Gradient High Performance Liquid Chromatographic Assay for Degradation Products of Adinazolam Mesylate in a Sustained Release Tablet Formulation

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A gradient high performance liquid chromatographic method was developed to determine degradation products of adinazolam mesylate in a sustained release tablet formulation. Sample preparations were chromatographed on a YMC-Basic column using a formate buffer/acetonitrile gradient with absorbance detection at 254 nm. Adinazolam mesylate was found to degrade at high relative humidity and temperature to form a major product, the 6-aminoquinoline analog, plus numerous other compounds. Five of these compounds were identified and their structures indicate that the solid-state degradation of adinazolam, in the presence of sufficient moisture, involves not only a hydrolytic mechanism, but also an oxidative mechanism. Potential process impurities were resolved from the drug and degradation products. Recovery was near 100% over the 0.5 to 10% range for the major degradate (6-aminoquinoline) and over the 0.5 to 1% range for the other analytes. The method was applied to tablet samples stressed at high relative humidity and temperature. The relative standard deviation of the assay for the 6-aminoquinoline was less than 2% and less than 13% for the minor components. Calculated mass balances (sum of adinazolam plus degradation products in the degraded tablet divided by the same sum in the undegraded tablet) were less than 100% and were dependent on the extent of degradation in the tablet. The average mass balance result obtained for samples that were an average of 9.5% degraded was 95.0 ± 1.5%. It is possible that the decrease in mass balance with increase in percent degradation may be explained by the formation of many components at trace levels due to degradation by various permutations of hydrolytic and oxidative reaction pathways.

KEY WORDS: adinazolam mesylate; gradient reversed-phase liquid chromatography; mass spectrometry; decomposition products assay.

INTRODUCTION

A sustained release (SR) tablet formulation containing adinazolam mesylate (ADM) is being investigated for the treatment of panic disorder. The structure of this compound, a triazolobenzodiazepine, and related compounds are shown in Figure 1. During the development of this product, the stability of ADM was investigated under severe conditions (40°C, 88% relative humidity, tablets stored in open containers). Under these conditions, ADM degrades in the tablet to produce a major degradation product and numerous minor products, while the drug alone is stable.

High performance liquid chromatography (HPLC) has been used extensively in the assay of benzodiazepines (1,2). For example, alprazolam, triazolam, and other related triazolobenzodiazepines in pharmaceutical products were assayed with a normal phase HPLC method (3). The concentration of adinazolam in plasma and rodent feed samples has been determined with isocratic reversed phase HPLC methods (4–6). Using similar isocratic methods to assay degraded SR adinazolam mesylate tablets resulted in degradation products with a wide range of polarities as indicate by chromatographic peaks eluting at the void volume and late in the chromatogram with significant tailing. This scenario is indicative of the classic general elution problem in liquid chromatography (7), and suggested that a gradient method would be more appropriate for the analysis of degraded samples.

Here we describe a gradient HPLC method that was developed to improve the resolution and detection of low level degradation products in SR adinazolam mesylate tablets. The method was designed to be compatible with thermospray mass spectrometry (MS). The structures of five of these degradation products were deduced from the molecular weight information provided by LC-MS analysis, and confirmed by comparing the chromatographic retention times of authentic compounds to that obtained in degraded samples. The chemical structure of the degradation products led us to postulate two degradation pathways (hydrolytic and oxidative) of ADM in the solid-state.

Quantitation of the low-level degradation products was performed using an adaptation of a technique known as "high-low" chromatography (8). A concentrated sample preparation was assayed using adinazolam mesylate at a concentration near that of the analytes as the standard and relative response factors were used to calculate the individual analyte concentrations. Details of the method development and results from the assay of degraded tablets are reported. Mass balance of the degraded samples and its implication relative to the proposed degradation pathways is also discussed.

MATERIALS AND METHODS

Materials

HPLC grade water and acetonitrile from Burdick and Jackson were used to prepare mobile phases. Ammonium formate and formic acid of reagent grade quality from Aldrich Chemical Co. were used to prepare the aqueous buffers. Authentic samples of adinazolam mesylate, potential degradation products and related compounds were obtained from The Upjohn Co. Structures and names of these compounds are given in Figures 1 and 4).

Instrumentation

A modular HPLC system consisting of two Waters pumps (model 510) plus an automated gradient controller (model 680), a Waters Wisp 712 autosampler (Millipore Corporation), and a UV Monitor D detector (Milton Roy, fixed

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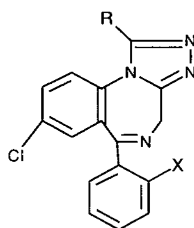

R	X	Common Name
$CH_2N(CH_3)_2$	H	adinazolam
CH_3	H	alprazolam
CH_3	Cl	triazolam
CH_2NH_3	H	N,N-didesmethyladinazolam

Fig. 1. Structures of adinazolam and related benzodiazepines.

wavelength, 254 nm) was used. A Hewlett Packard Series II 1090 Liquid Chromatograph with a DR5 ternary solvent delivery system and diode-array detection interfaced to a Chem Station was also used for some experiments. Both instruments were interfaced to an in-house computer for data collection.

Separations were obtained using a 4.6 mm ID × 25.0 cm YMC-Basic column (P.N. B-03-5 obtained from YMC, Inc.). Mobile phase A (20:80 acetonitrile: 0.05 M ammonium formate, pH = 4.5 with formic acid) and mobile phase B (80:20 acetonitrile:0.2 M ammonium formate, pH = 4.5 with formic acid) were preblended in separate bottles, filtered through a 0.45 μM nylon 66 filter, and degassed with vacuum and sonication prior to use. The apparent pH of mobile phase A was measured to insure that it was 4.8 ± 0.1. The apparent pH of mobile phase B was 6.3. One hundred (100) μL aliquots of the sample and standard preparations were chromatographed at ambient temperature using the following gradient conditions with detection at 254 nm and a flow rate of 1.0 ml/min: linear gradient from 0 to 50% B over 45 minutes; linear gradient from 50 to 100% B over 10 minutes; isocratic hold at 100% B for 5 minutes; linear reverse gradient from 100% B to 0% B. The column was equilibrated at initial conditions for at least twenty minutes between injections. A sodium nitrate solution (3 mg/ml in mobile phase A) was injected to determine the void retention time of the column.

Thermospray LC-MS was performed on selected samples using the described HPLC conditions. The thermospray vaporizer was set at 80°C and the block was set at 200°C. A post-column reverse gradient was run to maintain a constant solvent composition being introduced into the thermospray interface. Mass spectral data were obtained with a Finnigan TSQ-70 triple quadrupole (Q3MS only) with repetitive scan from 150 to 1500 AMU every second.

Stressed Sample Preparation

Degraded samples were prepared by placing tablets in open beakers in a sealed desiccator containing saturated aqueous KNO_3 solution. The samples were stored in a mechanical forced air oven (Thermolyne, model no. OV35135) set at 40°C. These conditions produced a relative humidity of about 88% (9). Samples were taken periodically over eight weeks and stored in sealed vials with teflon coated screw caps (Wheaton) in a refrigerator until time of assay.

Assay Procedures

Standard Solutions. For the quantitation of degradation products, about 2.5 mg of adinazolam mesylate reference material was dissolved in 100 ml of extraction solvent (87% v/v acetonitrile in deionized water). An aliquot of the stock standard solution was diluted 6:10 with extraction solvent. This solution was further diluted 2:25 with mobile phase A to produce a standard solution at a concentration of about 1 μg/ml (calculated as the free base). For the determination of ADM concentration, 15 mg of adinazolam mesylate reference material was dissolved in 10 ml of extraction solvent. This solution was diluted 2:25 with mobile phase A to produce a standard solution at a concentration of about 94 μg/ml (calculated as the free base).

Sample Preparation. Three tablets were placed in a 40 ml vial with a Teflon lined screw cap (Wheaton). Five glass balls (approximately 5 mm in diameter) and 30.0 ml of extraction solvent were added. The vials were sealed and shaken on a mechanical shaker for at least 25 minutes at high speed. The samples were centrifuged at about 2000 rpm for about 5 minutes. Aliquots of the supernatant were diluted with mobile phase A according to the tablet strength (for 7.5 mg tablets: 4:25 dilution; for 15 mg tablets: 2:25 dilution; for 30 mg tablets: 1:25 dilution).

Recovery Experiments. Mixed standard solutions of the identified degradation products (N-desmethyladinazolam (NDADM), triazolylbenzophenone (TBP), estazolam (ESTZ), and adinazolam 6-aminoquinoline (6AQ)) were prepared in the extraction solvent over the 4 to 40 μg/ml range. Aliquots of the standard solutions were added to placebo tablets to simulate the 0.1 to 1% range for NDADM, TBP, and ESTZ and 0.1 to 10% range for 6AQ. ADM was added to each spiked placebo to simulate the respective tablet strength (7.5, 15, and 30 mg). The final volume for each sample preparation was maintained at 10 ml using extraction solvent as diluent.

Sample Analysis. For the determination of degradation products, 100 μL aliquots of the sample preparation and a low concentration (1 μg/ml) ADM standard solution were chromatographed with the Waters HPLC system. For the determination of ADM, separate 100 μL aliquots of the same sample preparation and a high concentration (95 μg/ml) ADM standard solution were chromatographed with the Hewlett Packard HPLC system. This instrument has a shorter cell path length than the Waters instrument (6 vs. 10 mm) and the interface attenuated the signal by a factor of 50% to the computer. This allowed measurement of the ADM response in the sample preparation without dilution (this was not possible by just using the Waters instrument since the ADM response was greater than 1 absorbance unit and offscale for this computer system). An initial isocratic hold for 2.4 minutes was used with the Hewlett Packard 1090 instrument to account for the difference in gradient delay volume between the two instruments.

Calculations. The degradation products were identified using relative retention times measured with respect to the ADM peak. The areas of the analyte peaks were corrected

by dividing by the respective relative response factor. These factors were determined with authentic compounds (Table II). The concentration of analytes (μg/tablet) were then calculated using the 1 μg/ml ADM standard response data. The ADM concentration in the tablets was calculated using the 94 μg/ml standard response data. Unknown degradation products were assigned a relative response factor of 1. Results were calculated as the free base equivalent. Mass balance was calculated by summing the total mass detected (ADM plus degradation products) and dividing by the amount of ADM in an undegraded tablet.

RESULTS AND DISCUSSION

Development of the Chromatographic Separation

The YMC Basic C8 column gave the most promising peak shapes and selectivity in preliminary column screening, therefore this column was used to develop the gradient separation. Ammonium formate was used as the buffer to be compatible with LC-MS and preparative HPLC requirements. Acetonitrile and aqueous buffer were preblended in the A and B mobile phase reservoirs to maintain constant formate concentration. Figure 2 shows a typical chromatogram of a degraded tablet sample using the gradient conditions listed in the Experimental section. The adinazolam concentration had degraded by about 11% following exposure of the tablets to conditions of elevated temperature (40°C) and relative humidity (88%) when stored in open containers for a period of 2 weeks (Fig. 2). The final gradient conditions shown in Figure 2 were chosen based on several optimization experiments. In these experiments the effects of the formate concentration, the apparent pH of mobile phase A and B, and the gradient time on the retention of ADM and the major degradates (peaks 2 and 5–10) were examined. An increase in the formate concentration improved the resolution for some peak pairs but was limited to less than 0.08 M due to insolubility of the buffer above this concentration. Acceptable resolution and peak spacing were obtained at 0.04 moles/L ammonium formate. Figure 3 shows the effect of the apparent pH of mobile phase A on retention. Variation in pH of mobile phase A did not affect the retention of peaks 5 and 9, but did significantly affect the retention of peaks 2, 6, 7 (ADM), 8, and 10. Increasing the pH above 5.0 caused an increase in retention with some loss in resolution, while lowering the pH below 4 caused loss of resolution and hydrolysis of ADM to form the ring-open degradation product of adinazolam (4). Ring-open formation is a well-known reversible reaction of triazolobenzodiazepines that occurs in acidic solution. It is easily identified as peak 3 in Figure 2 by injecting an acidified solution of ADM. An apparent pH of 4.8 for mobile phase A gave a good separation of the analytes with negligible on-column ring-open formation. Retention of ADM and the major degradates was not significantly affected by variation of the pH of mobile phase B. A gradient time of 45 minutes (slope = 0.7% acetonitrile/min.) for the first linear segment gave adequate resolution and retention of early eluters with a run time of about 65 minutes per injection. Increasing the gradient time increased the retention without significant increase in resolution. Decreasing the gradient time caused loss of resolution of the early eluters.

Identification of Degradation Products

To determine the chemical structure of the degradates of adinazolam shown in Figure 2, selected undiluted sample extracts were screened using the gradient LC method coupled with thermospray (TSP) mass spectrometry. TSP sensitivity was limited by low column loading. It was determined that sample injections greater than 10 μL (15 μg) caused a significant reduction in chromatographic efficiency. Therefore, 8 μL (12μg) injections afforded a suitable compromise between LC-MS sensitivity and chromatographic performance. The peaks in the total ion current chromato-

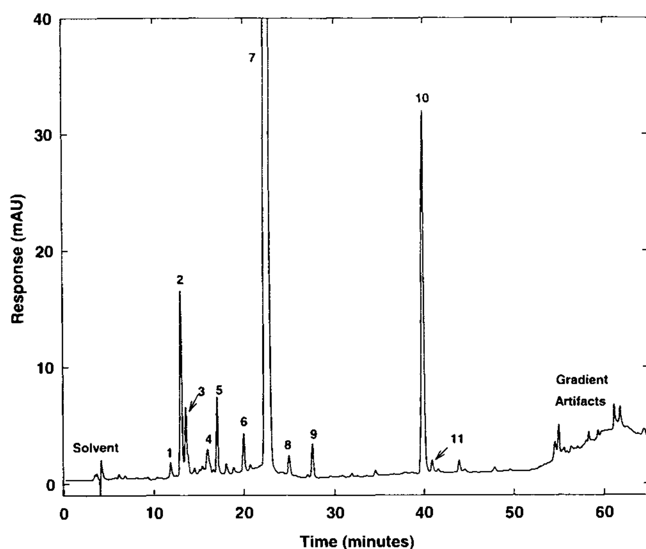

Fig. 2. Chromatogram of a degraded 30 mg/tablet sample preparation (adinazolam is about 11% degraded). See Table 1 for peak identifications. Scale expansion is about 25×.

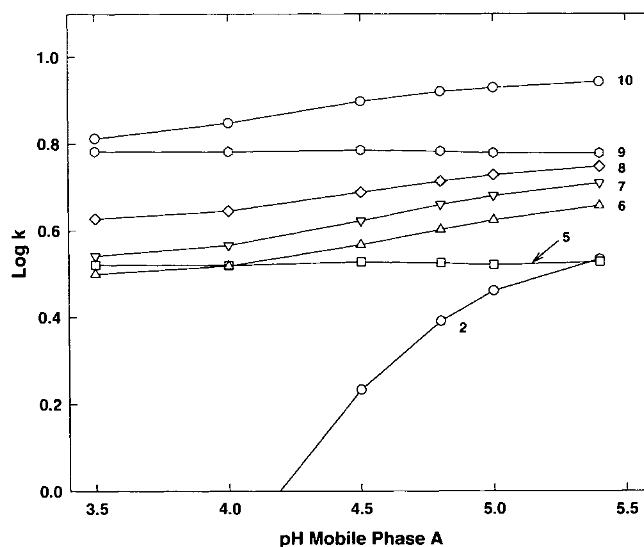

Fig. 3. Plots of capacity factor (k) for adinazolam and degradation products vs. the apparent pH of mobile phase A (the formate concentration was 0.04 moles/L, the apparent pH of mobile phase B was 6.3). See Table 1 for compound identifications.

gram corresponded to that obtained by UV detection, which indicates that no major degradation products which lack a chromophore are formed. Table I presents the molecular weight results obtained by LC-MS with proposed structural identification of 5 of the peaks based on logical chemical arguments. The identities of these 5 degradates of ADM, shown in Figure 4, were confirmed by chromatographing authentic materials and comparing both retention times and UV spectra to that obtained in the degraded samples (see Figure 7, which shows a chromatogram of a sample spiked with the degradation products and related compounds).

These data led us to postulate two degradation pathways of adinazolam in the solid state. The triazolylbenzophenone (TBP) and 6-aminoquinoline (6AQ) analogs, (peaks 6 and 10) form via the hydrolytic decomposition pathway outlined in Figure 5. These reactions were described previously for other benzodiazepines (10). Peak 3 is consistent with the ring-open form of adinazolam, resulting from the well-known acid-catalyzed hydrolysis of the diazepine ring nitrogen. A number of studies have been conducted that describe this reaction for related benzodiazepines (11–16). TBP and 6AQ are formed via the facile secondary degradation of this unstable "ring-open" benzophenone. Hydrolysis of the triazole ethylamine side chain and recyclization yield these products directly.

An oxidative degradation pathway starting with the formation of hydroperoxide intermediates is proposed in Figure 6 to explain the formation of N-desmethyladinazolam (NDADM) and estazolam (ESTZ) decomposition products (peaks 5 and 9). These components result from the secondary hydrolysis of the corresponding N-dimethyl side chain hydroperoxides with commensurate elimination of formaldehyde (for NDADM) and dimethylformamide (for ESTZ). The hydroperoxide intermediates would be unstable to TSP analysis and therefore would not produce molecular ions.

It should be noted that the oxidative decomposition products proposed can undergo secondary hydrolytic degradation and vice versa. Evidence of this is the identification of the 6-aminoquinoline analog of estazolam (ESTZ-6AQ, peak 11). The molecular weight and retention time of this compound is consistent with this assignment. Based on the retention time parallels between ESTZ and ESTZ-6AQ, and ADM and it's 6AQ analog, it is not unreasonable to expect

Table I. LC-MS Data for Degradation Products

Peak Number	Molecular Weight	Identification[a]
2	369	Unknown
3	369	Ring-open
4	397	Unknown
5	337	N-Desmethyladinazolam (NDADM)
6	340	Triazolylbenzophenone (TBP)
7	351	Adinazolam (ADM)
8	477	Unknown
9	294	Estazolam (ESTZ)
10	351	Adinazolam 6-aminoquinoline (6AQ)
11	294	Estazolam 6-aminoquinoline (ESTZ-6AQ)

[a] Structures of the degradation products are provided in Figures 4 and 5.

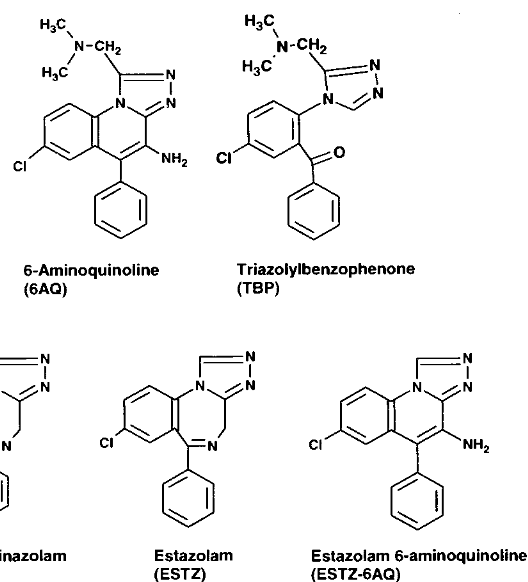

Fig. 4. Chemical structure of five degradation products of adinazolam.

that the low level peaks observed in the UV chromatograms of degraded samples (e.g., Figure 2) may be the corresponding analogs of NDADM and other unidentified components. Thus, secondary and higher order degradation by the permutation of hydrolytic and oxidative mechanisms may lead to a large number of trace components that are not detectable by conventional HPLC analysis.

Lucid structures could not be proposed for peaks 2, 4 and 8 based on molecular weight data alone. However, it was determined that peak 2, which is identical in molecular

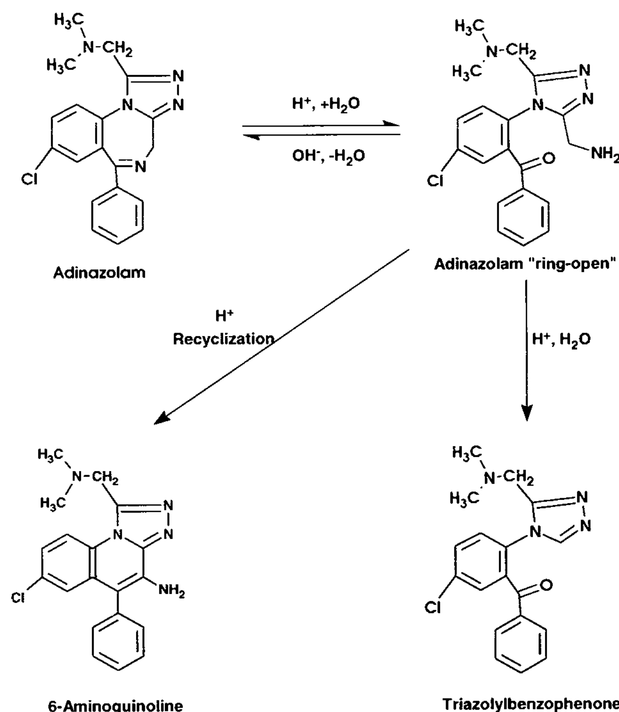

Fig. 5. The proposed acid catalyzed hydrolytic decomposition pathway of adinazolam in the sustained release formulation.

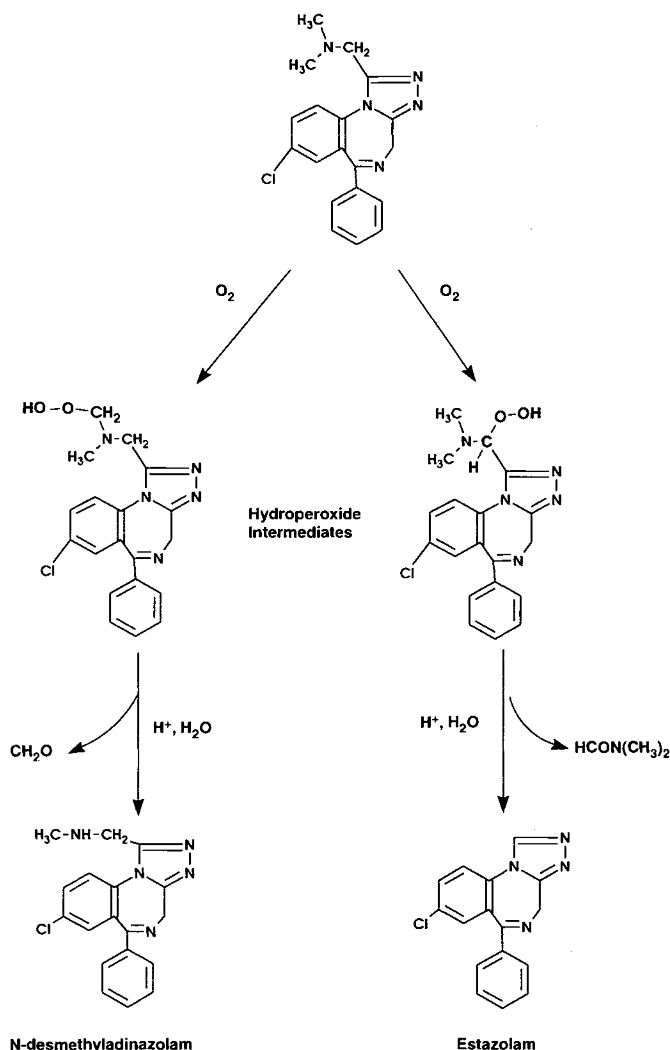

Fig. 6. The proposed oxidative decomposition pathway of adinazolam in the sustained release formulation.

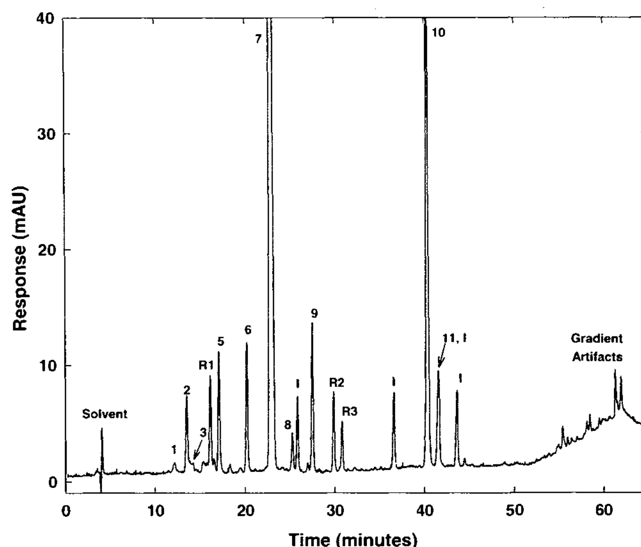

Fig. 7. Demonstration of method selectivity. Chromatogram of a sample extract spiked with degradation products and related compounds. 1. unknown. 2. unknown. 3. ring-open compound. 5. N-desmethyladinazolam. 6. Triazolylbenzophenone analog of adinazolam. 7. Adinazolam. 8. unknown. 9. Estazolam. 10. 6-aminoquinoline analog of adinazolam. 11. 6-aminoquinoline analog of estazolam. R1. N,N-didesmethyladinazolam. R2. Alprazolam. R3. Triazolam. I. process impurity. Scale expansion is about 10×.

weight to the ring-open compound, is in fact a unique decomposition product based on the distinct UV spectrum of this component (λ_{max} = 248 nm vs. λ_{max} = 260 nm for the ring-open compound).

Selectivity

In addition to selectivity for degradation products, the gradient method was evaluated for selectivity with respect to related triazolobenzodiazepines. Figure 7 shows a chromatogram of a sample preparation spiked with known degradation products, process impurities and other benzodiazepines (alprazolam, triazolam, and N-N-didesmethyladinazolam). All of the compounds were resolved except ESTZ-6AQ and a process impurity, which coeluted on most of the columns tested. The concentration of the process impurities are generally very low in the bulk drug, therefore interference in degraded samples is not likely. Between column variation in the separation (5 different column lots were tested) was negligible and there was good agreement between instruments

Method Validation

Peak area response at 254 nm for the known degradation products was linear in the 0.04 to 1.0 μg/ml range (0.04 to 10.2 μg/ml for ADM and 6AQ). This simulated the 0.04 to 1% range (10% for 6AQ) in a degraded sample. Relative response factors were calculated from the ratio of the slope of the analyte to that of the drug (see Table II). The estimated

Table II. Response Linearity Data at 254 nm for Known Degradation Products

Compound	Concentration range (μg/ml)	N	Slope ± SD	Intercept ± SD	F
NDADM	0.04–1.0	4	222630 ± 688	−191 ± 358	1.01
TBP	0.04–1.0	4	229500 ± 711	523 ± 369	1.04
ADM	0.04–10.2	6	221190 ± 260	358 ± 1131	—
ESTZ	0.04–1.0	4	264850 ± 346	346 ± 182	1.20
6AQ	0.04–10.0	6	250290 ± 196	−467 ± 836	1.13
ESTZ-6AQ	0.04–1.0	4	272540 ± 1382	919 ± 694	1.23

All of the intercepts were not significant (95% CI). R^2 = 1.000 for all the analytes. F = relative response factor.

detection limit for 6AQ, computed based on a response equivalent to 3 times the baseline noise, was about 0.005%.

The average recovery of adinazolam from spiked placebo was 100.4 ± 0.2% over the 48 to 101% of theory range. Recovery of NDADM, TBP, ESTZ, and 6AQ from spiked placebos are shown in Table III. Recovery was near 100% over the 0.5 to 10% range for the major degradate (6AQ). Similar accuracy was found for the other analytes at the 0.5 to 1% level, while lower recovery (94–96%) was found at the 0.1% level. These data indicated that the method had acceptable accuracy for this application.

Results for three samples assayed on several different days are shown in Table IV. The relative standard deviation (RSD) for the major degradation product (6AQ) was ≤2%, while generally less than 10% for the other degradates (except for peak 2 and ESTZ-6AQ). Calculated mass balances for these samples (sum of adinazolam plus degradation products in the degraded tablet divided by the same sum in the undegraded tablet) were less than 100% and were dependent on the extent of degradation in the tablet. Figure 8 shows the relationship between mass balance and percent degradation of adinazolam obtained from the assay of over 20 tablet samples. The average mass balance result obtained for samples that are an average of 9.5% degraded was 95.0 ± 1.5%. It is instructive to consider how many components would be required to account for the observed mass deficit. For this assay, a reasonable level below which peaks cannot be reliably quantified is 0.05%. It it is assumed that the 5% deficit in mass balance (for a sample that is 9.5% degraded) is due entirely to components at or below the quantitation limit, then 100 peaks would be required to account for this deficit. This is not an unusually high number if one considers that in a typical gradient chromatogram of a degraded sample, about 15 unknown peaks are quantified in addition to the five known degradation products. Therefore, it is possible that the decrease in mass balance with increase in percent degradation shown in Figure 8 may be explained by the formation of many components at trace levels due to degradation by various permutations of hydrolytic and oxidative reaction pathways. Another possible explanation that is still under investigation, is that the drug or one or more of the degradation products reacts with one of the excipients to form an insoluble adduct that is not extracted from the tablet matrix.

CONCLUSIONS

A gradient HPLC method was developed to determine the concentrations of low level degradation products in tablets containing adinazolam mesylate. In the presence of tablet excipients, high temperature and high relative humidity,

Table III. Recovery of Degradation Products from Spiked Placebo

Placebo formulation for indicated strength (mg/tab)	Percent of label	Mass (μg) Added	Mass (μg) Determined	Percent Recovery
		N-Desmethlyadinazolam (NDADM)		
7.5	1.0	60.8	60.8	100.0
15	0.1	12.1	11.8	97.5
	0.5	60.8	59.8	98.4
	1.0	121.6	119.4	98.2
30	1.0	231.3	227.6	98.4
		Mean ± SD		98.5 ± 0.9
		Triazolylbenzophenone analog (TBP)		
7.5	1.0	57.1	57.1	100.0
15	0.1	11.3	10.6	93.8
	0.5	57.1	57.0	99.8
	1.0	114.1	113.7	99.6
30	1.0	223.4	224.6	100.5
		Mean ± SD		98.7 ± 2.8
		Estazolam (ESTZ)		
7.5	1.0	48.8	48.9	100.2
15	0.1	8.4	8.4	100.0
	0.5	48.8	48.3	99.0
	1.0	97.6	97.3	99.7
30	1.0	197.9	198.3	100.2
		Mean ± SD		99.8 ± 0.5
		6-Aminoquinoline analog (6AQ)		
7.5	1.0	60.2	59.5	98.8
15	0.1	10.8	10.4	96.3
	0.5	60.2	59.3	98.5
	1.0	120.5	120.1	99.7
	5.0	588.1	589.9	100.3
	10.0	1176.2	1194.1	101.5
30	1.0	276.4	278.5	100.8
		Mean ± SD		99.4 ± 1.7

Table IV. Assay Results for Samples Stored at 88% RH at 40°C

	Sample A (3 weeks, 8% degraded, n = 6)		Sample A (6 weeks, 22% degraded, n = 9)		Sample B (3 weeks, 14% degraded, n = 6)	
Compound	μg/tablet ± SD	Percent of initial	μg/tablet ± SD	Percent of initial	μg tablet ± SD	Percent of initial
ADM	10502 ± 55	91.2	8977 ± 57	78.0	9814 ± 54	85.9
6AQ	232 ± 2	2.0	795 ± 4	6.9	460 ± 8	4.0
Unknown 2	77 ± 7	0.7	161 ± 21	1.4	106 ± 11	0.9
NDADM	42 ± 0.4	0.4	78 ± 1	0.7	57 ± 1	0.5
TBP	20 ± 1	0.2	54 ± 1	0.5	31 ± 1	0.3
Unknown 8	18 ± 1	0.2	53 ± 4	0.5	32 ± 2	0.3
ESTZ	23 ± 0.5	0.2	59 ± 1	0.5	32 ± 1	0.3
ESTZ-6AQ	6 ± 1	0.1	15 ± 7	0.1	10 ± 3	0.1
Unknown	114 ± 5	1.0	209 ± 11	1.8	165 ± 12	1.4
Sum	11034 ± 58		10401 ± 53		10707 ± 51	
% Mass Balance	96 ± 0.5	95.8	90 ± 0.5	90.4	94 ± 0.5	93.7

These samples originally contained 15 mg/tablet adinazolam mesylate. Results were calculated as the free base. Percent mass balance was calculated by dividing the total mass detected by the amount of adinazolam in an undegraded tablet. The mass detected for undegraded sample A was 11509 ± 49 μg/tablet and for sample B was 11425 ± 25 μg/tablet. Percent of initial was calculated by dividing the individual result by the respective initial value. SD = standard deviation.

adinazolam degrades to produce numerous degradation products. Five degradation products were identified by LC-MS analysis and confirmed by the chromatographic retention times of authentic compounds. Two solid-state degradation pathways based on hydrolytic and oxidative degradation mechanisms are proposed. The interplay between these two degradation pathways may account for the incomplete mass balance that is obtained at high levels of degradation.

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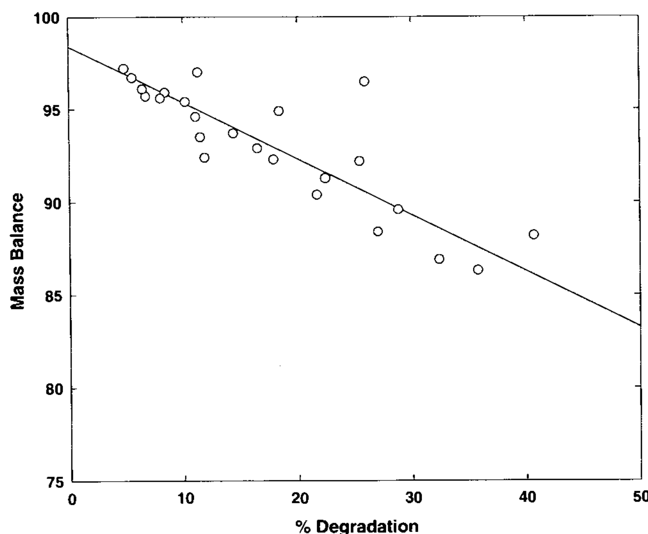

Fig. 8. Plot of mass balance as a function of % degradation.

REFERENCES

1. M. Japp, K. Garthwaite, A. V. Geeson, and M. D. Osselton. Collection of analytical data for benzodiazepines and benzophenones. *J. Chromatogr.* 439:317–339 (1988).
2. L. A. Berrueta, B. Gallo, and F. Vicente. Biopharmacological data and high-performance liquid chromatographic analysis of 1,4-benzodiazepines in biological fluids: a review. *J. Pharmaceutical & Biomedical Analysis* 10:109–136 (1992).
3. D. L. Theis and P. B. Bowman. Development of a liquid chromatographic method for the determination of triazolobenzodiazepines. *J. Chromatogr.* 268:92–98 (1983).
4. G. W. Peng. Assay of adinazolam in plasma by liquid chromatography. *J. Pharm. Sci.* 73:1173–1174 (1984).
5. G. J. VanGiessen, G. W. Peng, P. A. Bombardt, and F. L. Gilyard. High performance liquid chromatographic assay for adinazolam mesylate in rodent feed mixtures. *J. Liquid Chromatogr.* 9:1051–1064 (1986).
6. A. Lockniskar and D. J. Greenblatt. High-performance liquid chromatographic assay of adinazolam and its demethyl metabolite. *J. Chromatogr.* 424:215–217 (1988).
7. L. R. Snyder and J. J. Kirkland, *Introduction to Modern Liquid Chromatography*, John Wiley & Sons, Inc., 2nd edition, chap. 2, 1979.
8. E. L. Inman and H. J. Tenbarge. High-low chromatography: estimating impurities in HPLC using a pair of sample injections. *J. Chromatographic Sci.* 26:89–94 (1988).
9. J. A. Dean. *Lange's Handbook of Chemistry,* McGraw-Hill Book Company, 13th ed., 1985.
10. W. Mayer, S. Erbe, and R. Voight. Contribution to the analysis and stability of several pharmaceutically relevant benzodiazepines. *Pharmazie,* 27:32–42 (1972).
11. M. J. Cho, T. A. Scahill, and J. B. Hester, Jr. Kinetics and equilibrium of the reversible alprazolam ring-opening reaction. *J. Pharmaceutical Sci.* 72:356–362 (1983).
12. J. C. Vire and G. J. Patriarche. Polarographic behavior and degradation studies of triazolam. *J. Electroanal. Chem.* 214:275–282 (1986).

13. R. M. Jimenez, R. M. Alonso, F. Vicente, and L. Hernandez. Reversible ring-opening reaction of a triazolobenzodiazepine, triazolam, in acidic media. *Bull. Soc. Chim. Belg.* **96**:265–274 (1987).
14. R. M. Jimenez, E. Dominguez, D. Badia, R. M. Alonso, F. Vicente, and L. Hernandez. On the mechanism of hydrolysis of the triazolobenzodiazepine, triazolam. Spectroscopic study. *J. Heterocyclic Chem.* **24**:421–424 (1987).
15. R. M. Jimenez, R. M. Alonso, E. Oleaga, and F. Vicente. Polarographic study of the hydrolysis of a triazolobenzodiazepine, estazolam. *Bioelectrochemistry and Bioenergetics* **19**:533–539 (1988).
16. N. Inotsume and M. Nakano. Reversible ring-opening reactions of triazolobenzo- and triazolothieno-benzodiazepines in acid media at around body temperature. *Chem. Pharm. Bull.* **28**:2536–2540 (1980).