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Short Communication

The determination of fluconazole in rodent diet using solid-phase extraction and high-performance liquid chromatography

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Introduction

Fluconazole is a new drug effective orally [1] for the treatment of *Candida albicans* and dermatophyte fungal infections.

The objective of the work to be described was to develop a high sensitivity, specific and accurate HPLC method for the quantification of fluconazole in rodent feed at levels down to 4 mg/kg. This report describes a drug-feed analysis method for samples containing fluconazole in the concentration range 4–1000 mg/kg feed.

The two major problems to be overcome in the development of analytical procedures for drugs in animal feed are those of obtaining a final sample that is both sufficiently concentrated and sufficiently free from endogenous materials that the compound(s) of interest can be determined quantitatively. In the case of fluconazole, these problems are exacerbated by its very weak ultra-violet chromophore (λ max in methanol: 261 nm, ε = 710). In order to obtain sufficient sensitivity it was necessary to use end absorption at a much lower wavelength (210 nm) which, inevitably, increased the observed interferences from co-extracted feed components.

Solid phase extraction cartridges are designed to aid extraction and concentration of samples. To gain maximum selectivity advantage, particularly when liquid chromatography is to be used as the final step in the analysis, it is important to use a different chromatographic mode for the extraction step. If a cyanopropyl bonded-phase is used, then this can provide further selectivity advantages since, depending on which solvents are employed, normal-phase absorption and partition mechanisms are available as well as reversed-phase mechanisms [2–4]. This versatility of the cyanopropyl cartridge was exploited for the selective extraction of fluconazole prior to reversed-phase HPLC and to simultaneously transfer the fluconazole from the hydrophobic extraction solvent to an essentially aqueous phase, compatible with reversed-phase chromatography.

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Experimental

Chemicals and reagents

Methylene chloride and methanol were HPLC grade (Rathburn Chemicals, Peebles, Scotland) and the water used was freshly distilled from glass. Fluconazole was synthesized in the laboratories of Pfizer Central Research.

Chromatography system

Analyses were carried out using an Analyst 7800 liquid chromatograph (Laboratory Data Control, Stone, Staffs, UK) comprising a ConstaMetric III pump, SpectroMonitor IIID variable wavelength UV detector and a 60 position autosampler with a fixed volume loop injector. Chromatograms were recorded and integrated using a Chromatography Control Module (Laboratory Data Control).

Separations were carried out with a 10 cm × 2.1 mm i.d. stainless steel column packed with 5 μm Hypersil MOS (Hewlett–Packard, Winnersh, Berks, UK). The mobile phase was prepared by mixing water and methanol (80:20, v/v) which was then vacuum filtered through a membrane filter of pore size 0.4 μm (Nuclepore). A flow rate of 0.4 ml/min was employed and a detection wavelength of 210 nm was used.

Extraction assembly

The extraction assembly comprised a Bond Elut cartridge with solvent reservoir and a sintered glass funnel mounted on a side-arm flask using bungs and Bond Elut adaptors for airtight connections. The Bond Elut cartridges (Analytichem International) were 2.8 ml capacity and contained 500 mg of cyanopropyl silica.

Preparation of spiked feed samples

Feed samples of known fluconazole content were prepared by adding aliquots of a solution of fluconazole (20 μg/ml) in methylene chloride to weighed samples (5 g) of feed (type UAR AO4, obtained through Pfizer Central Research), contained in polypropylene Securitainers (Johnsen and Jorgenssen). These were capped and shaken vigorously to mix the contents. In this way feeds containing 4–1000 mg/kg fluconazole were prepared. These Securitainers were also used as extraction vessels for pre-blended feeds.

Extraction procedure

(a) *For feed samples containing* 0.004–0.2 g *fluconazole per kg feed.* A portion of the feed (5 g) was accurately weighed into the extraction vessel and extracted with methylene chloride (20 ml) at room temperature by stirring with a magnetic stirrer and follower for 30 min. A single cartridge was used for isolating the fluconazole and, following application of vacuum to the extraction assembly, was primed with methylene chloride (3 ml). The stirred suspension was quantitatively transferred to the extraction assembly using methylene chloride (12 ml) to wash out all solids from the extraction vessel into the filter and to mix the solids in the filter. Vacuum was maintained until the cartridge was dry. The filtrate was discarded and the filter funnel removed. The fluconazole was eluted from the cartridge under vacuum with methanol–water (35:65, v/v) into a collection tube using a minimum of 4 ml solvent for each 0.1 mg fluconazole to be eluted from the cartridge. The eluant was quantitatively transferred into an appropriately sized volumetric flask and made to volume with the same solvent to give a final solution concentration in the range 4–20 μg/ml.

(b) *For feed samples containing* 0.2–1.0 g *fluconazole per kg of feed.* A portion of the feed (5 g) was accurately weighed into an extraction vessel and extracted with methylene chloride (30 ml) at room temperature by stirring with a magnetic stirrer and follower for 30 min. Two cartridges in series were used for isolating the fluconazole and, following application of vacuum to the extraction assembly, were primed with methylene chloride (3 ml). The stirred suspension was quantitatively transferred as described in (a).

Each cartridge was eluted in turn under vacuum with 20 ml of methanol–water (35:65, v/v) into a collection tube. The eluants were quantitatively transferred into one 100 ml volumetric flask and made to volume with the same solvent. For feeds containing 0.4–1.0 g fluconazole/kg this solution was further diluted with chromatographic mobile phase to give final solution concentrations in the range 8–20 μg/ml.

Chromatographic procedure

Once stable chromatographic conditions were established, duplicate aliquots (10 μl) of the test solution and duplicate aliquots (10 μl) of an external standard solution of fluconazole were injected. The external standard was prepared by dissolving fluconazole (20 mg) in 100 ml of methanol–water (35:65, v/v) and then diluting with the same solvent to give a final concentration corresponding to that expected in the solution obtained from the test sample. Calculations were by the usual external standard procedure, using peak heights computed from the integrated chromatogram.

Results and Discussion

Chromatographic separation

Baseline resolution of fluconazole from endogenous material extracted from the feed was achieved with the mobile phase described with a retention time of 8 min for the fluconazole ($k' = 9$). Typical chromatograms are shown in Fig. 1.

Linearity and sensitivity

The linearity and sensitivity of the procedure were investigated by spiking blank feeds with aliquots of a solution of fluconazole in methylene chloride (20 mg/ml), allowing to dry and then extracting by the described procedure. The correlation coefficient for the peak heights against concentration of fluconazole was 0.99 ($n = 6$) over the range 4–20 μg/ml fluconazole in the final extract solution. The minimum concentration that can be reliably determined quantitatively using a 5 g sample of feed is 4 mg/kg. Concentrations of less than 4 mg/kg can be detected but quantitation was not established.

Recovery

The recovery during the extraction procedure was assessed by preparing individual samples of blend in the range 4–1000 mg/kg fluconazole. The results are shown in Table 1. The average recovery was 95.8% with no significant dependence on concentration.

Conclusion

The method described provides a rapid and convenient procedure for the determination of fluconazole in rodent diet over a wide concentration range. The advantages of using a disposable pre-column packed with cyanopropyl silica are two-fold. Firstly, the

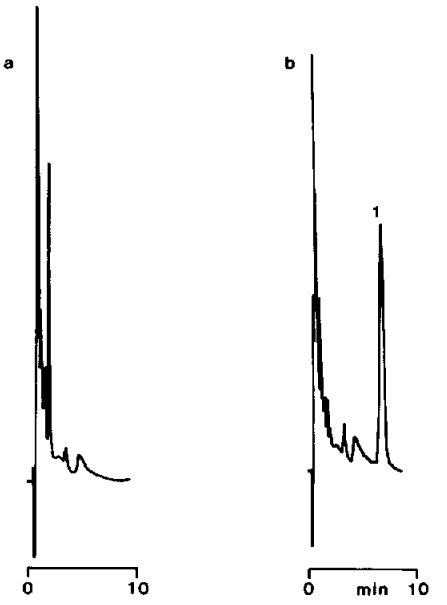

Figure 1
(a) Chromatogram of blank feed extract. (b) Chromatogram of an extract from feed containing 4 mg/kg fluconazole (1). For chromatographic conditions see text: unnumbered peaks are due to feed components.

Table 1
Evaluation of recovery of fluconazole from spiked rodent feed samples

Spiked dose level (mg/kg)	Mean fluconazole (mg/kg)	Mean recovery (%)
4.2	4.0	95.9
50.6	47.1	93.1
84.4	75.1	89.0
101.3	101.8	100.5
481	467	97.0
962	954	99.2

Mean recovery = 95.8%.
RSD = 4.4%.

cyanopropyl column confers additional selectivity on the procedure by complementing the separating power of the octyl-silica HPLC column. Secondly, since the pre-column can be used effectively in both normal and reversed-phase modes, it provides a very convenient method of quantitatively transferring an analyte from a lipophilic phase to an aqueous phase compatible with reversed-phase analytical chromatography.

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