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Short Communication

New high-performance liquid chromatographic method for the determination of alprazolam and its metabolites in serum: instability of 4-hydroxyalprazolam

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ABSTRACT

A high-performance liquid chromatographic method was developed for the determination of alprazolam (ALP) and its active metabolites, α-hydroxyalprazolam (AOH) and 4-hydroxyalprazolam (4OH) in human serum. During assay development, the instability of 4OH was revealed. Factors affecting stability of 4OH were then investigated. In this report, the assay methodology for the determination of ALP and AOH, the instability of 4OH, subsequent interference of 4OH breakdown products with AOH quantification, and factors affecting 4OH stability are described. The clinical significance of our findings are reported.

INTRODUCTION

Alprazolam (ALP) is a triazolobenzodiazepine used in the treatment of generalized anxiety [1], panic disorder [2], and depression [3]. It is extensively metabolized to at least 29 metabolites, two of which, α-hydroxyalprazolam (AOH) and

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4-hydroxyalprazolam (4OH), are pharmacologically active [4]. AOH, the major active metabolite, accounts for 15% of the urinary excretion of [^{14}C]ALP [4] and is approximately 60% as potent as alprazolam [5]. 4OH accounts for less than 1% of the urinary excretion of [^{14}C]ALP [4] and is approximately 20% as potent as alprazolam [5].

Several assays are available for the detection of ALP in human serum or plasma [6–8]. Simultaneous determination of ALP, AOH and 4OH concentrations in human plasma has been reported by Miller and DeVane [9] who used a reversed-phase high-performance liquid chromatographic (HPLC) method.

In this paper, we describe a new analytical method for determination of ALP and AOH in serum; in addition, we present evidence for the instability of 4OH, factors affecting 4OH stability, the interference of 4OH breakdown products with AOH quantification, and the significance of our findings.

EXPERIMENTAL

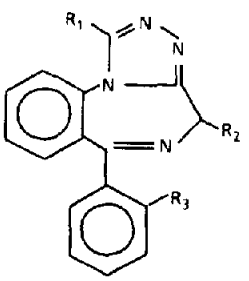
Apparatus

Chromatography was performed with an automated HPLC system consisting of a Waters (Millipore-Waters, Milford, MA, USA) Model 512 solvent delivery system, a Waters Model 1712 intelligent sample processor, a Supelco (Bellefonte, PA, USA) reversed-phase C_{18} column, 5 μm particle size, 15 cm × 4.6 mm I.D. × 6.35 mm O.D.), a Rainin (Woburn, MA, USA) Model ELD-1233 column heater, a Waters Lambda-Max Model 481 LC spectrophotometer (221 nm), and a Waters 740 data module.

Reagents and chemicals

Control reference standards of ALP, AOH, 4OH, and triazolam (TR) were supplied by The Upjohn Company (Kalamazoo, MI, USA). The chemical structures of each are shown in Fig. 1.

Hexyltriethylammonium phosphate (HTAP), 0.5 *M*, was obtained from Regis (Morton Grove, IL, USA). Methanol, methylene chloride, toluene, and water



ALP:	$R_1 = CH_3$	$R_2 = H$	$R_3 = H$
AOH:	$R_1 = CH_2OH$	$R_2 = H$	$R_3 = H$
4OH:	$R_1 = CH_3$	$R_2 = OH$	$R_3 = H$
TR:	$R_1 = CH_3$	$R_2 = H$	$R_3 = Cl$

Fig. 1. Structures of alprazolam (ALP), α-hydroxyalprazolam (AOH), 4-hydroxyalprazolam (4OH) and triazolam (TR).

were HPLC-grade reagents. Acetone, monobasic and dibasic sodium phosphate, ammonium hydroxide (2.0 *M*), and phosphoric acid were analytical-grade products.

Buffer, solutions and mobile phase

The mobile phase was composed of methanol–0.001 *M* phosphate buffer, and 0.003 *M* HTAP in water at pH 7.4 (40:60, v/v). The flow-rate was 2 ml/min at 35°C.

Standard curve preparation

Separate methanolic stock solutions of ALP, AOH, 4OH, and TR were prepared. TR stock solution was diluted with toluene to make a 1 μg/ml internal standard solution. Four working solutions (WS) were prepared in toluene and stored at −20°C until use. WS A contained 1 μg/ml ALP, 500 μg/ml AOH, and 500 μg/ml 4OH; WS B contained 200 ng of each analyte per ml; WS C contained 100 ng of ALP, AOH, and 4OH per ml; and WS D contained 20 ng of ALP, AOH, and 4OH per ml. Calibration curve standards were prepared by adding different volumes of the appropriate WS to a clean test tube, drying under nitrogen for 15 min at 50°C, adding 1 ml of blank human serum, and mixing the sample for 15 s by vortex. The final concentrations used in the calibration curves were 1–50 ng of ALP per ml, 1–25 ng of AOH per ml, and 1–25 ng of 4OH per ml.

Extraction procedure

The extraction procedure began with the addition of 25 μl of internal standard solution and 75 μl of ammonium hydroxide (0.1%, w/v). Samples were mixed by vortex for 30 s; 5 ml of methylene chloride and 5 ml of toluene were added. Samples were then capped, shaken for 15 min, and centrifuged for 10 min at 177 *g*. The aqueous layer was pipetted to waste; the test tube was then placed in a dry ice–acetone mixture for 30 s. With the residual aqueous layer frozen, the organic layer was decanted into a labeled conical tube and dried under nitrogen at 50°C. A 200-μl aliquot of mobile phase was added to each test tube, which was then mixed by vortex for 30 s and transferred to automatic injector vials; 125 μl were then injected onto the chromatographic system.

Calculations

The peak-height ratio (PHR)–concentration relationship was established for two curves (low curve: concentrations less than or equal to 10 ng/ml; high curve: concentrations greater then 10 ng/ml) using weighted least-squares regression (weight = $1/\text{concentration}^2$).

Validation

Six validation standard curves were analyzed to demonstrate standard response linearity and assay sensitivity. Standards were considered outliers and omitted from analysis by using Dixon's criteria for rejecting outliers [10].

Instability of 4OH

The instability of 4OH was first suspected when a standard mixture of ALP, AOH, 4OH, and TR, injected directly onto the column, produced extraneous peaks as the mixture aged. Moreover, the 4OH peak decreased in height and the AOH peak increased in height. A series of preliminary studies were completed to verify this observation and to evaluate the contribution of temperature, light, and pH on 4OH stability.

The first experiment was carried out by injecting a new solution of each analyte (ALP, AOH, 4OH, and TR) in mobile phase onto the HPLC system. These solutions were kept at room temperature for two weeks and reinjected. Because of obvious 4OH degradation, the effect of temperature and exposure to light on the stability of 4OH were determined in a second experiment. A 4OH solution prepared in mobile phase was divided into three aliquots. One was stored 4°C, one was stored at room temperature and a third was stored at room temperature protected from light. Two weeks later the solutions (containing $\sim$ 25 ng of 4OH) were reinjected.

Subsequently, the effect of pH on the stability of 4OH was determined. A new solution of 4OH in mobile phase was again prepared and separated into two autosampler vials. The pH of one solution was 7.4; the pH of the other was adjusted to 4.5 with 20% (w/v) phosphoric acid. After 30 min, a sample (containing $\sim$ 25 ng of 4OH) from each vial was injected onto the column.

Patient samples

Concentrations of ALP and AOH were determined in over 1000 serum samples collected from normal volunteers and dialysis patients receiving 0.5 or 2 mg of ALP [11]. Twenty-nine standard curves were analyzed during this four-month process; WS were prepared weekly during sample analysis.

RESULTS

Peaks of ALP, AOH, 4OH, and TR were separated during the chromatographic procedure. Fig. 2 illustrates a typical chromatogram after extraction of a calibration standard and a patient sample collected before and after alprazolam administration. The calibration curves were consistently linear and passed through the origin. A summary of the ALP and AOH results from standard curves determined during the validation procedure and during the analysis of 29 standard curves are listed in Table I. Overall accuracy and precision are within 10% for ALP and 19% for AOH.

Instability of 4OH

When chromatograms from injections made before and after two weeks of storage of solutions were compared, the solutions of ALP, AOH, and TR remained stable as indicated by a single peak. However, after storage for two

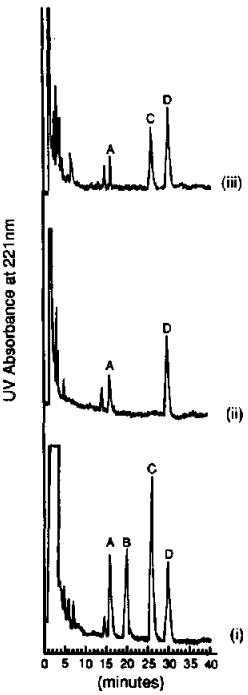


Fig. 2. Chromatogram following extraction of (i) a calibration curve standard (containing 25 ng of ALP, 12.5 ng of AOH and 12.5 ng of 4OH) and a patient sample collected (ii) before and (iii) after administration of 0.5 mg of alprazolam where (A) is 4-hydroxyalprazolam eluting at 16 min, (B) is α-hydroxyalprazolam eluting at 20 min, (C) is alprazolam eluting at 26 min, and (D) is triazolam eluting at 30 min. An unidentified peak coelutes with 4-hydroxyalprazolam in approximately 20% of samples as illustrated in (ii).

weeks, the 4OH peak decreased in height and two additional peaks, one of which coeluted with AOH, appeared (Fig. 3).

In the second experiment, the 4OH solutions which were kept at room temperature (in light and light-protected) appeared to break down similarly. The solution stored at 4°C deteriorated only slightly.

Injection of the solution maintained at a pH of 7.4 resulted in a single peak eluting at 14 min. The solution adjusted to a pH of 4.5 resulted in the elution of two peaks: one at 14 min as expected and the other at 6 min (Fig. 4).

Patient samples

Maximum ALP concentrations ranged from 5.28 to 14.43 ng/ml after 0.5 mg of ALP and from 17.96 to 51.15 ng/ml after 2 mg of ALP. AOH concentrations were less than 10% of ALP concentrations; maximum AOH concentrations ranged from < 1.00 to 3.80 ng/ml after 0.5 mg of ALP and from < 1.00 to 5.85 ng/ml after 2 mg of ALP [11]. There was no evidence of 4OH breakdown prod-

TABLE I

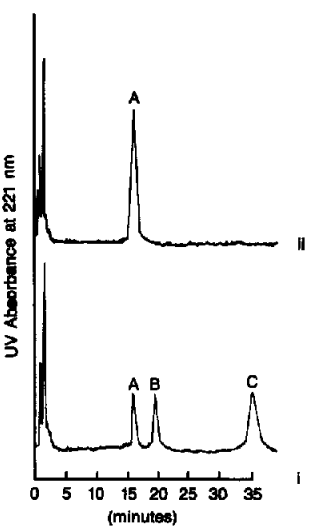
RESULTS FROM STANDARD CURVES

Predicted concentration (ng/ml)	Validation standard curves[a]		All standard curves[b]	
	Observed concentration (mean ± S.D.) (ng/ml)	C.V.[c] (%)	Observed concentration (mean ± S.D.) (ng/ml)	C.V.[c] (%)
Alprazolam				
1.0	1.00 ± 0.02	1.79	1.02 ± 0.02	2.36
7.5	7.59 ± 0.27	3.50	7.69 ± 0.50	6.51
25.0	24.76 ± 1.31	5.27	24.27 ± 1.24	5.09
50.0	51.22 ± 2.62	5.11	50.81 ± 2.05	4.03
α-Hydroxyalprazolam				
1.0	1.03 ± 0.03	2.45	1.04 ± 0.06	5.92
7.5	7.04 ± 0.89	12.63	7.97 ± 0.74	9.26
15.0	16.06 ± 0.63	3.94	15.50 ± 1.38	8.91
25.0	24.61 ± 0.68	2.75	24.18 ± 1.10	4.55

[a] Results from six standard curves.

[b] Overall results from 29 standard curves analyzed during sample analysis.

[c] Coefficient of variation.



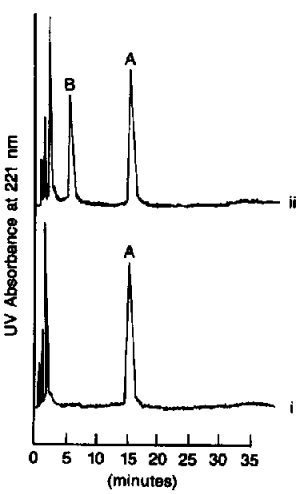


Fig. 3. Chromatogram of (i) a solution of 4-hydroxyalprazolam in mobile phase stored at room temperature for two weeks and (ii) a fresh solution of 4-hydroxyalprazolam in mobile phase. (A) is 4-hydroxyalprazolam and (B) and (C) are breakdown products of 4-hydroxyalprazolam.

Fig. 4. Chromatogram of (i) a fresh solution of 4-hydroxyalprazolam in mobile phase at pH 7.4 and (ii) a fresh solution of 4-hydroxyalprazolam in mobile phase at pH 4.5 (A) is 4-hydroxyalprazolam and (B) is a 4-hydroxyalprazolam breakdown product.

ucts in samples from standard curves. Peaks consistent with 4OH instability were present in fewer than 10% of serum samples from patients.

DISCUSSION

The analytical method described for determining ALP and AOH concentrations in serum is sensitive, accurate, and precise, and allows for the detection of 4OH degradation. The significance of our findings are discussed.

Analytical method

The accuracy and precision of this analytical method for ALP and AOH determination were within acceptable limits during the validation procedure and analysis of 29 standard curves. Miller and DeVane [9] described a reversed-phase HPLC method for simultaneous determination of ALP, AOH, and 4OH with a mobile phase composed of acetonitrile and phosphate buffer. These investigators reported similar results with an inter-day variability of 10% for ALP, 14% for AOH, and 19% for 4OH.

Evidence of 4OH instability

We are the first to report the instability of 4OH and its subsequent interference with the determination of AOH. Although 4OH instability has not been reported previously, Miller and DeVane [9] reported a high degree of inter-day variability for 4OH which is consistent with 4OH instability. The elution of 4OH breakdown products and potential interference with AOH determination in their assay cannot be predicted; however, it is likely that the instability of 4OH contributed to the high degree of inter-day variability in 4OH and AOH determination.

Factors affecting 4OH stability

Once the instability of 4OH was revealed, the contribution of temperature, pH, and exposure to light on the lack of 4OH stability was determined; these factors were chosen because of the ease of control during blood sample processing. Results from preliminary studies reported herein demonstrate that the instability of 4OH is not related to benzophenone formation or photo-decomposition but may be related to temperature. Benzophenone formation is secondary to a pH-dependent, ring-opening reaction common to triazolobenzodiazepines [12]. This reversible, ring-opening reaction is favored at an acidic pH; the closed ring form, however, is favored at a neutral to alkaline pH [12]. The breakdown product observed when changing the sample pH to 4.5 eluted at 6 min while the 4OH breakdown product, in our initial experiments, eluted after 16 min. Accordingly, these results indicate that ring opening of 4OH is not the mechanism for the breakdown of 4OH observed in our initial studies. Data from the solutions maintained at room temperature (in light and light-protected) and at 4°C demonstrate that controlling temperature, but not exposure to light, may be important in preserving the stability of 4OH.

Significance

To detect the significance of 4OH instability, the appearance of a 4OH breakdown product peak which elutes at 35 min (Fig. 3) was monitored. The appearance of this peak was also used to monitor potential interference with AOH concentration determination. Using this method, there was no evidence of 4OH breakdown resulting from the extraction procedure itself, nor was there evidence of 4OH breakdown from over 90% of the patient samples. Thus, the 4OH instability is not clinically significant and no further studies were completed to characterize the 4OH breakdown products.

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