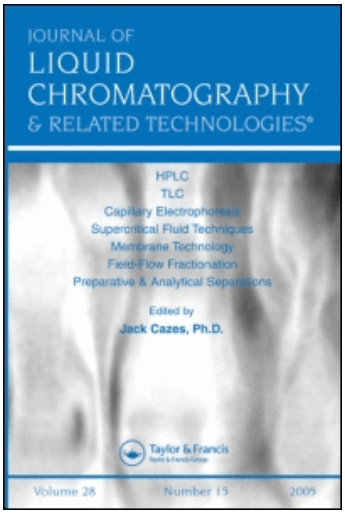

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A RAPID HPLC ANALYSIS OF DIAZEPAM IN ANIMAL FEED

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ABSTRACT

A rapid HPLC analysis is described for the methanolic extraction of diazepam from spiked animal feed. A short disposable reverse-phase extraction column is employed to remove interfering substances present in the animal feed. This is followed by direct injection of an aliquot into an analytical ODS column. The precision is better than 3.9% and the % recovery is 87.8 ± 0.8% resulting in a convenient, rapid method for the routine analysis of added diazepam in prepared animal diets.

INTRODUCTION

In a chronic diazepam spiked feed study, a method was needed to measure the concentration of diazepam fed to the experimental animals. An extensive literature search failed to produce a published method suitable for analysis of diazepam in feed. Excellent review articles by Tsuji (1) and Hailey (2) listed numerous spectrophotometric and GLC methods, developed to meet different analytical requirements. An attempt was made to modify the method of de Silva, Koechlin and Baker (3) but this classical organic extraction technique required considerable clean-up and had low recovery. HPLC methods using bonded phases (4,5) and UV detection have been used to measure diazepam and its metabolites in biological fluids. These methods employed an organic extraction, evaporation and reconstitution steps prior to injection into an analytical

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reverse-phase column. The use of disposable, reverse-phase, extraction columns (6,7) has offered speed, higher recoveries and superior resolution in the clean-up of biological fluids than the classical organic extraction methods. The benzodiazepines were selectively adsorbed into Bond Elut, C_{18}, disposable columns, from serum at pH = 9.0. Methanol was used to elute the benzodiazepines from the column, followed by injection into the analytical column. In our method, the diazepam in the feed was extracted directly into methanol, then the methanolic extract was passed through a "Baker", disposable extraction column, which retained the interfering, UV absorbing peaks present in the feed. The methanol eluent was then injected into the chromatographic system. Methanol was compatible with the mobile phase, thus eliminating time consuming evaporation and reconstitution, resulting in a rapid, convenient, highly reproducible analysis of diazepam in animal feed.

MATERIALS

Chemicals

Diazepam and ^{14}C-diazepam, labelled in the 5-position with a specific activity of 197 µCi/mg were obtained from Hoffman-LaRoche Limited (Vaudreuil, Quebec, Canada). The ODS bonded (1.0 ml) "Baker" disposable extraction columns, HPLC grade acetonitrile and methanol were purchased from the J.T. Baker Chemical Co. (Phillipsburg, N.J., U.S.A.). Atomlight L.S.C. cocktail was bought from New England Nuclear (Boston, Mass., U.S.A.). The animal feed used for the chronic animal experiments was Master Laboratory Mash, purchased from Ritchie Feed and Seed Ltd. (Ottawa, Ont., Canada).

METHODS

HPLC Analysis

A Varian Aerograph Series 4100 liquid chromatography system (Walnut Creek, CA, U.S.A.) was used to deliver the mobile phase (70% acetonitrile/30% H_2O), isocratically at a flow rate of 60 ml/hr, at ambient temperature. A six-port Valco injection valve with a 40 µl fixed

loop was used to introduce all the samples. Guard columns (5 cm Brownlee Labs RP-8) from Technical Marketing Associates Limited (Ottawa, Ont., Canada) were used to protect the 25 cm x 4.5 mm Dupont Zorbax ODS column purchased from Fisher Scientific Company (Ottawa, Ont., Canada). The effluent was monitored at 242 nm with a variable wavelength UV spectrophotometer. A strip chart recorder (Linear Instruments Corp., Irvine, CA, U.S.A.) was used to record the chromatographs. Peak heights were used to quantify diazepam concentrations.

Standard Solutions and Feed Standards

All diazepam standard solutions were made by serial dilution of freshly prepared 125 mg diazepam/100 ml methanol. Forty µl aliquots of these standard solutions were introduced into the HPLC system to produce the standard curve shown in Table 1.

For the preparation of spiked feed samples, diazepam in 5.0, 3.75, 2.5, 1.25 mg amounts was dissolved per g of corn oil and mixed with 25 g ground animal feed in a Janke & Kunkel KG blender (Johns Scientific, Toronto, Ont., Canada). This procedure simulated the actual feed-diazepam preparation used for the animal feeding study. The final corn oil content of each spiked sample was 4% w/w (corn oil/feed). These feed standards generated the data in Table 2.

General Procedure

Triplicate 300 mg samples of animal feed standards or samples with unknown diazepam content were placed in vials with teflon-lined screw caps. Each sample was shaken with 6.0 ml of methanol (1:20 w/v) on a Burrel Wrist Action Shaker (Burrell Corporation, Pittsburgh, PA, U.S.A.) for 15 minutes in a single extraction. The feed mixture was then centrifuged for 10 minutes at 2000 g in a Clinical Centrifuge (Damon/IEC, Needham Hts., Mass., U.S.A.). One ml of the clear supernatant from each sample was placed on a "Baker" disposable extraction column, which was first pre-conditioned with methanol and then with water. The 1.0 ml sample was completely pushed through with a gentle pressure of nitrogen.

TABLE 1

Precision Data of Diazepam Standard Solutions (n=9)

Amount Injected ng	Amount Found $\bar{X}$ ng	± S.D.	% C.V.
25	25	1.4	5.5
50	49	0.9	1.9
100	100	1.2	1.2
125	127	0.6	0.5
250	253	2.0	0.8
400	394	1.8	0.5
500	504	2.4	0.5
		Mean =	1.6

TABLE 2

Precision Data of Diazepam Spiked Feed Standards
n = 9 except *(n = 6)

Amount Diazepam Added mg/25 g Feed	Amount Diazepam Found mg/25 g Feed $\bar{X}$ mg	± S.D.	% C.V.
1.25	1.27	0.50	3.9
2.50	2.47	0.06	2.4
*3.75	3.72	0.06	1.6
5.00	5.02	0.11	2.2
		Mean =	2.5

The column was then rinsed with 100 µl methanol and the combined effluent was analysed by injecting a 40 µl aliquot on the analytical ODS column.

^{14}C% Recovery

^{14}C-diazepam was diluted with cold diazepam in 1 g of corn oil to give a specific activity of 7.5 µCi/5.0 mg of diazepam. This solution was

then mixed in a blender with 25 g of ground feed. This concentration of drug corresponded to the highest spiked feed standard (5 mg/25 g). Ten 300 mg samples of radioactive feed were weighed out, extracted, cleaned-up and each total eluent was collected in a glass vial, to which 15 ml of Atomlight liquid scintillation fluid was then added. A 1.0 ml aliquot of the 6.0 ml methanolic feed extract prior to its passage through the "Baker" disposable extraction column was also sampled to establish the efficiency of only the methanol extraction step.

All samples were subsequently counted on a Beckman LS 8100 liquid scintillation counter equipped with automatic quench correction.

RESULTS

The data shown in Tables 1, 2 were analysed by linear regression. The standard errors of the means at each concentration ranged from ±0.02 to ±0.80. Table 1 represents the diazepam standards in methanol injected directly into the chromatographic system. Table 2 shows the diazepam spiked feed standards. Both plots were highly linear with a correlation coefficient of 0.999 and had respective slopes ± S.E. of 0.3929 ± .003 and 0.3637 ± .005 with negligible intercepts in both cases.

The precision data (% C.V.) for the diazepam standard solutions injected directly into the mobile phase averaged 1.6% (Table 1). The average % C.V. of the spiked feed standards was 2.5% with a very low C.V. of 3.9% at the lowest concentration tried (Table 2). These data were used to calculate the concentration of diazepam in the feed during the chronic feeding experiment. These results indicate that this analysis of diazepam in feed is highly reproducible.

Some of the actual determinations of diazepam in the feed used during the chronic animal feeding experiment are shown in Table 3.

The recovery of the diazepam during the extraction procedure was determined with radio-labelled diazepam. The recovery of the initial methanolic extraction step alone was 96.9% and the total recovery for 10 samples was 87.8 ± 0.8%. This recovery compares well with the 88% to 95% values for diazepam extracted from plasma in HPLC analysis (6,7).

TABLE 3

Determination of "Unknown" Diazepam Spiked Feed Samples

(A) Actual Diazepam Found mg/25 g Feed	(E) Expected Concentration mg/25 g Feed	% A/E
0.45	0.45	100%
0.46	0.40	115%
2.34	2.50	94%
4.36	4.32	101%
37.80	40.00	95%
44.30	45.80	97%
	Mean =	100%

The chromatograms A,B (Figure 1) show that the clean-up procedure removes all interfering peaks from the complex feed mixture and that diazepam travels as a sharp band with a retention time of 7.8 min. The capacity factor k' was calculated to be 2.8.

DISCUSSION

Although methanol is not the solvent of choice for a lipophilic compound such as diazepam it was tried because a) methanol could be injected directly into a reverse-phase clean-up system without evaporation and re-constitution and b) because the feed did not float in methanol thereby producing a clear supernatant on centrifugation. Ethyl ether was tried but it is incompatible with a reverse-phase solvent system, and it produced a cloudy supernatant. Other methods (8) have used hot chloroform in a forward phase system but we found many more interfering peaks were extracted. Also the buoyancy of the feed in chloroform made it difficult to sample. The high recovery (96.9%) with methanol and its compatibility with reverse-phase chromatography made it an ideal solvent for extraction of diazepam from ground animal feed. Although acetonitrile is similar to methanol in polarity it failed to extract diazepam from feed.

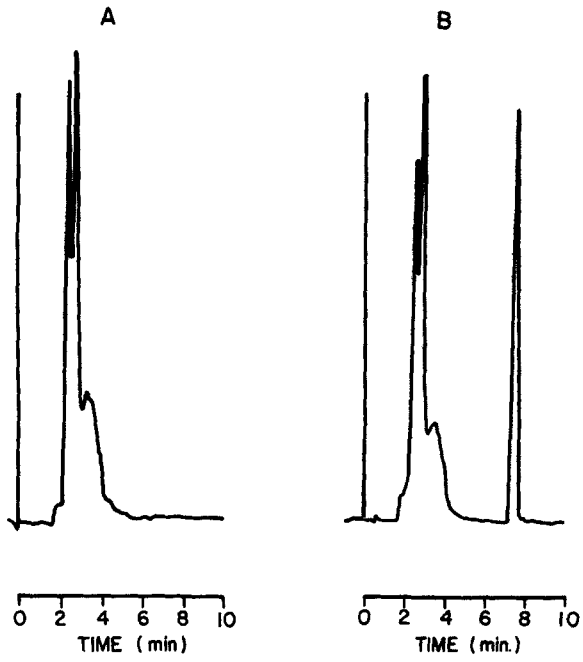

FIGURE 1. HPLC analysis of control feed (A), and diazepam-spiked feed (B), 3.75 mg/25 g, methanol extracts run in 70% acetonitrile/H_2O, at 60 ml/hr., attenuation 0.200 AUFS, at 242 nm.

The methanolic feed extract contained large peaks which masked the diazepam peak. Running the methanol extract through a short "Baker" disposable extraction column resulted in a clean, sharp, diazepam peak. The disposable clean up column retains most of the large, broad, interfering peaks, letting the diazepam through in the eluent. The methanolic extract when placed directly into a Dupont Zorbax column with a 70% acetonitrile/water mobile phase produced no chromatographic distortion. All our work reported was done on this column. Changing the guard column always restored the column to its original capabilities. The solvent system, 70% acetonitrile/30% H_2O, produced a very sharp peak which widened if we substituted 70% methanol.

This analytical procedure has proven to be a rapid, reliable and convenient method for monitoring diazepam in spiked animal feed. It is a method that could be readily adapted to the analysis of other drugs or contaminants in feed preparations.

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