary resolution, the next approach was " fine-tuning " the pressure programming. After systematic studies, the initial and final

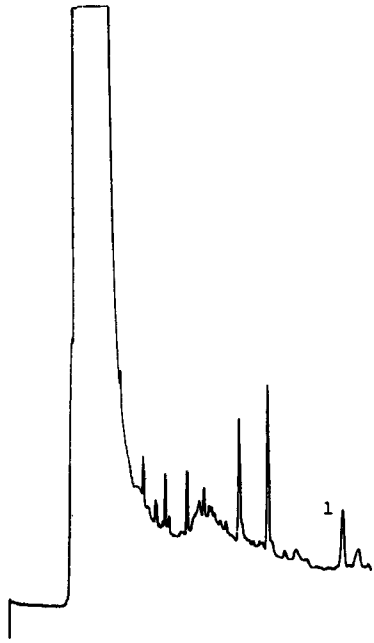


Figure 2: SFC chromatogram of a whole blood extract of a bone marrow tranplant patient with about 228 μg/L of CsA at 18.4 min. Pressure programming from 100 to 300 atmosphere at 10 atmos/min. Peak identification: 1, CsA.

pressures were chosen to be 175 and 250 atmosphere, with a " mini-step " pressure programming of 2 atmos/min. Figure 3 shows the successful resolution of CsA, peak 1 at about 35 min, after CsD the internal standard as peak 2 at 33 minutes. The extraction was performed with 4 mL of whole blood samples. The estimated CsA concentration was about 220 μg/L of whole blood from a bone marrow transplant patient. Since the therapeutic range of CsA for bone marrow transplant was proposed to be 150 to 400 μg/L,[13] this concentration represented a therapeutic concentration. Even though 4 mL of samples was used, both the apparently poor extraction recovery and poor FID response rendered this quantitation to be

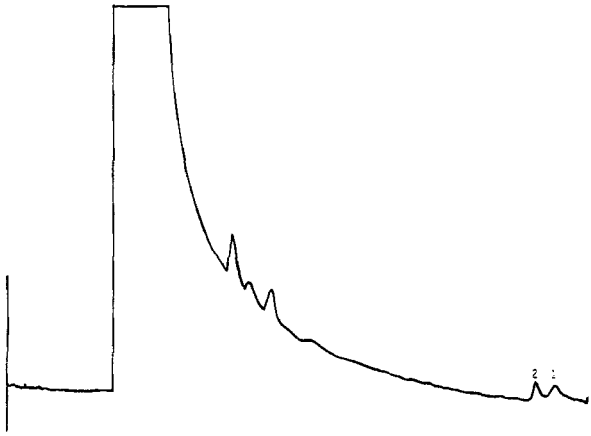


Figure 3: SFC chromatogram of a whole blood extract of a bone marrow transplant patient with about 220 µg/L of CsA . Pressure programming from 175 to 250 atmosphere at 2 atmos/min. Peak identification: 1. CsA at 35 min., and 2.CsD(Internal standard) at 33 min.

unacceptable. Sensitivity may be enhanced by using packed SFC column for increased loading capacity, other detectors with higher sensitivity, or by interfacing with mass spectrometer in the future.

However, from this study, the reversal of elution order of the " more polar " CsA and the " less polar " CsD in relation to RPLC confirmed the key observation on " normal-phase HPLC-like " selectivity of carbon dioxide and SB-biphenyl column as seen previously. This was also observed in the selectivity for analysis of FK 506 and its tautomer.

Figure 4A shows the SFC chromatogram of FK 506 drug standard solution as peak 1, and its tautomer, peak 2 with retention times of 17.5 and 17.8 minutes. The reversed order is shown in the HPLC chromatogram Figure 4B, FK 506 eluting after its tautomer at 5 and 4 minutes respectively. This elution order was also previously established by Friob et al [25]. Preliminary studies indicated serum

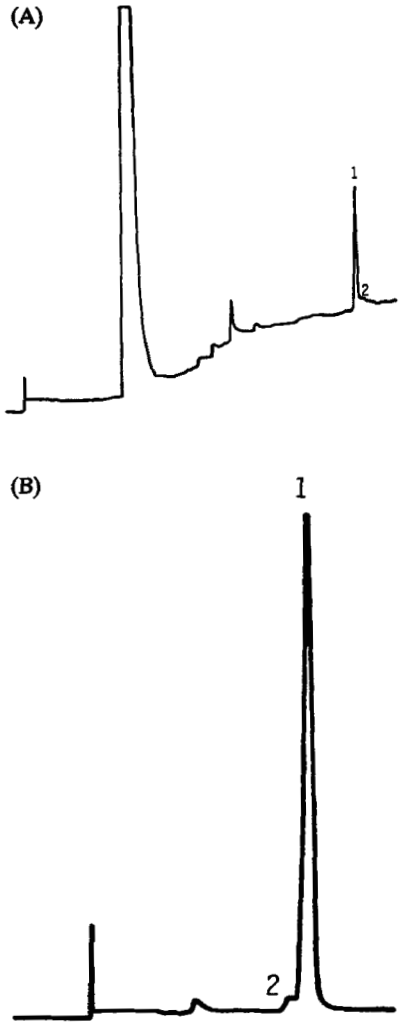


Figure 4: (A) SFC, and (B) HPLC chromatograms of FK 506. Peak identification: 1, FK 506; 2, FK 506 tautomer.

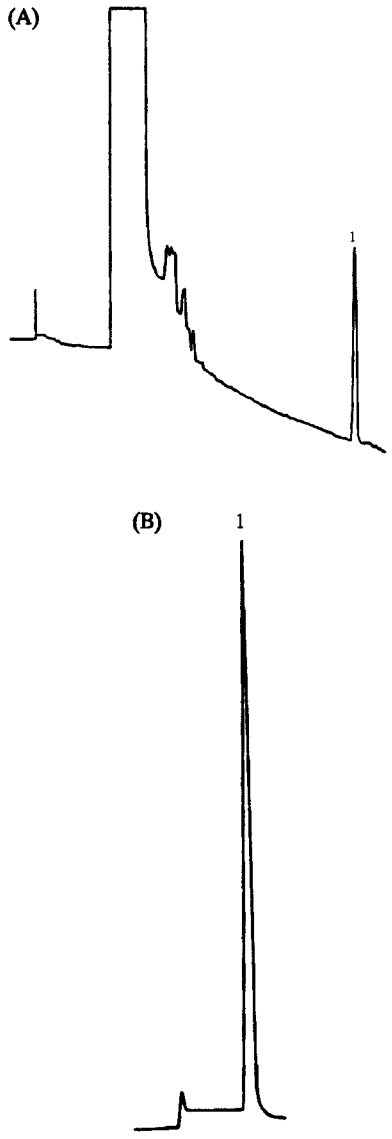


Figure 5: (A) SFC, and (B) HPLC chromatograms of rapamycin.

concentration of about 0.5 to 5 μg/L and whole blood concentrations of 5 to 20 μg/L [18]. Whole blood spiked at 20 μg/L did not reveal detectable signal. This was logical based on the previous experience with the CsA analysis of using open tubular capillary column.

In establishing the SFC selectivity of rapamycin, primary drug standard was also analyzed with a similar pressure program to FK 506. Figure 5 A and B show the SFC and RPLC chromatograms with retention times of 18.4 and 5 minutes respectively. Similar disappointing result was obtained from the analysis of whole blood samples spiked with rapamycin.

Conclusion

The present study established the selectivity of SFC analysis of immunosuppressants to be " normal -phase HPLC-like ". This selectivity was important in understanding the elution order and the design of the extraction protocol. The low extraction recovery and low sensitivity of the FID did not allow adequate quantitation of these drugs in whole blood, and these may be obviated by using packed SFC column, and high sensitivity techniques such as mass spectrometer in the future.

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