TECHNICAL NOTE

Weidong He,[1,3] *Ph.D.; Nicholas Parissis,*[1] *Ph.D.; and Trifon Kiratzidis,*[2] *M.D.*

Determination of Benzodiazepines in Forensic Samples by HPLC with Photo-Diode Array Detection*

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ABSTRACT: A high-performance liquid chromatographic (HPLC) method has been developed for the analysis of several benzodiazepines and some of their metabolites in blood, plasma and urine. The method included a liquid-liquid extraction with *n*-hexane:ethylacetate, a gradient elution on a C8 reversed phase column with a non-electrolyte eluent and a photo diode array detection. This allowed a rapid detection, a purity check, and identification as well as quantitation of the eluting peaks. The detection limit was 10 to 30 ng and the limit of quantitation was 0.05 μg/mL, using 1 mL of blood, plasma or urine. The procedure is applied routinely in forensic toxicological analyses involving blood, stomach content, urine and organ samples. About 30 positive cases are reported. The avoidence of the use of an electrolyte buffer in the eluent resulted in a robust procedure, free of technical problems and of long rinsing periods, suitable for routine use in forensic toxicology analysis involving blood, urine, stomach content and tissue samples.

KEYWORDS: forensic science, forensic toxicology, benzodiazepine, benzophenone, photo diode array detection, hydrolysis, high-performance liquid chromatography

Benzodiazepine drugs have hypnotic, anticonvulsant and tranquilizing properties (1) and are frequently encountered in emergency toxicology screening, drugs-of-abuse testing and forensic medicine examinations. Several analytical techniques for the isolation and quantitation of benzodiazepines in biosamples have already been published. These procedures have been reviewed by Sioufi and Dubois (2). Both thin-layer chromatography (TLC) and the immunoassay approach remain extremely useful for a first rapid screening. However, both techniques lack high specificity and in the case of immuno-analysis there is no discrimination between parent drug and metabolites (3–5). On the other hand, gas chromatography (GC) with flame-ionization (GC/FID) or mass-spectrometric detection (GC/MS) are much more sensitive and specific but they require complex equipment and derivatization procedures (6). In addition, the GC process can decompose thermolabile benzodiazepines such as oxazepam and chlordiazepoxide (7). The above-mentioned reasons resulted in a still-increasing popularity of HPLC for screening and quantitation of benzodiazepines in biosamples. Very often, however, the described procedures deal with the determination of only a single benzodiazepine and its metabolites (8–10). In simultaneous determinations of different benzodiazepines, single wavelength detection and identification of the eluting peaks based only on their retention behavior is very common (11–13). In postmortem matrices, however, numerous compounds do interfere in the chromatogram, leading to false positive results. To obtain more reliable peak identification criteria, additional information is necessary and more specific detection modes are recommended.

High-performance liquid chromatography (HPLC) combined with photo diode array detection is an extremely valuable solution as additional spectral information is obtained that is necessary for the identification of the eluting peaks (14). In this way, photo diode array detection is already applied for screening at multiple drug intoxicated cases (15–17). However, in the latter procedures, and also in a recent application to the analysis of benzodiazepines themselves (18), eluents always contain buffer salts that very often are known to cause technical problems such as crystal formation in connecting tubing and in the detector cell as well as damage to the pump seals.

We developed an HPLC procedure based on gradient elution and a reversed phase column using an eluent without any electrolyte buffer. The effluent is monitored by photo diode array detection with multi-wavelength, allowing both identification and quantitation of different benzodiazepines simultaneously in clinical or forensic samples.

Materials and Methods

Apparatus

A high-pressure gradient system was used, consisting of a HP 1090M liquid chromatograph and a HP 1040A diode array detector, a Rheodyne injector equipped with a 50-μL loop and a HP 79994A HPLC workstation (Hewlett Parkard, Palo Alto, CA). The column was a 100 mm × 3 mm inside diameter Chromsep glass column packed with ChromSpher C8, 5 μm (Cat. No. 28262) protected by a 10 mm × 2.0 mm inside diameter reversed phase guard column (Cat. No. 28141), all from Chrompack (Antwerp, Belgium).

[1] Laboratoy of Toxicology, University of Ghent, Ghent, Belgium.
[2] Veria Medical Center, Veria, Greece.
[3] Present address: Department of Organic Chemistry, University of Ghent, Ghent, Belgium.

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Reagents

All solvents and chemicals were of the highest grade from different manufacturers: isopropylamine (99%) from Acros (Geel, Belgium); methanol, *n*-hexane and ethylacetate from Romil Chemicals Ltd. (Loughborough, England); HPLC grade water from Prosan (Gent, Belgium); hydrochloric acid from UCB (Gent, Belgium) and potassium carbonate, potassium hydroxide from Merck AG (Darmstadt, Germany). The enzyme β-glucuronidase (EC 3.2.1.31 G0876) crude solution was purchased from Sigma Chemical Co. (St. Louis, MO).

Benzodiazepines used in the experiment were pure chemicals offered by Hoffmann-LaRoche (Basel, Switzerland and Brussel, Belgium), Upjohn (Kalamazoo, MI) and Fluka Chemie (Bornem, Belgium). The blank blood and plasma were provided by the blood bank of the university hospital (Gent, Belgium).

Stock Solutions

All benzodiazepine and benzophenone stock solutions were prepared in methanol (1 mg/mL), stored at 4°C and were found to be stable for several months. The working solutions were prepared by appropriate dilution of the stock solution with methanol:water, 50:50 (v/v).

Chromatographic Conditions

The gradient elution consisting of methanol(A)-water containing 0.125% (v/v) isopropylamine (B) was applied with the following profile: 0 to 2 min, 30% A; 2 to 3 min, from 30 to 43% A; 3 to 4 min, from 43 to 45% A; 4 to 5 min, from 45 to 48% A; 5 to 6.5 min, 48% A; 6.5 to 8.5 min, from 48 to 60% A; 8.5 to 10 min, from 60 to 75% A and 10 to 17 min, 75% A at a flow rate of 0.7 mL/min resulting in an average back pressure of 130 bar. The monitoring wavelength was set at 230 nm, bandwidth 10 nm, for the benzodiazepine determination and at 235 nm, bandwidth 10 nm, for the benzophenone detection with a reference wavelength at 550 nm, bandwidth 100 nm.

Sample Preparation Procedure

A 1 mL aliquot of whole blood, plasma or another biological matrix (homogenized stomach content, liver, or kidney) was taken for the analysis. To each aliquot, 2 μg triazolam was added as internal standard and a minimum of 0.5 mL 1 *M* K_2CO_3 was used to bring the sample under alkaline conditions. The extraction was performed with an 8 mL *n*-hexane: ethylacetate (7:3, v/v) mixture by rotary mixing for 2 min and then vortex mixing for 0.5 min. After centrifuging (5 min, 2000 rpm), the upper (organic) layer was transferred into a conical tube and evaporated to dryness under a gentle stream of nitrogen at 40°C. The residue was reconstituted in a 100 μL methanol: water (50:50, v/v) mixture and then centrifuged to become clear. If necessary, the sample may further be filtered with a syringe filter (Hewlett Packard, Part No. 5061-3366) prior to its injection into the chromatographic system.

A urine sample (1 mL) was acidified with 1.3 mL of 37% HCl and screw-stoppered. The hydrolysis step took place at 110°C on an aluminum block for 1 h. Thereafter, the sample was cooled down, and the pH was adjusted to 9 by addition of 10 *M* KOH and 5.4 μg of alprazolam was added as internal standard. The alkaline sample was extracted with 8 mL *n*-hexane by vortexing for 1 min. The organic layer was then evaporated to dryness under a flow of nitrogen in a water-bath (40°C) and reconstituted with 100 μL methanol-water (50:50, v/v) mixture.

An enzymatic digestion method was used for identification and quantification of the parent compounds from the benzodiazepine glucuronide or its conjugate usually present in urine. A 1 mL portion of urine was hydrolyzed at pH 5 by incubating with 0.1 mL of β-glucuronidase crude solution at 37°C for 12 h in a water-bath. The sample was then mixed with 2 μg triazolam (as internal standard), and alkalined by adding 0.5 mL of 1 *M* K_2CO_3. Further sample treatment was the same as the extraction procedure of the blood sample.

Method Evaluation

Calibration and Linearity—Calibration samples are prepared in blank blood, plasma and urine by addition of the appropriate amount of the respective benzodiazepines (maximum 2.00 μg/mL). Triazolam (20 μL of a 100 μg/mL methanolic solution) and alprazolam (20 μL of a 270 μg/mL methanolic solution) are used as internal standards for the different samples. The calibration samples are processed in the same way as the unknown samples. Calibration curves are obtained by plotting the peak height ratio of the analyte to that of the internal standard against the concentration of the respective benzodiazepines. The calibration curves are easily obtained by the use of the workstation-based least-square regression method.

Extraction Recovery—Extraction recovery is evaluated by comparing the slope of the calibration curve of the spiked sample with that obtained from the serial amounts of standard spiked in extraction solvent. For the acid hydrolysis of urine samples, the yield

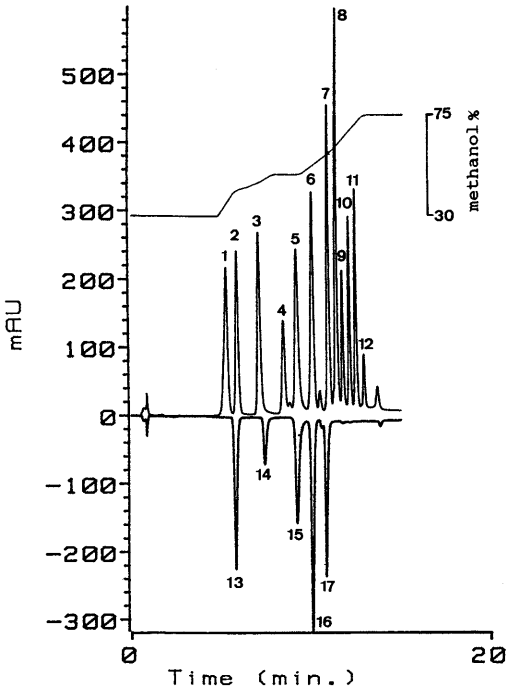

FIG. 1—*Representative chromatogram of standards (0.5 μg): 1 = 7-aminoflunitrazepam; 2 = nitrazepam; 3 = bromazepam; 4 = flunitrazepam; 5 = oxazepam; 6 = triazolam; 7 = nordazepam; 8 = diazepam; 9 = camazepam; 10 = clotiazepam; 11 = flurazepam; 12 = medazepam; 13 = clonazepam; 14 = 3-OH-flunitrazepam; 15 = lorazepam; 16 = alprazolam; 17 = lormetazepam. Chromatographic conditions are given in the text.*

TABLE 1—*Retention time of benzodiazepines and benzophenones.*

Benzodiazepine	T_R (min)	Benzophenone	Abbreviation	T_R (min)
Alprazolam	9.7	no change*		9.7
Bromazepam	7.3	2-(2-amino-5-bromo-benzoyl)-pyridine	ABBP	9.6
Brotizolam	10.2	---	---	---
Camazepam	11.7	2-methylamino-5-chloro-	MACB	12.9
Chlordiazepoxide	10.9	2-amino-5-chloro-	ACB	11.9
Clobazam	9.3	---	---	---
Clorazepate	1.9	2-amino-5-chloro-	ACB	11.9
Clotiazepam	12.1	---	---	---
Clonazepam	5.6	2-amino-5-nitro-2'-chloro-	ANCB	10.6
3-hydroxyl-clonazepam	2.3	---	---	---
7-amino-clonazepam	1.9	---	---	---
Cloxazolam	11.8	---	---	---
Diazepam	11.4	2-methylamino-5-chloro-	MACB	12.9
Flunitrazepam	8.5	2-methylamino-5-nitro-2'-fluoro-	MNFB	11.2
Desmethyl-7-amino-	2.4	2,5-diamino-2'-fluoro-	DAFB	3.5
7-amino-	5.3	2-methylamino-5-amino-2'fluoro-	MAFB	6.8
7-acetamido-3-hydroxyl-	5.5	2-methylamino-5-amino-2'fluoro-	MAFB	6.8
3-hydroxyl-	7.5	2-methylamino-5-nitro-2'-fluoro-	MNFB	11.2
Desmethyl-	5.6	2-amino-5-nitro-2'-fluoro-	ANFB	9.9
7-acetamido-	6.0	2-methylamino-5-amino-2'fluoro-	MAFB	6.8
7-acetamido-desmethyl-	4.0	2,5-diamino-2'-fluoro-	DAFB	3.5
Flurazepam	12.4	2-diethylamino-ethylamino-5-chloro-2'-fluro-	Alk-CFB	14.4
N-desalkyl-flurazepam	9.4	2-amino-5-chloro-2'-fluoro-	ACFB	11.7
Ketazolam	10.2	2-methylamino-5-chloro-	MACB	12.9
Lorazepam	8.9	2-amino-5,2'-dichloro-	ADB	12.0
Lormetazepam	10.5	2-methylamino-5,2'-dichloro-	MADB	12.8
Medazepam	12.9	2-methylamino-5-chloro-	MACB	12.9
Nitrazepam	6.3	2-amino-5-nitro-	ANB	10.6
Nordazepam	10.9	2-amino-5-chloro-	ACB	11.9
Oxazepam	8.9	2-amino-5-chloro-	ACB	11.9
Prazepam	12.4	2-cyclopropyl-methylamino-5-chloro-	CMCB	14.5
Temazepam	10.5	2-methylamino-5-chloro-	MACB	12.9
Triazolam	10.1	no change*		10.1
a-hydroxyl-triazolam	8.5	---	---	---

* unchanged by this method

of transformation of benzodiazepine to benzophenone has to be considered; for instance, the yield of diazepam converted completely to 2-aminomethyl-5-chloro-benzophenone is 86.3% by weight.

Sensitivity and Precision—The detection limit is estimated from samples spiked with decreasing benzodiazepine concentration and is the injected amount which results in a peak with a height equivalent to three times the standard deviation of the mean blank (n = 10) (19).

Within-day and day-to-day coefficients of variation are determined by replicate analysis of an aliquot of a sample either in the same run (within-day) or on separate days (day-to-day).

Results and Discussion

Figure 1 shows representative HPLC chromatograms obtained for a mixture of benzodiazepine standards and metabolites. The gradient elution on the reversed phase column with the eluent containing isopropylamine allowed the separation of several benzodiazepines and their metabolites together with triazolam as the internal standard. There is about a three-minute delay in this programming elution when the concentration of solvent is changed, which is estimated by the change of head pressure of column.

The quantitative evaluation is carried out in whole blood, plasma and urine using ten benzodiazepines: 7-amino-flunitrazepam, flunitrazepam, nitrazepam, bromazepam, oxazepam, nordazepam, diazepam, camazepam, flurazepam amd medazepam. The urine sample was treated with both acidic hydrolysis and enzymatic hydrolysis. The instrumental detection limit of these benzodiazepines is 10 to 30 ng. The lower limit of quantitation is 0.05 μg/mL (50 ppb). Bromazepam, flunitrazepam and flurazepam have relatively the worst sensitivities in the detection and this may be improved by the use of a monitoring wavelength at 242, 224 and 225 nm, respectively. The presented analytical procedure proved to be linear (squared correlation coefficient $r^2 > 0.99$, n = 7~9) in the examined range (0.05 to 2.0 μg/mL) for all benzodiazepines and their correspondent bezophenones: MAFB, ABBP, ANB, MNFB, ACB, MACB and Alk-CFB (for the abbreviations see Table 1). Samples containing amounts of a benzodiazepine over the working range are reanalyzed using a smaller sample aliquot.

Extraction of the samples with a mixture of *n*-hexane: Ethylacetate is preferred because it results in higher recoveries and in less interfering peaks than with diethylether or chloroform as extraction solvents (18). Furthermore, this mixture allows vortex-mixing without gel formation and the layers can easily be separated by centrifugation. For the extraction of benzophenones, pure *n*-hexane was used to decrease the extraction of hydrophilic impurities in urine and to help obtain cleaner extracts. The recoveries of benzodiazepines are summarized as: 62.2~105.1% (n = 10, SD = 5.54~9.44) for plasma, 74.6~96.1% (n = 10, SD = 6.44~12.13) for whole blood and 48.8~106.1% (n = 6, SD = 3.72~7.54) for urine treated by enzymatic hydrolysis. The recoveries of benzophenones are 60.1~109.8% (n = 10, SD = 4.76~12.72).

The within-day and day-to-day coefficients of variation (CV, %) are evaluated by spiking 0.25 or 1.5 μg of benzodiazepines in plasma, blood and urine. The obtained values are: within-day CVs for plasma 3.3~8.9% (n = 10), blood 1.8~8.9% (n = 10), urine 2.5~6.5 (n = 6) and day-to-day CVs for plasma 4.4~9.5% (n = 10), blood 2.2~9.9% (n = 10), urine 3.2~8.4% (n = 6). The respective CVs for urine treated by acidic hydrolysis are 4.3~12.2 and 6.6~12.9.

The analysis of blank blood specimens indicated that the co-extracted endogenous impurities were not interfering with the benzodiazepines (Fig. 2). In urine, after enzymatic hydrolysis, the impurities were eluting before the benzodiazepines. The benzodiazepines and some drugs chromatographed on the C8 column are listed in Tables 1 and 2, with the retention times and the absorption wavelengths at alkaline environment.

Identification of an eluting peak, based on the retention time and on the assumption that this peak is composed of only one single compound, is very weak. The use of a photo diode array detector, however, provides additional information about the peak purity and structure of the eluting compound. It also provides the possibility to quantitate every compound with the most suitable wavelengths. For example, the use of 373 nm instead of 235 nm for quantitation of MNFB (see Table 1, Fig. 4-I) easily eliminates the interference of impurities extracted from urine without decreasing absorption intensity.

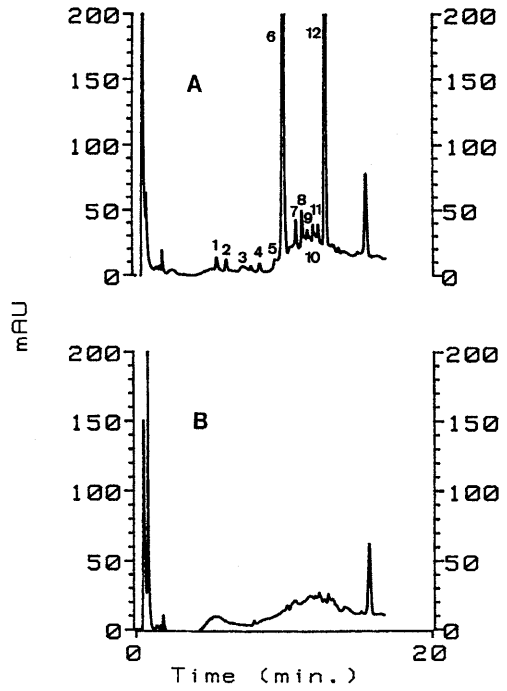

FIG. 2—*Chromatograms of spiked blood (top) and blank blood (bottom): 1 = 7-aminoflunitrazepam; 2 = nitrazepam; 3 = bromazepam; 4 = flunitrazepam; 5 = oxazepam; 6 = triazolam; 7 = nordazepam; 8 = diazepam; 9 = camazepam; 10 = clotiazepam; 11 = flurazepam; 12 = medazepam. Each peak is due to 0.05 μg/mL; except for peak 6 (2 μg/mL) and peak 12 (2 μg/mL). Conditions are given in the text.*

In more than 30 toxicological cases benzodiazepines were found to be positive (Table 3). In ten cases, more than one benzodiazepine was detected, for example, bromazepam with lormetazepam or diazepam or clorazepate, and diazepam with clorazepate. For the postmortem examination, urine, blood and some organs are normally suitable for the analysis. The ratio of the benzodiazepine concentration in urine to blood is especially useful for calculating the approximate time of poisoning. Acid hydrolysis is the usual digestion method of a urine sample, resulting in the conversion of a benzodiazepine to benzophenone. The disadvantage is that a parent compound is sometimes difficult to be confirmed (Table 1). Enzymatic hydrolysis is an additional method to help identify benzodiazepine in urine (22).

Figures 3 and 4 show representative examples of the application of the photo diode array detector in the identification of benzodiazepines in a urine sample pretreated with both acid hydrolysis and enzymatic hydrolysis. In this forensic case, the only information available was that the patient, Mr. V. H., had taken probably four Rohypnol^{R} (flunitrazepam) pills. Indeed, MNFB (benzophenone of flunitrazepam and 3-OH-flunitrazepam) was determined (Fig. 3-I, peak a; Fig. 4-I) in his urine sample after acidic hydrolysis, together with ACB (benzophenone of nordazepam, oxazepam, chlordiazepoxide and clorazepate) and MACB (benzophenone of medazepam, diazepam, temazepam, camazepam and ketazolam). By enzymatic hydrolysis, their parent benzodiazepines were found to be nordazepam, oxazepam, diazepam and temazepam. Flunitrazepam or 3-OH-flunitrazepam was not detected by this method, but 3-OH-flunitrazepam was determined (0.72 μg/mL urine) by running another published program (22). Thus, only the 3-hydroxy metabolite of flunitrazepam was found in the urine sample, which meant that flunitrazepam was almost completely metabolized. Temazepam and oxazepam were identified by comparison of their T_R and ultraviolet (UV) spectra and quantitated by re-extracting and chromtographing with correspondent standards. Figure 4-II shows one of the examples.

Triazolam and alprazolam are used as internal standards in blood, plasma and urine analysis. During acidic hydrolysis they do not degrade. They may alternatively be used depending on which one of them is positive in the sample.

Conclusions

As compared with other high-performance liquid chromatographic procedures for the determination of benzodiazepines, the presented method, based on a nonsalted eluent, is simpler and less subject to technical problems such as filter obstruction and pump piston damage. Also, the well-known long rinsing of the chromatographic system, to eliminate crystal deposit in connecting tubing and in the detector cell, was avoided.

Another remarkable point is the very low limit of quantitation (few ppb), which is much lower than the therapeutic range of the benzodiazepines (except flunitrazepam, for which method (22) is used) in plasma, allowing the method to be used also for therapeutic drug monitoring samples.

The filtration of the dirty extract prior to its injection into the

TABLE 2—*Retention time and UV absorption wavelength of drugs.*

Compound	T_R (min)	λp nm	λv nm
Glipizidum	2.47	252, 298	278
Acetanilide	2.62	240	
Nalorphine	3.43	252, 298	278
Guaiphesine	3.50	273	245
Naloxone	4.28	239(sh), 291	274
Ephedrine	6.09	257, 308	240
Trapridal	6.32	237, 285	271
Tryptamine	7.43	280(w)	248
Codeine	7.87	283, 240(s)	261
Hydrocodone	8.01	281	263
Oxycodone	8.09	281	263
Gliquidonum	8.50	256(sh), 314	282
Heroine	9.19	278	253
Ethylmorphine	9.53	283	265
Papaverine	9.83	239	258
Zolpidem	9.83	243, 315	270
Strychinine	10.55	254(w)	231
Nortilidine	10.68	258(s)	
Dothiepine(oxide)	10.72	246(s)	
Azaperone	11.44	249, 301	279
Cocaine	11.48	232	
Tryptizol	11.51	239	
Trazodone	11.53	253, 276(s), 315(w)	234, 283
Ethylamphetamine	11.54	235, 287	
Cinchonidine	11.55	227, (285, 300, 315)	
Methapyrilene	11.67	243, 309	
Quinine	11.76	232, 310(s), 279(s)	255, 302
XTC	11.76	235, 287	259
Cinconine	11.82	225, (283, 300, 315)	
Haloperidol	12.06	245	233
Piritramid	12.17		
Pentazocine	12.32	281	262
Trimipramine	12.40	287	241
Tilidine	12.46		
Pericyazine	12.50	272, 233	250
Propranolol	12.55	291(w)	247
Difenylamine	12.64	281	247
Depronal	12.82	258(s)	
Bezitramide	12.85	280	247
Propafenone	12.85	248, 305	273, 231
Mianserin	13.19	282(s)	
Etilefrine	13.27	270(w)	243
Anafranil	13.37	284(w)	251
Doxepine	13.48	293, 253(s)	274
Palfium	13.69		
Triprolidine	13.78	238, 282	265
Dothiepine	13.99	230, 270(s)	301, 252, 292
Nozinan	14.69	256, 308	280
Pizotifen	15.07	230, 260(s)	
Amitriptyline	18.87	239	
(-)-trans-Ù⁹-THC	17.08	(275, 282)	265
Maprotiline	18.73	273, 265	246
Nortriptyline	16.56	240	

λp= peak wavelength. λv = valley wavelength. sh= shoulder peak.
w= wide peak. s= small peak. ( )= group peak.

TABLE 3—*Results of analysis for benzodiazepine in blood and urine samples.*

Parent benzodiazepine	Concentration in blood(μg/ml) No. Cases	Mean	Range	Therapeutic/Toxic range* (μg/ml plasma)	Concentration in urine (μg/ml) No. Cases	Mean	Range	Excretion rate in urine (%) †
Diazepam	7	0.42	0.16 - 1.01	0.5-0.75 / 1.5-3.0	10	2.42	0.33- 6.72	70
Nordazepam	11	0.43	0.10 - 0.96	0.2-0.8 / 2.0	11	3.91	0.22-12.54	--
Bromazepam	7	0.34	0.11 - 0.58	0.08-0.17 / 0.25-0.50	3	1.84	0.76- 2.75	70
Clonazepam	3	0.30	0.073-0.35	0.03-0.06 / 0.1				
Nitrazepam	1	0.15		0.03-0.12 / 0.2-0.5				
Oxazepam	1	0.50		1-2 / 3-5				
Clobazam	1	0.37		0.1-0.4 / ----				
Flurazepam	1	0.77		0.001-0.01 / ----	1	4.0		60 %
Flurintrazepam				0.005-0.015 / 0.05	1	0.67		< 1 % ‡
Temazepam					1	0.71		

* based on [20]. † based on [21]. ‡ < 1 % as unchanged drug.

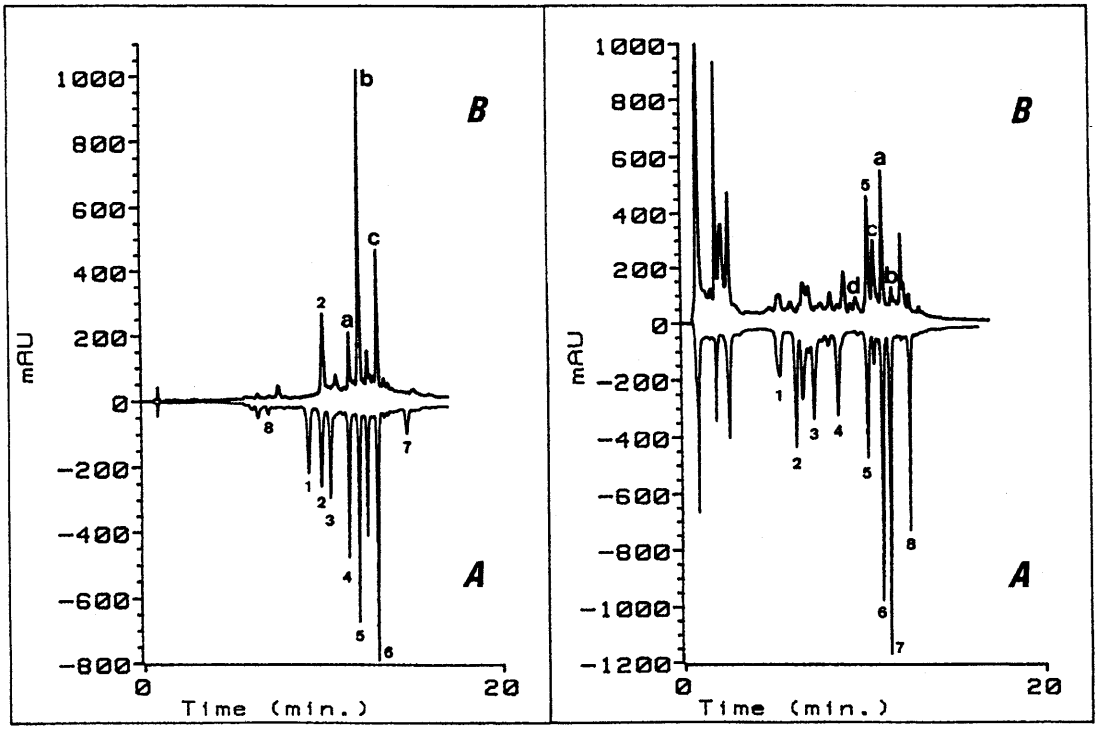

FIG. 3—*Chromatograms of urine sample from a toxicological case. Left, after acid hydrolysis of: (A) Drug-free urine spiked with correspondent benzodiazepines (2.0 μg/mL). 1 = ABBP; 2 = alprazolam (internal standard); 3 = ANB; 4 = MNFB; 5 = ACB; 6 = MACB; 7 = Alk-CFB; 8 = MAFB. (B) urine sample of patient (1 mL). Peak identification: a = MNFB (0.61 μg/mL); b = ACB (2.64 μg/mL); c = MACB (0.88 μg/mL). Right, after enzymatic hydrolysis: (A) drug-free urine spiked with following benzodiazepines (2.0 μg/mL). 1 = 7-aminoflunitrazepam; 2 = nitrazepam; 3 = bromazepam; 4 = flunitrazepam; 5 = triazolam (internal standard); 6 = nordazepam; 7 = diazepam; 8 = flurazepam. (B) Urine sample of patient (1 mL). Peak identification: a = nordazepam (1.21 μg/mL); b = diazepam (0.11 μg/mL); c = temazepam (0.71 μg/mL); d = oxazepam (0.30 μg/mL). Conditions are given in the text. For the abbreviations see Table 1.*

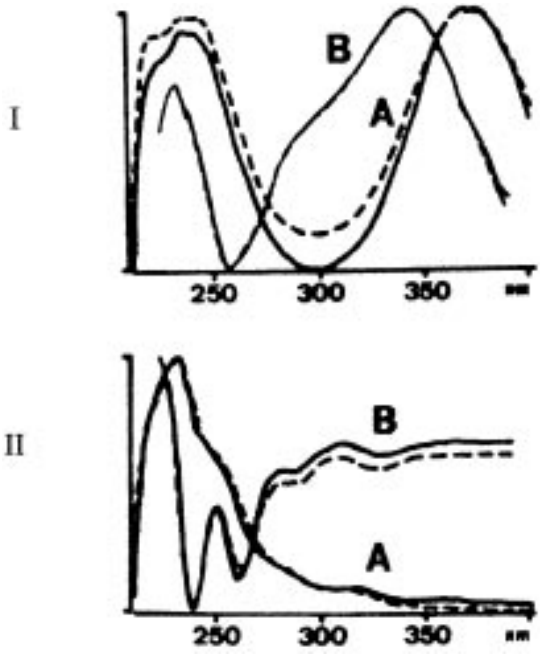

FIG. 4—*Examples of peak identification of benzodiazepine and benzophenone by photo diode array detection from a positive case (the same as in Fig. 3): the UV spectra (A) and the first derivative UV spectra (B) of MNFB (I) and temazepam (II) from standard (—) and urine sample (---).*

system was necessary to protect the column's long life. It was required also to change the guard column every 20 injections. The retention behavior of each benzodiazepine by this elution program was tested for stability (CV: 0.16 to 1.2%, n = 50). The column could be used for more than 400 injections of sample extract and still maintain its stable column efficiency.

Acknowledgments

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References

1. Shader RI, Greenblatt DJ. Clinical implications of benzodiazepine pharmacokinetics. Am J Psych 1977;134:652–6.
2. Sioufi A, Dubois JP. Chromatography of benzodiazepines. J Chromatogr 1990;531:459–80.
3. Inoue T, Niwaguchi T. Determination of nitrazepam and its main metabolites in urine by thin-layer chromatography and direct densitometry. J Chromatogr 1985;339:163–9.
4. Manchon M, Verdier MF, Pallud P, Vialle A, Beseme F, Bienvenu J. Evaluation of EMIT-TOX enzyme immunoassay for the analysis of benzodiazepines in serum: usefulness and limitations in an emergency laboratory. J Anal Toxicol 1985;9:209–12.
5. Beck O, Lafolie P, Hjemdahl P, Borg S, Odelius G, Wirbing P. Detection of benzodiazepine intake in therapeutic doses by immunoanalysis of urine: two techniques evaluated and modified for improved performance. Clin Chem 1992;38:271–5.
6. Jones CE, Wians FH, Martinez LA, Merrit GJ. Benzodiazepines identified by capillary gas chromatography-mass spectrometry, with specific ion screening used to detect benzophenone derivatives. Clin Chem 1989;35:1394–8.
7. Joyce JR, Bal TS, Ardrey RE, Stevens HM, Moffat AC. The decomposition of benzodiazepines during analysis by capillary gas chromatography/mass spectrometry. Biomed Mass Spectrom 1984; 11:284–9.
8. Heizmann P, Geschke R, Zinapold K. Determination of bromazepam in plasma and of its main metabolites in urine by reversed phase high performance liquid chromatography. J Chromatogr 1984;310:129–37.
9. Kunsman GW, Manno JE, Przekop MA, Manno BR, Llorens KA, Kunsman CM. Determination of temazepam and temazepam glucuronide by reversed-phase high performance liquid chromatography. J Chromatogr 1991;568:427–36.
10. Fernandez P, Hermida I, Bermajo AM, Lopez-Rivadulla M, Cruz A, Concheiro L. Simultaneous determination of diazepam and its metabolites in plasma by high performance liquid chromatography. J Liq Chromatogr 1991;14:2587–99.
11. Gill R, Law B, Gibbs JP. High performance liquid chromatography systems for the separation of benzodiazepines and their metabolites. J Chromatogr 1986;356:37–46.
12. Moore CM, Sato K, Katsumata Y. Rapid monitoring of benzodiazepines by using on-line column switching HPLC. Clin Chem 1991; 37:804–8.
13. Boukhabza A, Lugnier AAJ, Kintz P, Mangin P. Simultaneous HPLC analysis of the hypnotic benzodiazepines nitrazepam, estazolam, flunitrazepam, and triazolam in plasma. J Anal Toxicol 1991;15:319–22.
14. Alfredson T, Sheehan T. Recent developments in multichannel, photodiode-array, optical LC detection. J Chromatogr Sci 1986;24: 473–82.
15. Bogrsz M, Wu M. Standardized HPLC/DAD system based on retention indices and spectral library, applicable for systematic toxicological screening. J Anal Toxicol 1991;15:188–97.
16. Hayashida M, Nihira M, Watanabe T, Jinno K. Application of a computer-assisted high-performance liquid chromatographic multiwavelength ultraviolet detection system to simultaneous toxicological drug analysis. J Chromatogr 1990;506:133–43.
17. Turcant A, Premel-Cabic A, Cailleux A, Allain P. Toxicological screening of drugs by microbore high performance liquid chromatography with photodiode-array detection and ultraviolet spectral library searches. Clin Chem 1991;37:1210–5.
18. Pulpolo PR, Pothier ME, Volpicelli SA, Flood JG. Single procedure for detection, confirmation and quantification of benzodiazepines in serum by liquid chromatography with photo diode-array detection. Clin Chem 1991;37:701–6.
19. Commission decision. Official Journal of the European Communities No. L223, 11.8.1987;18–36.
20. Uges DRA, Lijst van klinisch farmaceutische en toxicologische bepalingen: Apotheek Academisch Ziekenhuis Groningen 1987.
21. Moffat AC, Jackson JV, Moss MS, Widdop B, editors. Clarck's Isolation and Identification of Drugs, 2nd ed. London: The Pharmaceutical Press, 1986.
22. He W, Parissis N. Simultaneous determination of flunitrazepam and its metabolites in plasma and urine by HPLC/DAD after solid phase extraction. J Pharm Biomed Anal 1997;16(4):707–14

Additional information and reprint requests:
Weidong He
Department of Organic Chemistry
University of Ghent
Coupure Links 653, B4
B-9000 Ghent, Belgium