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Solid-phase extraction with supercritical fluid elution as a sample preparation technique for the ultratrace analysis of flavone in blood plasma

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ABSTRACT

A new sample preparation technique, solid-phase extraction with supercritical fluid elution, was developed for the selective isolation of ultratrace levels of drugs from plasma. Plasma samples spiked with a drug were applied to octadecylsilane cartridges and the cartridges were then washed, briefly dried and directly fitted into cells for subsequent supercritical fluid elution. The absolute recovery was studied by using a radiolabeled model compound. The extraction selectivity was examined by chromatographing the extracts with a reversed-phase high-performance liquid chromatographic method with ultraviolet detection. The effects of extraction pressure and the length of capillary restrictors on drug recovery were examined in order to determine the optimal conditions for supercritical fluid elution. The performance of the method was compared to that of conventional solid-phase extraction in terms of recovery, selectivity, precision and accuracy of analysis. Flavone was used as the model compound and dog plasma as the biological matrix for these studies.

INTRODUCTION

Solid-phase extraction (SPE) cartridges have been increasingly used to prepare samples for the ultratrace analysis of drugs and metabolites in biological fluids [1–5]. The procedure is required not only for sample clean-up, but also for concentration of analytes in the sample prior to chromatographic analysis. The SPE cartridges are typically plastic tubes packed with stationary phase particles, most commonly octadecylsilane (ODS). Sample preparation based on SPE usually involves five steps: (i) loading samples onto the cartridges; (ii) washing the cartridges with proper solutions to remove interfering components; (iii) eluting the adsorbed analytes from the cartridges with an eluent; (iv) taking the collected effluent to dryness; and (v) redissolving the residue in a proper solvent prior to chromatographic analysis. Although this procedure is widely used and largely successful, step iv can be very time-consuming depending on the percentage of water in the eluent. Additionally, SPE normally involves the use of a considerable amount of organic solvents, which results in significant purchase and disposal costs.

Recently, we have demonstrated that supercritical fluids (SF) can be used as an alternative eluent for SPE [6]. The methodology was referred to as SPE with SF elution (SPE–SFE). Specifically, once a sample is loaded, the ODS packing material inside the SPE cartridge is transferred to an SF extraction cell. The analyte (a tertiary amine drug metabolite) was then quantitatively eluted from the ODS packing material with SF CO_2 with modifiers. The extracts were analyzed in an interference-free manner by gas chromatography–mass spectrometry. Some of the potential advantages of the approach include rapid removal of the effluent, minimum use of organic sol-

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vents and potentially different selectivities relative to traditional SPE.

In this paper we report our efforts to further develop the approach into a more routine sample preparation technique for the ultratrace analysis of drugs and metabolites in biological fluids. Flavone (2-phenyl-4*H*-1-benzopyran-4-one) was chosen as a model neutral compound and dog plasma as the sample matrix. Flavone is a member of the flavonoids, a group of naturally occurring compounds [7,8]. Analogues of flavone have been used for treating tumors [9]. Plasma samples spiked with ultratrace levels of flavone were loaded onto ODS cartridges. The cartridges were then briefly vacuum-dried and directly fitted into SFE cells for subsequent elution with SF. The extraction recovery was studied using ^{14}C-labeled flavone. The selectivity, precision and accuracy of SPE–SFE relative to conventional SPE was examined using reversed-phase high-performance liquid chromatography (HPLC) with UV detection. The effects of restrictor length, extraction pressure and extraction time on the SPE–SFE procedure were also examined.

EXPERIMENTAL

Chemicals

Distilled, deionized water was from a Barnstead NANOpure II system (Dubuque, IA, USA). Methanol (HPLC grade) was from J. T. Baker (Phillipsburg, NJ, USA). Flavone, [^{14}C]flavone and 2′-methylflavone (2′-mF) were synthesized at Procter & Gamble, Miami Valley Laboratories. Dog plasma was obtained from Pel-Freez (Rogers, AR, USA). SFC-grade CO_2 with 5% methanol was purchased from Scott Specialty Gases (Plumsteadville, PA, USA). ODS cartridges (1.0 ml size) packed with 100 mg of packing material were obtained from J. T. Baker.

SFE–SPE recovery studies

Cumulative recovery. The cumulative percentage recovery of [^{14}C]flavone was measured against extraction time at various pressures and using different lengths of capillary restrictors. Blank dog plasma was spiked with [^{14}C]flavone (50 ng/ml). The plasma sample (1.0 ml) was then applied to an ODS cartridge which had been conditioned with 2.0 ml of methanol followed by 2.0 ml of water–methanol (95:5, v/v). The cartridge was then washed with 2.0 ml of water–methanol (95:5, v/v). The outside of the cartridge was washed with acetone to remove ink and other materials. It was then vacuum-dried (5 min) using a Baker-10 SPE system (J. T. Baker) equipped with a vacuum pump. The cartridge was fitted into a 3.47-ml Keystone SFE cell (Keystone Scientific, Bellefonte, PA, USA) as shown schematically in Fig. 1. A fused-silica capillary with a 25 μm I.D. (Valco Instruments, Houston, TX, USA), either 25 cm or 50 cm long, was used as the restrictor. The cartridge was then eluted with CO_2–5% methanol at fixed pressures (100, 150 and 200 bar) and 50°C using a Dionex SFE-703 instrument (Dionex, Salt Lake City, UT, USA). The percentage recovery was measured against extraction time by continuously collecting the extracted [^{14}C]flavone into a series of test-tubes (100 mm × 16 mm) containing 2.0 ml of methanol using 2-min collection intervals. The [^{14}C]flavone collected in each tube was

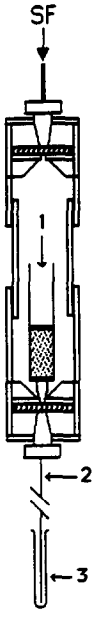

Fig. 1. Schematic diagram of SPE–SFE cell: 1 = SPE cartridge; 2 = capillary restrictor; 3 = extract collection test-tube.

counted on a Packard Model 2000CA liquid scintillation analyzer (Packard Tri-Carb, Downers Grove, IL, USA). The cumulative extraction recovery was obtained by summing up the radioactivities of the methanol solutions and comparing it to that of an equivalent standard solution.

Absolute recovery. The percentage recoveries of [^{14}C]flavone at different spiked plasma concentrations (10, 50 and 250 ng/ml) were measured using the same procedures as described above, except only one test-tube was used to collect the whole extraction. In some instances, the SPE–SFE extracts were collected by "dry-collection" without using any methanol. The following SFE conditions were used: 150 bar, 50°C and 10 min extraction time with a 25-cm restrictor. The flow-rate of expanded gases was *ca.* 80 ml/min under these conditions.

SPE–SFE selectivity, accuracy and precision studies

Blank dog plasma and blank dog plasma spiked with 50 ng/ml internal standard (2′-mF) and flavone (10, 50 and 250 ng/ml) were processed the same way as described for the absolute recovery study above. After completion of the extractions, the methanol solutions were blown to dryness under nitrogen. The residues were redissolved in 0.25 ml of the mobile phase (methanol–water, 65:35, v/v). The reconstituted samples were analyzed by HPLC–UV as described below.

Solid–phase extraction

Absolute recovery. Blank dog plasma was spiked with [^{14}C]flavone (10, 50 and 250 ng/ml). The plasma sample (1.0 ml) was then applied to an ODS cartridge which had been conditioned with 2.0 ml of methanol followed by 2.0 ml of water–methanol (95:5, v/v). The cartridge was washed with 2.0 ml of water–methanol (95:5, v/v). The cartridge was then eluted with 2.0 ml of methanol–water (85:15, v/v). The eluent was collected and the absolute recovery of the [^{14}C]flavone was calculated as described for SPE–SFE.

Selectivity, accuracy and precision studies. Blank dog plasma and blank dog plasma spiked with flavone (10, 50 and 250 ng/ml) and internal standard (50 ng/ml) were prepared. The plasma samples (1.0 ml) were then processed as described above. The collected eluent was then taken to dryness under a stream of nitrogen and redissolved in 0.25 ml of the mobile phase. The reconstituted sample was analyzed by HPLC–UV as described below.

Preparation of standards

Flavone standard solutions covering the concentration range from 5 to 500 ng/ml were prepared in the mobile phase. The standards were aliquoted (1.0 ml) into test-tubes containing 50 ng of 2′-mF.

HPLC analysis

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) UVIS 200 detector and a Hewlett-Packard 3390A integrator. The chromatograms were collected using a PE Nelson (Cupertino, CA, USA) data system (Turbochrom). The column was a Waters (Milford, MA, USA) Nova-Pak C_{18} (150 mm × 3.9 mm I.D. 4 μm particle size). The mobile phase was methanol–water (65:35, v/v) and the flow-rate was 0.75 ml/min. An injection volume of 50 μl was used. The wavelength of the detector was set at 300 nm.

Standard curves were obtained by plotting the peak-area ratio (peak area flavone/peak area 2′-mF) *versus* the concentration of flavone. The concentration of flavone in plasma samples was determined from the peak-area ratio of the sample by interpolation from the linear regression curve.

RESULTS AND DISCUSSION

An ideal sample preparation methodology would be rapid, give quantitative recovery of the analyte(s) of interest and selectively isolate the analyte(s) from interfering endogenous components. A short sample preparation time increases sample throughput, while a high analyte extrac-

tion recovery improves the limit of detection and precision of the analysis. A high degree of selectivity in the sample preparation step reduces the resolution requirements of the subsequent chromatographic system.

Optimization of extraction recovery

The study was designed to determine the optimum conditions for maximizing extraction recovery while keeping the extraction time short. The percentage recovery of [^{14}C]flavone was measured against extraction time at three different extraction pressures (100, 150 and 200 bar) using two different lengths of restrictors (25 and 50 cm) with a constant temperature of 50°C.

The cumulative percentage recovery of [^{14}C]flavone *versus* extraction time for the 50-cm restrictor at the three different extraction pressures is shown in Fig. 2. The extraction time required for quantitative recovery was *ca.* 8, 16 and 40 min for extraction pressures of 200, 150 and 100 bar, respectively. The increase in extraction time with decreasing pressure was caused by two factors. First, decreasing pressure slows down the flow-rate of SF and consequently it takes a longer time to pass the same mass of SF through the cartridge. Secondly, decreasing pressure causes the density or the solvating power of the SF to decrease. As a result, the distribution coefficient of flavone on the ODS packing increases, which means that it takes a longer time to elute flavone off the packing material.

The cumulative percentage recovery of [^{14}C]flavone *versus* extraction time for the 25-cm restrictor is shown in Fig. 3. The extraction time required for quantitative recovery was *ca.* 5, 8 and 20 min for extraction pressures of 200, 150 and 100 bar, respectively. The extraction time required with the 25-cm restrictor was shorter than for the 50-cm restrictor at all extraction pressure. Since the distribution coefficient of flavone for a fixed pressure and temperature is constant, the shorter extraction times for the 25-cm restrictor was due to the increase in the flow-rate of SF relative to the 50-cm restrictor. These results suggest that a high extraction pressure and a short restrictor are desirable for optimization of extraction time and recovery. However, increasing the extraction pressure may result in increased extraction of potentially interfering matrix components. Also care must be exercised in the analyte trapping technique used when short restric-

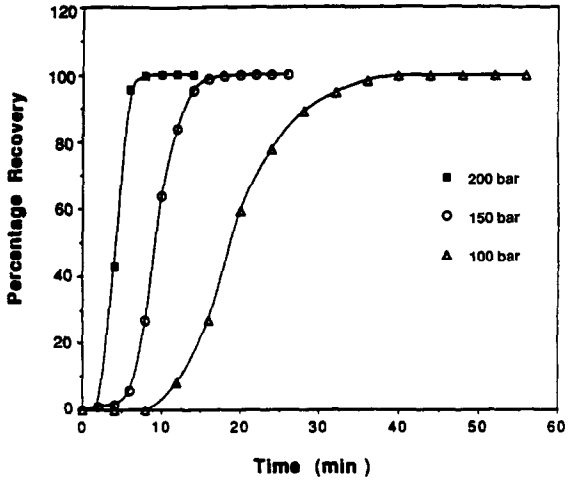

Fig. 2. SPE–SFE cumulative recovery of [^{14}C]flavone from plasma sample applied to ODS cartridges and extracted with CO_2–5% methanol *versus* extraction time. Restrictor length, 50 cm.

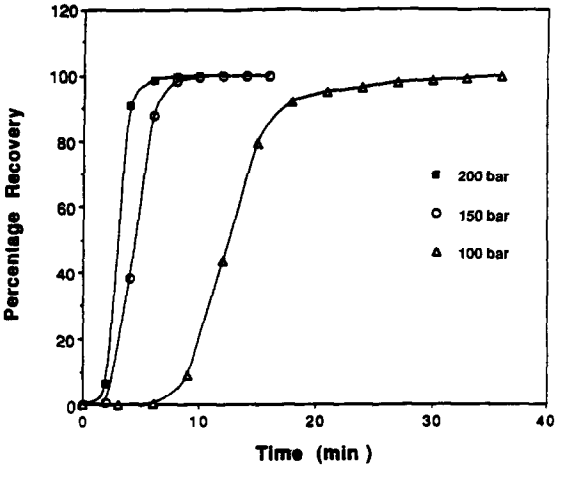

Fig. 3. SPE–SFE cumulative recovery of [^{14}C]flavone from plasma sample applied to ODS cartridges and extracted with CO_2–5% methanol *versus* extraction time. Restrictor length, 25 cm.

tors are employed. The high flow-rate of expanded gas resulting from the use of short restrictors may cause inefficient trapping of the analyte.

The conditions chosen as optimal for the remainder of the SPE–SFE work were: a 150-bar extraction pressure, a 50°C extraction temperature, a 25-cm-long restrictor and a 10-min extraction time. These conditions were chosen based on the fact that quantitative recovery of the flavone was obtained with a relatively low pressure (150 bar) and in a reasonably short time (8 min).

Absolute recoveryt of flavone under optimal conditions

The recovery of [^{14}C]flavone from spiked plasma samples under the optimal conditions by SPE–SFE and SPE was examined at three different flavone concentrations (10, 50 and 250 ng/ml). The recovery of the [^{14}C]flavone from plasma by both SPE–SFE and SPE is shown in Table I. Quantitative recovery of [^{14}C]flavone was obtained by both techniques across the concentration range examined. The relative standard deviations for the SPE–SFE procedure were somewhat higher than those obtained by SPE. For SPE–SFE, experimental results showed that the extracts could be effectively collected by "dry-collection" without using any methanol.

Extraction selectivity

The selectivity of the SPE–SFE and conventional SPE sample preparation procedures was compared using reversed-phase HPLC. The HPLC eluent was monitored at 300 nm which is the maximum absorption wavelength for flavone. Typical chromatograms obtained by SPE–SFE extraction of blank ODS cartridges, ODS cartridges spiked with blank dog plasma and ODS cartridges spiked with dog plasma containing 10 ng/ml flavone and 50 ng/ml internal standard (2'-mF) are shown in Fig. 4. The corresponding chromatograms for extraction by traditional SPE are shown in Fig. 5. Interference-free detection of both flavone and 2'-mF was obtained by both extraction procedures. However, some differences in the extraction characteristics of the two techniques can be seen. First, the extraction of the blank ODS cartridges by SPE–SFE resulted in the extraction of a component with a retention time of 15.5 min, while the SPE procedure did not extract this component. Also, the SPE–SFE procedure extracts more materials from the blank cartridges that elute from the HPLC column in the 1–3 min time range than SPE. Transferring the ODS packing directly into the SFE cell before extraction gave similar profiles to

TABLE I

EXTRACTION RECOVERY OF [^{14}C]FLAVONE

Concentration (ng/ml)	Recovery (mean ± S.D., n = 3) (%)	
	SPE–SFE	SPE
10	94.5 ± 4.5	100.8 ± 1.0
50	96.1 ± 1.8	99.0 ± 0.1
250	88.9 ± 6.1	91.3 ± 1.1

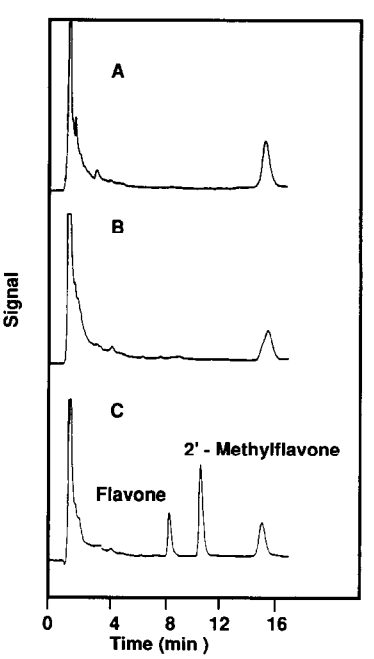

Fig. 4. HPLC of SPE–SFE extracts of plasma samples. (A) Blank ODS cartridge; (B) ODS cartridge loaded with blank plasma; (C) ODS cartridges loaded with plasma spiked with 10 ng/ml flavone and 50 ng/ml 2'-methylflavone.

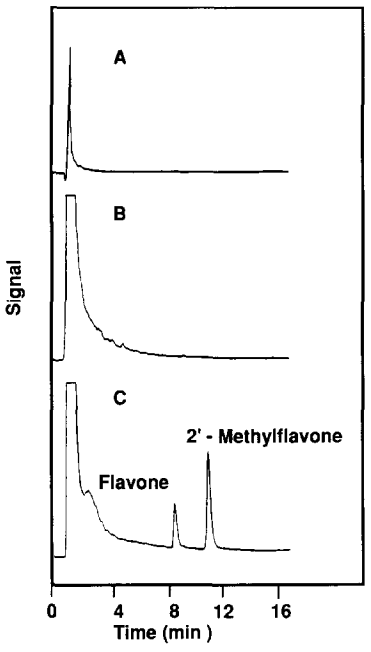

Fig. 5. HPLC of SPE extracts of plasma samples. (A) Blank ODS cartridge; (B) ODS cartridge loaded with blank plasma; (C) ODS cartridges loaded with plasma spiked with 10 ng/ml flavone and 50 ng/ml 2'-methylflavone.

those obtained by traditional SPE. Apparently, the SF (CO_2–5% methanol) extracts some materials from the plastic cartridges. Use of glass or PTFE-lined tubes for SPE–SFE would eliminate this problem. Secondly, when plasma samples were extracted the SPE procedure gave much broader initial peak(s) in the retention time range from 1 to 4 min relative to the SPE–SFE procedure. Apparently, the SPE eluent (methanol–water, 85:15) is stronger than the SF (5% methanol–CO_2) and eluted more polar substances from the plasma matrix.

Accuracy and precision of SPE–SFE and SPE

The applicability of SPE–SFE as a sample preparation technique for ultratrace analysis was compared to traditional SPE by analyzing dog plasma samples spiked with flavone (10, 50 and 250 ng/ml). The spiked samples were processed by both techniques in parallel and then analyzed by HPLC. The results of the HPLC analysis following SPE–SFE and traditional SPE sample preparation are shown in Table II. Accuracy for SPE–SFE and SPE ranged from 101.0 to 108.8% and 100.5 to 104.0%, respectively. The precision of both methods was good with R.S.D. values ranging from 1.0 to 3.0% and 1.0 to 2.0% for SPE–SFE and SPE, respectively.

CONCLUSION

We have demonstrated that an SF can be used as an effective alternative eluent for SPE of a neutral compound from ODS cartridges. The performance of an SF is comparable to that of traditional aqueous–organic solvents used for SPE in terms of absolute recovery, selectivity, precision and accuracy. However, an advantage of using an SF in contrast to using an aqueous–organic eluent is the easier removal of eluent after the extraction. Also, since the solvating power of an SF is related to its density, it is potentially possible to adjust the selectivity of the SPE–SFE through controlling the pressure and temperature of the extraction.

TABLE II

ANALYSIS OF FLAVONE IN PLASMA (n = 3)

Concentration spiked (ng/ml)	SPE–SFE		SPE	
	Concentration found (mean ± S.D.) (ng/ml)	Recovery (mean ± R.S.D.) (%)	Concentration found (mean ± S.D.) (ng/ml)	Recovery (mean ± R.SD.) (%)
10	10.1 ± 0.3	101.0 ± 3.0	10.4 ± 0.1	104.0 ± 1.0
50	54.4 ± 0.5	108.8 ± 1.0	51.3 ± 0.3	102.6 ± 1.0
250	255.6 ± 4.5	102.4 ± 1.8	251.3 ± 5.2	100.5 ± 2.0

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