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A comparison between solid phase extraction and supercritical fluid extraction for the determination of fluconazole from animal feed

S. Khundker [a], J.R. Dean [a,*], P. Jones [b]

[a] *Department of Chemical and Life Sciences, University of Northumbria at Newcastle, Ellison Building, Newcastle upon Tyne, NE1 8ST, UK*

[b] *Analytical Chemistry Department, Pfizer Central Research, Sandwich, Kent CT13 9NJ, UK*

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Abstract

The application of supercritical fluid extraction with carbon dioxide and modified carbon dioxide for the determination of fluconazole from an animal feed was studied. A fractional factorial design approach was used to examine the significant experimental variables for quantitative extraction of fluconazole. Gas chromatography with either flame ionisation or mass selective detection was used for quantitation of the extracts. The results indicated that modifier (methanol) had the greatest effect on the recovery of fluconazole from the animal feed.

Keywords: Supercritical fluid extraction; Fluconazole; Animal feed; Fractional factorial design; Gas chromatography

1. Introduction

In chronic toxicity studies, administration of drug substances to laboratory animals may involve incorporating the drug into the animals' feed [1]. The determination of the drug substance level in the feed matrices is necessary to monitor dosage levels, verify dose uniformity throughout the feed mix and confirm the drug's stability. Animal feeds are complex mixtures of proteins, lipids, glucicide, cellulose and mineral matter [2] and, if coextracted, the components can interfere in the determination of the analyte of interest. Various means of sample preparation have been investigated to extract drug substances from feed prior to assay. These methods include liquid-solid extraction [3], solid-phase extraction [4], Soxhlet extraction [5] and liquid-liquid extraction [6]. The use of supercritical fluid extraction to determine analytes from animal feed matrices has been investigated only briefly for several pharmaceutical compounds [7-9]. The present method for the isolation of drugs from feed involves time consuming extraction/cleanup steps which also introduce analytical uncertainty and a limited ability to automate such assays. Supercritical fluid extraction offers a simpler isolation procedure with the potential for in situ cleanup of analytes from complex matrices and the possibility of automation. This paper focuses on the use of supercritical fluid extraction for the extraction of fluconazole from animal feed matrix prior to chromatographic determination.

Fluconazole (UK-49588; 2-(2,4-difluoropheny-1,3-bis(1*H*-1,2,3-triazol-1-yl)-2-propanol; Fig. 1) is a triazole compound with potent

* Corresponding author.

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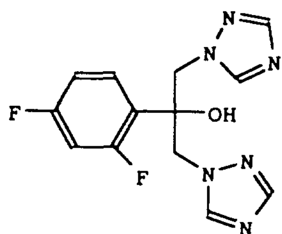


Fig. 1. Structure of fluconazole.

antifungal activity which is currently being used effectively for the treatment of Candida albicans and dermatophyte fungal infections [4]. The published method available for the determination of fluconazole from animal feed is a reverse-phase high-performance liquid chromatography (HPLC) technique involving solid-liquid extraction cleanup and concentration followed by a chromatographic separation on a C_8 column [4]. The aim of this study was to investigate whether a simpler procedure could be developed which was less time consuming and suitable for gas chromatographic analysis for improved selectivity and sensitivity.

Supercritical fluid extraction (SFE) is a relatively new technique for sample preparation that exploits the properties of a substance at temperatures and pressures above the critical point. Carbon dioxide is usually the most favourable substance for analytical scale extraction owing to its low critical temperature (31°C) and moderate critical pressure (1073 psi). Supercritical fluids offer advantages over other extracting liquids in that their properties, namely density, solvating power, viscosity and solute diffusivity, can be varied by controlling the applied pressure, leading to a greater selectivity, rapid mass transfer and higher flow rates compared with liquids. Further, the solvent strength of supercritical fluids can be enhanced by adding suitable modifiers. The addition of modifiers, such as methanol, is known to overcome matrix effects caused by the solutes being strongly bound to the matrix and also to increase the polarity of the supercritical fluid.

In the studies undertaken, extractions were performed with supercritical carbon dioxide in the dynamic mode, and the SFE parameters (pressure, temperature, time of extraction and %modifier) were varied to determine their influence on the recovery of fluconazole from the feed at different spiking levels. An experimental design approach (fractional factorial design) was employed to identify the significant variables for quantitative extraction of fluconazole from the feed. The SFE extracts were analysed by gas chromatography (GC) using two different detectors, as the GC method gave greater selectivity for separation of fluconazole from coextractives compared to the HPLC method [4].

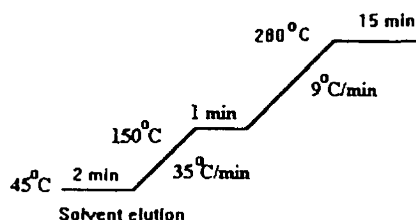
2. Experimental

2.1. Instrumentation

The HPLC system consisted of a Gilson model 305 master pump and slave pump, Gilson 805 S monometric module, Gilson 811 B dynamic mixer, Anachem OMRON E5CS column oven, Jasco UV 975 UV/vis detector, and LDC-Milton Roy Integrator.

Chromatography was performed at 35°C using a 10 cm × 2 mm, 5 μm C_8 Spherisorb analytical column. The mobile phase was methanol-water (25:75, v/v). It was filtered and degassed by vacuum and sonication. The mobile phase flow rate was 0.4 ml min^{-1}. The column eluent was monitored for UV absorption at a wavelength of 210 nm.

The GC system used was a MEGA series Carlo Erba system (CU600) with a flame ionisation detector. All samples (2 μl) were injected using on-column injection into a 15 m long × 0.3 mm diameter capillary column coated with DB5 stationary phase of 0.25 μm thickness. Propiophenone was used as the internal standard. The temperature ramp used for the GC programme was as follows:



The GC-MSD system consisted of a Hewlett Packard 5890 series 2 GC with 5972 MSD. Samples were injected on-column with an Hewlett Packard 7673 autosampler. The GC conditions were the same as described above and the separation used a 25 m long × 0.22 mm diameter capillary column coated with a film of BPX5 stationary phase of 0.25 μm thickness.

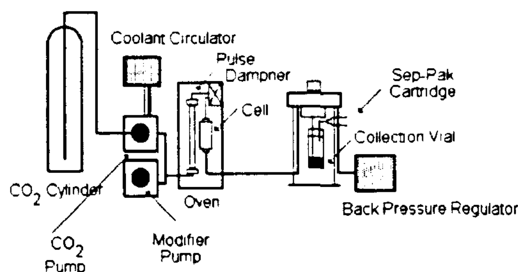


Fig. 2. Schematic diagram of supercritical fluid extraction apparatus.

Single ion monitoring was carried out with ions being monitored at mass 77 and 105 u for propiophenone, and 127 and 224 u for fluconazole. The peak area ratios of fluconazole and the internal standard were used for quantitation. The extraction recovery was determined by comparing the peak area ratios of the extracted samples with that of a 100% standard.

Supercritical fluid extraction was undertaken on a Jasco Supercritical fluid extraction system (Fig. 2). The Jasco system operates with two reciprocating pumps, a master pump (Jasco 880-PU) fitted with a cooling jacket on the pump head and a second pump for the addition of organic modifier. The extraction cell (2.0 ml capacity) is inserted into the thermostatically controlled oven (Jasco 860-CO). Extractions were performed in the dynamic mode, where a fresh supply of carbon dioxide is continuously swept through the extraction cell, prior to depressurisation, which is achieved using an oscillating variable restrictor (back pressure regulator, Jasco 880-81). The extracted analyte was collected in a screw cap glass vial (25 ml) with rubber septum at the top. A metal extension to the restrictor was used to pierce the rubber septum and allow collection directly in the collection solvent, methanol. A hypodermic needle with a cyanopropyl Bond-Elute cartridge attached also pierced the septum. This had two functions: first, to allow carbon dioxide to vent to the atmosphere; second, to remove residual analyte from the venting gaseous carbon dioxide. After each extraction, the cartridge was back-flushed with a suitable solvent to ensure quantitative recovery of the extracted analyte. This method of collection has been previously identified to be essential for the quantitative recovery of analytes [10–13].

2.2. Reagents

Fluconazole reference standard was obtained from Pfizer Central Research (Sandwich, Kent, UK). For the HPLC analysis, stock solutions of fluconazole (1000 $\mu g\ ml^{-1}$) were prepared in methanol–water (75:25, v/v) and stored at 4°C. Working solutions containing 2, 5, 8, 10, 12 and 15 $\mu g\ ml^{-1}$ fluconazole were prepared by diluting the stock solution. For GC analysis, stock solutions of fluconazole were prepared (10 000 $\mu g\ ml^{-1}$) with an internal standard (250 000 $\mu g\ ml^{-1}$) and stored at 4°C. Working standards were prepared by serial dilution over the range 50–400 $\mu g\ ml^{-1}$. An aliquot (20 µl) of internal standard was added to each of the working standards and the extracts analysed by GC. Methanol and water of HPLC grade were used throughout. Supercritical fluid grade carbon dioxide (Air Products, Sunderland) of certified purity 99.995% was used for extraction.

2.3. Solid phase extraction methodology

A fluconazole standard of concentration 20 $\mu g\ ml^{-1}$ in dichloromethane was prepared which corresponded to a spike level of 40 mg drug kg^{-1} of feed. The following procedure was followed for the extraction. A portion of feed (5 g) was accurately weighed in a glass jar and spiked with 10 ml of the fluconazole standard (20 $\mu g\ ml^{-1}$ in dichloromethane). The jar was capped, shaken vigorously and the unopened container then left overnight to allow the solvent to evaporate. The spiked samples were extracted with 20 ml of dichloromethane at room temperature by stirring with a magnetic stirrer for 1.5 h. The slurry mixture was vacuum filtered and the filtrate was quantitatively transferred to two cyanopropyl (500 mg) SPE cartridges (Bond-Elute, Jones Chromatography Ltd., Hengoed, UK) of 2.8 ml capacity, connected in series using a manual adapter and preconditioned with dichloromethane. The washings were collected and put through the cartridges again to ensure that all of the fluconazole was trapped on the chromatographic packing material. Both cartridges were then left to dry under vacuum, the eluent was discarded and fluconazole was eluted with 20 ml of solvent $MeOH/H_2O$ (35/65). The eluent was quantitatively transferred into a 25 ml volumetric flask and made up to volume with the appropriate solvent. The procedure was repeated five times and a blank extraction was

also prepared. All extracts were analysed by HPLC with UV/vis detection using a seven-point calibration plot with a correlation coefficient, r, of 0.9979.

2.4. Supercritical fluid extraction methodology

The extraction efficiency of SFE for the recovery of fluconazole from rodent feed was determined at two levels, 10 g of drug kg^{-1} of feed and 500 mg kg^{-1} of feed. The spiked feed samples of known fluconazole content were prepared by adding aliquots of the fluconazole standard in dichloromethane to a 1 g weighed sample of animal feed in a glass jar. These jars were capped, shaken vigorously and the contents left to dry overnight in an open jar. The spiked sample was quantitatively transferred to a cell and the extraction was carried out, using a fractional factorial design, to investigate SFE variable dependence. The flow-rate of extraction was maintained constant at 2 ml min^{-1} throughout the experiment. The collection vial contained a few drops of methanol and two cyanopropyl Bond Elute cartridges were placed in series at the exit to prevent any loss of fluconazole. Following extractions, all extracts were made up to volume in a 25 ml volumetric flask. The SFE extracts obtained at the 500 mg of drug kg^{-1} of feed level were analysed by GC/MS, while the extracts obtained at the 10 g of drug kg^{-1} of feed were analysed by GC with flame ionisation detection. All experiments were done in duplicate.

3. Results and discussion

Previously reported work [4] on the use of SPE followed by HPLC with UV detection for the isolation and determination of fluconazole from animal feed was done at the 40 mg kg^{-1} level. The methodology was repeated in this study to allow a direct comparison with SFE. The isolation and cleanup offered by SFE proved to be insufficient to eliminate extraneous material which coextracted with methanol-modified supercritical CO_2. The nature of the coextracted material prevented quantitative analysis by HPLC with UV detection. It had been noted previously that fluconazole could be analysed using GC [14-16]. Therefore, quantitative isolation by SFE followed by GC with either FID or MSD has been evaluated. However, in order not to be sensitivity limited (particularly in the case of GC with FID detection) the spiking level of fluconazole was done at the 10 g kg^{-1} (high) level. An intermediate spiking level of 500 mg kg^{-1} was also prepared and extracted using methanol-modified supercritical CO_2. These extracts were analysed by GC with MSD detection. It is apparent, that the inherent sensitivity of GC-MSD will allow lower spiking levels to be analysed.

3.1. SPE-HPLC

The extracts obtained from the SPE were visibly clean and the results obtained gave a mean fluconazole recovery of 99.1% (98.1%, 100.7%, 97.9%, 100.2%, 98.7%). The method described provides a convenient way of determining fluconazole quantitatively. The final solution obtained using this method was both sufficiently concentrated and also free from coextractives to make it compatible with HPLC analysis.

3.2. SFE of fluconazole from animal feed

In order to maximise the information available from the minimum number of experimental results, the use of an experimental design approach was pursued. A fractional design was selected [17]. This involved the use of a simple model of the form

$$Y = b_0 + b_1v_1 + b_2v_2 + b_3v_3 + b_4v_4 \quad (1)$$

where Y is the response (percentage extracted); v_1, v_2, v_3 and v_4 are the four main variables selected, i.e. pressure, temperature, time of extraction and percentage methanol, respectively; b_1, b_2, b_3 and b_4 are the parametric coefficients; b_0 is the intercept.

Owing to the presence of coextractives from the animal feed matrix, when methanol modifier was present, the use of HPLC with UV detection for the analysis of extracts was impractical. However, it had been reported that fluconazole could be analysed by GC [14-16]. In order to facilitate analysis and detection at high and intermediate spiking levels, it was necessary to use GC separation with either flame ionization or mass selective detection. The calibration plot for GC-FID showed acceptable linearity over the chosen range (50-400 μg ml^{-1}) with a correlation coefficient (r) of 0.974. The peak height of either the sample or standard was used for quantitation. Baseline resolution of fluconazole, from the endogenous

Table 1
Percentage recovery data for the GC-FID analysis of fluconazole ($10\ g\ kg^{-1}$ of feed) from an animal feed using a fractional factorial design. All experiments were done in duplicate

Pressure ($kg\ cm^{-2}$)	Temperature (°C)	Time (min)	% MeOH	% Recovery
250	80	40	20	87.0
250	80	5	2	76.6
250	40	5	20	79.0
110	80	5	20	90.9
110	40	40	20	80.5
110	40	5	2	16.9
110	80	40	2	22.3
250	40	40	2	27.3
170	65	20	10	97.4

Table 2
Percentage recovery data for the GC-MSD analysis of fluconazole ($500\ mg\ kg^{-1}$ of feed) from an animal feed using a fractional factorial design. All experiments were done in duplicate

Pressure ($kg\ cm^{-2}$)	Temperature (°C)	Time (min)	% MeOH	% Recovery
250	80	40	20	99.6
250	80	5	0	5.3
250	40	5	20	82.7
110	80	5	20	99.7
110	40	40	20	72.5
110	40	5	0	5.1
110	80	40	0	16.8
250	40	40	0	5.8
170	65	20	10	100.3

material co-extracted from the feed, was achieved under the conditions described at a retention time of 19 min.

Spiked fluconazole animal feed was prepared according to the above procedure and a fractional factorial design carried out at two different concentration levels. The extraction time was extended, within the design, from 20 to 40 min in order to compensate for any potential matrix effects. The results obtained for both levels of feed are shown in Tables 1 and 2. It should be noted that the percentage of methanol was considered at three levels, 0, 10 and 20%, in the case of the $500\ mg\ kg^{-1}$ level, whereas at the $10\ g\ kg^{-1}$ level it was considered at 2, 10 and 20%. Multilinear regression was used to calculate the coefficients in Eq. (1) [18]. The coefficients are reported in Tables 3 and 4. Significance is determined by a t-test with a probability (p) of 0.05 at the 95% confidence level with four degrees of freedom. In Tables 3 and 4, a coefficient with a p-level of less than 0.05 or a t-test of greater than 2.78 will be considered significant. In both cases, the results obtained indicate that the percentage of methanol was the only significant variable for the extraction of fluconazole from the animal feed matrix. The difference in the b coefficients (-0.3647 and -0.0419 for the $10\ g\ kg^{-1}$ and $500\ mg\ kg^{-1}$ spiking levels, respectively) reflects the influence that the percentage of methanol-time of extraction interaction has on extraction recovery. The consequence of the addition of a small amount of methanol (2%) as opposed to CO_2 only had a pronounced effect on extraction recovery (Tables 1 and 2). The model for the $10\ g\ kg^{-1}$ extraction results is

$$\begin{aligned}\text{Percentage recovery of fluconazole} &= -4.751 + 0.097(\text{pressure}) \\ &+ 0.509(\text{temperature}) \\ &- 0.365(\text{time of extraction}) \\ &+ 2.647(\text{percentage methanol})\end{aligned}$$

$r = 0.8535$ ($r^2 = 0.7286$) at the 95% confidence interval.

However, the model for the $500\ mg\ kg^{-1}$ results is as follows:

Table 3
Coefficients of regression and standard error of linear Eq. (1) for the GC-FID analysis of fluconazole from animal feed (10 g kg^{-1} level)

Variable	Descriptor	Beta	Standard error	b	Standard error	$t(4)$	p-level
Pressure	c_1	0.212	0.261	0.097	0.120	0.812	0.462
Temperature	c_2	0.317	0.261	0.509	0.419	1.215	0.291
Time	c_3	−0.198	0.261	−0.365	0.480	−0.760	0.489
% MeOH	c_4	0.739	0.261	2.647	0.933	2.837	0.047

Table 4
Coefficients of regression and standard error of linear Eq. (1) for the GC-MSD analysis of fluconazole from animal feed (500 mg kg^{-1} level)

Variable	Descriptor	Beta	Standard error	b	Standard error	$t(4)$	p-level
Pressure	c_1	0.020	0.246	0.011	0.136	0.089	0.940
Temperature	c_2	0.182	0.215	0.405	0.479	0.847	0.445
Time	c_3	−0.016	0.214	−0.042	0.546	−0.077	0.943
% MeOH	c_4	0.907	0.214	4.062	0.958	4.239	0.013

$$\begin{aligned}\text{Percentage recovery of fluconazole} &= -11.813 + 0.010(\text{pressure}) \\ &+ 0.405(\text{temperature}) \\ &- 0.042(\text{time of extraction}) \\ &+ 4.062(\text{percentage methanol})\end{aligned}$$

$r = 0.9199$ ($r^2 = 0.8462$) at the 95% confidence interval.

A typical response surface was generated for fluconazole (Fig. 3) showing the effect of temperature and percentage methanol, as modifier, on extraction recovery. The percentage extraction efficiency repeatability for the isolation and cleanup of fluconazole from animal feed followed by GC with either FID or MSD detection was determined to be 87.0% (RSD 8.4%) at the 10 g kg^{-1} level and 91.0% (RSD = 13.2%) at the 500 mg kg^{-1} level. In each case the number of replicates was ten.

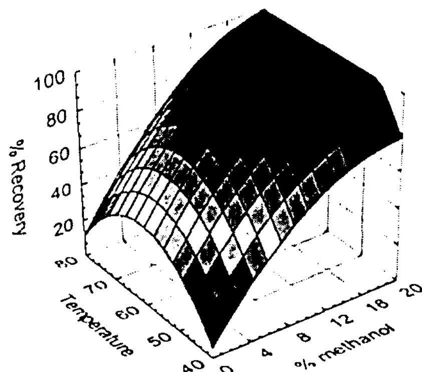


Fig. 3. Response surface for fluconazole showing the effect of temperature and percentage modifier on extraction efficiency.

The recovery of fluconazole from animal feed was determined, using a fractional factorial design approach, to be dependent upon the addition of modifier in the extraction fluid. The modifier's function is thought to be two-fold. Firstly, the modifier increases the polarity of the extraction fluid and thus competes with the drug for the active site on the matrix. Secondly, the modifier acts to decrease the adsorption of solutes onto the surface of the matrix by increasing the swelling of the matrix, which in turn increases the likelihood of solute diffusion out of the matrix.

4. Conclusions

A comparison of SPE with SFE for the isolation and cleanup of fluconazole from animal feed has been made. The inter-dependence of the extraction procedure on the method of analysis and determination was effectively demonstrated. The unselective nature of SFE, when methanol-modified supercritical CO_2 was used, prevented analysis by HPLC with UV detection owing to coextractives. Quantitative recovery of fluconazole was possible using GC with either FID or MSD detection at the 10 000 mg kg^{-1} and 500 mg kg^{-1} spike levels, respectively. However, the inherent sensitivity of GC-MSD should allow lower spike levels to be quantified. SPE-HPLC was shown to be an effective method of isolating and analysing

fluconazole from animal feed at a low spike level (40 mg kg^{-1}). In addition, the time taken by the SPE-HPLC method was shorter than that for the SFE-GC method (SPE-HPLC, SPE, ≈20 min; HPLC, 10 min compared to SFE-GC; SFE, 40 min; GC, 25 min).

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