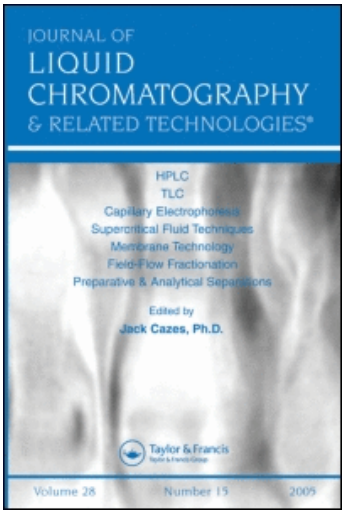

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR ADINAZOLAM MESYLATE IN RODENT FEED MIXTURES

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ABSTRACT

Adinazolam mesylate was recovered from the feed mixtures by repeated extraction with a pH 4 citrate buffer. The pooled extracts were diluted with water when necessary and mixed with an equal volume of an acetonitrile solution of internal standard (naphthalene). After mixing and centrifugation, the clear supernates were separated for chromatography. The chromatography was carried out on a reverse phase column using a mixture of phosphate buffer, acetonitrile and methanol in the volume ratios of 85:40:20 as mobile phase. Adinazolam and the internal standard were eluted from the columns at about 11.7-12 and 15.50-17.5 minutes, respectively.

The peak height ratios and adinazolam mesylate concentrations showed excellent linearity ($r > 0.999$). Extracts of blank feed samples did not show interference to the assay. Assay results with satisfactory accuracy and precision were obtained. The methodology was applicable for the assay of the drug-feed mixtures containing 0.02 to 10.0 mg of adinazolam mesylate per gm of feed.

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INTRODUCTION

Adinazolam mesylate, a new triazolobenzodiazepine with a chemical structure similar to alprazolam and triazolam (1-3), has shown in animal tests pharmacological activities indicative of both anxiolytic and antidepressant properties (4-6). Adinazolam mesylate is currently undergoing clinical trials for efficacy in the treatment of depression in humans. Initial test results show that patients with major depressive disorders were successfully treated with adinazolam mesylate and that the onset of therapeutic responses was rapid (7).

Adinazolam mesylate has also been undergoing chronic toxicity studies in animals. In a study in rats, adinazolam mesylate was administered to the test animals as drug-feed mixtures. Similar administration of drug-feed mixtures has been utilized to evaluate the chronic toxicity, of triazolam (8), acecainide (9,10), and procainamide (9,10). To assure the integrity of such studies, and to meet the current Good Laboratory Practice (GLP) regulations, a high performance liquid chromatographic (HPLC) method was developed for the assay for adinazolam mesylate in rodent feed mixtures. This methodology was utilized to assay for potency, uniformity, and stability of adinazolam mesylate in the rodent feed mixtures to support the chronic toxicity study in the animals.

EXPERIMENTAL

Reagents

Acetonitrile and methanol used in this study were distilled-in-glass UV grade. Reagent grade naphthalene, citric acid monohydrate, sodium hydroxide, and ammonium dihydrogen phosphate were used as received. The drug-feed mixtures were prepared by mixing adinazolam mesylate (The Upjohn Company) with commercial rodent

feed (Certified Rodent Chow 5002 Meal®, Ralston Purina Co., St. Louis, MO) in a "V" shell mechanical blender. Citrate buffers were prepared by dissolving 0.05 M citric acid monohydrate in 800 mL of water, adding a solution of 1M NaOH to adjust to the desired pH, and adding water to make a 1L solution. Citrate buffer solutions with pH 3, 4, and 5 were used in this study. A solution of naphthalene in acetonitrile (10 μg/mL) was prepared for use as internal standard solution. A phosphate buffer was prepared by dissolving 0.05 M ammonium dihydrogen phosphate in water, adjusting to pH 5.0 with a solution of 1 M NaOH, and adding water to make a 1L solution. The phosphate buffer was used to prepare the mobile phase for HPLC.

Extraction of Feed Samples

Accurately weighed 1.0 gm portions of the feed samples were placed into 16 x 125 mm culture tubes equipped with polytef-lined screw caps. To these tubes were added 1 mL aliquots of water and 9 mL aliquots of pH 4 citrate buffer. These tubes were tightly capped and mixed briefly on a vibro-mixer to expel trapped air and then allowed to stand for 15 min. These samples were then shaken on a horizontal shaker for 15 min followed by centrifugation for 10 min at ~2000 rpm. The supernates were decanted into 50 mL centrifuge tubes. The feed samples which became pellets upon centrifugation were extracted three more times with 10 mL aliquots of pH 4 citrate buffer. For each extraction, the feed pellets were broken up by mixing briefly on a vibro-mixer before shaking the samples on the horizontal shaker. The supernates of the repeat extracts were pooled and mixed with the corresponding initial extracts.

The pooled extracts were diluted with water, when necessary, to make the concentrations of adinazolam mesylate in the diluted extracts in the range of about 1.0 to 10.0 μg/mL. Aliquots of these sample solutions were mixed with an equal volume of the internal standard solution. These mixtures were vortexed, allowed to stand for

5 min, and centrifuged for 10 min at ~2000 rpm. The supernates were transferred to injection vials for chromatographic analysis.

Calibration standards of the feed samples were prepared by supplementing 1 gm portions of the blank rodent feed with 1 mL aliquots of stock solutions of adinazolam mesylate in water in the concentration range of 0.1-10 mg/mL. These standards were extracted with 9 mL pH 4 citrate buffer followed by three additional extractions using the same procedure as for the feed samples.

Chromatography

The prepared feed samples and calibration standards were chromatographed on a prepacked reverse phase column (μ-Bondapak C_{18}, Waters Associates or Lichrosorb EC RP-8, EM Laboratories, Merck) with a precolumn (Bondapak C_{18}/Corasil). The mobile phase, which consisted of phosphate buffer, acetonitrile, and methanol in the volume ratios of 85:40:20, was delivered using a suitable solvent pump at a flow rate of 2.2 mL/min. The prepared samples were injected using an automated injector with a 50 μL sample loop. The column effluent was monitored with an UV detector (Spectromonitor III, LDC, Riviera Beach, FL) to measure absorbance at 254 nm. Peak height measurements obtained from the chromatograms on a strip chart recorder or a computer data system were used for quantitation. The concentrations of the drug in the rodent feed mixtures were calculated based on the calibration standard curves with correction for the dilution factors, mass of the feed samples, and purity of the drug standard.

RESULTS AND DISCUSSION

Extraction of Adinazolam Mesylate from Feed Mixtures

During the development of this assay methodology, adinazolam mesylate was recovered from the feed matrices by a single extraction

with water (1 gm feed extracted with 10 ml water). The slopes of the calibration curves prepared from blank feed samples supplemented with adinazolam mesylate by single extraction with water were about 48% of the slopes of calibration curves prepared from standard solutions of adinazolam mesylate in water using an identical concentration of internal standard. This indicated that the absolute extraction recovery of adinazolam was low.

Since the extraction recovery of adinazolam from the feed mixtures after a single extraction with water was relatively low, the feed/water mass ratio could be critical for the extraction and quantitation of the drug. This was confirmed by the results of the experiment in which duplicate samples of 0.5, 1.0 and 1.5 gm of the drug-feed mixture containing 0.1 mg adinazolam mesylate per gram of feed were assayed. The average amounts of the drug found were about 126, 100 and 84% of the theoretical amounts for the 0.5, 1.0 and 1.5 gm samples, respectively. Therefore, the amounts of adinazolam mesylate partitioned into water from the feed samples which were quantifiable showed an inverse relationship with the amounts of the feed in the samples. In another experiment, duplicates of 0.5, 1.0 and 1.5 gm of blank feed samples were supplemented with 0.1 mg adinazolam mesylate and analyzed. The average amounts of drug found were about 125, 97 and 83% of the theoretical amount for the 0.5, 1.0 and 1.5 gm samples, respectively. These results also showed that the amount of adinazolam mesylate partitioned into the water was reduced by an increase in the amounts of feed in the samples and was increased by a reduction in the amounts of feed in the samples. These results are depicted graphically in Figure 1.

To evaluate the tolerable range allowed for sample size variations using a single extraction with water, samples of a feed lot containing 1.0 mg adinazolam mesylate per gm feed were analyzed with sample sizes varied from 0.5 to 1.5 gm. The results indicated that a deviation

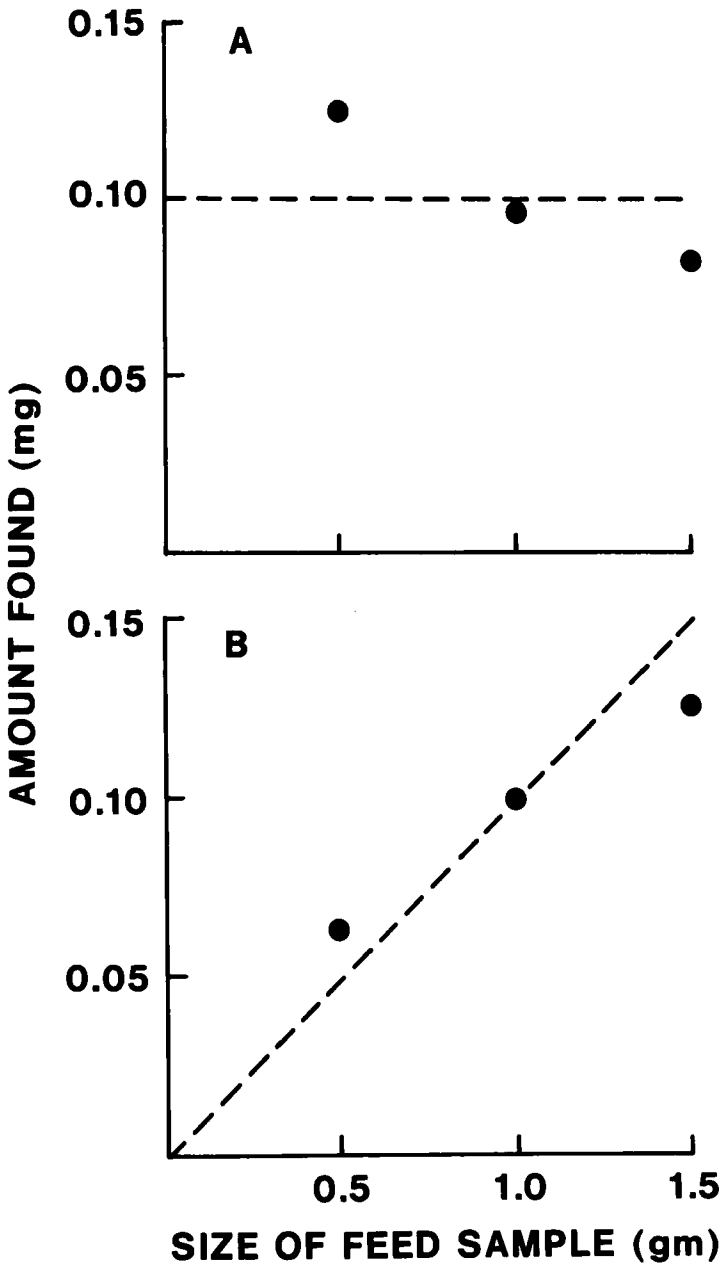

Figure 1. Effects of feed sample sizes on the extraction recovery of adinazolam mesylate. (The broken lines indicate expected values, the dots are experimental values, 1 gm feed sample extracted with 10 mL water).

of ± 10% in sample size gave assay results which were within about ± 6% of the theoretical concentrations.

Using the single extraction procedure, the analysis of freshly prepared lots of drug-feed mixture yielded satisfactory results while drug-feed mixtures which had been stored yielded non-reproducible results with concentrations often substantially lower than the corresponding theoretical concentrations. This occurred despite keeping the sample weights constant. Since adinazolam mesylate is chemically stable when stored in solid state and in solution, it was thought that these erratic and lower assay results might be caused by the difficulties in the extraction with water to recover adinazolam from the feed mixture and that adinazolam mesylate penetrated into the feed particles upon storage and thus became unavailable or only slowly available for the extraction into water. Based on the above assumptions and to overcome the difficulties encountered by the single extraction procedure, multiple extractions to recover adinazolam from feed were investigated. Table 1 summarizes the results of the multiple extraction procedures using water and citrate buffers at pH 3, 4 and 5. Quantitative recovery of adinazolam in the concentration range of 1.0 or 10.0 mg/gm appeared to be obtained by three or four extractions with the citrate buffers and water. The apparent recoveries that exceeded 100% were probably due to experimental errors. Although the extraction recovery of the first extraction appeared to be higher with a lower pH of the extractant, the citrate buffer solution with pH 3 could not be used because of potential degradation of adinazolam at pH 3 by the hydrolysis of the azomethine linkage in the benzodiazepine ring structure. The buffer solution with pH 4 was chosen as the extraction solvent and satisfactory assay results have been obtained.

Without treatment with acetonitrile, the aqueous extracts of the feed samples became cloudy and a precipitate settled upon standing. This inevitably caused blockage of the precolumn upon

TABLE 1

Effect of the pH of the Solvents on the Multiple Extraction Recovery of Adinazolam Mesylate from Spiked Rodent Feed

Theoretical Concentration	Order of Extraction	Adinazolam Extracted, % of Theoretical Concentration[a] Water	pH 3	pH 4	pH 5
1.0 mg/gm	1	56.5	79.6	77.5	68.5
	2	25.8	25.2	29.8	28.1
	3	16.6	10.8	8.7	8.7
	4	11.4	0	7.9	7.6
	Total	110.3	115.6	123.9	112.9
5.0 mg/gm	1	55.4	75.6	75.1	64.4
	2	22.6	20.5	24.0	23.9
	3	12.2	5.8	8.2	11.9
	4	7.4	1.4	3.3	5.4
	Total	97.6	103.3	110.6	105.6
10.0 mg/gm	1	58.7	79.2	75.5	68.2
	2	22.1	22.4	23.1	25.7
	3	10.9	7.0	8.8	10.8
	4	6.1	2.4	3.4	5.1
	Total	97.8	111.0	110.8	109.8

[a] Each extract was chromatogarphed separately. Extraction solvents were water and citrate buffer at pH 3, 4, and 5.

chromatography. This problem was circumvented by addition of acetonitrile to the final diluted extracts to precipitate proteinaceous materials. Acetonitrile has been used to precipitate plasma proteins to prepare plasma samples for HPLC (11). The resulting solutions showed no apparent physical change up to 72 hours at ambient temperature (22°C). Direct extraction of the feed samples with acetonitrile-water mixture yielded extracts which showed very high background interferences in the chromatographic separation.

Internal Standard

Naphthalene was used as the internal standard because of its desirable retention in the chromatographic separation. The reason

that the internal standard was not added directly to the feed samples before or during the extraction was that naphthalene partitioned predominantly into the feed matrices in the aqueous suspension of feed because of its poor solubility in water. The internal standard compensated for any variations in volume of samples injected for chromatographic separation.

Chromatography

The prepared samples were successfully chromatographed on prepacked reverse phase columns from two manufacturers using a mixture of 85:40:20 (v/v) phosphate buffer, acetonitrile and methanol as mobile phase. Adinazolam and the internal standard were eluted from a μ-Bondapak C_{18} column (30 cm x 3.9 mm I.D.) at the retention times of about 11.7 and 17.5 minutes, respectively, and from the Lichrosorb EC RP-8 column (25 cm x 4.6 mm I.D.) at about 12 and 15.5 minutes, respectively. Blank rodent feed extracts showed no interferences in the chromatographic separations. Representative chromatograms of the extracts of a drug-feed mixture and of a blank feed sample are shown in Figures 2 and 3 obtained from C_{18} and RP-8 columns, respectively. The chromatographic conditions were a modification of the procedure reported for the assay of adinazolam in plasma [12].

Calibration Curves

Analysis of calibration standards showed excellent linearity ($r > 0.999$) between the peak hight ratios and the concentrations of adinazolam mesylate in the range from about 0.02 to 10.0 mg per gram feed. These standards were appropriately diluted after extraction so that the concentrations of the drug in the prepared samples were less than 10 μg/mL. Separation on both C_{18} and RP-8 columns generated satisfactory calibration curves. For example, when the same set of the calibraton standards were chromatographed, calibration curves of $Y = 1.618X - 0.019$ ($r = 0.9998$) and $Y = 1.368X -$

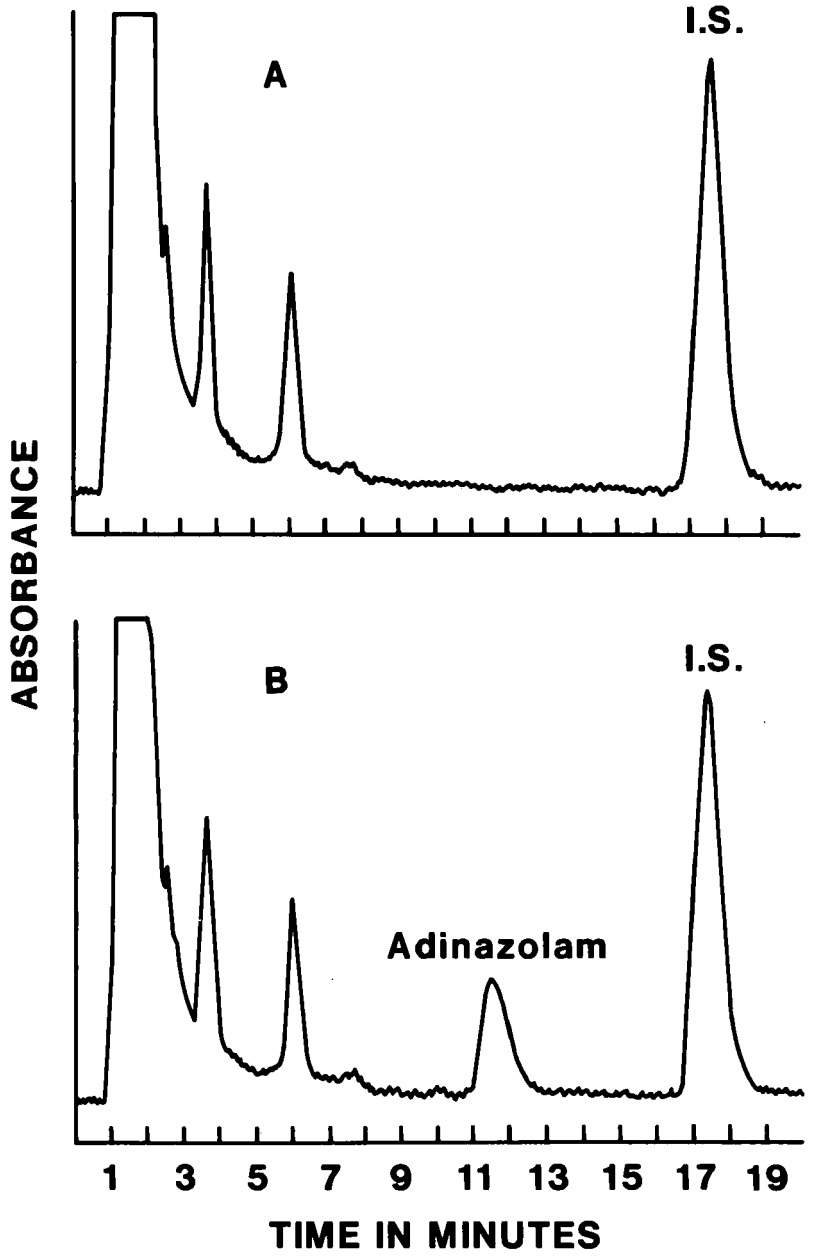

Figure 2. Chromatograms of the extracts of (a) a blank feed sample and (b) a feed sample containing 0.1961 mg adinazolam mesylate per gm feed mixture. Separated on a μ-Bondapak C_{18} column. I.S. = internal standard.

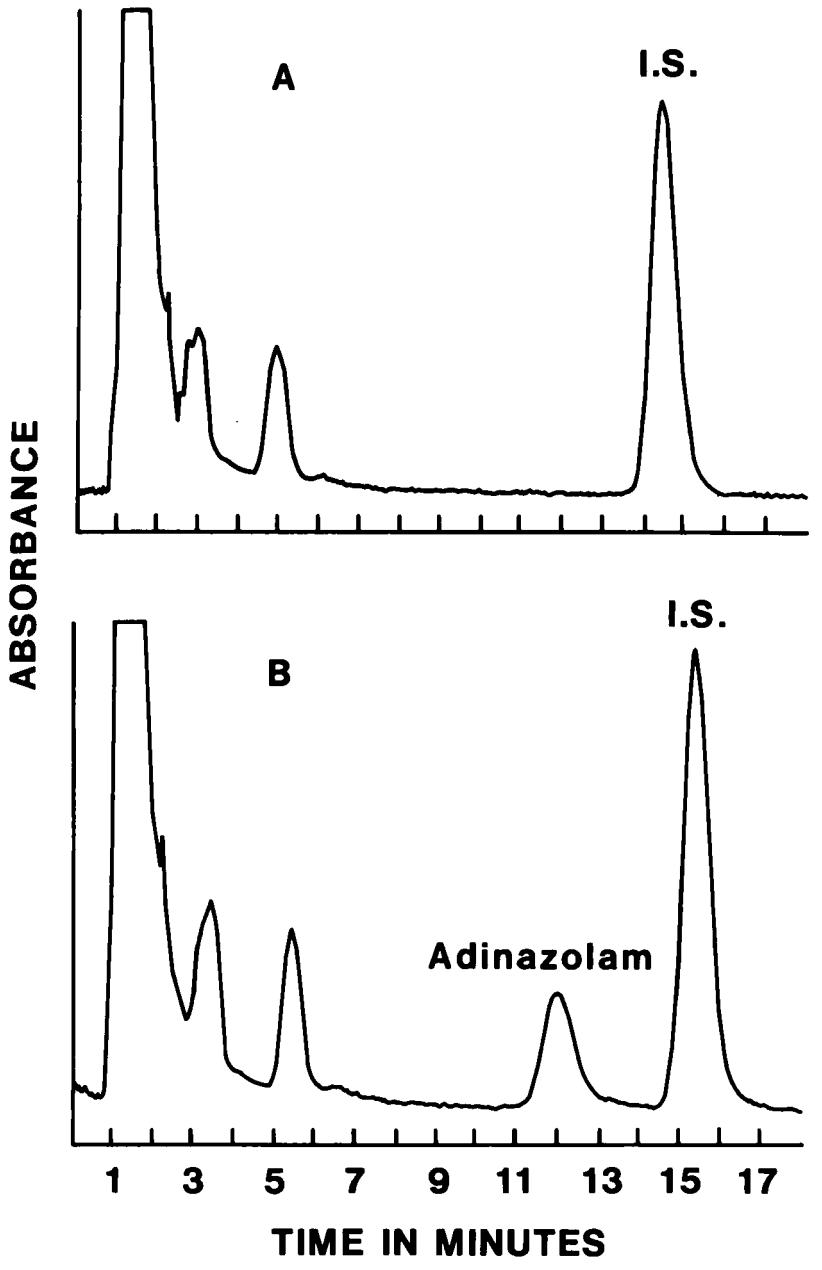

Figure 3. Chromatograms of the extracts of (a) a blank feed sample and (b) a feed sample containing 0.1961 mg adinazolam mesylate per gm feed mixture, separated on an EC RP-8 column. I.S. = internal standard.

0.0164 ($r = 0.9998$) were obtained using C_{18} and RP-8 columns, respectively. The intercepts were not statistically different from zero ($p > 0.05$).

Accuracy and Precision

Blank feed samples supplemented with 0.075 to 10 mg adinazolam mesylate per gram were analyzed to determine the precision and accuracy of the assay. The concentrations found were from 102.5 to 108% of the corresponding theoretical concentrations and the relative standard deviations (RSD) varied from 0.08 to 2.47%.

Samples from pre-mixed feed lots containing adinazolam mesylate at 0.1 to 5.0 mg/gm were analyzed. The concentrations found were 91.5, 99.5, 92.8, 91.8 and 104.3% of theoretical concentrations corresponding to the samples of 0.1, 0.2, 0.6, 1.0, and 5.0 mg/gm lots, respectively. The replicate assays showed RSDs of 2.72 to 5.46%. Essentially reproducible results were obtained on chromatography using μ-Bondapak C_{18} and Lichrosorb EC RP-8 columns.

Uniformity and Stability of Adinazolam Mesylate in Drug-Feed Mixtures

The uniformity of distribution of adinazolam mesylate in drug-feed mixtures was determined. Four 30 gm portions of the feed mixtures were weighed out separately. Each portion was put into a separate pile and sub-divided into 4 approximately equal parts. Each of these parts was then again sub-divided into 4 parts to give a total of 16 piles for each portion or a total of 64 separate piles from the 4 portions. A 1.0 gm sample was weighed from 12 (out of 16) randomly selected piles from each portion and assayed. Half of the samples from each portion were assayed on Day 1 by one operator and the other half on Day 2 by a second operator. The RSDs of the six samples from each portion of 30 gm samples were less than 6%. The overall RSDs were about 4%. These results indicate drug uniformity in the feed mixtures.

TABLE 2

Stability of Adinazolam Mesylate in Certified Rodent Chow at Ambient Temperature

Days on Stablity	Theoretical 5 mg/gm Found (mg/gm)	% To (N)	Theoretical 0.1 mg/g Found (mg/gm)	% To (N)
0	4.90 ± 0.25 (To)	100 ± 5.3 (5)	0.094 ± 0.005 (To)	100 ± 5.3 (5)
33	4.67 ± 0.16	95.2 ± 3.5 (5)	0.090 ± 0.007	95.7 ± 7.8 (5)
42	4.45 ± 0.24	90.7 ± 5.3 (5)	0.086 ± 0.005	91.5 ± 5.8 (5)
60	4.34 ± 0.28	88.8 ± 6.4	0.072 ± 0.004	76.6 ± 5.6 (8)

Stability of adinazolam mesylate in the drug-feed mixtures was studied by preparing mixtures containing 5.0 mg and 0.1 mg of adinazolam mesylate per gram of feed. Portions of each mixture were stored at ambient temperature in open nalgene bottles in an animal room in order to mimic the conditions of use. At periodic intervals portions were removed, placed in amber glass bottles, and stored at -20°C until analysis. Table 2 gives the values obtained from the HPLC analysis of the stability samples. Linear regression analysis of the stability data indicate that the slope (of concentration *vs.* time) was not significantly different from zero between 0 and 60 days for the 0.1 mg/gm samples ($p > 0.11$). The 5 mg/gm samples did have a slope significantly different from zero between 0 and 60 days ($p < 0.025$) when linear regression analysis was performed. Study of Table 2 indicates that both the 0.1 mg/gm and 5.0 mg/gm samples show an apparent monotonic decrease in potency over time. This trend suggests that the nonsignificant slope for the 0.1 mg/gm sample is likely due to the scatter of the data.

The use of the stability data in Table 2 gives conservative estimates of drug-feed potency over time. These data indicate that a manufacture and use interval of up to 40 days would insure a minimum potency of 90% of the initial concentration. Although it was not certain that the apparent loss of potency of adinazolam mesylate upon

long term (>40 days) storage of the feed mixtures was due to actual degradation of the drug or due to the penetration of the drug into the feed matrices and thus could not be extracted with the citrate buffer, the above criterion of using the drug-feed mixtures within 40 days of their preparation was adopted in the chronic toxicity study to assure that adequate dose levels were given to the test animals.

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