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Short Communication

Supercritical fluid extraction as a sample preparation technique for the direct isolation of drugs from plasma prior to analysis

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Abstract

Supercritical carbon dioxide was used for the direct extraction of drugs from plasma prior to analysis. The supercritical fluid was directly passed through plasma samples spiked with either a neutral (flavone) or an acidic (ketorolac) drug. The addition of an antifoam agent to the plasma prior to extraction was required to avoid restrictor plugging caused by denaturation of the plasma proteins by the supercritical fluid. The effluent from the extraction cell was bubbled through a small volume of methanol or into an empty tube to trap the extracted drug. The effect of extraction pressure and time on absolute recovery were examined. The absolute recovery, selectivity, precision and accuracy of the supercritical fluid extraction approach was compared to conventional liquid–liquid extraction using reversed-phase HPLC with ultraviolet detection.

1. Introduction

The analysis of drugs in biological fluids provides critical information necessary to evaluate the safety, efficacy and mechanism of action of pharmaceuticals [1]. Modern high-resolution chromatographic techniques combined with selective detectors are widely used for the determination of drugs in biological matrices. However, even with the high degree of selectivity obtainable with these chromatographic techniques sample preparation is still usually required prior to analysis. Sample preparation may be necessary to remove interfering endogeneous matrix components or to concentrate the sample prior to analysis. Additionally, sample preparation may be required to remove high-molecular-mass plasma proteins which can foul the instrumentation or to improve the stability of the analyte.

A number of techniques including liquid–liquid extraction (LLE), solid-phase extraction, microdialysis, ultrafiltration and solvent precipitation have been employed as sample preparation steps prior to analysis. Although developed before the turn of the century, LLE is still widely used as a sample preparation technique for biological fluids largely because it is a relatively simple procedure to perform. There are, however, a number of disadvantages associated with conventional LLE. Water immiscible organic solvents are required which may result in cost, safe handling and disposal issues. LLE techniques also tend to be labor intensive, as well as

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difficult and expensive to fully automate. Additionally, emulsion formation can complicate the sample preparation process.

Recently supercritical fluid extraction (SFE) has been shown to be a promising alternative to traditional extraction methods [2,3]. Supercritical fluids can provide increased extraction efficiencies and give at least an order-of-magnitude increase in the rate of extraction. The solvating strength of a supercritical fluid can be adjusted over a wide range through variation of the extraction temperature and pressure. Modifiers, such as methanol, can also be employed to change the solvating strength, as well as improve the desorption capabilities of a supercritical fluid. Additionally, the most commonly used supercritical fluid, CO_2, is relatively inexpensive, fairly nontoxic and is easily removed following extraction.

To date, most of the published literature on SFE has dealt with the application of the technique for the extraction of solid and semisolid sample matrices. However, several approaches for the extraction of liquid samples have been investigated. In one approach the aqueous matrix is removed prior to extraction by either freeze-drying [4], use of an adsorbent like anhydrous sodium sulfate [5] or through matrix isolation on solid-phase cartridges [6,7]. A few reports have investigated the use of SFE for the direct extraction of a liquid matrix. Hedrick and Taylor [8,9] extracted triprolidine, phenol, and phosphonate from water solutions by passing a supercritical fluid through the solutions. Ong and coworkers [10] extracted high levels of cholesterol from a plasma matrix with a supercritical fluid. In this report we describe the direct extraction of trace levels of a neutral (flavone) and an acidic (ketorolac) drug (see Fig. 1) from a plasma matrix using supercritical CO_2. The addition of an antifoam agent prior to extraction was critical to the successful SFE isolation of the drugs. The absolute recovery, selectivity, accuracy and precision of the SFE approach was compared to traditional LLE for the analysis of plasma drug levels using HPLC with UV detection (HPLC-UV).

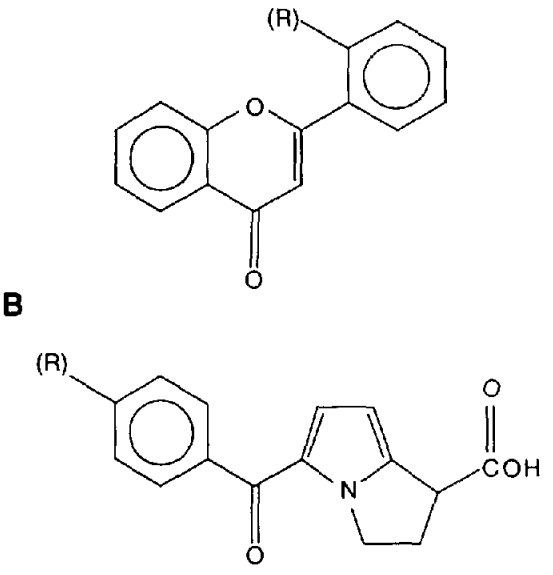

Fig. 1. Structures of (A) flavone, R = H and 2-methylflavone (2-MF), R = CH_3; and (B) ketorolac, R = H and *p*-fluoroketorolac (*p*-FKR), R = F.

2. Experimental

2.1. Chemicals

Distilled/deionized water was from a Barnstead Nanopure II system (Dubuque, IA, USA). Methanol (HPLC grade) and formic acid (reagent grade) were from J.T. Baker (Phillipsburg, NJ, USA). Flavone, ^{14}C-flavone and 2'-methylflavone (2-MF, Fig. 1) were synthesized at Procter and Gamble, Miami Valley Laboratories (Cincinnati, OH, USA). Ketorolac, *p*-fluoroketorolac (*p*-FKR, Fig. 1) and ^{14}C-ketorolac (Fig. 1) were obtained from Syntex Research (Palo Alto, CA, USA). Dog plasma was from Pel-Freez (Rogers, AR, USA). SFC-grade CO_2 was purchased from Scott Specialty Gases (Plumsteadville, PA, USA). Antifoam reagent (1520-US) was obtained from Dow Corning (Midland, MI, USA).

2.2. SFE cumulative recovery for ^{14}C-flavone

In one study the cumulative percentage recovery of ^{14}C-flavone from water, plasma and a

water/plasma mixture was determined *vs.* extraction time. One milliliter of water, blank dog plasma or a blank dog plasma/water mixture (1:2, v/v), each containing 250 ng ^{14}C-flavone/ml, were individually mixed with 20 ml of antifoam reagent. A volume (0.4 ml) of each solution was placed in individual Keystone Scientific (Bellefonte, PA, USA) SFE cells (0.85 ml) for extraction. A fused-silica capillary of 25 cm × 25 μm I.D. (Valco Instruments, Houston, TX, USA) was used as the restrictor. The samples were extracted with CO_2 at 60°C and 30 MPa using a Dionex SFE-703 instrument (Dionex, Salt Lake City, UT, USA). The end of the restrictor was placed in a test tube (16 × 100 mm) containing 2 ml of methanol at room temperature. The percent recovery of ^{14}C-flavone was measured against extraction time by continuously collecting the CO_2 effluent into a series of test tubes. The amount of ^{14}C-flavone in each tube was determined by liquid scintillation counting on a Packard Model 2000CA liquid scintillation analyzer (Packard Tri-Carb, Downers Grove, IL, USA). The cumulative extraction recovery was obtained for each sample at a given pressure by summing the radioactivities obtained from the methanol solutions for that sample and comparing it to the counts obtained for an equivalent standard solution.

The effect of extraction pressure *vs.* time was examined in a similar manner using blank dog plasma (1.0 ml), containing 250 ng/ml ^{14}C-flavone, mixed with 20 μl antifoam reagent and 2.0 ml of water. A volume of this solution (0.4 ml) was extracted as described above using extraction pressures of either 10, 15 or 30 MPa and a constant extraction temperature of 60°C. The CO_2 effluent for each extraction pressure was collected for a given period of time and counted as described above.

2.3. SFE absolute recovery for ^{14}C-flavone and ^{14}C-ketorolac

For flavone, samples were prepared by mixing blank dog plasma (1.0 ml), spiked with either 25, 100 or 250 ng of ^{14}C-flavone/ml, with 20 μl of antifoam reagent and 2.0 ml of water. An aliquot (0.4 ml) of the mixed sample was placed in a 0.85-ml Keystone cell and extracted as described above, except only one test tube containing 2.0 ml of methanol was used to collect the entire extraction for a given sample. In some instances, the SFE extracts were collected by dry-collection into an empty test tube. The extraction was done with CO_2 (30 MPa and 60°C) using an extraction time of 20 min and a 25 cm × 25 μm I.D. restrictor. The flow-rate of expanded gases was approximately 130 ml/min under these conditions. The collected effluent was analyzed by liquid scintillation counting and compared to a similar standard spiked directly into methanol.

The absolute recovery of the acidic drug, ketorolac, was also examined. Blank dog plasma (1.0 ml) spiked with 25 ng ^{14}C-ketorolac/ml was mixed with 20 μl of antifoam reagent and 2.0 ml of water. An aliquot (0.4 ml) of the mixed sample was placed in a 0.85-ml Keystone cell equipped with a 25 cm × 25 μm restrictor and extracted with CO_2. The extraction was done at 30 MPa and 60°C for 20 min and the CO_2 effluent was passed through 2.0 ml of methanol contained in a test tube (16 mm × 100 mm). The collected effluent was analyzed by liquid scintillation counting and compared to a similar standard spiked directly into methanol.

2.4. SFE selectivity, accuracy and precision for flavone and ketorolac

For the flavone studies, blank dog plasma (1.0 ml) and blank dog plasma (1.0 ml) spiked with internal standard (50 ng 2-MF/ml) and flavone (25, 100 and 250 ng/ml) were mixed with 20 μl of antifoam reagent and 2.0 ml of water as described above. An aliquot of each sample (0.4 ml) was extracted in a 0.85-ml cell using CO_2 (30 MPa and 60°C) for 20 min. The restrictor (25 cm × 25 μm) was placed in a test tube (16 mm × 100 mm) containing 1.0 ml of methanol. After completion of the extraction, the methanol solution was taken to dryness under N_2. The residue

was dissolved in 0.2 ml of HPLC mobile phase and analyzed by HPLC-UV as described below.

For the ketorolac studies, blank dog plasma (1.0 ml) and blank dog plasma (1.0 ml) spiked with internal standard (50 ng *p*-FKR/ml) and ketorolac (25 ng/ml) were mixed with 20 μl of antifoam reagent and 2.0 ml of water as described above. An aliquot (0.4 ml) of each sample was extracted in a 0.85-ml cell as described above for flavone. After completion of the extraction, the methanol solution was taken to dryness under N_2. The residue was reconstituted in 0.1 ml of mobile phase and analyzed by HPLC-UV as described below.

2.5. LLE absolute recovery for ^{14}C-flavone

Blank dog plasma was spiked with various concentrations of ^{14}C-flavone (25, 100 or 250 ng/ml). The spiked dog plasma (1.0 ml) was mixed with 20 μl of antifoam reagent and 2.0 ml of water. An aliquot of the mixed sample (0.4 ml) was pipetted into a screw top test tube (16 mm × 100 mm) for extraction. Then 4.0 ml of either hexane or methylene chloride was added to the test tube and the tube was capped and mixed for 10 min by inversion. The test tube was then centrifuged for 3 min at 200 *g*. The organic layer was removed and placed in a scintillation vial for analysis by liquid scintillation counting. The absolute recovery was determined by comparison to an equivalent standard solution.

2.6. LLE selectivity, accuracy and precision for flavone

Blank dog plasma and blank dog plasma spiked with internal standard (50 ng 2-MF/ml) and flavone (25, 100 and 250 ng/ml) were processed in the same manner as described above for the LLE absolute recovery study. After completion of the extraction, the organic layer was taken to dryness under N_2. The resulting residue was reconstituted in mobile phase and analyzed by HPLC-UV as described below.

2.7. HPLC-UV methods for flavone and ketorolac

The HPLC system consisted of a Milton Roy (Riviera Beach, FL, USA) constaMetric III pump, a Perkin-Elmer (Norwalk, CT, USA) ISS-100 autosampler, a Linear (Reno, NV, USA) UV-Vis 200 detector and a Perkin-Elmer Nelson (Cupertino, CA, USA) Turbochrom data system. The separation was done on a Waters Associates (Milford, MA, USA) Nova-Pak C_{18} column (150 × 3.9 mm I.D., 4 μm particle). For the flavone analysis a mobile phase of methanol–water (65:35, v/v) was used at a flow-rate of 0.5 ml/min. The injection volume was 25 μl and the detection wavelength was 300 nm. For the ketorolac analysis a mobile phase of water–acetonitrile–formic acid–triethylamine (70:30:0.032:0.047, v/v) was used at a flow-rate of 0.75 ml/min. The injection volume was 50 μl and the detection wavelength was 317 nm.

Standard solutions of flavone and ketorolac were prepared in methanol over concentration ranges from 5 to 500 ng/ml and 5 to 100 ng/ml, respectively. Standard curves were obtained by plotting the peak-height ratio (analyte peak height/internal standard peak height) *vs.* the concentration of the standard. The concentration of flavone or ketorolac in a plasma sample was determined from the sample peak-height ratio by interpolation from the linear regression curve.

3. Results and discussion

3.1. Recovery of flavone

The extraction cell design used for these studies is shown in Fig. 2. The supercritical CO_2 was introduced into the bottom of the cell through a stainless steel tube inserted from the top of the cell. The restrictor entered from the bottom of the cell and was positioned near the top of the cell. The supercritical fluid exited the stainless steel tube, passed through the liquid sample and exited at the top of the cell through the restrictor. Initial attempts to directly extract

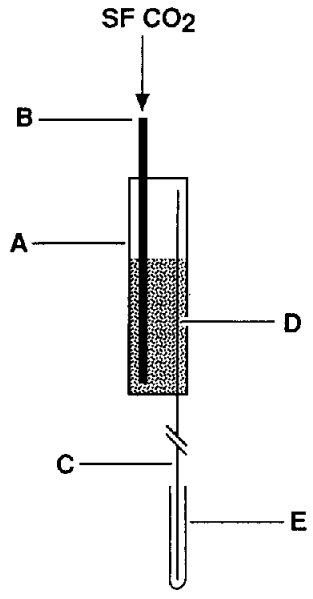

Fig. 2. Cell design used for SFE of plasma samples. (A) SFE cell, (B) stainless steel tubing, (C) capillary restrictor, (D) plasma sample, and (E) test tube.

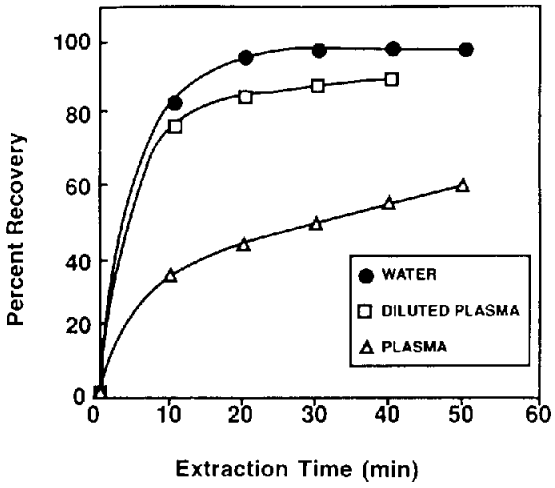

Fig. 3. SFE cumulative recovery of ^{14}C-flavone from liquid matrices extracted with CO_2 (30 MPa and 60°C) as a function of time.

a liquid plasma sample resulted in almost immediate plugging of the restrictor. It is likely that the restrictor plugging was caused by denaturation of the plasma proteins due to the passage of the supercritical fluid. This is analogous to the frothing observed in plasma samples when nitrogen is bubbled through the plasma causing denaturation of the plasma proteins. The addition of a commercial food grade antifoam agent was found to eliminate denaturation of plasma proteins caused by the passage of nitrogen gas through the plasma. Addition of the same antifoam agent to the plasma prior to SFE eliminated the restrictor plugging problem and was essential for the successful extraction of any plasma sample. For all subsequent work the antifoam agent was added to samples prior to extraction.

The absolute recovery of ^{14}C-flavone from plasma, plasma diluted with water and distilled water following extraction with supercritical CO_2 (30 MPa and 60°C) was examined *vs.* extraction time (Fig. 3). The absolute recovery of ^{14}C-flavone from water was quantitative and reached completion by 30 min. In contrast, the absolute recovery of ^{14}C-flavone from undiluted plasma was significantly lower than from pure water at each time point and only reached 60% after extraction for 50 min. The decreased rate and extent of extraction of ^{14}C-flavone in plasma *vs.* distilled water may be due to binding of flavone by the plasma proteins. Dilution of the plasma with two volumes of water resulted in both a faster rate of extraction and a higher absolute recovery (85%) of ^{14}C-flavone. Plasma diluted with two volumes of water was used for the remainder of the studies. Additionally, it was found that the analyte could be collected into an empty tube with absolute recoveries identical to those obtained using methanol in the tube.

The effect of extraction pressure, at a constant temperature of 60°C, on the absolute recovery of ^{14}C-flavone from diluted plasma samples was examined. The percent of ^{14}C-flavone recovered at each time point increased with increasing pressure (Fig. 4). The best recovery was achieved at an extraction pressure of 30 MPa. Pressures greater than 30 MPa were not examined due to leakage of the extraction cells above this pressure. Therefore, the optimal obtainable extraction conditions used for the

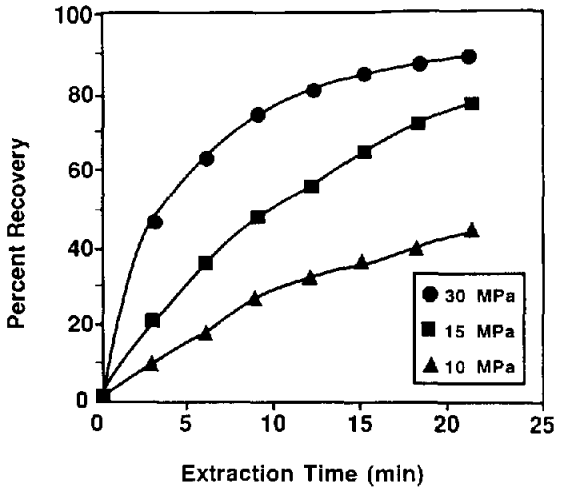

Fig. 4. SFE recovery of ^{14}C-flavone from diluted plasma samples extracted with CO_2 (60°C) as a function of extraction pressure and time.

remainder of the studies were 30 MPa, 60°C and 20 min, respectively.

3.2. Absolute recovery of flavone and ketorolac

The absolute recovery of ^{14}C-flavone (25, 100 and 250 ng/ml) from diluted plasma samples by SFE under the optimal extraction conditions (60°C, 30 MPa and 20 min collection) was compared to conventional LLE (Table 1).

The absolute recovery of ^{14}C-flavone by LLE was less than 80% with methylene chloride and ranged from 83 to 92% with hexane. The absolute recovery by SFE ranged from 85 to 98% across the concentration range examined and was comparable to that obtained with hexane. All three extractions gave reproducible absolute recoveries of ^{14}C-flavone as judged by the small %R.S.D. values obtained for triplicate extractions. The absolute recovery of ^{14}C-ketorolac from diluted plasma by SFE was also examined using the optimal extraction conditions determined for flavone. The absolute recovery of ketorolac was 80% with a %R.S.D. of 11.8% ($n = 3$) at a spiked plasma level of 25 ng/ml.

3.3. Extraction selectivity

The selectivity of SFE relative to LLE for the preparation of flavone plasma samples was examined using HPLC-UV. Diluted blank plasma and diluted blank plasma spiked with flavone (25 ng/ml) and with the 2-MF internal standard (50 ng/ml) were extracted by both procedures. SFE was done under the optimal conditions and LLE was done with both hexane and methylene chloride as the extraction solvents. Typical chromatograms obtained for samples prepared by SFE and LLE are shown in Fig. 5. For all

Table 1
Extraction recovery of ^{14}C-flavone and ^{14}C-ketorolac from plasma

Spiked concentration (ng/ml)	Average absolute percent recovery (%R.S.D.) [a]		
	Hexane extraction	Methylene chloride extraction	SFE
Flavone			
25	92 (5.0)	80 (7.1)	98 (5.2)
100	84 (2.4)	79 (2.5)	85 (5.8)
250	83 (7.8)	72 (4.3)	87 (3.8)
Ketorolac			
25			80 (11.8)

[a] Averages are for triplicate sample preparations and analysis.

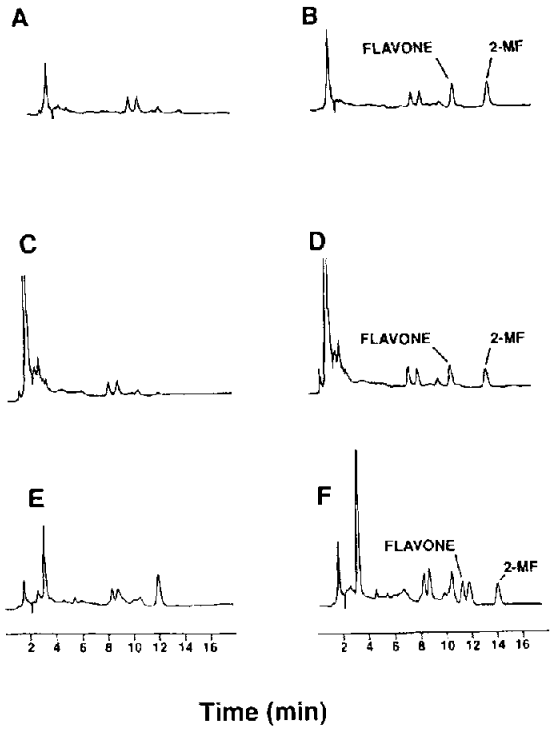

Fig. 5. Chromatograms for diluted blank plasma extracted by (A) hexane LLE, (C) methylene chloride, and (E) SFE. HPLC-UV chromatograms for diluted plasma spiked with flavone (25 ng/ml) and 2-MF (50 ng/ml) and extracted by (B) hexane LLE, (D) methylene chloride LLE, and (F) SFE.

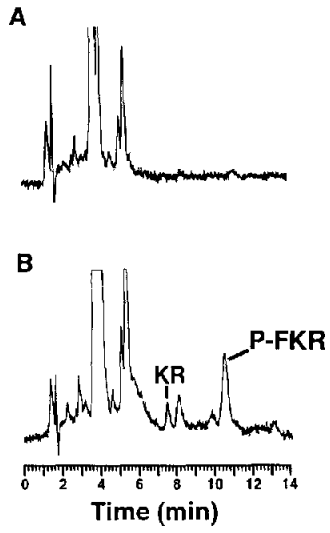

Fig. 6. Chromatograms for SFE of (A) diluted blank plasma and (B) diluted blank plasma spiked with KR (25 ng/ml) and *p*-FKR (50 ng/ml). KR = ketorolac.

extractions, the blank plasma was free of interferences in the retention time range of flavone and 2-MF. However, the LLE extraction resulted in more selective extraction based on the smaller number of closely eluting components observed in the LLE *vs.* SFE sample chromatograms. A component eluting near the flavone peak was present for both SFE and LLE and somewhat hindered the quantification of flavone. The selectivity of SFE for the analysis of ketorolac was also examined under the optimal extraction conditions used for flavone. Chromatograms for diluted blank plasma and for diluted blank plasma spiked with ketorolac (25 ng/ml) and the *p*-FKR internal standard (50 ng/ml) are shown in Fig. 6. The blank plasma extract was free of interfering components at the retention time of ketorolac and *p*-FKR. The selectivity of LLE was not examined for ketorolac.

3.4. Accuracy and precision

The accuracy and precision of the SFE and LLE approach were compared for the analysis of diluted plasma samples spiked with flavone (25, 100 and 250 ng/ml) and the 2-MF internal standard (50 ng/ml). The results of the HPLC-UV analysis of triplicate SFE and LLE spiked plasma sample preparations are shown in Table 2. The accuracy and precision of the flavone analysis, as measured by the average found concentration and the %R.S.D. for replicates, was similar for the SFE and LLE sample preparation approaches. The accuracy and precision of the SFE approach was also examined for diluted blank plasma samples spiked with ketorolac (25 ng/ml) and the *p*-FKR internal standard (50 ng/ml). The results of the HPLC-UV analysis of triplicate SFE sample preparations of the spiked plasma are shown in Table 2. The accuracy and precision at this low concentration were both excellent.

Table 2
Analysis of spiked plasma samples following LLE and SFE [a]

Spiked concentration (ng/ml)	Average found (ng/ml) (%R.S.D.)		
	Hexane	Methylene chloride	SFE
Flavone			
25.0	26.1 (2.7)	26.5 (0.4)	28.0 (2.0)
100	105 (2.0)	101 (1.7)	112 (3.8)
250	246 (1.3)	270 (6.3)	260 (8.5)
Keterolac			
25.0			24.6 (3.1)

[a] Averages are for the analysis of triplicate samples.

4. Conclusions

SFE has been demonstrated to be a viable sample preparation technique for the isolation of drugs from a liquid plasma matrix prior to trace analysis. The addition of an antifoam agent was found to be critical to the successful use of a supercritical fluid for the extraction of analytes directly from the plasma matrix. SFE was shown to give absolute analyte recoveries comparable to those obtained by LLE. Additionally, SFE was shown to give similar method selectivity, accuracy and precision as the more traditional LLE technique. In contrast to conventional LLE where large amounts of water immiscible organic solvents are used, the SFE approach is carried out with a relatively nontoxic eluent, supercritical CO_2.

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