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Supercritical fluid extraction method development for extraction of an experimental HIV protease inhibitor drug from animal feed

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Abstract

Supercritical fluid extraction (SFE) was evaluated as a sample preparation procedure for the recovery of experimental drugs from animal feed preparations that are generated during long-term toxicology studies. A commercially available supercritical fluid extractor was utilized to develop and validate an off-line procedure for the recovery of an experimental HIV protease inhibitor drug from animal feed. Extracts were analyzed with a conventional reversed-phase HPLC method. Elements of the SFE method developed that are described include optimization of the system temperature and selection of the extraction media modifier. The study emphasized the performance of two-day precision and accuracy studies. Precision and accuracy studies were carried out with SC-52151 levels of 0.05, 0.1 and 1.0% (w/w) and used an internal standard quantitation format. Also, the study utilized a relatively large analytical scale extraction vessel size of 10 ml to accomodate 6 g animal feed samples.

Keywords: Supercritical fluid extraction; Animal feed; Experimental drug; HIV protease inhibitor

1. Introduction

Toxicological studies for experimental drugs are often performed with animal feed spiked with known quantities of the compound of interest. Since good laboratory practices (GLPs) require that the concentrations and stability of the experimental drugs in the animal feed sample be determined as the toxicological study is being performed, the determination of experimental drug levels in animal feed samples is a commonly encountered problem in the pharmaceutical industry laboratory. Owing to the chemical complexity of animal feed matrices, the quantitative recovery of drug substances from animal feed can be problematic. A variety of classical sample preparation approaches have been utilized, including liquid–solid and liquid–liquid extraction and semi-preparative liquid chromatographic clean-up procedures [1].

Studies, which evaluated the use of supercritical fluid extraction (SFE) as a sample procedure for the recovery of the synthetic prostaglandin misoprostol from drug formulation materials were re-

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ported previously [2,3]. In a continuing effort to evaluate SFE as a sample preparation procedure for pharmaceutical development applications, off-line SFE has now been applied to the determination of SC-52151 (Fig. 1) in animal feed samples. SC-52151 is an experimental HIV protease inhibitor drug that has recently undergone clinical trials. The intent of this study was to evaluate SFE as a sample preparation procedure for animal feed samples by attempting to develop and validate an off-line SFE procedure which could be used to perform analyses of animal feed samples from GLP toxicology studies for SC-52151. Extracts were analyzed using conventional reversed-phase HPLC conditions. Three previous reports have summarized results pertaining to the use of SFE for the recovery of drugs and/or vitamins from animal feed [4–6] and have been summarized previously [7]. Most recently Messer et al. [6] described the use of SFE for the recovery of atovaquone from animal feed. The atovaquone study evaluated the recovery of the drug with SFE using 250 and 500 mg animal feed samples with drug concentrations ranging from 0.3 to 1%. This study emphasized the performance of two-day precision and accuracy studies, which were implemented to demonstrate that the SFE–HPLC procedure is suitable for supporting GLP toxicology studies conducted at Searle. The precision and accuracy studies were carried out with SC-52151 levels of 0.05, 0.1 and 1.0% (w/w) and used an internal standard quantitation format. Also, the study utilized a relatively large analytical scale extraction vessel size of 10 ml to accomodate 6 g animal feed samples.

2. Experimental

2.1. Materials and reagents

Samples of SC-52151 were prepared by the Chemical Sciences Department of Searle Research and Development. The HPLC assay value for the SC-52151 lot used in the current study was > 98%. Benzophenone and sodium 1-pentanesulfonate salt were purchased from Aldrich Chemical. Ottawa sand was purchased from Fischer Scientific. All HPLC mobile phase components were of reagent grade and used as purchased. All SFE experiments were completed with supercritical fluid-grade carbon dioxide purchasd from Scott Specialty Gases. Modifiers for SFE experiments were HPLC-grade solvents. Purina rodent chow (No. 5002) was used for all experiments.

2.2. SFE experiments

All SFE experiments were completed with a Dionex Model 703 supercritical fluid extractor equipped with a Model 703M co-solvent addition module. Extractions were completed as single-channel, single-cell extractions. Experiments were performed with extraction cells purchased from Dionex. The SFE conditions for rodent chow analyses are listed in Table 1. "Synthetic" spiked rodent chow samples were made by adding 4 g of rodent chow, SC-52151 and the internal standard (benzophenone) to the cell followed by manual shaking of the capped cell. After shaking, an additional 2 g of rodent chow was added to the cell followed by additional shaking and installation in the Dionex 703 extractor. Amounts of 3, 6 and 60 mg of SC-52151 were added to make 0.05, 0.1 and 1% (w/w) spiked rodent chow samples, respectively. Amounts of 2, 4 and 40 mg of internal standard were added to the 0.05, 0.1 and 1% (w/w) spiked samples, respectively. The internal standard (benzophenone) level was maintained at 67% of the SC-52151 concentration to approximate a 1:1 chromatographic response for analyte and internal standard. After completion of the SFE experiment, the ethanol collection solvent was transferred into a volumetric flask and diluted

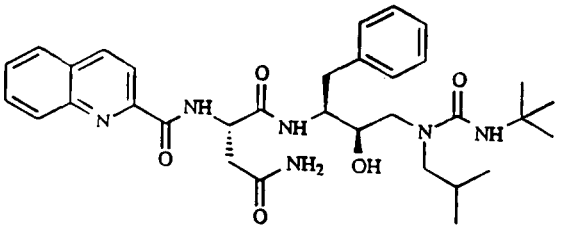

Fig. 1. Structure of SC-52151.

Table 1
SFE Conditions for SC-52151 extraction

Instrument	Dionex Model 703
Extractant	Carbon dioxide containing 10% ethanol
Cell volume	10 ml
Pressure	330 atm
Oven temperature	45°C
Restrictor	250 mL min^{-1}
Restrictor temperature	50°C
Vial temperature	0°C
Collection solvent	13 ml of ethanol
Sample quantity	ca. 6 g of animal feed
Extraction time	60 min

to volume with ethanol. The collection solvent from the 0.5 and 0.1% (w/w) samples were transferred into a 50 ml volumetric flask and the collection solvent from the 1% (w/w) samples were transferred into a 100 ml volumetric flask. A portion of the enthanol solution containing the extractants was transferred into an autosampler vial for injection into the HPLC system.

$$\frac{\text{SC-52151 conc.}_{\text{known}}/\text{benzophenone conc.}_{\text{known}}}{\text{SC-521451 area}_{\text{known}}/\text{benzophenone area}_{\text{known}}} = \frac{\text{SC-52151 conc.}_{\text{unknown}}/\text{benzophenone conc.}_{\text{unknown}}}{\text{SC-521451 area}_{\text{unknown}}/\text{benzophenone area}_{\text{unknown}}}$$

2.3. HPLC system

All HPLC experiments were performed with a system consisting of a Hewlett-Packard Model 1090 liquid chromatograph equipped with a Kratos Model 783 variable-wavelength detector. Reversed-phase HPLC conditions were as follows: column, Supelco C8 DB (250 mm × 4.6 mm i.d.; 5 μm particle size); mobile phase flow-rate, 1.0 ml min^{-1}; injection volume, 10 μl and detection wavelength, 205 nm. Each liter of aqueous mobile phase A contained 6.9 g of sodium 1-pentanesulfonate and 1 ml of 85% phosphoric acid. Mobile phase B consisted of 62% (v/v) methanol and 38% mobile phase A. The mobile phase program was as follows: 0–25 min, 100% A; 25–40 min, 100% B; and 40–50 min, 100% A.

Standard concentrations for direct injection into the liquid chromatograph were the concentrations for a sample with 100% recovery. For example, the SC-52151 concentrations for the 0.5, 0.1 and 1% (w/w) level analyses were 0.6, 0.12 and 0.60 mg mg^{-1}, respectively. Internal standard quantitation was based on the following expression:

Statistical calculations were performed with Minitab release 6.1.1, VAX/VMS version. Chromatographic integration and data management were completed with the TURBOCHROM 3 Multitasking Data System.

3. Results and discussion

3.1. SFE conditions

The SFE conditions that were used for the determination of SC-52151 in animal feed are shown in Table 1. Several SFE parameters were studied during the SFE method development, including extraction media modifier, collection solvent, extraction duration and extraction cell temperature. Preliminary experiments focused on the selection of an effective carbon dioxide modifier and collection solvent for the recovery of SC-52151. To minimize sample complexity, SC-52151 was suspended in Ottawa sand in the SFE cell for initial experiments. Also, preliminary experiments were implemented with a 0.5 ml cell

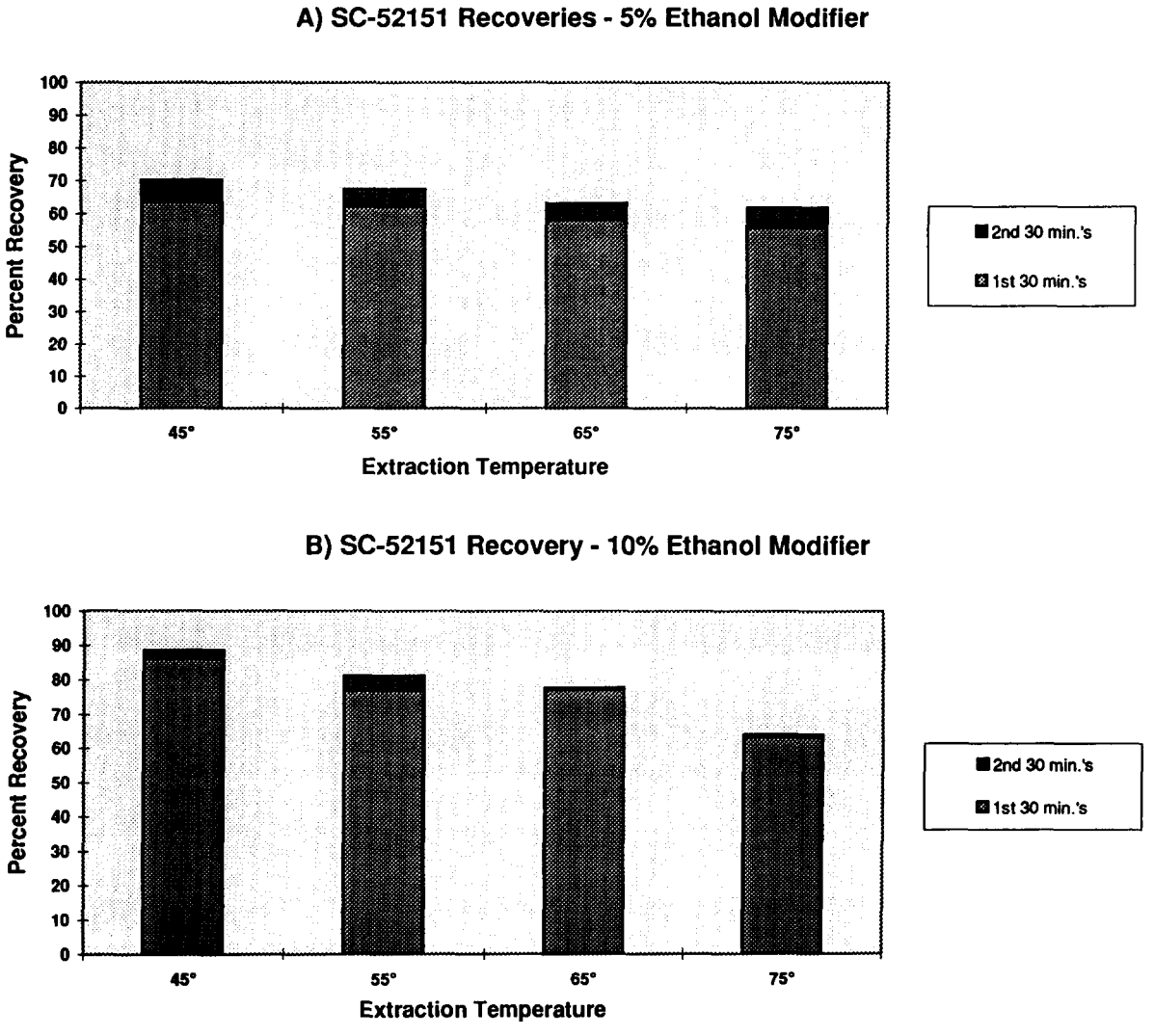

Fig. 2. Extraction recoveries for SC-52151 from animal feed spiked at the 0.05% level.

with 1 mg of sample. SC-52151 was known to have a poor aqueous solubility and a relatively high solubility in ethanol. It was observed that the use of 10% ethanol as the carbon dioxide modifier and the use of ethanol as a collection solvent during a 10 min extraction yielded recoveries over 98% when the sample was suspended in Ottawa sand. Substitution of methanol as a modifier of as collection solvent lowered the recoveries to < 30%.

Additional method development results are shown in Fig. 2. The experiments summarized in Fig. 2 were completed with a 10 ml extraction cell using 6 g of sample. Fig. 2A shows the cumulative recovery of SC-52151 from animal feed samples spiked at the 0.05% level during two successive 30 min extractions using 5% ethanol as a modifier. Extractions were completed at four temperatures ranging from 45 to 75°C. The recoveries ranged from 61.8 to 70.1% and decreased as the temperature increased. Fig. 2B summarizes the results of extractions under the same conditions as in Fig.

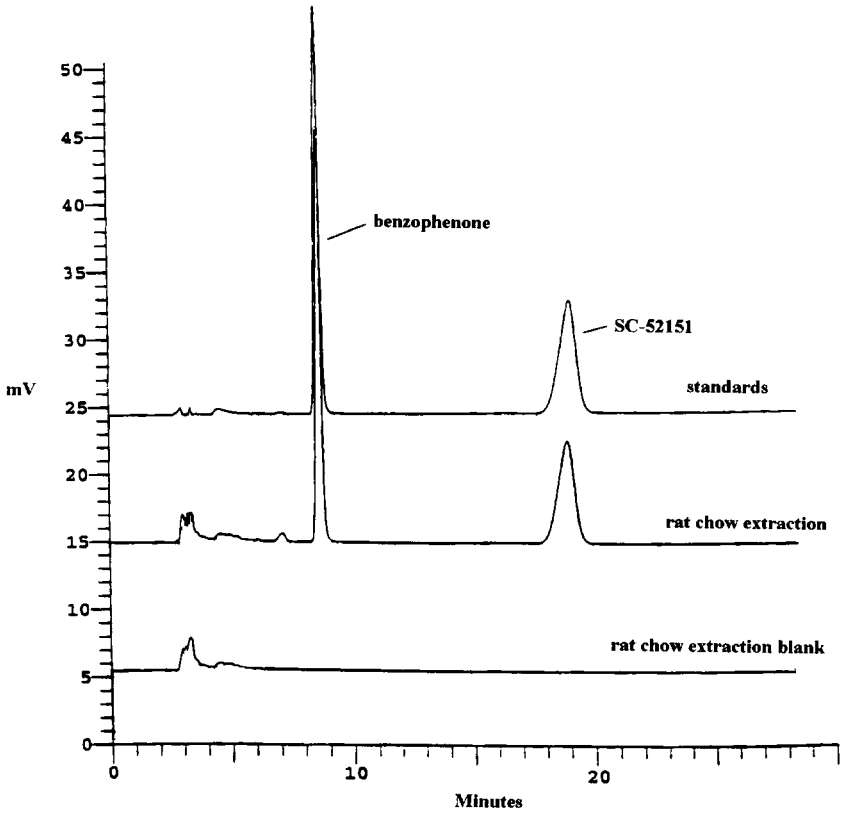

Fig. 3. Reversed-phase HPLC traces of SFE animal feed extracts. Chromatographic conditions: column, Supelco C8 DB (250 mm × 4.6 m i.d.; 5 μm particle size); mobile phase flow-rate, 1.0 ml min^{-1}; injection volume, 10 μl; detection wavelength, 205 nm; mobile phase, 0.04 M sodium pentanesulfonate.

2A except that the ethanol modifier concentration was increased to 10%. The recoveries ranged from 64.3 to 88.5% and were highest at 45°C. The experiments summarized in Fig. 2 supported the use of 10% ethanol as a modifier and an extraction temperature of 45°C. They also supported the use of a 60 min extraction period since an additional 2–3% of the analyte were recovered during the second 30 min of extraction at 45°C with 10% ethanol modifier. Additional important elements of the SFE conditions summarized in Table 1 are the cell size and sample size. The intent was to use a large enough cell volume to accomodate 5–6 g of animal feed, which is a typical animal feed sample size in the authors department. Accomodating 5–6 g of feed required the use of a 10 ml cell. All extractions were carried out as single-channel extractions since it was found that simulationeous extractions with a relatively large cell volume and quantities yielded poor precision and apparent cell-to-cell sample contamination.

Representative chromatograms of SFE extracts obtained under the conditions in Table 1 for animal feed and animal feed spiked with SC-52151 are shown in Fig. 3. The spiking level for SC-52151 was 0.05%. Comparison of the chromatograms reveals that coextraction of animal feed components and SC-52151 did occur; however, the reversed-phase chromatographic conditions resolved SC-52151 from the coextractants. An additional component of the extract was benzophenone, which was used as an internal standard for the determination of SC-52151 in the

Table 2
SC-52151 and benzophenone recoveries from animal feed

Day	Target SC-52151 level in animal feed (%)	Benzophenone recovery (%)[a]	SC-52151 recovery (%)[a]	
			External standard method	Internal standard method
1	1	86.7	86.1	99.3
2	1	83.2	81.5	97.9
1	0.10	86.5	85.2	98.6
2	0.10	85.9	84.2	98.1
1	0.05	88.1	86.3	98.0
2	0.05	84.2	84.2	100.0

[a] Mean values for three determinations.

animal feed extracts. The reversed-phase HPLC conditions that were utilized for the analysis of the SFE extracts were optimized to provide resolution of SC-52151 and benzophenone while providing symmetrical peak shapes and a relatively short run time. It should be noted that an additional peak was evident in the SC-52151 sample extract at approximately the 1% level (normalized) and with a retention time of 7 min. A trace component with the same retention was also recovered from the blank rat chow extract. Two possible effects may have caused the appearance of an additional peak in the SC-52151 extract: (1) slight degradation of SC-52151 during the sample mixing or SFE procedure or (2) enhanced extraction of rat chow component due to the presence of SC-52151 in the sample.

3.2. Internal standard

Since all of the method development experiments generated recoveries of <90% for SC-52151, an internal standard quantitation format was adopted for the SFE-HPLC procedure. Several compounds were considered as internal standards and benzophenone was selected as it is stable, inexpensive and commercialy available in high purity and, more important, it generated chromatographic figures of merit that were similar to those observed for SC-52151 with the reversed-phase chromatographic conditions that were being utilized for the analysis of the animal feed extracts (see Fig. 3). An additional important factor was that the recoveries for SC-52151 and benzophenone were closely correlated over an extended concentration/spiking range. Table 2 (columns 3 and 4) shows the recoveries of SC-52151 and benzophenone recorded on different days at concentrations of 0.05, 0.1 and 1% (w/w). The values in Table 2 were generated versus an external standard. The external standard recovery ranges for SC-52151 and benzophenone were 81.5–86.3% and 83.2–88.1%, respectively. The recovery values for SC-52151 and benzophenone had a correlation coefficient of 0.9149. Table 2 also summarizes (column 5) the recovery levels for SC-52151 that were generated using an internal standard rather than an external standard. The recovery increases considerably, with the range of values increasing from 81.5–86.3% to 97.9–100.0%. It should be noted that the quantity of benzophenone added to the sample was 67% of the level of SC-52151 so that the ratio of chromatographic area responses for analyte and internal standard approximated 1:1.

3.3. Validation studies

Validation studies for the animal feed SFE–HPLC procedure characterized the linearity of response for SC-52151 and benzophenone under the HPLC conditions that were used for the extract analysis, the accuracy of recovery values and the within- and between-day precision for tripli-

Table 3
Regression analysis for SC-52151 and internal standard HPLC responses

Target level in animal feed (%)		Range evaluated[a] ($\times 10^{-2}$) (mg ml^{-1})	Intercept ($\times 10^{3}$)	Slope ($\times 10^{6}$)	Correlation coefficient	Range of recoveries (%)[b]
0.05	SC-52151	1.5–7.5	−2.62	3.496	0.99993	99.4–100.6
	Benzophenone	0.99–4.9	0.42	5.463	0.99987	99.4–101.0
0.10	SC-52151	3.2–15.7	−2.34	3.445	0.99997	98.8–100.5
	Benzophenone	2.0–9.9	1.16	5.336	0.99998	99.5–100.4
1	SC-52151	15–75	14.12	3.363	0.99996	99.5–100.7
	Benzophenone	9.9–49.4	63.68	4.896	0.99967	96.2–101.6

[a] Represents five standards with 25–125% of target concentrations.
[b] Recovery for back-calculated concentrations.

cate analyses at the three different levels. Initial validation experiments assessed the linearity of response for SC-52151 and benzophenone over a concentration range of 25–125% of the target concentrations in the accuracy study. Regression analyses of the data (Table 3) revealed that the system yielded a linear response for each of the analyte and internal standard concentration ranges which "bracketed" the 100% recovery concentration range for the 0.5, 0.1 and 1% (w/w) samples.

Table 4 summarizes accuracy results for the SC-52151 recoveries observed with spiking concentrations of 0.05, 0.1 and 1% (w/w). The recoveries in Table 4 are the mean value for triplicate determinations and were generated using the internal standard quantitation format. The mean recoveries for each of the two-day experiments at three different levels ranged from 97.9 to 100.0%. The two-day mean values each of the three levels ranged from 98.3 to 99.0%. The recoveries were consistent over the 0.05–1.0% level. Precision data are also summarized in Table 4, which tabulates within-day, between-day and total RSDs for each of the three spiking levels. The within-day RSDs ranged from 2.44 to 3.54% and the between-day RSDs from 0.0 to 0.34%. The most significant component of the imprecision was the within-day variation.

4. Conclusions

These investigations are preliminary; however, they demonstrate that SFE can be a viable sample preparation procedure for animal feed samples from toxicology studies. Precision and accuracy data generated for the recovery of SC-52151 in the current study are consistent with the data generated with more conventional sample preparation procedures for other compounds in development at Searle. Future studies in this area will

Table 4
SC-52151 recovery: accuracy and precision study

Target SC-52151 level in animal feed (%)	Recovery (%)			Within-run RSD (%)	Between-run RSD (%)	Total RSD (%)
	Day 1 ($n = 3$)	Day 2 ($n = 3$)	Mean for Day 1 and 2			
1	99.3	97.9	98.6	2.47	0.00	2.47
0.10	98.6	98.1	98.3	3.54	0.00	3.54
0.05	98.0	100.0	99.0	2.44	0.34	2.46

concern the application of SFE to other problems and compounds at Searle. Future efforts will focus on enhancing the absolute recovery of analyte during SFE experiments and systematic approaches to SFE method development.

References

[1] J. Luch and S. Ahuja, in S. Ahuja, (Ed.), The Ultratrace Analysis of Pharmaceuticals and Other Compounds of Interest, Wiley-Interscience, New York, 1986, pp. 273–293.
[2] D. Roston, Drug Dev. Ind. Pharm., 18 (1992) 245–255.
[3] D. Roston, J. Sun, P. Collins, W. Perkins and S. Tremont, J. Pharm. Biomed. Anal., 13 (1995) 1513–1520.
[4] M. Schneiderman, A. Sharma and D. Locke, J. Chromatogr. Sci., 26 (1988) 458–462.
[5] D. Messer and L.T. Taylor, J. High Resolut. Chromatogr., 15 (1992) 238–241.
[6] D. Messer, L. Taylor, W. Weiser, J. McRae and C. Cook, Pharm. Res., 11 (1994) 1545–1548.
[7] D. Messer, L. Taylor, W. Moore and W. Weiser, Ther. Drug. Monit. 15 (1993) 581–587.