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Journal of Chromatography B, 765 (2001) 187–197

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Simultaneous determination of fifteen low-dosed benzodiazepines in human urine by solid-phase extraction and gas chromatography–mass spectrometry

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Received 13 July 2001; received in revised form 10 September 2001; accepted 18 September 2001

Abstract

A gas chromatographic–mass spectrometric method was developed for the simultaneous analysis of 15 low-dosed benzodiazepines, both parent compounds and their corresponding metabolites, in human urine. The target compounds are alprazolam, α-hydroxyalprazolam, 4-hydroxyalprazolam, flunitrazepam, 7-aminoflunitrazepam, desmethylflunitrazepam, flurazepam, hydroxyethylflurazepam, nitrogen-desalkylflurazepam, ketazolam, oxazepam, lormetazepam, lorazepam, triazolam and α-hydroxytriazolam. Nitrogen-methylclonazepam is used as the internal standard. The urine sample preparation involves enzymatic hydrolysis of the conjugated metabolites with *Helix pomatia* β-glucuronidase for 1 h at 56°C followed by solid-phase extraction on a phenyl-type column. The extracted benzodiazepines are subsequently analyzed on a polydimethylsiloxane column using on-column injection to enhance sensitivity. The extraction efficiency exceeded 80% for all compounds except for oxazepam, lorazepam and 4-hydroxyalprazolam which had recoveries of about 60%. The LODs ranged from 13 to 30 ng/ml in the scan mode and from 1.0 to 1.7 ng/ml in the selected ion monitoring (SIM) mode. Linear calibration curves were obtained in the concentration ranges from 50 to 1000 ng/ml in the scan mode and from 5 to 100 ng/ml in the SIM mode. The within-day and day-to-day relative standard deviations at three different concentrations never exceeded 15%.

Keywords: Benzodiazepines

1. Introduction

Benzodiazepines are among the most widely prescribed drugs, being used in the treatment of stress, anxiety, sleep disorders, muscle spasms and seizures. Many patients develop a dependence on these drugs which are often involved in intoxications. Consequently, benzodiazepines are frequently encountered both in clinical and forensic toxicological analyses.

For identification purposes, urine is the preferred matrix as the concentrations of benzodiazepines and their metabolites are higher in urine than in plasma. This is especially relevant for the low-dosed compounds. Benzodiazepines are extensively metabolized and many metabolites are excreted in urine as glucuronide conjugates. Cleavage of these conjugates by acid hydrolysis is fast and therefore often applied

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0378-4347/01/$ – see front matter
PII: S0378-4347(01)00419-4

but decomposes the benzodiazepine molecules to benzophenones [1–3]. In this way unequivocal identification is impaired as some compounds yield common benzophenones. During enzymatic hydrolysis, which is a more gentle procedure, benzodiazepines remain intact. The commonly used β-glucuronidases are produced from different sources like snail intestinal juice (*Helix pomatia*), bovine liver and bacteria (*Escherichia coli*), and the reported hydrolysis conditions also vary widely [4–7].

A large number of analytical methods have been published for the determination of benzodiazepines. Most of these methods can only be applied to the analysis of either parent compounds alone or one parent benzodiazepine and its corresponding metabolites. Traditional liquid–liquid extraction (LLE) techniques are still very popular and solvents used to extract benzodiazepines include chloroform [8,9], diethylether [10,11], *n*-butyl acetate [12,13] or mixtures of different solvents [14,15]. Extractions are mostly performed under slightly alkaline conditions (pH 9–10) obtained with dilute sodium hydroxide or sodium carbonate, –phosphate and –borate buffers.

Existing solid-phase extraction procedures for benzodiazepines cover a broad range of apolar bonded-phase cartridges: C_{18} [16,17], C_8 [18] or C_2 [19,20]. Mixed-phase Bond Elut Certify columns are also commonly used, as well for gas chromatography (GC) [21,22] as for high-performance liquid chromatography (HPLC) applications [23,24].

As none of the sample preparation methods described in the literature proved satisfactory, our aim was to develop a new, sensitive and simple extraction procedure using phenyl-type solid-phase extraction columns for the simultaneous determination of the following low-dosed benzodiazepines and their corresponding metabolites in human urine: ketazolam (Solatran, Unakalm), oxazepam; flunitrazepam (Rohypnol, Hypnocalm), 7-aminoflunitrazepam, desmethyl-flunitrazepam; flurazepam (Staurodorm), hydroxyethylflurazepam, *N*-desalkylflurazepam; lormetazepam (Loramet), lorazepam (Serenase, Temesta); alprazolam (Xanax), 4-hydroxyalprazolam, α-hydroxyalprazolam; triazolam (Halcion) and α-hydroxytriazolam. Unequivocal identification of each benzodiazepine is guaranteed by the combination of enzymatic hydrolysis and mass spectrometric detection.

2. Experimental

2.1. Solvents and reagents

Standards of ketazolam, alprazolam, 4-hydroxyalprazolam, α-hydroxyalprazolam, triazolam and α-hydroxytriazolam were a gift from Upjohn (Kalamazoo, MI, USA). Flunitrazepam, desmethyl-flunitrazepam, 7-aminoflunitrazepam, 7-aminodesmethyl-flunitrazepam, and the internal standard *N*-methylclonazepam were a gift from Hoffman-La Roche (Basel, Switzerland) and flurazepam and hydroxyethylflurazepam were a gift from Madaus-Therabel (Brussels, Belgium). *N*-Desalkylflurazepam was obtained from Mikromol (Teltow, Germany). Oxazepam and lorazepam were purchased from Sigma (Bornem, Belgium). All standards were more than 99% pure and used without further purification. *N*-Methylclonazepam was used as the internal standard.

Ethyl acetate and methanol were obtained from Sigma–Aldrich (Bornem, Belgium), water was from Merck (Darmstadt, Germany) and acetonitrile was obtained from Prosan (Merelbeke, Belgium). All solvents used were HPLC grade. β-Glucuronidase (EC 3.2.1.31), type HP-2 from *H. pomatia* (127 300 U/ml) was from Sigma–Aldrich. Sodium acetate was from UCB (Leuven, Belgium) and acetic acid, sodium hydroxide, disodium hydrogenphosphate 2-hydrate, sodium dihydrogenphosphate 1-hydrate, pyridine and acetic anhydride were purchased from Merck.

2.2. Preparation of standards and buffer solutions

Individual stock solutions of 1.0 mg/ml were prepared in a methanol–ethyl acetate (20:80, v/v) mixture. Working solutions containing 20 ng/μl of each drug were prepared by repeated dilutions of the stock solutions with ethyl acetate.

Sodium acetate buffer (pH 4.5) for hydrolysis: to 4.15 ml of 2 *M* sodium acetate (164.1 g $C_2H_3NaO_2$/l), 5.85 ml of 2 *M* acetic acid was added and the solution was made up to 100 ml with water.

Phosphate buffer (pH 6.8) for extraction: to 24.5 ml of 0.2 *M* Na_2HPO_4 (35.61 g $Na_2HPO_4 \cdot 2H_2O$/l), 25.5 ml of 0.2 *M* NaH_2PO_4 (27.60 g $NaH_2PO_4 \cdot$

H_2O/l) was added and the solution was made up to 100 ml with HPLC-grade water.

2.3. Instrumentation and chromatographic conditions

The GC–MS instrument consisted of a HP 6890 Series gas chromatograph coupled to a HP 5973 mass-selective detector (Avondale, PA, USA). The chromatographic system was a Restek (Bellefonte, PA, USA) hydroguard guard column (5 m×0.32 mm I.D.) coupled to an SGE (Achrom, Zulte, Belgium) BP1 capillary column (30 m×0.25 mm I.D., 0.25 μm film thickness; nonpolar polydimethylsiloxane phase) with a Universal angled press-tight connector (Restek). On-column injections were performed with a HP 7683 autosampler (ALS). A 5-μl syringe was used and the injection volume was 1 μl. The injector temperature was set at 70°C and the flow-rate was maintained at 1.2 ml/min using helium as the carrier gas. The oven temperature was programmed as follows: the initial temperature was set at 65°C, held for 1 min and ramped at 15°C/min to 250°C where it was held for 8 min, ramped at 10°C/min to 300°C and held for 2 min. The transfer line temperature was set at 300°C. The mass-selective detector was used in the electron impact (EI) scan or multiple ion monitoring mode (SIM), at low resolution. The atomic mass units (u) of the target and qualifier ions used for the target compound analysis are reported elsewhere [25].

2.4. Enzymatic hydrolysis

To 1 ml of urine in a 15-ml amber centrifuge tube, 20 μl of the *N*-methylclonazepam internal standard solution (at 2.5 ng/μl for SIM and at 25 ng/μl for scan analyses) was added. Amber glass was used throughout the entire analysis as some of the examined benzodiazepines are described as being photosensitive [10,16]. The urine was buffered with 2 ml 0.2 *M* sodium acetate buffer (pH 4.5) and 5500 U of *H. pomatia* β-glucuronidase were added. The tubes were mixed vigorously and incubated at 56°C for 1 h. After centrifugation at 2500 rpm for 10 min, the supernatant was transferred to a 15-ml amber centrifuge tube. To each tube 10 μl of 1 *M* sodium hydroxide and 2 ml phosphate buffer (pH 6.8) were added prior to solid-phase extraction.

2.5. Solid-phase extraction

Bond Elut phenyl (PH) solid-phase extraction cartridges were from Varian (Middelburg, The Netherlands). The solid-phase extraction was performed using a Chromabond vacuum manifold (Merck) for the simultaneous preparation of 12 samples. The solid-phase extraction cartridges were preconditioned with two 1-ml portions of methanol, water and phosphate buffer, pH 6.8. The prepared urine sample was then applied and was allowed to pass through the column at a constant pressure of −2 kPa corresponding to a flow-rate of approximately 0.5 ml/min. The vacuum was then increased to −20 kPa and the sorbent was washed with 1 ml of HPLC-grade water and two 250-μl aliquots of acetonitrile–water (30:70, v/v). The vacuum was kept at maximum (−70 kPa) during 20 min in order to dry the disk completely. Finally, the vacuum was released and benzodiazepines were eluted with 1 ml of methanol into amber collection tubes. The eluate was evaporated under a gentle stream of nitrogen prior to derivatization. The time needed for the simultaneous extraction of 12 samples is approximately 40 min.

2.6. Derivatization

To the dried extract 200 μl of pyridine–acetic anhydride (1:1, v/v) was added. The sample was incubated at room temperature for 20 min. Subsequently, the reagents were evaporated under a low stream of nitrogen and the residue was redissolved in 20 μl of ethyl acetate and 1 μl was injected on-column. The results of the optimization experiments are already described in a previous report [25].

2.7. Calibration

Urine standards were prepared by mixing human blank urine with acetate buffer. The diluted samples were then spiked with the selected benzodiazepines to the concentrations of 20, 50, 100, 500 and 1000 ng/ml (scan) and 2, 5, 10, 50 and 100 ng/ml (SIM) each, and with the appropriate internal standard

solution. The validation was done in six series of experiments.

3. Results and discussion

Enzymatic hydrolysis conditions were optimized using a urine sample from a patient on lormetazepam prescription. Absolute recoveries could not be determined because of the unavailability of the benzodiazepine–glucuronide conjugate standard. In four series of experiments the following parameters were optimized: enzyme activity, hydrolysis pH, temperature and incubation time. In the first series of experiments 1-ml aliquots of urine were adjusted to pH 5.0 and incubated at 56°C for 2 h with increasing amounts of β-glucuronidase (0, 500, 2000, 4500, 5000, 5500, 8000, 10 000 U added). Optimal results were obtained with the addition of 5500 U and this activity was kept constant during all further experiments. In the second series of experiments the urine sample was buffered to pH 4.0, 4.5, 5.0 and 5.5 with the sodium acetate buffer and to pH 6.0 with the phosphate buffer and subsequently incubated at 56°C for 2 h. An optimal recovery was obtained at pH 4.5. Thirdly, enzymatic hydrolysis was performed for 2 h at 22, 37, and 56°C. Chromatograms of these experiments are illustrated in Fig. 1. As expected, the optimal reaction temperature was 56°C. Finally, the incubation time was varied and the reaction was performed at pH 4.5 and 56°C during 1, 2, 4, 6 or 8 h. An optimal recovery was already reached after 1 h of incubation. These results are in accordance with those reported by Meatherall for other urinary benzodiazepines [26].

The applicability of several reported LLE procedures for the extraction of the 15 selected benzodiazepines from urine was then evaluated. Extractions with *n*-hexane or methyl *tert*-butyl ether (MTBE) yielded low recoveries for all compounds, while ethyl acetate and chloroform extracts were too dirty for on-column injection. Moreover the limit of detection (LOD) was substantially increased due to contamination of the retention gap. Better results were obtained with MTBE–chloroform (2:1, v/v) mixtures. Although variations in extraction pH influenced the results significantly, these modified LLE procedures were never satisfactory for the 15 selected benzodiazepines as a group.

The first step in the optimization of a solid-phase extraction procedure was the selection of the type of cartridge to be used. As mentioned in the introduction, the most frequently used solid-phase types for benzodiazepines are reversed-phase and mixed-phase extraction columns. In our experience, reversed-phase columns yielded excellent recoveries for the parent compounds but for several important metabolites recoveries were below 50%. We also investigated the applicability of the mixed-phase type extraction columns because the selected compounds have a large polarity range. However, because small amounts of NH_4OH are needed to displace the metabolites from the column, the eluate contains water and this is absolutely unacceptable for GC analysis. With the on-column injection technique, water directly injected into the retention gap resulted in a destruction of the deactivation layer. Even the hydroguard guard column used, was not resistant to the direct injection of this large amount of water.

Several other commercial sorbents [CH (cyclohexyl), CN (cyanopropyl), C_{18}-OH, Abselut Nexus, HCX (octyl), PH] were therefore evaluated on their ability to retain the selected compounds. In our hands, satisfying results for all parent compounds and their corresponding metabolites could only be obtained with the PH-type extraction columns. Optimization of the extraction procedure was performed by varying the extraction pH and the washing and elution solvents. The main criteria used for this optimization were recoveries and absence of interfering peaks.

Varying the pH of the urine sample between 5 and 11 significantly influenced the recoveries of benzodiazepines as well as the background noise in the corresponding chromatogram. At low pH-values recoveries were acceptable only for the parent compounds, while at high pH-values dissolution of the silica-sorbent resulted in rapid deterioration of the chromatographic performance. The optimum extraction pH was found to be 6.8. Washing of the solid-phase cartridge with 1 or 2 ml deionized water and 250–500 μl of a methanol–water (20:80) mixture [27–29] is usually recommended for adequate removal of matrix interferences from the column. However, several tests revealed that these eluates

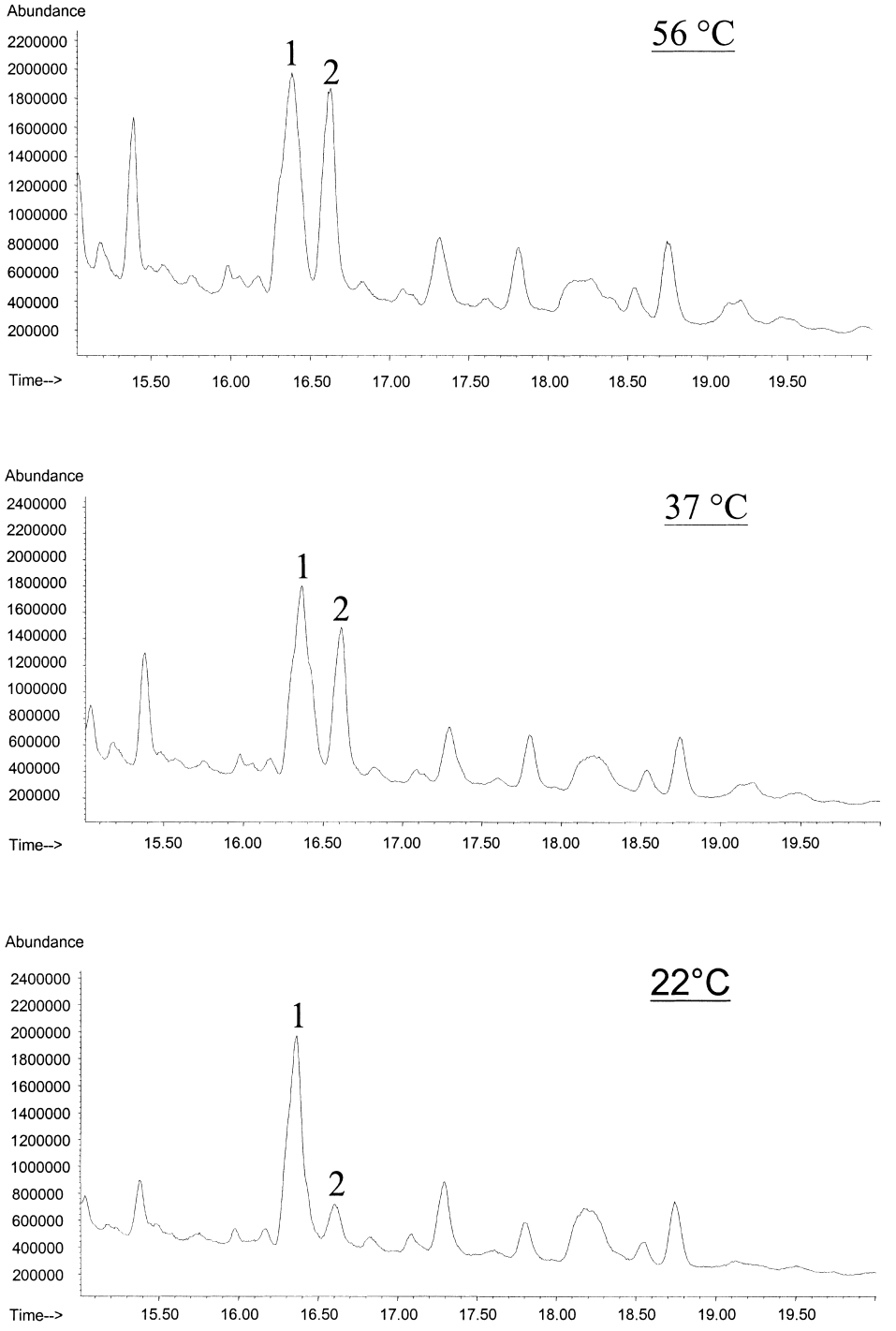

Fig. 1. Influence of the reaction temperature during hydrolysis with *H. pomatia* β-glucuronidase. 1=Internal standard, 2=lormetazepam. The enzyme activity is 5500 U/ml urine, hydrolysis is performed at pH 4.5 for 2 h.

were too dirty for on-column injection. Acceptable recoveries for all selected benzodiazepines and adequate purification of urinary matrix compounds were obtained by washing the extraction columns with two 250-μl portions of acetonitrile–water (30:70). Higher concentrations of organic solvent yielded low recoveries especially for the more polar metabolites.

Drying of the column was the next critical stage in the extraction procedure as no water was allowed in the chromatographic step. Removal of water is mostly carried out by applying a small volume of methanol (50 μl) [30] or a larger volume of *n*-hexane (1 ml) [31]. Both methods were evaluated but were found not applicable because several benzodiazepines were partially eluted with the washing solvents. However, optimal column drying was achieved by application of maximum vacuum for about 20 min.

Finally, several solvents were tested to elute the benzodiazepines from the solid-phase PH cartridge. Ethyl acetate and *n*-hexane were not suitable as too many matrix interferences were co-extracted. On the other hand, elution with two 250-μl portions of methanol yielded clean extracts and good recoveries for all selected compounds. However, analysis of underivatized extracts, is required for the determination of the metabolites of flunitrazepam [25], as desmethylflunitrazepam is degraded by acetylation and 7-aminoflunitrazepam is converted into 7-acetamidoflunitrazepam, another endogenous urinary metabolite of flunitrazepam. Direct injection of this underivatized methanolic eluate was not possible as methanol injection is not compatible with the chromatographic system used and results in split peaks. Therefore, the extracts had to be evaporated and reconstituted in ethyl acetate. In Fig. 2 a representative scan chromatogram of an extracted blank urine sample is shown. Interferences from endogenous substances or possibly co-administered drugs were not detected.

The final extraction procedure was validated. The calibration curves (i.e., peak area ratios of each benzodiazepine to the internal standard against the amount of each benzodiazepine added) showed excellent linearity over the concentration ranges of 50–1000 ng/ml urine in the scan mode and 5–100 ng/ml urine in the SIM mode. The correlation coefficient of all individual curves exceeded 0.995. The LOD was estimated as three times the signal-to-noise ratio and was determined for all compounds by spiking urine with decreasing concentrations until an equivalent response was observed. The obtained values are listed in Tables 1 and 2. The limit of quantitation (LOQ) is the lowest concentration that can be measured on the standard curves with accept-

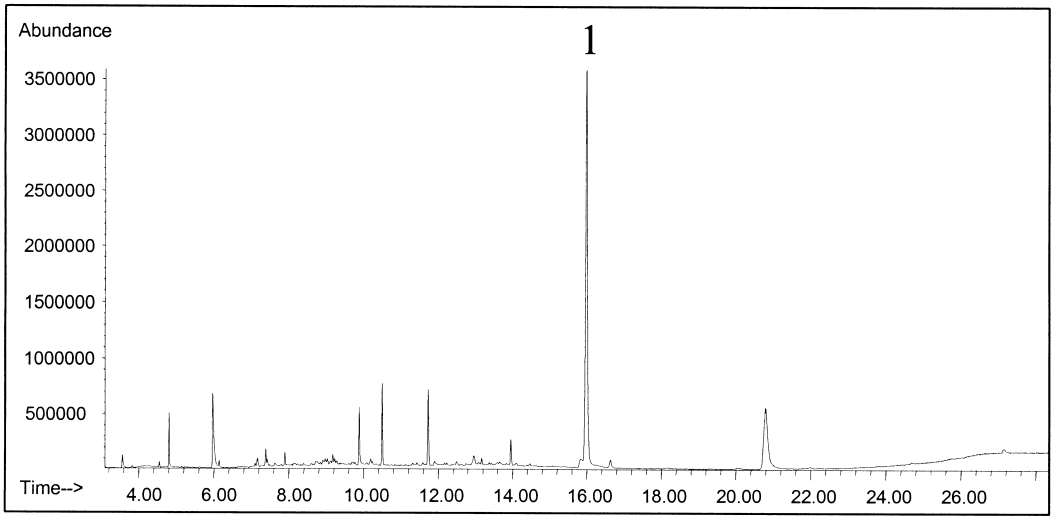

Fig. 2. Chromatogram of a blank urine sample spiked with internal standard. 1=Nitrogen-methylclonazepam.

Table 1
Calibration data of the examined compounds in the scan mode ($n=6$)

Compound	Scan mode				
	Data points	Linearity	r^2	SD (on slope)	LOD (ng/ml)
Alprazolam	5	$y=0.0019x+0.018$	0.9996	0.0001	16
α-Hydroxyalprazolam	5	$y=0.0017x+0.0269$	0.9981	0.0001	25.5
4-Hydroxyalprazolam	5	$y=0.0010x-0.0163$	0.9974	0.0001	15
Flunitrazepam	5	$y=0.0019x+0.031$	0.9973	0.0001	13
7-Aminoflunitrazepam	5	$y=0.0012x-0.0363$	0.9987	0.0001	18
Desmethylflunitrazepam	5	$y=0.0014x-0.0031$	0.9988	0.0001	18
Flurazepam	5	$y=0.0018x+0.0115$	0.9987	0.0001	16
Hydroxyethylflurazepam	5	$y=0.0025x-0.0123$	0.9994	0.0001	17.5
N-Desalkylflurazepam	5	$y=0.0020x+0.0114$	0.9989	0.0002	13
Ketazolam	5	$y=0.0027x-0.0347$	0.9972	0.0002	16
Lormetazepam	5	$y=0.0024x+0.0201$	0.9973	0.0001	18
Lorazepam	4	$y=0.0002x-0.0016$	0.9994	0.0001	24
Oxazepam	4	$y=0.0005x-0.0137$	0.9975	0.0001	30
Triazolam	5	$y=0.0017x-0.0172$	0.9984	0.0001	14
α-Hydroxytriazolam	5	$y=0.0027x-0.0581$	0.9988	0.0001	17

able reproducibility (RSD<15%) and was ≤5 ng/ml for all compounds in the SIM mode, while in the scan mode the LOQ was ≤50 ng/ml except for oxazepam, lorazepam and 4-hydroxyalprazolam (LOQs of about 80 ng/ml). As compared to the results obtained by other GC–MS procedures the sensitivity for the parent compounds and the metabolites was found to be either comparable or better [31–33].

Recoveries were calculated by comparing the peak area ratios from the compounds to the internal standard in an extracted urine sample to those of unextracted reference standards. Drug-free urine samples were spiked with the selected benzodiazepines at concentrations of 5, 100 and 1000 ng/ml. Six samples of each concentration were analyzed and the results are summarized in Table 3. Although the recoveries obtained for oxazepam, lorazepam and 4-hydroxyalprazolam are significantly lower (±60%) than those for the other compounds, they are still

Table 2
Calibration data of the examined compounds in the SIM mode ($n=6$)

Compound	SIM mode				
	Data points	Linearity	r^2	SD (on slope)	LOD (ng/ml)
Alprazolam	5	$y=0.0252x+0.0272$	0.9995	0.0007	1.2
α-Hydroxyalprazolam	5	$y=0.0222x-0.0059$	0.9993	0.0007	1.6
4-Hydroxyalprazolam	5	$y=0.0230x-0.0178$	0.9987	0.0008	1.3
Flunitrazepam	5	$y=0.0343x+0.0043$	0.9985	0.0009	1.1
7-Aminoflunitrazepam	5	$y=0.0242x-0.0392$	0.9992	0.0005	1.2
Desmethylflunitrazepam	5	$y=0.0109x+0.0106$	0.9989	0.0003	1.2
Flurazepam	5	$y=0.1054x-0.0826$	0.9996	0.0023	1.1
Hydroxyethylflurazepam	5	$y=0.0381x+0.0351$	0.9993	0.0007	1.1
N-Desalkylflurazepam	5	$y=0.0404x+0.0081$	0.9985	0.0008	1.1
Ketazolam	5	$y=0.0639x-0.0725$	0.9973	0.0009	1.1
Lormetazepam	5	$y=0.0244x+0.0125$	0.9991	0.0007	1.1
Lorazepam	5	$y=0.0093x-0.0034$	0.9973	0.0004	1.6
Oxazepam	5	$y=0.0076x-0.0047$	0.9985	0.0004	1.7
Triazolam	5	$y=0.0344x-0.0408$	0.9993	0.0006	1.1
α-Hydroxytriazolam	5	$y=0.0281x-0.0298$	0.9988	0.0007	1.2

Table 3
Recoveries for the examined compounds at three different concentrations ($n=6$)

	5 ng/ml		100 ng/ml		1000 ng/ml	
	Recovery (%)	SD	Recovery (%)	SD	Recovery (%)	SD
Alprazolam	92.67	10.04	99.27	5.16	94.80	3.04
α-Hydroxyalprazolam	96.71	8.54	100.29	6.04	100.55	6.33
4-Hydroxyalprazolam	61.14	4.75	61.31	7.24	63.00	5.16
Flunitrazepam	83.14	2.58	89.31	1.73	89.73	5.87
7-Aminoflunitrazepam	80.67	2.35	82.46	2.35	87.93	3.98
Desmethylflunitrazepam	93.38	7.07	96.67	6.59	99.16	4.30
Flurazepam	87.11	7.22	90.68	5.81	85.47	1.91
Hydroxyethylflurazepam	81.22	6.92	79.62	2.07	80.65	5.55
N-Desalkylflurazepam	89.72	3.59	93.06	5.96	91.24	8.64
Ketazolam (as diazepam)	97.60	3.12	99.07	8.94	100.02	8.51
Lormetazepam	87.93	6.12	96.33	4.09	93.73	4.57
Lorazepam	58.87	6.50	60.86	8.27	64.71	1.96
Oxazepam	59.14	7.65	61.32	3.70	64.72	2.82
Triazolam	92.34	2.35	96.83	7.20	92.15	2.09
α-Hydroxytriazolam	98.44	7.61	101.20	5.78	101.78	4.31

acceptable and reproducible. For all other benzodiazepines the recoveries are high (≥79%) irrespective of the concentration.

Finally, the within-day and day-to-day variations were evaluated at three different concentrations in the scan and the SIM modes. The results are given in Tables 4 and 5 and are expressed as the relative standard deviation (RSD) at each level. The within-day RSDs ranged from 7.53 to 14.51% at 5 ng/ml, from 2.53 to 8.26% at 20 ng/ml, and from 2.36 to 6.47% at 100 ng/ml in the SIM mode. In the scan mode the within-day RSDs ranged from 5.00 to 14.01% at 50 ng/ml, from 2.59 to 13.59% at 200 ng/ml, and from 1.94 to 9.47% at 1000 ng/ml. The day-to-day RSDs ranged from 5.49 to 15.63% at 5 ng/ml, from 3.75 to 11.69% at 20 ng/ml, and from

Table 4
Within-day precision data of the examined compounds at concentrations of 5, 20, 100 ng/ml in the SIM mode and at 50, 200 and 1000 ng/ml in the scan mode

Compound	Relative standard deviation (%)					
	SIM ($n=6$)			Scan ($n=6$)		
	5 ng/ml	20 ng/ml	100 ng/ml	50 ng/ml	200 ng/ml	1000 ng/ml
Alprazolam	14.51	5.56	3.39	10.12	4.79	3.20
α-Hydroxyalprazolam	8.83	2.60	4.11	11.01	6.02	6.29
4-Hydroxyalprazolam	7.78	8.26	3.77	/	8.62	8.18
Flunitrazepam	8.10	4.33	4.03	14.01	6.54	1.94
7-Aminoflunitrazepam	9.70	2.92	2.42	9.05	2.85	4.53
Desmethylflunitrazepam	11.54	4.60	3.93	10.25	6.82	7.13
Flurazepam	8.29	7.50	2.55	5.00	6.41	2.23
Hydroxyethylflurazepam	8.52	4.32	2.63	8.46	2.59	6.88
N-Desalkylflurazepam	7.84	4.00	2.36	12.37	6.41	9.47
Ketazolam (as diazepam)	7.53	3.20	2.79	9.80	9.03	8.51
Lormetazepam	8.63	6.96	3.43	9.41	4.24	4.88
Lorazepam	11.05	3.60	5.21	/	13.59	3.03
Oxazepam	12.93	3.70	6.47	/	6.03	4.36
Triazolam	10.10	2.53	2.40	11.30	7.43	2.27
α-Hydroxytriazolam	7.73	7.58	3.57	11.92	5.71	3.47

Table 5
Day-to-day precision data of the examined compounds at concentrations of 5, 20, 100 ng/ml in the SIM mode and at 50, 200 and 1000 ng/ml in the scan mode

Compound	Relative standard deviation (%)					
	SIM (n=6)			Scan (n=6)		
	5 ng/ml	20 ng/ml	100 ng/ml	50 ng/ml	200 ng/ml	1000 ng/ml
Alprazolam	13.51	11.69	4.50	13.51	9.68	6.11
α-Hydroxyalprazolam	11.66	4.90	4.01	10.31	4.86	4.59
4-Hydroxyalprazolam	8.98	5.78	8.82	/	8.67	7.45
Flunitrazepam	11.12	8.59	4.71	11.12	10.35	2.41
7-Aminoflunitrazepam	12.95	7.34	2.59	12.81	5.87	4.01
Desmethylflunitrazepam	10.31	5.57	5.19	10.31	8.09	1.94
Flurazepam	6.10	3.75	3.73	8.13	2.98	4.19
Hydroxyethylflurazepam	5.49	5.52	3.25	12.09	6.12	2.32
N-Desalkylflurazepam	12.90	7.63	3.12	12.91	6.75	8.45
Ketazolam (as diazepam)	9.02	5.29	4.15	7.92	7.63	2.58
Lormetazepam	11.23	9.49	3.00	9.50	7.47	6.12
Lorazepam	15.63	10.21	6.51	/	4.87	3.46
Oxazepam	10.86	6.53	5.13	/	8.86	2.72
Triazolam	13.56	10.89	4.88	13.56	4.30	6.42
α-Hydroxytriazolam	11.13	11.63	5.25	13.96	6.55	7.43

2.59 to 8.82% at 100 ng/ml in the SIM mode. In the scan mode the day-to-day RSDs ranged from 7.92 to 13.96% at 50 ng/ml, from 2.98 to 10.35% at 200 ng/ml, and from 1.94 to 8.45% at 1000 ng/ml.

In order to evaluate the developed method on real samples, about 300 urine samples collected from healthy subjects with a high prevalence of benzodiazepine (mis-)use were analyzed. No analytical or chromatographic problems were encountered, demonstrating the robustness of the procedure. At least 15 samples could be analyzed before the retention gap showed deterioration. Contamination of the

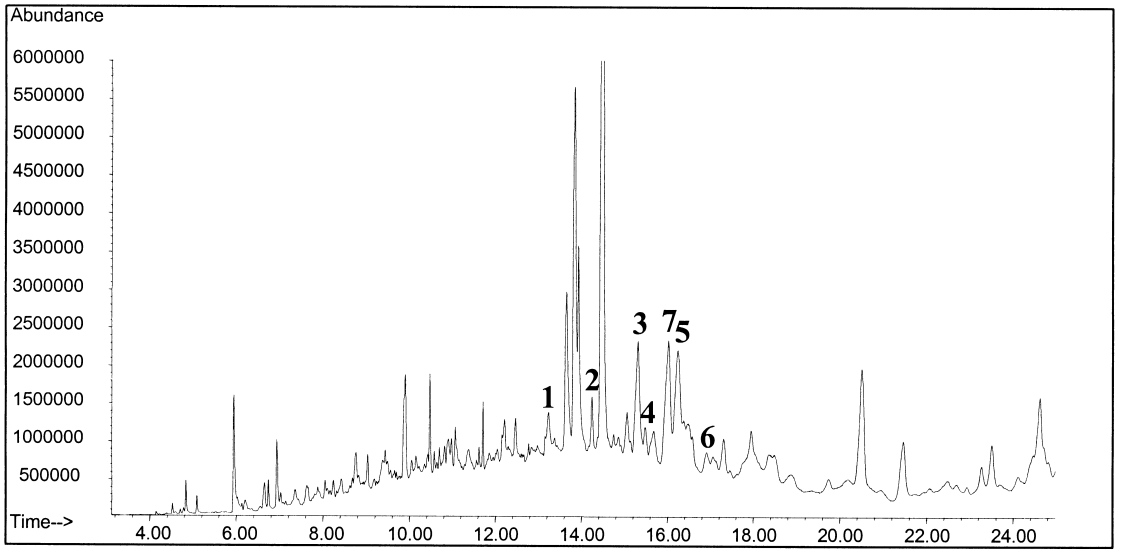

Fig. 3. TIC chromatogram of an extracted urine sample containing: 1=nordiazepam, 2=clorazepate-metabolite, 3=temazepam, 4=oxazepam (285 ng/ml), 5=lormetazepam (331 ng/ml), 6=diazepam-metabolite, 7=internal standard.

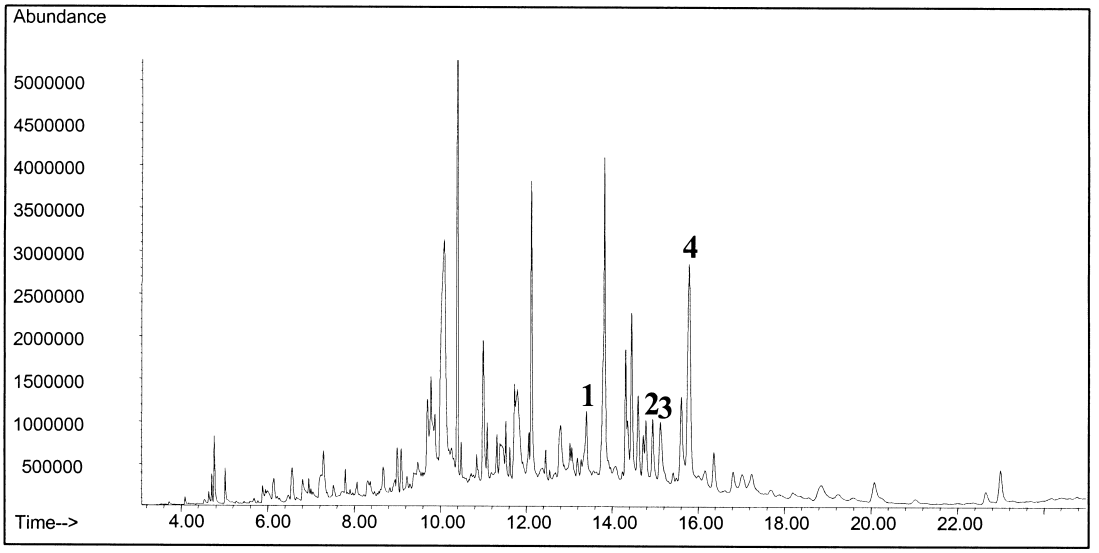

Fig. 4. TIC chromatogram of an extracted urine sample containing: 1=nordiazepam, 2=clobazam-metabolite, 3=temazepam, 4=internal standard.

separation column did not occur. As only 1 μl of the extracts was injected, short sections of the retention gap were flooded and chromatographic performance could be restored by removing 20 cm of the pre-column at the injector-site. Figs. 3 and 4 are total ion current (TIC) chromatograms of two of these collected samples. The first sample contains six benzodiazepines: nordiazepam and another metabolite of diazepam, a clorazepate metabolite, temazepam, oxazepam and lormetazepam. The measured concentrations were as follows: oxazepam (285 ng/ml), lormetazepam (331 ng/ml). The other compounds were not quantified as the method was not validated for these benzodiazepines.

In the second sample nordiazepam, a clobazam metabolite and temazepam were identified.

4. Conclusion

The optimized enzymatic hydrolysis conditions, followed by the developed phenyl-phase extraction and acetylation procedures provided satisfying recoveries of all selected benzodiazepines. The obtained extracts were suitable for on-column injection. This injection technique in combination with GC–MS proved to be very sensitive and selective for the determination of 15 commonly used low-dosed benzodiazepines in human urine samples.

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