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Supercritical fluid extraction of selected pharmaceuticals from water and serum

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Abstract

Selected drugs from benzodiazepine, anabolic agent and non-steroidal anti-inflammatory drug (NSAID) therapeutic classes were extracted from water and serum using a supercritical CO_2 mobile phase. The samples were extracted at a pump pressure of 329 MPa, an extraction chamber temperature of 45°C, and a restrictor temperature of 60°C. The static extraction time for all samples was 2.5 min and the dynamic extraction time ranged from 5 to 20 min. The analytes were collected in appropriate solvent traps and assayed by modified literature HPLC procedures. Analyte recoveries were calculated based on peak height measurements of extracted vs. unextracted analyte. The recovery of the benzodiazepines ranged from 80 to 98% in water and from 75 to 94% in serum. Anabolic drug recoveries from water and serum ranged from 67 to 100% and 70 to 100%, respectively. The NSAIDs were recovered from water in the 76 to 97% range and in the 76 to 100% range from serum. Accuracy, precision and endogenous peak interference, if any, were determined for blank and spiked serum extractions and compared with classical sample preparation techniques of liquid–liquid and solid-phase extraction reported in the literature. For the benzodiazepines, accuracy and precision for supercritical fluid extraction (SFE) ranged from 1.95 to 3.31 and 0.57 to 1.25%, respectively ($n=3$). The SFE accuracy and precision data for the anabolic agents ranged from 4.03 to 7.84 and 0.66 to 2.78%, respectively ($n=3$). The accuracy and precision data reported for the SFE of the NSAIDs ranged from 2.79 to 3.79 and 0.33 to 1.27%, respectively ($n=3$). The precision of the SFE method from serum was shown to be comparable to the precision obtained with other classical preparation techniques.

Keywords: Diazepam; Oxazepam; Temazepam; Nordiazepam; Prazepam; Fluoxymesterone; Nortestosterone; Methyltestosterone; Stanozolol; Testosterone; Methandrostenolone; Zeranol; Fenbufen; Indomethacin; Ketoprofen; Tolmetin; Benzodiazepine; Anabolic agents; Non-steroidal anti-inflammatory drugs

1. Introduction

The pharmaceutical analyses of biological fluids provide the information necessary for evaluating the safety, therapeutic effect and mechanism of action of a variety of drugs [1]. While modern high-resolution chromatographic techniques are available for analysis, sample preparation is an important step in the assay. Various techniques including liquid–liquid extraction, solid-phase extraction, soxhlet extraction, microdialysis, ultrafiltration and solvent precipitation have been used for sample preparation prior to analysis. In most biological fluid assays, the majority of analysis time is spent preparing the sample. The analytical laboratory of today is under considerable pressure to increase sample throughput and lower

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assay costs. One way to achieve this goal is to develop quick and inexpensive sample preparation procedures. Supercritical fluid extraction (SFE) is an attractive option as it considerably reduces sample preparation time and provides efficient analyte recovery from liquid, solid and semisolid samples. The most unique feature of SFE is the use of a supercritical fluid mobile phase which may be adjusted for solubilizing power by varying density via pressure or temperature modifications [2].

To date, most studies involving the SFE of pharmaceuticals have applied the technique to the extraction of solid and semisolid dosage forms and bulk drug substances. Lawrence et al. [3] employed SFE in extracting benzodiazepines from the matrices of standard dosage forms. Tena et al. [4] demonstrated an improved SFE of sulphonamides from solid supports using supercritical CO_2 and methanol modified CO_2 as mobile phases [4]. Howard et al. [5] used SFE for the sample preparation of sustained release felodipine tablets. Extractions of felodipine from the tablet matrix required moderate modifier concentrations (8.7%, v/v methanol in CO_2) and was shown to be an accurate and precise sample preparation alternative to liquid–liquid extraction of felodipine tablets with an 80% reduction in solvent consumption.

Supercritical fluid extraction has also been applied to the analysis of pharmaceuticals in serum and plasma. Ndiomu et al. [6] reported that SFE was more efficient in recovering morphine from freeze-dried serum using supercritical CO_2 as the mobile phase. Liu and Wehmeyer [7] investigated the solid-phase extraction of flavone from plasma with supercritical fluid elution and showed that conventional solid-phase extraction and solid-phase extraction with supercritical fluid elution were comparable. The serum blank using supercritical fluid extraction showed more peaks than the standard solid-phase extraction blank, but the recoveries and precisions of both methods were comparable. Liu and Wehmeyer [8] also showed the direct SFE analysis of flavone from diluted serum with no solid support matrix. The SFE method was compared to liquid–liquid extraction of a serum sample for accuracy and precision. The SFE serum blanks gave more endogenous peaks on the HPLC chromatogram, but the SFE recoveries were slightly higher than those from liquid–liquid extraction with comparable precision data.

In this paper, supercritical fluid extraction of water and serum samples containing each of three therapeutic classes of drugs were analyzed by HPLC chromatography with UV detection. The water and serum samples were immobilized on celite and supercritical CO_2 was used as the mobile phase in each extraction. The SFE results were compared to literature data of solid-phase or liquid–liquid extraction sample preparation techniques for accuracy, precision and lack of interference from endogenous serum components.

2. Experimental

2.1. Reagents and chemicals

Oxazepam was obtained from Wyeth Laboratories (Philadelphia, PA, USA). Diazepam was obtained from Hoffman-La Roche (Nutley, NJ, USA). Zeranol was supplied by Pitman-Moore (Terre Haute, IN, USA). Naproxen sodium was a USP reference standard (Rockville, MD, USA). Stanozolol was obtained from Sterling-Winthrop Research Institute (Rensselaer, NY, USA). Temazepam, nordiazepam, prazepam, fluoxymestrone, nortestosterone, testosterone, methandrostenolone, methyltestosterone, tolmetin, ketoprofen, fenbufen and indomethacin were all purchased from Sigma Chemical Company (St. Louis, MO, USA).

Supercritical fluid chromatography/extraction grade carbon dioxide was obtained from Air Products (Atlanta, GA, USA). The SFE wet support (celite, Cat. No. 68-3867-010) was purchased from Isco (Lincoln, NE, USA). Deionized-distilled water was obtained by a filtration system from Continental Water Systems (Roswell, GA, USA). HPLC grade methanol, acetonitrile, glacial acetic acid and hydrochloric acid were purchased from J.B. Baker (Phillipsburg, NJ, USA). Serachem Plus Clinical Chemistry Control Serum Unassayed Level 1 was purchased from Instrumentation Laboratories (Lexington, MA, USA).

2.2. Instrumentation

Supercritical fluid extraction was performed on an Isco Model SFX 2-10 supercritical fluid extractor

(Cat. No. 67-9000-040) equipped with 1.5 ml/min linear coaxially heated capillary restrictors and a temperature controller (Cat. No. 68-3960-001). Isco 2.5-ml stainless steel extraction cartridges (Cat. No. 68-3867-002) were used to load the sample into the extraction chamber.

Chromatography was performed on an isocratic HPLC system consisting of a Micromeritics Model 760 HPLC solvent delivery system, a Model 728 autosampler equipped with a 100-μl loop (Norcross, GA, USA), and a Kratos Spectroflow Model 757 UV–Vis variable-wavelength detector (Ramsey, NJ, USA). The analytical wavelengths were 254 nm (benzodiazepines), 247 nm (anabolic agents) and 240nm (NSAIDs). Data acquisition was performed on a Hewlett-Packard Model HP-3395 Integrator (Palo Alto, CA, USA).

An ODS-1 column (Spherisorb 5 μm, 250×4.6 mm I.D., Alltech, Deerfield, IL, USA) was used for both the benzodiazepine and NSAID separations. A silica column 250×4.6 mm I.D. and a 3-μm particle size (Phenomenex, Torrance, CA, USA) was used for the anabolic agents. The columns were maintained at an ambient temperature of 23±1°C.

2.3. Preparation of HPLC mobile phases

The HPLC mobile phases were modified from literature sources to fit the chromatographic parameters for each class of drugs. The mobile phase for the benzodiazepines was composed of methanol–deionized water–glacial acetic acid in the ratio 63:35:2 (v/v) [9] at 1.0 ml/min. The mobile phase for the anabolic agents consisted of 0.05 *M* aqueous monobasic sodium phosphate adjusted to pH 3.0 with concentrated phosphoric acid–acetonitrile (85:15, v/v) [10] at 0.8 ml/min. The mobile phase for the NSAIDs was acetonitrile–deionized water–glacial acetic acid (45:55:0.4, v/v) [11] at 1.0 ml/min. Each mobile phase was filtered through a 0.45-μm nylon-66 filter (MSI, Westborough, MA, USA) and degassed by sonication prior to use.

2.4. Preparation of stock and standard solutions

Stock solutions of the benzodiazepines (diazepam, oxazepam, temazepam, nordiazepam and prazepam), the anabolic agents (fluoxymestrone, nortestosterone, methyltestosterone, stanozolol, testosterone, methandrostenolone and zeranol), and the NSAIDs (fenbufen, naproxen, indomethacin, ketoprofen and tolmetin) were prepared at 20 μg/ml concentrations in methanol and further diluted to 1 μg/ml to investigate matrix interactions and solvation of the analytes. A combined 20 μg/ml stock solution of nordiazepam and diazepam and a 20 μg/ml solution of prazepam (internal standard) were prepared in methanol for the nordiazepam–diazepam calibration curve. A combined 5 μg/ml combined stock solution of nortestosterone and testosterone and a 4 μg/ml solution of a methyltestosterone solution (internal standard) were prepared in methanol for the nortestosterone–testosterone calibration curve. A 5 mg/ml combined stock solution of ketoprofen and fenbufen and a 2 mg/ml solution of tolmetin (internal standard) were prepared in methanol for the ketoprofen–fenbufen calibration curve.

2.5. Preparation of calibration curves

The nordiazepam–diazepam stock solution was diluted with methanol and aliquots (100 μl) of the dilutions were added to 2-ml volumetric tubes along with 50 μl prazepam solution. After evaporation to dryness with a nitrogen stream, water or serum was added to volume and vortexed for 30 s to give final concentrations of 100, 500 and 1000 ng/ml for the nordiazepam–diazepam calibration curve. Spiked water and serum samples (250 and 750 ng/ml) were also prepared in the same manner for accuracy and precision data. The final concentration of the internal standard was 500 ng/ml.

The 5 μg/ml combined stock solution of nortestosterone–testosterone was diluted with methanol and aliquots (100 μl) of the dilutions were added to 2-ml volumetric tubes along with the 50 μl of methyltestosterone stock solution. After evaporation to dryness with a nitrogen stream, water or serum was added to volume and vortexed for 30 s to give final concentrations of 10, 50 and 250 ng/ml for the nortestosterone–testosterone calibration curve. Spiked water and serum samples (25 and 100 ng/ml) were prepared in the same manner for accuracy and precision data. The final concentration of the internal standard was 100 ng/ml in each solution.

The 5 mg/ml combined stock solution of ketoprofen–fenbufen was diluted with methanol and

aliquots (100 μl) of the dilutions were added to 2-ml volumetric tubes along with the 50 μl of tolmetin stock solution and 50 μl of 6 *M* HCl. After evaporation to dryness with a nitrogen stream, water or serum was added to volume and vortexed for 30 s to give the final concentrations of 5, 50 and 250 μg/ml for the ketoprofen–fenbufen calibration curve. Spiked water and serum samples (25 and 100 μg/ml) were also prepared in the same manner to measure accuracy and precision data. The final concentration of the internal standard in each solution was 50 μg/ml.

2.6. Preparation of samples for SFE

A 1-ml volume of each water and serum sample was added to approximately 1 g of celite wet support contained in an evaporation dish. In the case of the NSAIDs, 50 μl of 6 *M* HCl was added to the serum. The samples were then dried in an oven at 45°C for 30 min with some stirring to ensure even drying. The dried samples were then transferred into 2.5-ml extraction cartridges with the aid of a small glass funnel and a spatula.

2.7. Supercritical fluid extraction method

The samples were extracted with supercritical carbon dioxide at an extraction chamber temperature of 45°C, a restrictor temperature of 60°C and a pump pressure of 329 MPa. Static (2.5 min) and 10-min dynamic extraction times were used for the anabolic agents and 2.5-min static and 20-min dynamic extraction times were employed for the NSAIDs and benzodiazepines. The mobile phase for each HPLC drug system served as the solvent trap for the extraction. Solvent trap volumes of 2, 1 and 10 ml were used for the benzodiazepines, anabolic agents, and NSAIDs, respectively. After SFE, each extract was transferred with washes to clean volumetric flasks of the same size, allowed to stand for 5 min to equilibrate to ambient temperature, and brought to volume with mobile phase. Aliquots (100 μl) were then injected into the HPLC system and the concentration of each analyte was calculated from the respective standard curve.

3. Results and discussion

In our initial investigations, one or two model drugs from the benzodiazepine, anabolic agent and NSAID classifications were spiked onto a celite support and extracted at the 1 μg/ml level by SFE to establish the experimental parameters most affected by the extraction process. Based on the experiments performed, a pump pressure of 329 MPa, a 45°C extraction chamber temperature, a 60°C restrictor temperature and a 2.5-min static extraction time were held constant for each of the extractions. The dynamic extraction time was most affected by the extraction process and was optimized for each of the extraction procedures.

A drug-free water sample was extracted by the SFE process to determine if celite was a viable matrix support for the samples. It was found that there were little or no interfering peaks in the HPLC chromatograms. Thus celite was selected as a very appropriate support for the samples.

No attempt was made to compare times required for batch samples by SFE vs. solid-phase extraction or liquid–liquid extraction. The SFE apparatus used in this study only processed two samples at a time. Minimum SFE trap volumes of 2, 1 and 10 ml were selected for benzodiazepines, anabolic agents and NSAISs, respectively, based on maximum drug recoveries while maintaining high HPLC sensitivity without the need for any additional sample concentration steps.

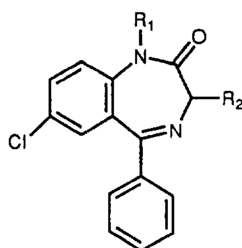
Table 1 shows the extraction efficiencies obtained for the benzodiazepines and Fig. 1 illustrates the chemical structures of the analytes. The highest percent recoveries from serum occurred using a 20-min dynamic extraction time. The serum data indicate that the polarity and protein binding of the benzodiazepines effect the efficiency of extraction using CO_2 as the mobile phase. The most polar analyte, oxazepam, gave the lowest percent recovery from serum at 75.13%, and less polar analytes, such as temazepam, nordiazepam, diazepam and prazepam, had higher recoveries of 93.95, 90.04, 92.44 and 93.77%, respectively. Temazepam had a slightly higher extraction efficiency than nordiazepam, and is slightly more polar. Both temazepam and nordiazepam are approximately 97% protein bound, indicating that the interactions be-

Table 1
SFE of benzodiazepines from water and serum compared to liquid–liquid extraction from serum

Analyte[a]	Recovery %				
	SFE				Liquid–Liquid[b]
	Water		Serum		Serum
	10 min[c]	20 min	10 min	20 min	
Diazepam	92.65±1.06[d]	97.52±0.44	91.62±1.59	92.44±0.64	94.4
	(1.14)[e]	(0.45)	(1.73)	(0.69)	–[f]
Oxazepam	93.58±3.14	96.66±1.21	44.71±1.27	75.13±0.60	100.0
	(3.35)	(1.25)	(2.81)	(0.80)	–[f]
Temazepam	92.07±2.99	88.77±0.33	92.84±1.90	93.95±0.60	–[g]
	(3.24)	(0.37)	(2.05)	(0.64)	
Nordiazepam	87.59±2.03	79.69±0.60	84.06±1.13	90.04±0.88	99.8
	(2.31)	(0.76)	(1.35)	(0.98)	–[f]
Prazepam	88.79±1.66	82.87±1.27	90.42±1.06	93.77±1.27	–[g]
	(1.87)	(1.53)	(1.17)	(1.35)	

[a] Analyte concentration was 1 μg/ml for SFE and 0.6 μg/ml for liquid–liquid extraction.
[b] Ref. [9].
[c] Dynamic extraction time.
[d] Mean±S.D. based on $n=3$.
[e] %R.S.D.
[f] R.S.D. not available.
[g] Data not available.

tween the serum proteins and the hydroxyl group of temazepam may be more easily disrupted with CO_2 than the interactions between the serum proteins and the secondary amide of nordiazepam [12]. The SFE efficiency is dependent on the solubility of a drug in carbon dioxide and the manner in which the carbon dioxide disrupts the interactions between analyte,



Diazepam	$R_1 = CH_3$	$R_2 = H$
Oxazepam	$R_1 = H$	$R_2 = OH$
Temazepam	$R_1 = CH_3$	$R_2 = OH$
Nordiazepam	$R_1 = H$	$R_2 = H$
Prazepam	$R_1 = CH_2$–cyclopropyl	$R_2 = H$

Fig. 1. Chemical structures of benzodiazepines studied.

serum protein and celite support. The SFE efficiencies of the least polar analytes, diazepam and prazepam, were very similar although diazepam is slightly more protein bound (99 vs. 97%) [12]. An attempt was made to increase the extraction of oxazepam from a spiked serum sample using a 5% methanol modified CO_2 mobile phase. This resulted in a cloudy extract which could have possibly plugged the restrictor, so CO_2 containing methanol was abandoned as a more efficient extraction solvent.

Two suggestions to use anhydrous inorganic salts in the extraction cartridge to absorb moisture and thus eliminate sample drying time and to lypholyze the sample prior to extraction to eliminate moisture were not investigated. It was thought that strong interactions might occur on the surface of the inorganic salts that may prevent the carbon dioxide from solvating the analyte. The lyophization process is too slow and tedious to be an effective sample preparation step.

Table 1 also includes literature liquid–liquid extraction data for benzodiazepines [9]. The SFE data of the benzodiazepines, except oxazepam, was comparable to the reported extraction data. Ratnaraj et. al. reported a general R.S.D. of ±5% for triplicate

extractions. The SFE serum R.S.D.s were in the range of 0.63 to 1.35%. Thus, the SFE method provides somewhat greater precision at lower recoveries.

HPLC chromatograms of serum blanks from liquid–liquid extraction and SFE are shown in Fig. 2A and B. The liquid–liquid extraction gave a cleaner extract than SFE, but the SFE blank was comparable to that of the liquid–liquid extraction as there were no serum peaks interfering with the analytes of interest. A chromatogram of a spiked serum sample extracted by SFE containing nordiazepam (750 ng/ml), diazepam (750 ng/ml), and the internal standard, prazepam (500 ng/ml), is also shown in Fig. 2C. The parameters for this extraction were set at 329 MPa and 45°C with 2.5-min static and 20-min dynamic extraction times. A 2-ml aliquot of the HPLC mobile phase served as the solvent trap.

The calibration curves for the selected benzodiazepines were constructed by plotting nordiazepam and diazepam at concentrations of 100, 500 and 1000 ng/ml with an internal standard, prazepam, at 500 ng/ml in each sample. To monitor accuracy and precision, spiked samples of nordiazepam and diazepam were analyzed at the 250 and 750 ng/ml levels. Linear regression analysis of peak height vs. Nordiazepam concentration showed a correlation coefficient of 0.9997 with a slope of 0.004 and a y-intercept of 0.06. The spiked samples of nordiazepam gave a percent error of 3.31% at 250 ng/ml with an R.S.D. of 1.25%, and a percent error of 2.72% at 750 ng/ml with an R.S.D. of 0.57% ($n=3$). Linear regression analysis of the standard curve data for diazepam gave a correlation coefficient of 0.9982 with a slope of 0.003 and a y-intercept of −0.01. The spiked samples of diazepam gave a percent error of 2.89% at 250 ng/ml with an R.S.D. of 0.78%, and a percent error of 1.95% at 750 ng/ml with an R.S.D. of 0.59% ($n=3$). These results showed that the SFE of the benzodiazepines was linear over the 100–1000 ng/ml range typically utilized in therapeutic drug monitoring of nordiazepam and diazepam.

Table 2 shows the SFE efficiency data of the anabolic agents, fluoxymestrone, nortestosterone, methyltestosterone, stanozolol, testosterone, methandrostenolone and zeranol from spiked water and serum samples at 5-, 10- and 20-min dynamic extraction times. Fig. 3 shows the chemical structures for the anabolic agents. It was found that the 10-min dynamic extraction time was the most efficient for the extraction from serum. Zeranol and fluoxymestrone were exceptions; they were most efficiently recovered at a 20-min dynamic extraction

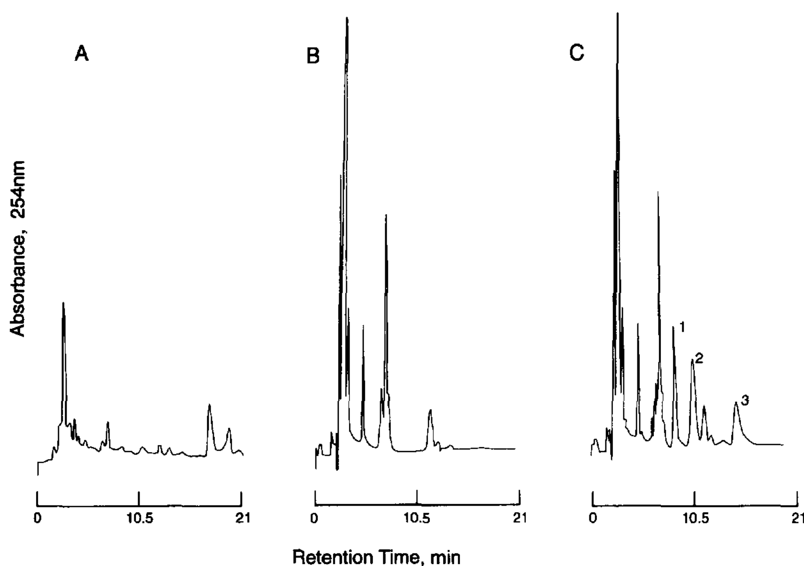


Fig. 2. Typical HPLC chromatograms of (A) blank serum from liquid–liquid extraction [9], (B) blank serum from SFE and (C) SFE of serum containing 750 ng/ml nordiazepam (1), diazepam (2) and 500 ng/ml prazepam (3).

Table 2
SFE of anabolic agents from water and serum compared to solid-phase extraction from serum

Analyte[a]	Recovery %						
	SFE						Solid phase[b]
	Water			Serum			Serum
	5 min[c]	10 min	20 min	5 min	10 min	20 min	
Fluoxymestrone	96.49±0.95[d]	100.37±0.65	98.68±1.33	48.12±1.63	90.66±0.72	100.26±0.41	103.80±6.33
	(0.98)[e]	(0.65)	(1.35)	(3.38)	(0.79)	(0.40)	(2.80)
Nortestosterone	90.47±0.12	98.85±0.77	92.74±2.91	86.87±0.76	100.08±0.75	98.48±1.17	102.40±6.56
	(0.14)	(0.78)	(3.14)	(0.87)	(0.75)	(1.18)	(3.08)
Methyltestosterone	88.87±0.45	91.94±0.55	79.16±2.60	87.90±0.94	91.19±0.23	87.35±3.48	96.30±7.92
	(0.51)	(0.60)	(3.28)	(1.07)	(0.25)	(3.99)	(3.24)
Stanozolol	66.23±0.33	70.57±1.10	63.83±1.62	54.40±1.61	69.80±1.55	50.20±0.49	103.00±7.38
	(0.50)	(1.55)	(2.54)	(2.95)	(2.22)	(0.98)	(3.54)
Testosterone	89.16 0.62	92.88±107	81.55±3.82	86.29±0.22	93.87±1.00	91.92±2.56	102.00±7.03
	(0.70)	(1.15)	(4.68)	(0.25)	(1.07)	(2.78)	(3.48)
Methandrostenolone	92.57±0.42	93.67±2.49	89.29±0.46	90.43±0.48	95.35±1.51	96.27±2.56	101.30±9.97
	(0.45)	(2.66)	(0.52)	(0.53)	(1.59)	(2.66)	(3.45)
Zeranol	66.94±2.00	62.14±1.60	43.76±0.44	36.35±1.46	67.86±3.39	79.57±1.95	97.20±10.60
	(2.98)	(2.58)	(1.00)	(4.01)	(4.99)	(2.45)	(4.01)

[a] Analyte concentration was 1 μg/ml for SFE and 0.05 μg/ml for solid-phase extraction.
[b] Ref. [10].
[c] Dynamic extraction time.
[d] Mean±S.D. based on $n=3$.
[e] %R.S.D.

time. An increase in extraction efficiency occurred when the dynamic extraction time was changed from 5 to 10 min indicating the need for more CO_2 to remove the analytes from the celite support. A further increase to 20 min, however, showed a decrease in recovery, indicating a possible loss of analyte from the solvent trap. A loss of analyte can be due to the mechanical bubbling of the CO_2 mobile phase as it flows into the solvent trap causing vapor formation which, in turn, causes nonvolatile drug loss with the mechanical overflow of the vapor from the open solvent trap. Stanozolol followed this pattern with 70% recovery at 10 min; the extraction efficiency of the other drugs ranged from 91 to 100%.

The chemical SFE differences between zeranol and stanozolol and the other anabolic agents paralleled the structural differences. Zeranol has no parent steroid backbone and the four hydroxyl groups, two of which are acidic, increase its polarity and affinity for the celite support relative to the other compounds. Stanozolol has a pyrazole ring system in addition to the parent steroid backbone. The pyrazole ring may aid in increasing the polarity of the compound, thereby decreasing its recovery when using the CO_2 mobile phase, or it may interact more strongly with serum proteins and silanol groups on the celite support than do the other anabolic agents. Similar results were obtained in SFE with spiked water samples, but the zeranol and fluoxymestrone extraction results were more closely related than those of the other anabolic drugs with the highest percent extraction at 10 min. Anabolic agents exhibit minimal protein binding as reflected by the similarity in the SFE data for both water and serum. Table 2 shows solid-phase extraction data for the anabolic compounds reported by Lampert and Stewart [10]. The SFE technique compared favorably with solid-phase extraction of serum samples spiked with anabolic agents. The SFE of these compounds from serum samples using a 10-min dynamic extraction time gave R.S.D.s ranging from 0.25 to 4.99%, which were comparable to the R.S.D.s of the solid-phase extractions, which varied from 2.8–4.01%.

Fig. 4A and B shows an HPLC comparison of the serum blanks derived from solid-phase extraction

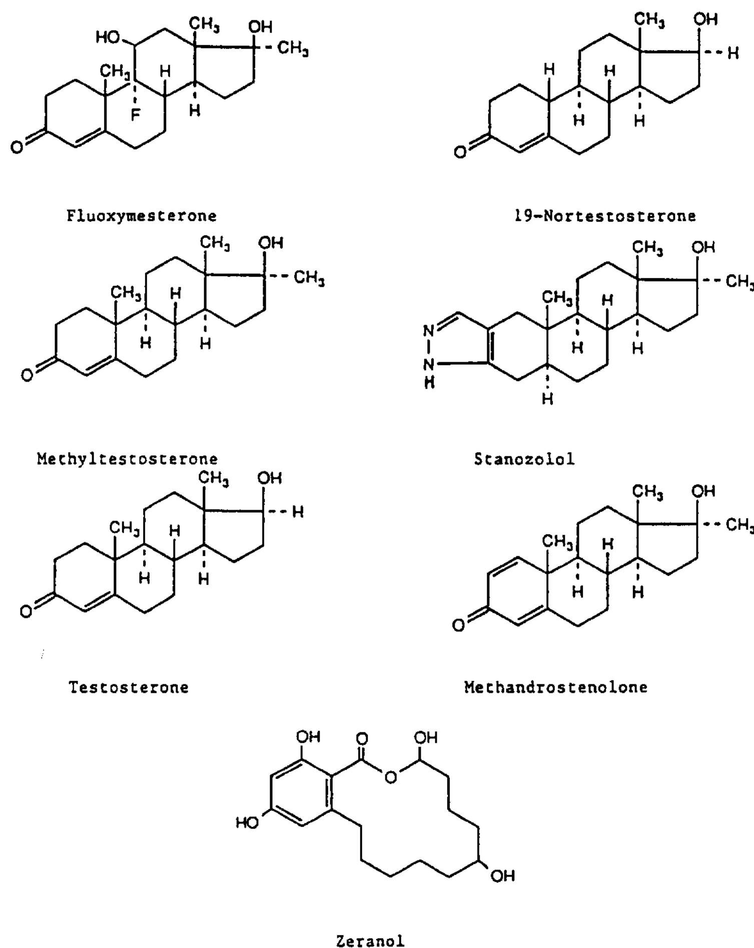


Fig. 3. Chemical structures of anabolic agents studied.

and SFE. The chromatograms for both sample preparation techniques were similar. The chromatogram of SFE of a spiked serum sample containing nortestosterone (50 ng/ml), testosterone (50 ng/ml) and the internal standard, methyltestosterone (100 ng/ml), is also shown in Fig. 4C. The parameters for the extraction were set at 329 MPa and 45°C with 2.5-min static and 10-min dynamic extraction times. A 1-ml aliquot of the HPLC mobile phase served as the solvent trap.

The standard curve for selected anabolic compounds was constructed by plotting nortestosterone and testosterone at concentrations of 10, 50 and 250 ng/ml with the internal standard, methyltestosterone, at 100 ng/ml in each sample. Accuracy and precision data of the extraction were monitored using spiked samples at concentrations of 25 and 100 ng/ml. The linear regression analysis of peak height vs. concentration for nortestosterone gave a correlation coefficient of 0.9999 with a slope of 0.03 and a y-intercept of 0.05. The spiked samples of nortestosterone gave a percent error of 4.03% at 100 ng/ml with an R.S.D. of 1.26%, and a percent error of 4.84% at 25 ng/ml with an R.S.D. of 2.07% ($n=3$). The linear regression analysis of the calibration curve for testosterone had a correlation coefficient of 0.9999 with a slope of 0.02 and a y-intercept of 0.04. The spiked samples for testosterone showed a percent error of 7.84% at 100 ng/ml with an R.S.D. of 2.78%, and a percent error of 5.64% at 25 ng/ml with an R.S.D. of 0.66% ($n=3$). These results showed that the SFE of the anabolic agents was

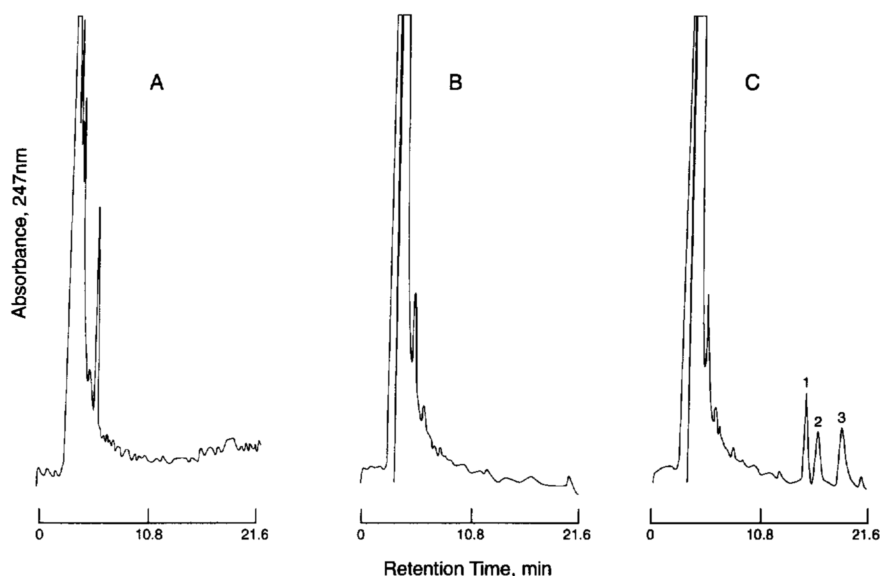


Fig. 4. Typical HPLC chromatograms of (A) blank serum from solid-phase extraction [10], (B) blank serum from SFE and (C) SFE of serum containing 50 ng/ml of nortestosterone (1), testosterone (2) and 100 ng/ml methyltestosterone (3).

linear over the 10–250 ng/ml concentration range typically utilized in the therapeutic drug monitoring of nortestosterone and testosterone.

Table 3 shows the results of the SFE of NSAIDs from spiked water and serum compared to the liquid–liquid extraction method reported by Omile et

Table 3
SFE of NSAIDs from water and serum compared to liquid–liquid extraction from serum

Analyte[a]	Recovery %					
	SFE					Liquid–liquid[b]
	Water		Serum			Serum
	10 min[c]	20 min	10 min	20 min	20 min[d]	
Fenbufen	94.71±1.79[e]	91.84±2.11	54.95±0.65	88.97±2.31	100.57±1.13	–[g]
	(1.89)[f]	(2.30)	(1.18)	(2.59)	(1.12)	
Naproxen	97.58±0.46	97.42±2.13	11.36±0.29	35.80±0.37	100.30±1.06	100.0
	(0.48)	(2.18)	(2.53)	(1.04)	(1.06)	–[h]
Indomethacin	70.66±0.66	66.03±0.28	64.18±1.38	71.74±1.58	75.96±0.96	70.0
	(0.93)	(0.42)	(2.15)	(2.20)	(1.26)	–[h]
Ketoprofen	98.85±0.38	97.14±1.18	56.49±0.25	94.60±0.59	100.67±0.50	100.0
	(0.39)	(1.21)	(0.45)	(0.63)	(0.50)	–[h]
Tolmetin	47.64±0.47	88.09±0.66	13.37±0.59	23.95±0.16	78.43±0.29	–[g]
	(0.98)	(0.74)	(4.40)	(0.68)	(0.37)	

[a] Analyte concentration was 1 μg/ml for SFE; concentration data is unavailable for liquid–liquid extraction.
[b] Ref. [13].
[c] Dynamic extraction time.
[d] 50 μl of 6 *M* HCl added to sample.
[e] Mean±S.D. based on $n=3$.
[f] %R.S.D.
[g] Data not available.
[h] R.S.D. not available.

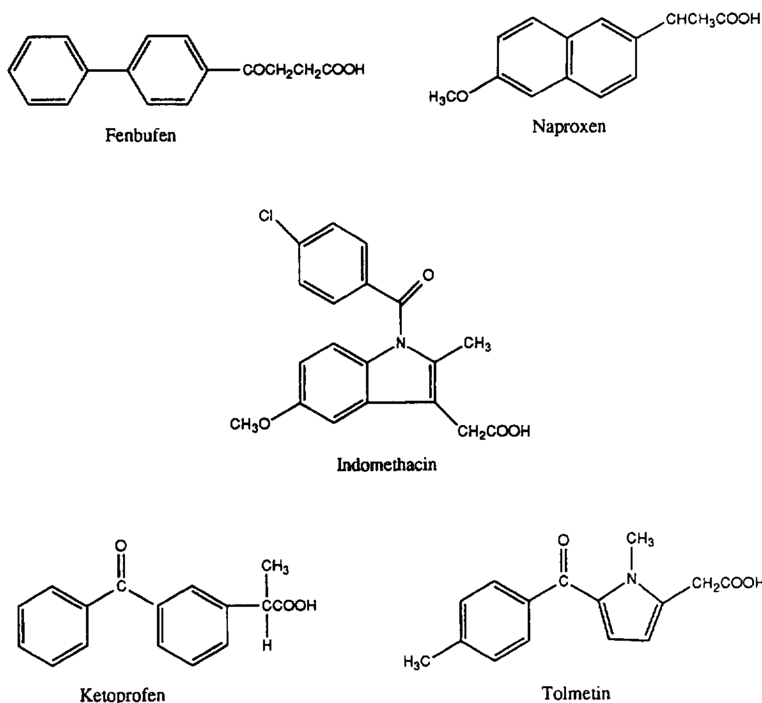


Fig. 5. Chemical structures of NSAIDs studied.

al. [13]. Fig. 5 shows the chemical structures of the NSAIDs analyzed. The SFE results from ketoprofen, fenbufen and indomethacin at 20-min dynamic extraction times were comparable to the liquid–liquid extraction data. However, the recoveries for naproxen and tolmetin for SFE at 20-min dynamic extraction times were much lower compared to liquid–liquid extraction. In order to increase the recoveries of tolmetin and naproxen, 50 μl of 6 *M* HCl was added to each serum sample prior to extraction. The extraction results are summarized in Table 3. All NSAIDs studied showed an increase in extraction efficiency upon the addition of acid. The increase in extraction efficiency was attributed to the unionized form of these drugs being less protein bound and less polar, or less tightly bound to the celite support. All of the NSAIDs studied were reported to be 95–99% protein bound in serum [12]. The data in Table 3 may be explained by the structural variation of these drugs. Naproxen, fenbufen, and ketoprofen have a carboxylic acid moiety upon which their polarity is based. Fenbufen and ketoprofen also have a ketone group, and naproxen has an ether group. Tolmetin contains a tertiary amine which, in the ionized form, may bind more strongly to serum proteins or to the ionized silanol groups on the surface of the celite support causing a decrease in SFE recovery. The amide group of indomethacin may increase the drug's polarity as well as its interactions with serum and/or the celite support causing a decrease in analyte recovery. The other NSAID compounds lacking one or more nitrogens in their structure were extracted quantitatively from serum containing 6 *M* HCl indicating that polarity, serum protein interaction, and celite support interaction are all important factors in determining the extraction efficiency from spiked serum samples. The differences in the SFE data of water vs. serum indicated that serum proteins greatly increased the interactions between NSAIDs and the matrix.

Fig. 6A and B compare the chromatograms of a serum blank prepared by liquid–liquid extraction reported by Omile et al. [13] to a serum blank prepared by SFE with a 20-min dynamic extraction time and containing 50 μl of 6 *M* HCl. The SFE serum blank was comparable to the liquid–liquid

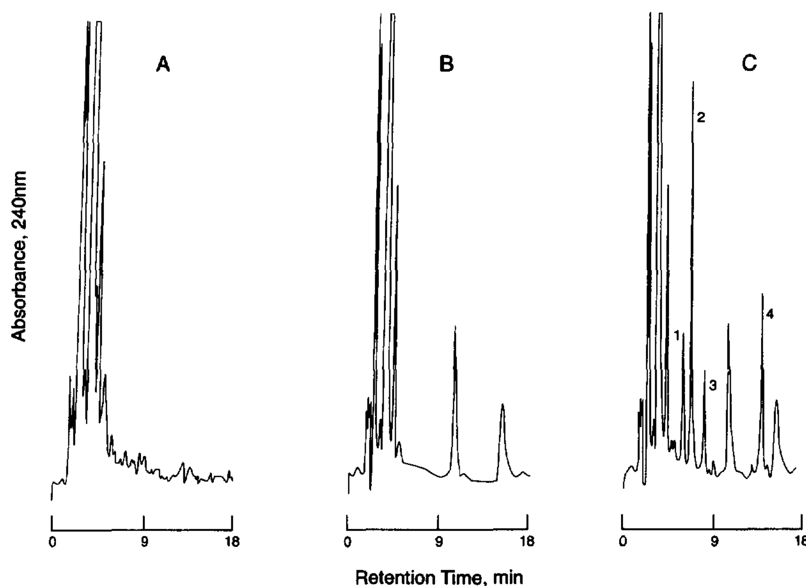


Fig. 6. Typical HPLC chromatograms of (A) blank serum from liquid–liquid extraction [13], (B) blank serum from SFE and (C) SFE of serum containing 1 μg/ml of tolmetin (1), ketoprofen (2), fenbufen (3) and indomethacin (4).

blank. There were no interfering peaks from serum in the SFE blank. A chromatogram of a spiked serum sample prepared by SFE containing tolmetin, ketoprofen, fenbufen and indomethacin at concentrations of 1 μg/ml is also shown in Fig. 6C. The parameters for the extraction were set at 329 MPa and 45°C with 2.5-min static and 20-min dynamic extraction times. A 10-ml aliquot of the HPLC mobile phase served as the solvent trap.

The calibration curve for selected NSAIDs was constructed by plotting ketoprofen and fenbufen at concentrations of 5, 50 and 250 μg/ml with an internal standard, tolmetin, at 50 μg/ml in each sample. The accuracy and precision of the extraction were monitored using spiked serum samples at 25 and 100 μg/ml concentrations. The linear regression analysis of peak height vs. analyte concentration for ketoprofen gave a correlation coefficient of 0.9999 with a slope of 0.16 and a y-intercept of 0.92. The spiked serum samples of ketoprofen gave a percent error of 3.79% at 25 μg/ml with an R.S.D. of 1.03%, and a percent error of 2.97% at 100 μg/ml with an R.S.D. of 0.33% (n=3). The linear regression data of the standard curve for fenbufen gave a correlation coefficient of 0.9999 with a slope of 0.02 and a y-intercept of −0.01. The spiked serum samples gave a percent error of 2.79% at 25 μg/ml with an R.S.D. of 1.27%, and a percent error of 3.34% at 100 μg/ml with an R.S.D. of 0.67% (n=3). These results showed that the SFE of serum samples containing ketoprofen and fenbufen is linear within the reported therapeutic range of 5–250 μg/ml.

In summary, the results of this study present SFE as a viable sample preparation technique for the therapeutic drug monitoring of serum for selected benzodiazepines, anabolic agents and NSAIDs. The HPLC chromatograms of the serum blanks derived from the SFE method and the recovery data were comparable to those derived from existing classical techniques of liquid–liquid or solid-phase extraction with little or no interfering peaks from serum. Also, the precision of the SFE method from serum was shown to be comparable to the precision obtained with the classical preparation techniques.

References

[1] A. Yokobi, J.P. Skelly and V.K. Batra, Toxicokinetics and New Drug Development, Pergamon Press, New York, 1989.

[2] M.L. Lee and K.E. Markides (Editors), Analytical Supercritical Fluid Chromatography and Extraction, Chromatography Conferences, Inc., Provo, UT, 1990.

[3] J.K. Lawrence, A.K. Larson, Jr. and I.R. Lebbett, Anal. Chim. Acta, 288 (1994) 123.
[4] M.T. Tena, M.D. Luque-de-Castro and M. Valcarcel, Chromatographia, 40 (1995) 197.
[5] A.L. Howard, M.C. Shah, M.A. Brooks, J.T. Strode III and L.T. Taylor, J. Pharm. Sci., 83 (1994) 1537.
[6] D.P. Ndiomu and C.F. Simpson, Anal. Chim. Acta, 213 (1988) 237.
[7] H. Liu and K.R. Wehmeyer, J. Chromatogr., 577 (1992) 61.
[8] H. Liu and K.R. Wehmeyer, J. Chromatogr. B, 657 (1994) 206.
[9] N. Ratnaraj, V.D. Goldberg, A. Elyes and P.T. Lascelles, Analyst, 106 (1981) 1001.
[10] B.L. Lampert and J.T. Stewart, J. Liq. Chromatogr., 12 (1989) 3231.
[11] B. Levine and Y.H. Caplan, Clin. Chem., 31 (1985) 346.
[12] A.C. Moffat, J.V. Jackson, M.S. Moss and B. Widdop (Editors), Clark's Isolation and Identification of Drugs, The Pharmaceutical Press, London, 2nd ed., 1986.
[13] C.I. Omile, I.R. Lebbett and B. Danech, Chromatographia, 22 (1986) 187.