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Short Communication

Confirmation of benzodiazepines in urine as trimethylsilyl derivatives using gas chromatography–mass spectrometry

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Abstract

A confirmation procedure for the identification and quantitation of various benzodiazepines in urine is presented. The urine sample is first hydrolyzed enzymatically because of the glucuronide conjugation of some benzodiazepine metabolites, then extracted using bonded-phase columns. After elution into an organic solvent, the samples are evaporated, converted to the trimethylsilyl ether derivatives and analyzed by electron ionization GC–MS. Quantitation was performed using selected-ion monitoring for each benzodiazepine using prazepam as the internal standard. The method provides excellent linearity and sensitivity for the trimethylsilyl derivatives.

1. Introduction

Benzodiazepines represent a relatively large group of compounds often used as anti-anxiety agents in the treatment of psychiatric disorders. These compounds are widely prescribed and often misused socially. Their therapeutic and toxic effects on humans have been well documented. Because of the increasing potency of new benzodiazepines, *e.g.* alprazolam and triazolam, highly sensitive procedures are required for their identification and quantitation. Benzodiazepines in urine are usually screened using immuno assays [1,2] and often confirmed using HPLC [3–7] or GC [8,9]. However, the most widely accepted and mandated chemical test for the confirmation of presumed positive samples is gas chromatography–mass spectrometry (GC–MS) [10–12]. Many benzodiazepines possess dual pK_a values and are relatively polar compounds, especially the desmethyl and hydroxylated metabolites. Therefore, derivatization is necessary to improve their chromatography. GC–MS analysis of benzophenone derivatives has been reported [13]. Although the trimethylsilyation derivative is subject to hydrolysis with time, it is easy to synthesize.

Various extraction procedures have been reported but they often lack the specificity and sensitivity required by laboratories and certifying agencies. The use of bonded–phase columns has improved the cleanliness of urine samples while maintaining high recovery and rapid extraction [14]. The co-polymeric Clean Screen columns provide added reproducibility and efficiency in the extraction of benzodiazepines which are analyzed by GC–MS.

Urine was selected because the Department of Transport now requires the determination of

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barbiturates and benzodiazepines as well as NIDA-5 in urine samples.

This paper provides a simple extraction and analysis method for the more difficult benzodiazepines and their metabolites.

2. Experimental

2.1. Reagents

All solvents and reagents used in the extraction were either ACS or HPLC grade. Standards for the various benzodiazepines were purchased from Radian Corporation, (Austin, TX, USA). Sylon BFT (BSTFA and TMCS, 99:1) was obtained from Supelco (Belle Fonte, PA, USA), in 1 ml ampules. Clean Screen bonded phase extraction columns and the vacuum manifold were obtained from United Chemical Technologies (Bristol, PA, USA).

2.2. Hydrolysis and extraction

Urine aliquots (5 ml) were initially hydrolyzed by adding β-glucuronidase solution (5000 F units/ml *Patella vulgata* in 0.1 *M* acetate buffer, pH 5.0, 2 ml) and heating at 65°C for 3 h. Enzyme hydrolysis was chosen over acid hydrolysis because of the lack of specificity with the latter. Some benzodiazepines convert to the same benzophenone when acid hydrolysis is employed. After hydrolysis, the samples were allowed to cool at room temperature and internal standard was added. The Clean Screen DAU columns were conditioned with methanol, (3 ml), deionized water (3 ml) and 0.1 *M* phosphate buffer pH 6.0 (1 ml). The columns were not allowed to dry out after the buffering step and the samples were poured into the expanded reservoir, 10-ml capacity columns (part No. ZSDAU020). The samples were aspirated slowly at 1–2 ml/min using a vacuum manifold system set at 10–17 kPa. The columns were washed with deionized water (2 ml) and 20% acetonitrile in 0.1 M phosphate buffer (pH 6.0) (2 ml). The columns were allowed to dry for 5 min using full vacuum and finally washed with hexane (2 ml). Elution was accomplished with ethyl acetate (3 ml) and the samples were subsequently evaporated to dryness at 40°C in a TurboVap water bath system from Zymark (Hopkinton, MA, USA).

After sample evaporation ethyl acetate (50 μl) was added to each tube and gently swirled. Sylon BFT (50 μl) was added to each sample and vortex mixed for 15–20 s. The tubes were capped and placed in a 70°C heating block for 20 min. After removal and cooling to room temperature, the samples were transferred to microvial inserts and injected onto the GC–MS system.

2.3. Analysis

GC–MS analysis was performed on a Hewlett-Packard 5890 gas chromatograph interfaced to an HP Model 5971A mass spectrometer. The GC column used for analysis was an Ultra I (12 m × 0.2 mm I.D., 0.33 μm film thickness) (Hewlett-Packard, Wilmington, DE, USA). All samples were automatically injected using a 7673A Hewlett-Packard autosampler. A 2-μl injection volume was programmed using the splitless mode of injection with an injection temperature of 250°C. Helium was used as the carrier gas with a linear velocity of approximately 35 cm/s. The detector temperature was held at 300°C. Initial oven temperature was 100°C for 0.00 min; ramped to 200°C at a rate of 50°C per min then ramped to 300°C at a final ramp rate of 20°C per min. The final temperature of 300°C was held for 3 min for a total run time of 10 min. The mass spectrometer was autotuned with perfluorotributylamine on each day of operation. Selected-ion monitoring was performed using the following ions: desalkylflurazepam, 359, 341, 245; nordiazepam, 341, 342, 343; oxazepan, 429, 430, 313; diazepam, 256, 383, 221; lorazepam, 429, 430, 347; nitrazepam, 352, 353, 306; temazepam, 343, 283, 257; clonazepam, 387, 352, 306; prazepam, 269, 324, 241; α-hydroxyalprazolam, 381, 396, 383; α-hydroxytriazolam, 415, 417, 430. All ions were monitored simultaneously.

The trimethylsilyl derivative was chosen because of its compatability with the silicone-treated capillary column. The derivative en-

hances column life as opposed to causing a detrimental effect.

3. Results and discussion

Recovery was determined by spiking blank urine samples with known concentrations of various benzodiazepines, then comparing peak-area ratios to an unextracted benzodiazepine standard known to contain 200 ng/ml. A single-point calibration forced through the origin was established at the beginning of each batch of samples. "Target" software from Thru-Put Systems automatically performed these functions using the computer data system. Blank urine extracts were free from interfering peaks.

In order to determine which factors in the extraction process were critical to extract cleanliness and recovery of the benzodiazepines, several parameters were evaluated. The first was to determine which initial pH and wash pH would be ideal for compound recovery, secondly, to determine which eluting solvent provides maximum recovery as well as cleanliness for GC–MS quantitation.

3.1. pH range effect

The application pHs ranged from 3.0 to 9.0 and the wash pHs ranged from 4.5 to 9.3. After evaluating several pH effects on the recovery of the benzodiazepines, it was determined that the overall optimum conditions occurred at pH 5–6 for recovery and cleanliness (Fig. 1C,D) except for flurazepam. This particular compound preferred basic conditions for the initial sample pH as well as the wash pH solution (Fig. 1E,J). While the recovery of flurazepam improved under these conditions, the recoveries of the other benzodiazepines decreased. Flurazepam metabolizes easily to desalkylflurazepam and later to hydroxyethylflurazepam. pH conditions established in the range of 5–6 provided excellent recovery of the desalkylflurazepam metabolite, exceeding 90%.

Deionized water was also evaluated assuming a pH 7.0; however, the pH of water changed daily and this did affect the between-run reproducibility.

An application pH of 5 was chosen so that hydrolysed samples did not have to be re-buffered prior to extraction. The wash pH of 6 gave reproducible results and clean extracts.

3.2. Eluting solvent effect

Eluting solvents of ethyl acetate, methylene chloride, hexane–ethyl acetate (50:50, v/v) and methylene chloride–isopropanol (80:20, v/v) were evaluated.

The primary goal was to extract as many benzodiazepines as possible from a single sample. The benzodiazepines are classified as weak bases with a wide range of pK_a values, many possessing dual values. Additionally, the extraction variability of tertiary, secondary and primary amines had to be considered.

Oxazepam, for example, is the 3-hydroxy metabolite of nordiazepam with dual pK_a values of 1.7 and 11.6 and is classified as a secondary amine. The compound is relatively polar so that it will not be miscible with many organic solvents, and extraction efficiency will be determined by how the extraction procedure effects these two pK_a values.

The Clean Screen DAU phase is consistent with a C_8 hydrophobic and a benzene sulfonic acid cation exchanger. In order for the cation-exchange function to perform correctly, a weakly basic drug must be ionized by pH adjustment to less than the compound's pK_a value. The general rule for ion exchange is to adjust the pH 2 units less than a compound's pK_a for 99% ionization. In the case of oxazepam, the sample must be applied at, or the column washed with a pH solution of less than 1.7 by 2 units. This could not be accomplished without some detrimental effect to the extraction column. Although ion-exchange mechanisms tend to perform more reproducibly and are generally cleaner chromatographically, they could not be used to extract benzodiazepines as a group. Therefore, the only other option for bonded-phase extraction was to incorporate the C_8 hydrophobic function on the copolymer. The only reason for

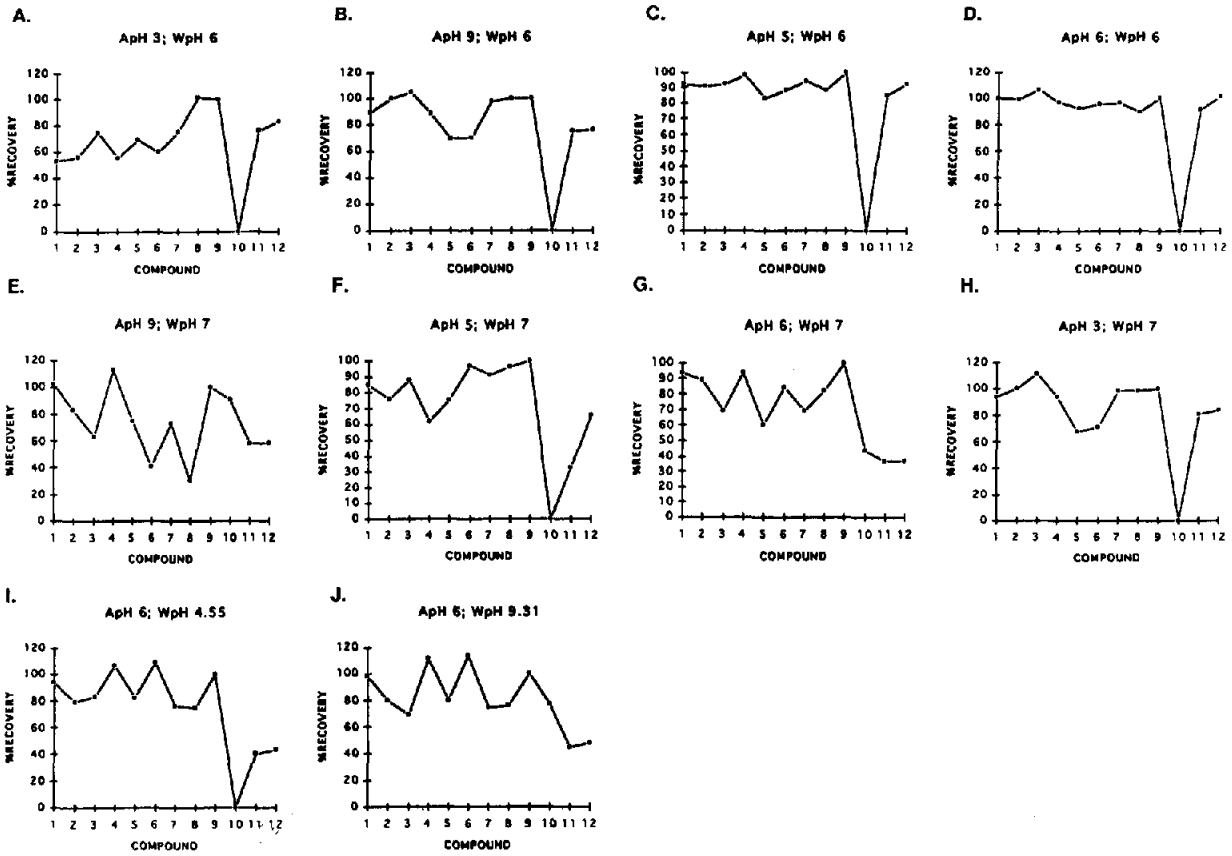

Fig. 1. ApH = pH of sample application; WpH = pH of column wash; Peaks: 1 = nordiazepam; 2 = desalkylfluroazepam; 3 = oxazepam; 4 = diazepam; 5 = lorazepam; 6 = nitrazepam; 7 = temazepam; 8 = clonazepam; 9 = prazepam (I.S.); 10 = flurazepam; 11 = α-hydroxyalprazolam; 12 = α-hydroxytriazolam; WpH 6 = 20% acetonitrile in 0.1 *M* (pH 6.0) phosphate buffer; WpH 7 = 20% acetonitrile in H_2O (pH 7.0); WpH 4.55 = 20% acetonitrile in 0.1 *M* monobasic phosphate buffer; WpH 9.31 = 20% acetonitrile in 0.1 *M* dibasic phosphate buffer.

continuing the use of the DAU copolymer was consistency of column. All our other NIDA-5 and barbiturate procedures are run on this column so the "one column for all" approach was adopted.

Since a hydrophobic mechanism for retention and elution was the only option available for the simultaneous extraction of benzodiazepines, solvent polarity was an important issue. Most organic solvents are capable of breaking hydrophobic retention allowing compounds to be eluted from the sorbent bed. More polar solvents like methanol and isopropanol will disrupt hydrophobic retention, and cause elution of many polar interfering substances as well as the compounds of interest. For this reason we evaluated several moderately polar–non-polar organic solvents and mixtures.

Ethyl acetate, methylene chloride, hexane–ethyl acetate (50:50, v/v) and methylene chloride–isopropanol (80:20, v/v) were evaluated. All of these solvents and solvent mixtures performed well and background noise was kept to a minimum. Hexane–ethyl acetate (50:50, v/v) gave slightly lower recoveries, possibly due to the increased solubility of the benzodiazepines in the more polar ethyl acetate as opposed to the hexane–ethyl acetate mixture. Methylene chloride or mixtures of methylene chloride–isopropanol provided excellent recovery and

cleanliness. However, many laboratories are reluctant to use halogenated solvents in their extraction procedures. For these reasons, we determined ethyl acetate to be the best choice for benzodiazepine elution from a hydrophobically bound sorbent. A sample chromatogram is shown in Fig. 2.

3.3. Extraction data

The described procedure using an application pH of 5, a wash pH of 6 and ethyl acetate as the elution solvent, gave the following recoveries (mean ± C.V.): desalkylflurazepam 92.1 ± 7.3%; nordiazepam 91.3 ± 6.2%; oxazepam 92.1 ± 8.6%; diazepam 98.4 ± 3.4%; lorazepam 83.0 ± 6.4%; nitrazepam 88.3 ± 7.1%; temazepam 94.3 ± 7.2%; clonazepam 88.1 ± 4.7%; α-hydrozyalprazolam 85.0 ± 4.9%; α-hydroxytriazolam 92.3 ± 8.1% ($n = 10$).

The extraction method was linear for all benzodiazepines in the range 20–1000 ng/ml and the limit of detection was 5 ng/ml for desalkylflurazepam, nordiazepam, lorazepam and temazepam; 10 ng/ml for oxazepam, diazepam, nitrazepam and clonazepam; 15 ng/ml for α-hydroxyalprazolam and α-hydroxytriazolam. The assay was consistent on a day-to-day basis with variations less than 9.9%.

Prazepam was used as the internal standard for all benzodiazepines with a retention time of 6.08 min. This was the same as the retention time of clonazepam but the different selected ions in MS-SIM made quantitation possible.

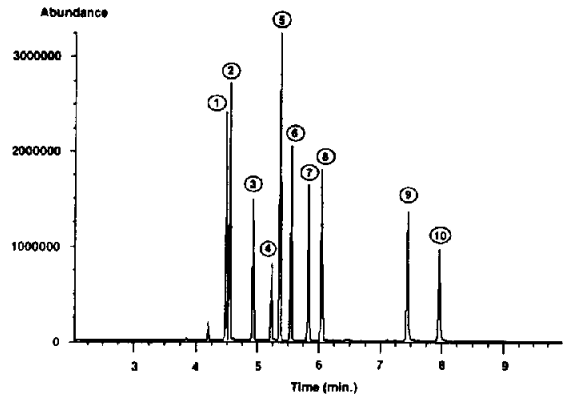

Fig. 2. Chromatogram of extracted benzodiazepines (200 ng/ml). Peaks: 1 = desalkylflurazepam (4.49 min); 2 = nordiazepam (4.54 min); 3 = oxazepam (4.95 min); 4 = diazepam (5.24 min); 5 = lorazepam (5.39 min); 6 = nitrazepam (5.56 min); 7 = temazepam (5.86 min); 8 = clonazepam (6.08 min); 9 = α-hydroxyalprazolam (7.48 min); 10 = α-hydroxytriazolam (8.00 min).

4. Conclusion

The bonded-phase extraction procedure presented has been found to accurately isolate several benzodiazepines commonly analyzed in laboratories today. The method combines bonded-phase technology with a single-step derivatization for GC–MS analysis that can be easily performed in any well equipped laboratory. Recovery and cleanliness are maintained while providing reproducible, quantitative results.

The uniqueness of this extraction is its ability to isolate metabolites of alprazolam and triazolam. Previous methods have isolated the parent drug which is rapidly metabolized and difficult to detect in urine. α-Hydroxyalprazolam, α-hydroxytrizolam, desalkylflurazepam and oxazepam have a longer half-life than the parent compound and are therefore more easily quantitated.

Unfortunately, we did not have access to "real" samples to determining the concentration–time curve after therapeutic dosing.

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