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Sensitive gas chromatographic–mass spectrometric screening of acetylated benzodiazepines

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Abstract

GC–MS screening conditions were developed for 15 low-dosed benzodiazepines, covering alprazolam, flunitrazepam, flurazepam, ketazolam, lorazepam and triazolam, and the corresponding metabolites α-hydroxyalprazolam, 4-hydroxyalprazolam; 7-aminoflunitrazepam, desmethylflunitrazepam, 7-aminodesmethylflunitrazepam; hydroxyethylflurazepam, *N*-desalkylflurazepam; oxazepam and α-hydroxytriazolam, respectively. Benzodiazepines are analyzed on a polydimethylsiloxane column in both the scan and the multiple ion monitoring modes using on-column injection to attain maximal sensitivity. The reactive compounds are acetylated with pyridine and acetic anhydride for 20 min. The derivatives are stable for at least 4 days. The relative standard deviation observed with standard compounds at the low nanogram-level ranged from 1.13 to 4.87% within-day and from 1.12 to 4.94% between-day. Unequivocal identification potential, high chromatographic resolution and sensitivity are combined with minimal thermal degradation. The presented screening conditions provide the basis for a unique routine screening method for low-dosed benzodiazepines with a broad polarity range. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Derivatization, GC; Pharmaceutical analysis; Benzodiazepines

1. Introduction

Benzodiazepines are widely prescribed drugs used as anxiolytics, sedative hypnotics, anticonvulsants and muscle relaxants. Apart from their therapeutic applications, benzodiazepines are often abused by drug addicts. As a consequence, this class of drugs and their metabolites are frequently present in both clinical and forensic cases. Although the first benzodiazepines were first marketed over 40 years ago, the screening of especially the low-dosed compounds remains an analytical challenge. Indeed, low nanogram levels of benzodiazepines and their metabolites often remain undetected by traditional enzyme-multiplied immunoassay (EMIT) or cannot be confirmed by high-performance liquid chromatographic (HPLC) or gas chromatographic (GC) screening techniques, respectively [1–4].

Several parameters complicate the analysis of

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benzodiazepines. A first complicating factor is the wide range of therapeutic doses used for these compounds. An overview of the different classes of benzodiazepines, their therapeutic dose and corresponding plasma concentration is given in Table 1. The concentration of the parent compounds and their respective metabolites in biological fluids can differ by several orders of magnitude depending on the type of drug and its dosage. In addition the metabolization of each benzodiazepine is characterized by large inter-individual differences.

A second complicating factor is the chemical heterogeneity of benzodiazepines. Benzodiazepines have a large part of their structure in common but due to a wide polarity difference they show a different chromatographic behavior, limiting the number of compounds that can be chromatographed simultaneously. Although numerous methods exist for the determination of benzodiazepines, few of them can be applied for the simultaneous analysis of both parent compounds and their corresponding metabolites [5–8].

The chromatographic techniques commonly used for the determination of benzodiazepines are HPLC with ultraviolet (UV) detection [9,10], GC with electron-capture detection (ECD) [6,11] and gas chromatography–mass spectrometry (GC–MS) [15,18]. When comparing HPLC with GC, HPLC lacks the sensitivity required for measurement of therapeutic plasma concentrations of low-dosed benzodiazepines [12]. Moreover, the UV spectra of the benzodiazepines are very similar. As a consequence, the identification of benzodiazepines, e.g., in putrefied forensic samples is hampered by spectral interferences from coextracted impurities.

Although GC–ECD provides excellent sensitivity an important drawback is that identification is based solely on retention time. For toxicological purposes GC–MS provides a superior alternative as additional spectral information is combined with excellent resolution and sensitivity. However, next to the described advantages, there are also disadvantages inherent to GC analysis of benzodiazepines. The more polar compounds, per definition mostly metab-

Table 1
Overview of the different classes of benzodiazepines, their therapeutic doses, and both therapeutic and toxic plasma concentrations [31–34]

Compound	Dose (mg)	$t_{1/2}$ (h)	Proprietary names	Theraputic concentration (μg/ml)	Toxic concentration (μg/ml)
Short-acting ($t_{1/2}$<6 h)					
Clorazepate dipotassium	15–30	1–2	Tranxene, Unitranxene	0.02–0.2	2
Ketazolam	15–60	1–3	Solatran, Unakalm	0.001–0.02	
Prazepam	10–20	1–3	Lysanxia	0.2–0.7	1
Triazolam	0.125–0.25	2–5	Halcion	0.002–0.02	
Intermediate-acting ($t_{1/2}$ 6–24 h)					
Alprazolam	0.75–3	6–20	Alprazolam, Xanax	0.005–0.05	0.1–0.4
Bromazepam	4.5–18	8–22	Lexotan, Anxiocalm	0.08–0.2	0.3–0.4
Brotizolam	0.25	4–10	Lendormin	0.001–0.02	
Clobazam	20–30	10–32	Frisium	0.1–0.4	
Clotiazepam	10–15	3–15	Clozan	0.1–0.7	
Flunitrazepam	0.5–1	10–20	Hypnocalm, Rohypnol	0.005–0.015	0.05
Loprazolam	0.5–1	11–20	Dormonoct	0.003–0.01	
Lorazepam	2.5–5	10–40	Serenase, Temesta	0.08–0.25	0.3–0.5
Lormetazepam	1–2	10–15	Loramet, Stilaze	0.005–0.025	
Oxazepam	7.5–45	6–20	Oxazepam, Seresta	0.2–1.5	2
Temazepam	20	6–25	Euhypnos, Levanxol	0.02–0.15	1
Tetrazepam	25–100	10–26	Myolastan	0.05–0.6	
Long-acting ($t_{1/2}$>24 h)					
Clonazepam	0.5–8	20–60	Rivotril	0.01–0.08	0.1
Ethylloflazepate	2	75–120	Victan		
Flurazepam	13.5–27	40–120	Staurodorm	0.02–0.1	0.2–0.5

olites, display rather poor peak shapes and require derivatization prior to analysis. In addition, we have recently reported the importance of the type of mass spectrometer for the analysis of some benzodiazepines [13]. In our hands excessive peak tailing was observed for alprazolam and triazolam with quadrupole ion-trap (IT) MS, excluding its use for these important low-dosed benzodiazepines.

The purpose of this work was to establish a novel quadrupole GC–MS screening procedure for the simultaneous analysis of especially low-dosed benzodiazepines, both parent compounds and their major urinary metabolites. The method was developed for those compounds that are low-dosed and most representative of the European market, although many other benzodiazepines can be analyzed under the same conditions. The compounds studied were ketazolam (Solatran, Unakalm), oxazepam; flunitrazepam (Rohypnol, Hypnocalm), 7-aminoflunitrazepam, desmethylflunitrazepam, 7-aminodesmethylflunitrazepam; flurazepam (Staurodorm), hydroxyethylflurazepam, *N*-desalkylflurazepam; lorazepam (Serenase, Temesta); alprazolam (Xanax), 4-hydroxyalprazolam, α-hydroxyalprazolam; triazolam (Halcion) and α-hydroxytriazolam. With this selection the most frequently prescribed low-dosed benzodiazepines are covered. Although ketazolam is not a low-dosed benzodiazepine, it was included in our study because its steady-state plasma concentrations are in the low-nanogram range. Moreover, its major metabolite oxazepam is also formed during the metabolization of several related higher-dosed benzodiazepines like diazepam (Valium) and nordiazepam (Calmday). Consequently, the developed screening method allows the analysis of these higher dosed benzodiazepines as well.

2. Experimental

2.1. Solvents and reagents

Ethyl acetate and methanol were HPLC grade and obtained from Sigma–Aldrich (Bornem, Belgium). Pyridine and acetic anhydride were both obtained from Merck (Darmstadt, Germany). Standards of ketazolam, alprazolam, 4-hydroxyalprazolam, α-hydroxyalprazolam, triazolam and α-hydroxytriazolam were a gift from Upjohn (Kalamazoo, MI, USA). Flunitrazepam, desmethylflunitrazepam, 7-aminoflunitrazepam, 7-aminodesmethylflunitrazepam, and *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 (I.S.).

2.2. Preparation of standards

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

2.3. Instrumentation and chromatographic conditions

The GC–MS system consisted of a HP 6890 Series gas chromatograph coupled to a HP 5973 mass-selective detector (Avondale, PA, USA). The chromatographic system consisted of a Restek (Bellefonte, PA, USA) hydroguard guard column (5 m×0.32 mm I.D.) coupled to a 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 (SIM) modes at low resolution. The retention times of the drugs ex-

Table 2
Retention times before and after eventual derivatization, target and qualifier ions after eventual derivatization of the examined compounds

Compound	t_R before (min)	t_R after (min)	Target ion	Qualifier ions
Alprazolam	21.49	21.49	279.10	308.00–204.10
α-Hydroxyalprazolam	–	24.33	323.10	366.10
4-Hydroxyalprazolam	–	24.45	295.10	281.10–323.10
Flunitrazepam	16.21	16.21	312.10	285.10–266.10–238.10
7-Aminoflunitrazepam	16.35	21.26	325.20	297.20–255.10–306.10
Desmethylflunitrazepam	17.07	–	298.10	271.10–224.10
7-Aminodesmethylflunitrazepam	16.98	22.45	311.10	283.10–240.10
Flurazepam	18.65	18.65	86.10	
Hydroxyethylflurazepam	–	17.74	314.10	287.10–346.10–374.10
N-Desalkylflurazepam	14.67	14.67	259.05	288.00
Ketazolam (as diazepam)	14.67	14.67	256.00	283.00
Lorazepam	14.35	17.82	291.00	320.00–362.00
Oxazepam	14.10	16.13	257.00	286.00–328.00
Triazolam	23.23	23.23	313.00	342.00–238.05
α-Hydroxytriazolam	–	25.88	357.10	400.10
N-Methylclonazepam	18.23	18.23	328.10	294.10–302.10–248.10

amined and the atomic mass units (u) of the target and qualifier ions used for the target compound analysis are listed in Table 2.

2.4. Optimization experiments

For the optimization of the derivatization conditions, the influence of three parameters was examined: the ratio of the pyridine–acetic anhydride reagents, the reaction temperature and the reaction time. The following conditions were evaluated: ratios of 1:2, 2:3, 1:1, 3:2 and 2:1; reaction temperatures at room temperature, 35, 45, 55, 65 and 75°C; reaction times of 5, 10, 15, 20, 25, 30, 35 and 40 min.

The acetylation procedure was validated using standard solutions of the different compounds.

Stability: The stability of the derivatives and the non-derivatized benzodiazepines was evaluated on 4 consecutive days. The samples remained in the tray of the ALS in an air-conditioned room at 18°C and were stored in amber vials protected from light as at least some of the examined benzodiazepines are described as being photosensitive [28,29].

Linearity: Calibration standards were prepared for the scan mode (2.5, 5, 10, 20 and 30 ng/μl) and for the SIM mode (0.1, 0.25, 0.5, 1, 2.5 and 5 ng/μl) measurements. Calibration curves were obtained by plotting the target ion response ratio of the analyte to the internal standard, against the concentration ratio.

Within- and between-day precision: Within-day precision was determined by analyzing samples with three different concentrations: 5, 10 and 20 ng/μl. Ten samples of each concentration were analyzed. Between-day precision was measured on samples with the same concentrations during 10 separate days. The results are expressed as the relative standard deviation (RSD) at each level.

3. Results and discussion

A substantial effort was put into the development and subsequent optimization of the chromatographic conditions. On-column injection was chosen to improve sensitivity and to avoid thermal degradation. On-column injection is best performed at a column temperature beneath the boiling point (b.p.) of the injection solvent. Ethyl acetate (b.p.=76.5–77.5°C) has a good intermediate polarity and is therefore suited as a solvent for all the benzodiazepines used. An important advantage of setting the initial temperature below the b.p. is the solvent trapping effect resulting in a sharp chromatographic peak shape. After injection, the oven temperature has to be increased slowly to allow full trapping [14]. The final temperature needed for elution of all benzodiazepines was 300°C resulting in an elegant total run time of 28.33 min.

To improve the lifetime of the analytical column a retention gap was used. The difference between the internal diameter (I.D.) of the retention gap and the separating column has to be small to avoid band broadening effects [14]. With the 0.22-mm I.D. capillary column used, the largest acceptable I.D. of the retention gap was 0.32 mm. The deactivation type of the retention gap was found to influence the resolution. Polar, apolar and intermediate polarity retention gaps were examined and the best results were obtained with the latter type. However, when water-saturated ethyl acetate was used as an injection solvent, the chromatographic response showed a very large increase in noise eluting prior to the analytes (results not shown). The peak shapes deteriorated after a few injections of water-containing solutions, most likely due to destruction of the deactivation layer by the condensed water. The selection of a hydroguard guard column provided a solution for this problem.

As mentioned, the more polar benzodiazepines require derivatization prior to GC analysis as they remain undetectable under the described chromatographic conditions without derivatization: α-hydroxyalprazolam, α-hydroxytriazolam, 4-hydroxyalprazolam and hydroxyethylflurazepam. Moreover, although underivatized oxazepam and lorazepam are detected, they are characterized by a bad peak shape.

To overcome these problems and to increase the sensitivity of all compounds when using mass spectrometric detection, several derivatization procedures described in the literature were evaluated. The most frequently used derivatives are trimethylsilyl (TMS) [15–17] and alkyl [18–20] derivatives. However, TMS derivatives are not stable as they are extremely prone to hydrolysis making them unsuitable for routine analyses [19–21]. Another drawback is that these derivatives are injected onto the GC–MS system in excess silylating reagent, resulting in a severe contamination of the column. When non-derivatized benzodiazepines are injected onto such a contaminated column the limit of detection (LOD) increases significantly. As we were interested in the analysis of low-dosed benzodiazepines and injections were performed with on-column injection to improve sensitivity, this derivatization procedure could not be considered for our purposes.

Alkylation is another derivatization procedure frequently described for benzodiazepines. Both methylation and propylation were evaluated. These derivatives were stable for several days and showed an improved peak shape with respect to the underivatized analogues. However, an extra extraction step was required following derivatization, which increased the LOD. Moreover, hydroxyethylflurazepam, α-hydroxyalprazolam, 4-hydroxyalprazolam and α-hydroxytriazolam were not derivatized and remained undetectable in the screening method.

A third procedure for derivatization concerns acetylation as reported for alprazolam and its metabolites [22], and for benzophenones generated after acid hydrolysis of several benzodiazepines [23,24]. As some benzodiazepines yield a common benzophenone hydrolysis product, identification of the parent compound becomes almost impossible when a hydrolysis step precedes chromatographic analysis. Acetylation with pyridine and acetic anhydride provided a possible solution to solve the problems encountered with silylation and alkylation. Indeed, an additional extraction step was no longer needed as the reagents could easily be evaporated under nitrogen. Secondly, all those compounds that did not chromatograph underivatized (i.e., hydroxyethylflurazepam, 4-hydroxyalprazolam, α-hydroxyalprazolam and α-hydroxytriazolam) or showing an unsatisfactory peak shape (i.e., oxazepam and lorazepam) had a hydroxyl-functional group that could be acetylated (Fig. 1).

The acetylation procedure was optimized using a representative set of five standard compounds: hydroxyethylflurazepam, 7-aminoflunitrazepam, 4-hydroxyalprazolam, α-hydroxyalprazolam and α-hydroxytriazolam. 7-Aminoflunitrazepam was included in the set as amino functional groups are also derivatized. The structures of the obtained derivatives are shown in Fig. 1. Based on an evaluation of the recovery and the reproducibility the final parameters chosen were: a reagent ratio of pyridine–acetic anhydride (1:1, v/v), a reaction temperature of 18°C (air conditioned-stabilized room temperature), and a reaction time of 20 min. These fast and mild conditions were then successfully applied to the other 10 benzodiazepines. An overview of the retention times before and after eventual derivatization is given in Table 2. Representative chromatograms

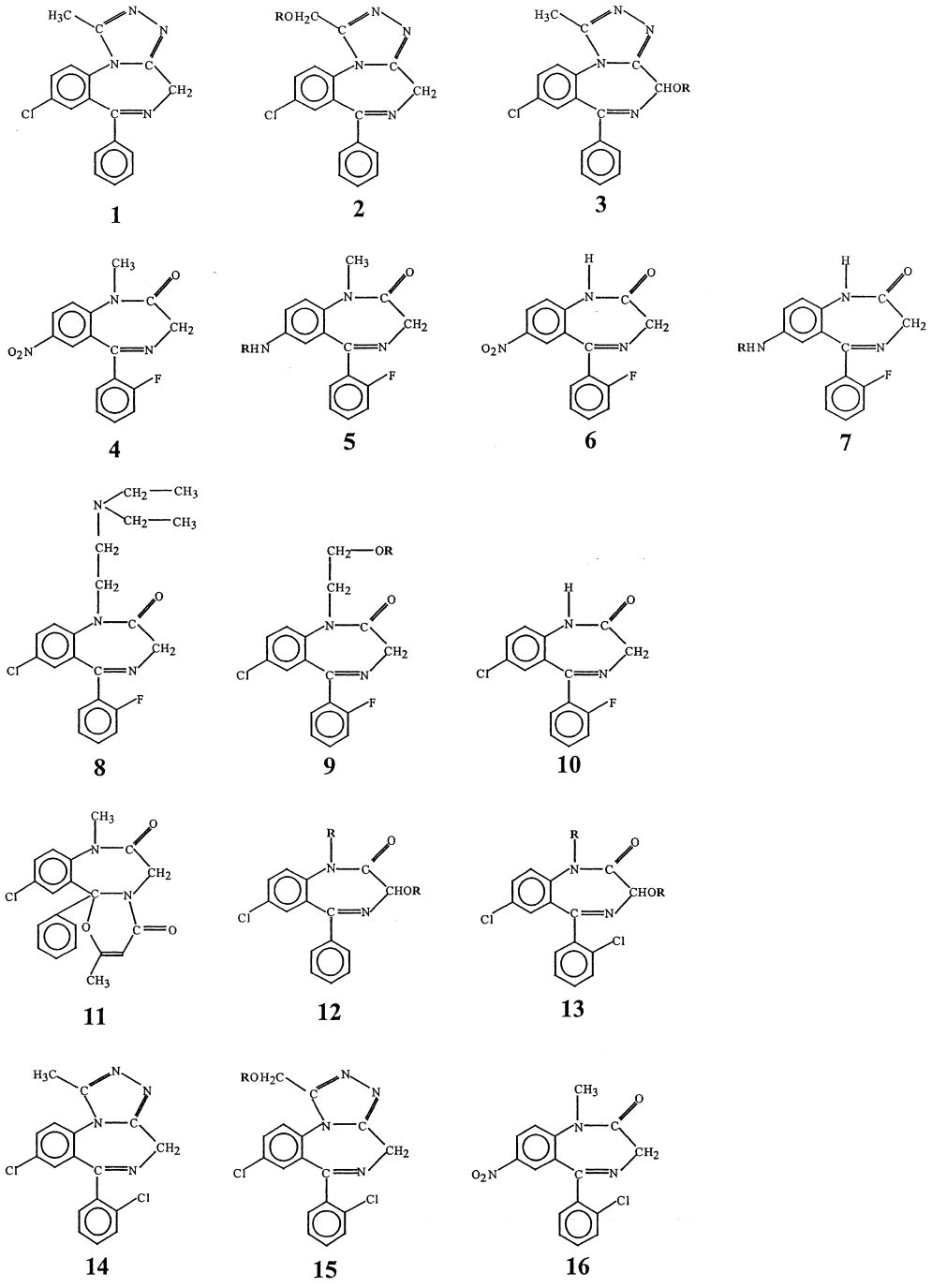

Fig. 1. Structures of the examined compounds before (R=H) and after (R=$COCH_3$) eventual derivatization: 1=alprazolam, 2=α-hydroxyalprazolam, 3=4-hydroxyalprazolam, 4=flunitrazepam, 5=7-aminoflunitrazepam, 6=desmethylflunitrazepam, 7=7-aminodesmethylflunitrazepam, 8=flurazepam, 9=hydroxyethylflurazepam, 10=*N*-desalkylflurazepam, 11=ketazolam, 12=oxazepam, 13=lorazepam, 14=triazolam, 15=α-hydroxytriazolam, 16=*N*-methylclonazepam (I.S.).

of all compounds before and after derivatization are shown in Fig. 2.

For some of the analyzed substances specific remarks have to be made. Importantly, ketazolam, flunitrazepam, flurazepam, *N*-desalkylflurazepam, alprazolam, triazolam and *N*-methylclonazepam remained underivatized because they lack a reactive functional group in their chemical structure (Fig. 1). These compounds were also not degraded under the described conditions. One exception concerns ketazolam, which is systematically decomposed to diazepam upon GC analysis [25]. Even with the mild on-column injection technique applied in our method, this thermal degradation could not be avoided. As a result, the best way to differentiate ketazolam from diazepam by GC analysis is through estimation of its metabolite ratios. This indirect determination of ketazolam is feasible because the major blood metabolite observed following ingestion of ketazolam is nordiazepam, while only traces of diazepam can be found. In urine, the predominant metabolite is oxazepam. In contrast, after diazepam intake both diazepam and nordiazepam can be found in blood, whereas both nordiazepam and oxazepam are major urinary metabolites [30].

Oxazepam and lorazepam have been described as

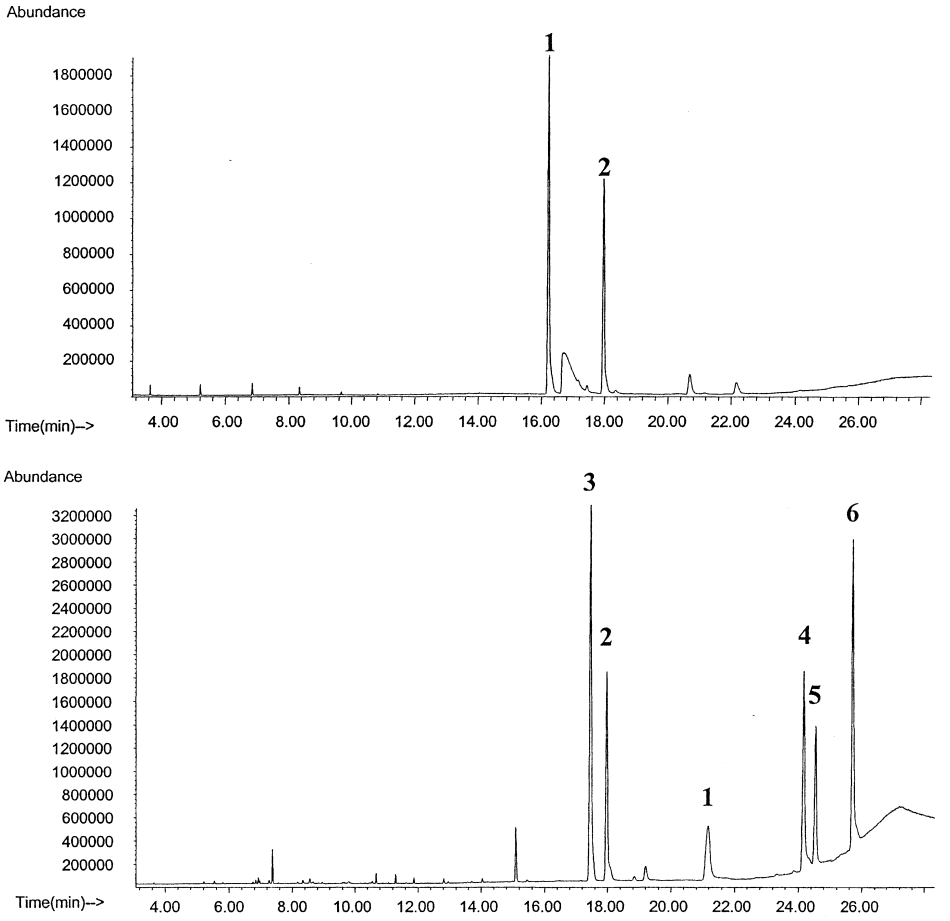

Fig. 2. Representative chromatograms of all examined compounds before (upper trace) and after (lower trace) eventual derivatization. (a) 1=7-Aminoflunitrazepam, 2=*N*-methylclonazepam (I.S.), 3=hydroxyethylflurazepam, 4=α-hydroxyalprazolam, 5=4-hydroxyalprazolam, 6=α-hydroxytriazolam. (b) 1=Oxazepam, 2=lorazepam, 3=ketazolam, 4=7-aminodesmethylflunitrazepam, 5=*N*-methylclonazepam, 6=flurazepam. (c) 1=*N*-Desalkylflurazepam, 2=flunitrazepam, 3=desmethylflunitrazepam, 4=*N*-methylclonazepam, 5=alprazolam, 6=triazolam.

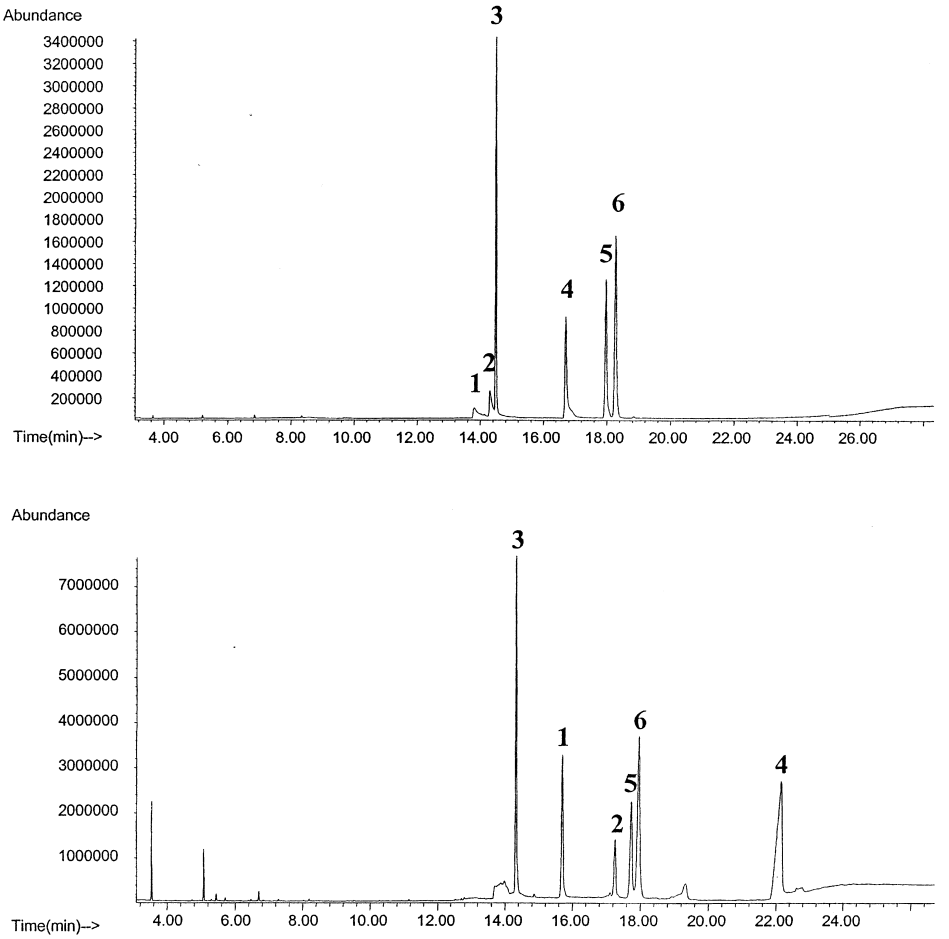

Fig. 2. (*continued*)

thermolabile compounds [26,27]. This thermal decomposition involves on-column rearrangement of the seven-membered ring to a six-membered ring, followed by the loss of a water molecule to form a stable aromatic structure. However, under our conditions this decomposition could be avoided by acetylation of the α-OH– and NH– groups prior to GC analysis. The mass spectra of these diacetylated compounds are shown in Fig. 3. Nuclear magnetic resonance (NMR) spectrometry confirmed that the derivatization procedure employed indeed yields the diacetylated benzodiazepines. In the case of lorazepam, for example, the ^{1}H-NMR spectrum revealed the presence of two CH_3CO peaks: one at 2.21 ppm, the other at 2.55 ppm (both integrate for three protons). Additionally, the exchangeable proton signals assigned to 3-OH and N(1)H in the spectrum of lorazepam (at 6.37 ppm and 10.83 ppm, respectively) were absent in the spectrum of the acetylated derivative, while the resonance of C(3)H shifted downfield by ±1.2 ppm (singlet at 6.04 versus doublet at 4.85).

On the other hand, extensive degradation of desmethylflunitrazepam was caused by the acetylation reagent. Increasing the ratio of pyridine–acetic anhydride to 7:1 was performed in an attempt to prevent this degradation. However, under these milder conditions other compounds (i.e., oxazepam, lorazepam, 4-hydroxyalprazolam and 7-aminodesmethylflunitrazepam) were not completely deriva-

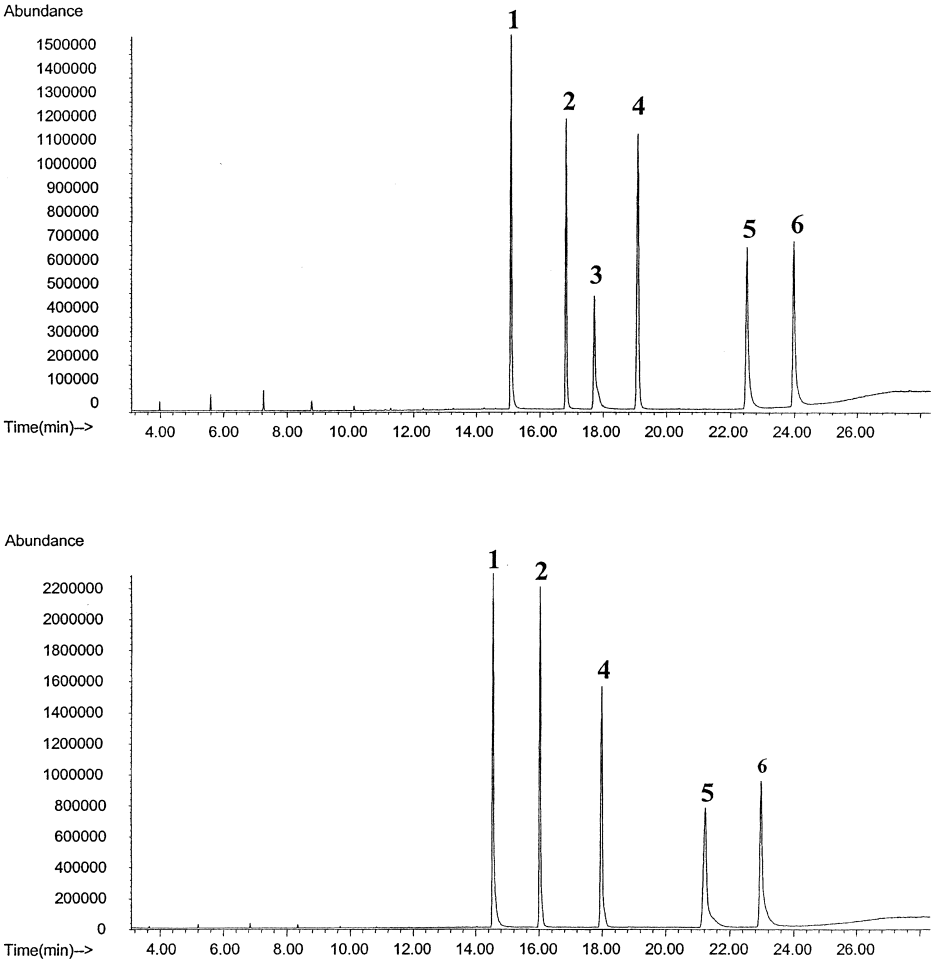

Fig. 2. (*continued*)

tized. Therefore, the presence of desmethylflunitrazepam has to be determined by an additional injection of an underivatized sample. Indeed, although desmethylflunitrazepam was degraded by acetylation it is characterized by an excellent peak shape prior to derivatization.

Other problem compounds with respect to direct determination are 7-aminoflunitrazepam and 7-aminodesmethylflunitrazepam which were converted into 7-acetamidoflunitrazepam and 7-acetamidodesmethylflunitrazepam, respectively. This is a problem as the latter two derivatization products also occur as endogenous urinary metabolites of flunitrazepam. However, the contribution of 7-aminoflunitrazepam and 7-aminodesmethylflunitrazepam to their respective 7-acetamido peaks can easily be evaluated by additional injection of an underivatized sample as described for desmethylflunitrazepam.

After the optimization of the derivatization conditions, further experiments were performed to check the stability of the derivatives and of the non-derivatized benzodiazepines. All compounds except for desmethylflunitrazepam were derivatized and injections were performed on 4 consecutive days. The results are shown in Figs. 4 and 5. All tested substances were stable when stored under these conditions, showing minimal losses of up to 2% during the examined period. Only hydroxy-

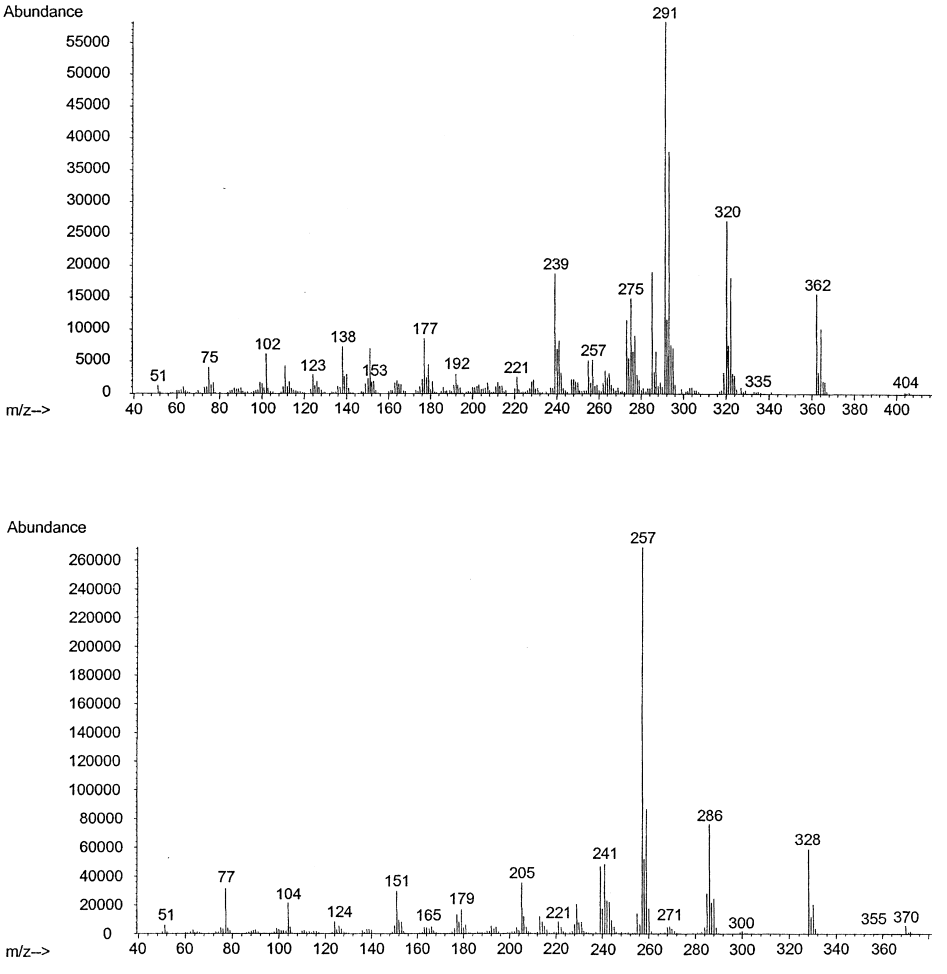

Fig. 3. MS spectra of derivatized oxazepam (bottom) and lorazepam (top).

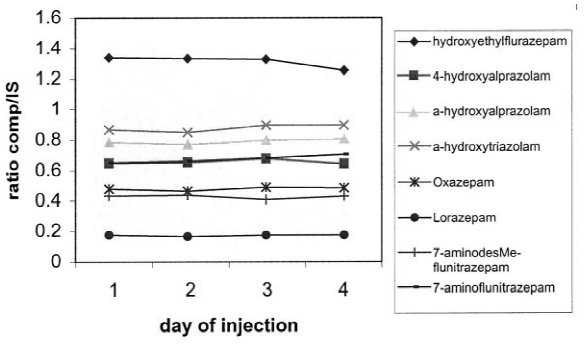

Fig. 4. Stability of the derivatized benzodiazepines.

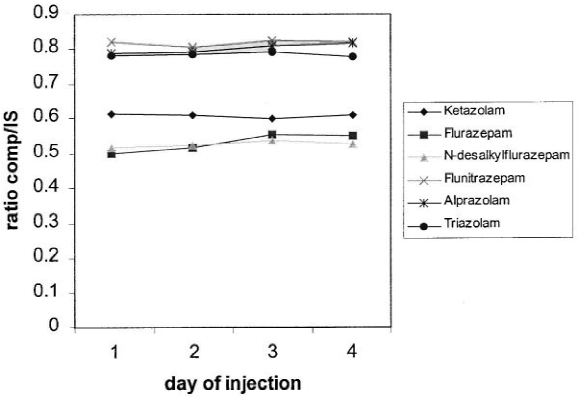

Fig. 5. Stability of the non-derivatized benzodiazepines.

Table 3
Calibration data of the examined compounds in the scan mode

Compound	SCAN mode					
	Data points	Slope	SD	Intercept	SD	r^2
Alprazolam	5	2.11	0.27	−1.7	0.33	0.997
α-Hydroxyalprazolam	5	9.93	0.78	−0.39	0.16	0.995
4-Hydroxyalprazolam	4	4.44	0.17	−0.84	0.38	0.996
Flunitrazepam	5	2.17	0.30	−1.09	0.62	0.997
7-Aminoflunitrazepam	5	7.61	0.88	−1.44	0.43	0.996
Desmethylflunitrazepam	5	1.53	0.53	−1.57	0.94	0.995
7-Aminodesmethylflunitrazepam	4	3.82	0.61	−1.7	0.27	0.996
Flurazepam	5	10.88	1.09	−0.58	0.87	0.997
Hydroxyethylflurazepam	5	8.67	0.66	0.47	0.05	0.997
N-Desalkylflurazepam	5	2.52	0.24	−0.49	0.54	0.999
Ketazolam	5	2.59	0.13	−0.4	0.33	0.998
Lorazepam	4	0.65	0.06	−0.68	0.18	0.997
Oxazepam	5	2.62	0.10	−1.51	0.36	0.995
Triazolam	5	2.24	0.32	−1.24	0.40	0.997
α-Hydroxytriazolam	5	10.41	1.02	−1.08	0.92	0.996

ethylflurazepam shows a loss of 5.5% during the fourth day.

The limit of quantitation (LOQ) varied between 2.5 and 5 ng/μl in the scan mode and between 0.1 and 0.5 ng/μl in the SIM mode. The linearity was also evaluated and the obtained calibration data are summarized in Tables 3 and 4. The standard deviations (SDs) of the slope and intercept are satisfactory and the correlation coefficient of the linear regression curves consistently exceeded 0.995.

Finally, the precision was evaluated and the obtained data are summarized in Table 5. The within-day RSDs ranged from 1.13 to 4.40% at 5 ng/μl, from 1.85 to 4.76% at 10 ng/μl, and from 2.75 to 4.87% at 20 ng/μl, respectively. The day-to-day RSDs ranged from 3.22 to 4.94% at 5 ng/μl, from 3.55 to 4.88% at 10 ng/μl, and from 1.12 to 4.80% at 20 ng/μl, respectively.

The developed analysis conditions have been extended to other benzodiazepines than the low-

Table 4
Calibration data of the examined compounds in the SIM mode

Compound	SIM mode					
	Data points	Slope	SD	Intercept	SD	r^2
Alprazolam	6	2.37	0.21	−0.5	0.04	1.000
α-Hydroxyalprazolam	6	1.63	0.14	−0.2	0.03	0.996
4-Hydroxyalprazolam	5	4.61	0.24	−0.3	0.02	0.995
Flunitrazepam	6	1.95	0.16	−0.2	0.02	0.995
7-Aminoflunitrazepam	6	3.34	0.21	−0.6	0.03	0.995
Desmethylflunitrazepam	6	1.64	0.12	−0.04	0.02	0.996
7-Aminodesmethylflunitrazepam	5	1.98	0.11	−0.03	0.01	0.997
Flurazepam	6	2.23	0.15	−0.8	0.13	0.997
Hydroxyethylflurazepam	6	3.89	0.22	−0.1	0.05	0.998
N-Desalkylflurazepam	6	2.38	0.21	−0.08	0.03	1.000
Ketazolam	6	4.48	0.53	−0.06	0.02	1.000
Lorazepam	5	3.96	0.24	−0.06	0.03	1.000
Oxazepam	6	1.03	0.16	−0.05	0.02	0.995
Triazolam	6	2.61	0.14	−0.4	0.12	0.998
α-Hydroxytriazolam	6	5.5	0.21	−0.1	0.04	0.997

Table 5
Within- and day-to-day precision data of the examined compounds at concentrations of 5, 10 and 20 ng/μl, respectively

Compound	RSD (%)					
	Within-day variation (n=10)			Day-to-day variation (n=10)		
	5 ng/μl	10 ng/μl	20 ng/μl	5 ng/μl	10 ng/μl	20 ng/μl
Alprazolam	3.29	3.77	4.50	4.79	4.61	4.28
α-Hydroxyalprazolam	2.43	1.85	4.87	3.99	4.79	4.60
4-Hydroxyalprazolam	1.44	3.00	2.75	3.56	4.59	4.50
Flunitrazepam	2.79	3.03	3.94	3.22	3.82	1.99
7-Aminoflunitrazepam	1.13	3.76	4.35	4.78	4.21	4.02
Desmethylflunitrazepam	3.84	3.46	4.55	4.42	3.76	4.72
7-Aminodesmethylflunitrazepam	4.93	2.89	4.73	4.64	4.03	4.62
Flurazepam	3.24	4.18	3.12	4.48	3.67	4.80
Hydroxyethylflurazepam	4.40	1.86	4.32	4.03	3.55	4.57
N-Desalkylflurazepam	3.37	2.74	3.65	3.59	4.22	1.12
Ketazolam (as diazepam)	3.54	4.76	4.64	4.94	3.97	4.18
Lorazepam	3.84	3.17	4.25	4.27	4.88	3.72
Oxazepam	2.09	4.03	4.65	4.10	4.17	3.47
Triazolam	3.20	2.97	3.79	3.59	4.19	4.12
α-Hydroxytriazolam	3.02	3.39	4.51	4.10	4.54	3.47

dosed benzodiazepines originally selected. The additionally checked benzodiazepines are: bromazepam, brotizolam, camazepam, clobazam, clonazepam, clotiazepam, cloxazolam, diazepam, halazepam, lormetazepam, medazepam, nitrazepam, nordazepam, prazepam and temazepam. These standards were injected before and after derivatization and the observed retention times are given in Table 6.

Table 6
Retention times before and after eventual derivatization of some other benzodiazepines

Compound	Proprietary names	t_R before (min)	t_R after (min)
Bromazepam	Bromidem, Lexotan	16.19	16.19
Brotizolam	Lendormin	23.64	23.64
Camazepam	Albego, Limpidon	22.10	23.22
Clobazam	Frisium	15.65	15.65
Clonazepam	Rivotril	19.35	19.35
Clotiazepam	Clozan	15.36	15.36
Cloxazolam	Akton	17.23	17.23
Diazepam	Diazepam	14.66	14.66
Halazepam	Paxipam	13.58	13.58
Lormetazepam	Loramet, Loranka	17.14	18.61
Medazepam	Nobrium, Raporan	13.36	13.36
Nitrazepam	Mogadon, Nitraphar	17.99	–
Nordazepam	Calmday	15.00	14.09
Prazepam	Lysanxia	–	16.79
Temazepam	Euhypnos, Levanxol	16.04	17.21

The developed method has been preliminary applied to toxicological urine and blood samples. For this purpose, a new extraction procedure was used, involving solid-phase extraction on Bond Elut phenyl cartridges (Varian). The biological applicability for screening purposes and the optimization of the extraction procedure will be the subject of a forthcoming paper. Chromatograms of postmortem urine and blood sample extracts, respectively, in the full scan and SIM modes are presented in Fig. 6a and b.

4. Conclusions

Novel GC–MS screening conditions for benzodiazepines have been developed, allowing the simultaneous analysis of both parent compounds and their corresponding metabolites with a large polarity range. The described acetylation procedure is elegant and validation results are satisfactory. The obtained

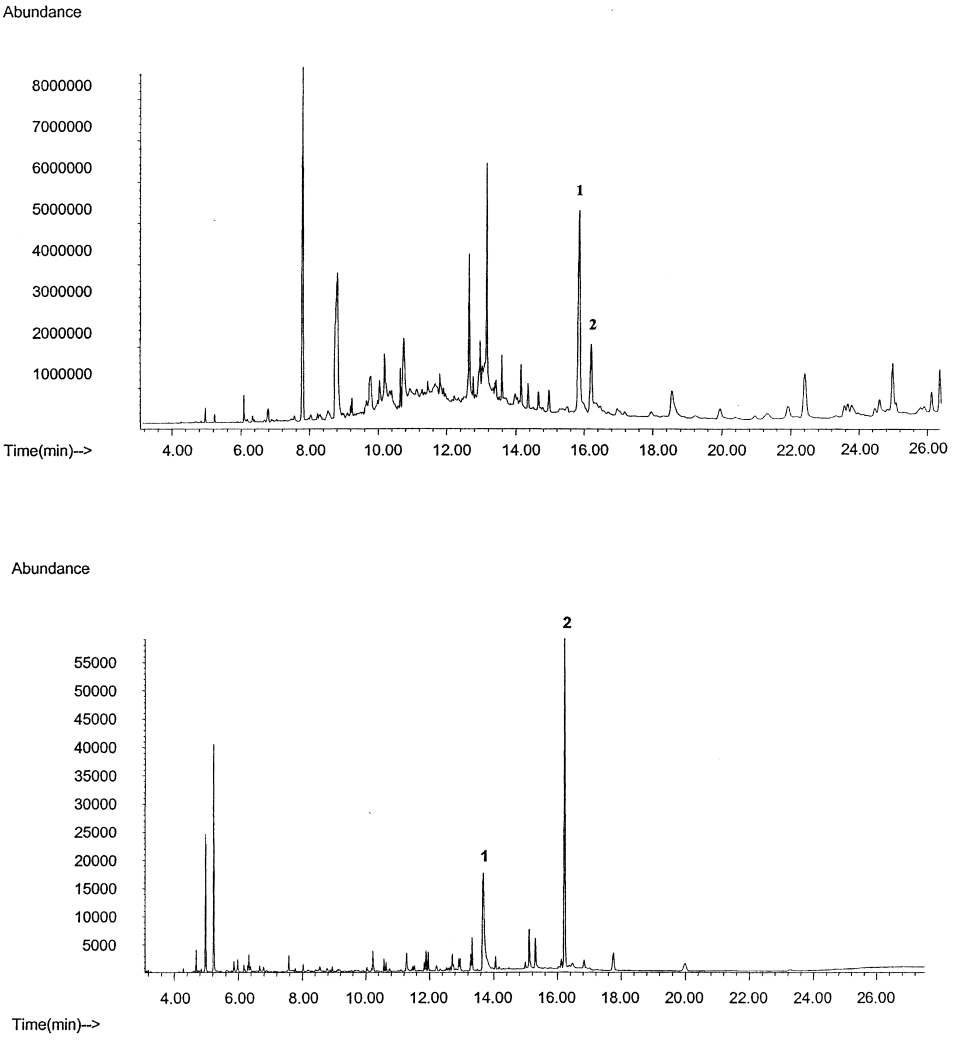

Fig. 6. Representative chromatograms of a urine and blood sample extract, respectively. (a) Total ion current (TIC) of an extracted postmortem urine sample; 1=hydroxyethylflurazepam (150 mg/ml), 2=*N*-methylclonazepam. (b) SIM chromatogram of an extracted postmortem blood sample; 1=*N*-desalkylflurazepam (28.6 mg/ml), 2=*N*-methylclonazepam.

derivatives are stable for at least 4 days making the method very suitable for routine analyses.

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