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Determination of 14 benzodiazepines and hydroxy metabolites, zaleplon and zolpidem as *tert*-butyldimethylsilyl derivatives compared with other common silylating reagents in whole blood by gas chromatography–mass spectrometry

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Abstract

The most common commercially available silylating reagents, *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA), *N*,*O*-bis-(trimethylsilyl)trifluoroacetamide + 1% trimethylchlorosilane (BSTFA + 1% TMCS) and *N*-methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) were evaluated to achieve optimal derivatization conditions for analyzing various benzodiazepines based on gas chromatography–electron impact ionization–mass spectrometry (GC–EI–MS). Sensitivity, repeatability, retention times and stability of the derivatives, as well as specificity of mass fragmentation, were studied in detail. Also other parameters affecting the derivatization chemistry of benzodiazepines are discussed. *tert*-Butyldimethylsilyl (TBDMS) derivatives proved to be more stable, reproducible and sensitive than corresponding trimethylsilyl (TMS) derivatives for the GC–EI–MS analysis of benzodiazepines. Based on the TBDMS derivatives, a rapid, reliable, sensitive and quantitative GC–MS method was developed for the determination of 14 benzodiazepines and two hydroxy metabolites, as well as two non-benzodiazepine hypnotic agents, zolpidem and zaleplon, using 500 μl of whole blood. The method was completely validated in terms of accuracy, intra- and interday precision, limit of detection (LOD), limit of quantitation (LOQ), linearity, selectivity and extraction efficiency; these were all within the required limits, except for the accuracy of nitrazepam at a medium concentration level.

Keywords: GC–MS; Silylation; Benzodiazepines; Zolpidem; Zaleplon

1. Introduction

Benzodiazepines and hypnotic agents are frequently prescribed drugs for treating a wide range of medical and psychiatric disorders. Benzodiazepines are anticonvulsive, centrally muscle relaxing, sedative hypnotics and anxiolytic agents, with a varying duration of action and potencies in these categories. After the first benzodiazepine (chlordiazepoxide) was introduced on the markets over 40 years ago, they have widely replaced other anxiolytes, such as barbiturates and meprobamate, which carried a greater risk of dependence and toxicity, and soon became the most prescribed psychoactive drugs in the world [1]. However, benzodiazepines can also cause severe dependence, and they are commonly misused by the persons with alcohol problems or multiple substance abuse [2,3]. Furthermore, several studies have indicated that benzodiazepines lower psychomotor performance, and are therefore a risk factor in traffic safety, especially when abused with illicit drugs and/or alcohol [4,5]. Short-acting non-benzodiazepine hypnotic agents, such as zolpidem and zaleplon, are also target analytes, e.g. in forensic and clinical toxicology, due to their various side-effects including impairment of psychomotor performance [6–8], additive influence with other central

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nervous system (CNS) depressants (e.g. benzodiazepines and alcohol) and the risk of fatal overdose [9].

Numerous analytical methods have been published for the analysis of a single benzodiazepine or a selected group of these analytes, and a few for non-benzodiazepine hypnotic agents. The published procedures nevertheless lack simultaneous determination of both groups. We have previously presented a method for simultaneous screening of these drugs in whole blood using GC in combination with both MS and electron capture (ECD) detection [10]. The majority of the compounds were quantitatively analyzed, and for others semi-quantitative results were obtained. Recently, Kratzsch et al. presented quantitative (except bromazepam) determination of the analytes from plasma samples using atmospheric pressure chemical ionization (APCI) liquid chromatography–mass spectrometry (LC–MS) [11]. Giroud et al. used the same analytical technique for the simultaneous quantitative analysis of zolpidem and zaleplon, but did not include benzodiazepines [12]. Recent quantitative methodology for the analysis of benzodiazepines in serum, plasma or whole blood includes dual-column GC [13], GC–MS [14–17], GC–tandem mass spectrometry (GC–MS/MS) [18,19], liquid chromatography (LC) [20,21], liquid chromatography–mass spectrometry (LC–MS) [22–26] and liquid chromatography–tandem mass spectrometry (LC–MS/MS) [27–30]. However, GC–MS based techniques remain a method of choice for many routine laboratories due to the separation efficiency, versatility, ease of operation and maintenance, as well as lower costs of the analyses and investment expenses of an analytical system compared to LC–MS/MS. Moreover, the unexpected consequences of matrix-dependent ion suppression complicate the optimization of LC–MS/MS techniques, especially when using electrospray ionization (ESI), but also with APCI [31–33].

In GC based analytical techniques of benzodiazepines, the derivatization of polar functional groups containing reactive hydrogen atoms is of great importance. For example, unspecific interactions with column phase material and peak tailing are usually avoided, and additionally, increased thermal stability, sensitivity and more specific mass fragments of the target analytes in mass spectra are generally achieved. Silylating reagents are commonly used for derivatization because they are versatile, easy to prepare and can be injected, unlike many other derivatizing agents, directly without removing the excess reagent into the GC–MS system.

In general, TBDMS derivatives formed by MTBSTFA have superior properties compared to other silylated derivatives. For example, they are reported to have more specific mass fragmentation and higher *m*/*z* values in EI mass spectra, their hydrolytic stability is greater, and thus TBDMS derivatives are less sensitive to moisture [34]. Benzodiazepines form highly sensitive and stable TBDMS derivatives that have appropriate GC–EI–MS properties. For example, unlike many other compounds, all active hydroxyl and secondary amine groups of these benzodiazepines silylated with MTBSTFA rather than MSTFA in our two-reagent, large-scale semiquantitative/quantitative blood screening method, indicating stable TBDMS rather than TMS derivative formation [10]. Moreover, in the studied derivatization procedures MTBSTFA has shown superior properties to various other analytes including fatty acids [35], endocrine disruptors [36], substituted phenols [37], herbicides [38] and non-steroidal anti-inflammatory drugs (NSAIDs) [39]. However, BSTFA including typically 1% TMCS as a catalyzing agent, has been the most commonly used derivatization reagent for analyzing benzodiazepines in biological matrices [40].

Therefore we systematically evaluated the most common commercially available silylating reagents to form optimal derivatives for the GC–EI–MS analyses of benzodiazepines and their hydroxy metabolites. Various reaction parameters affecting derivatization chemistry were studied and/or discussed in detail. Furthermore, the most appropriate derivatization reagent was chosen and a sensitive, rapid, economical as well as universal routine application for simultaneous determination of different benzodiazepines and non-benzodiazepine hypnotic agents in whole blood was developed.

2. Experimental

2.1. Chemicals and reagents

Chlordiazepoxide, diazepam, nitrazepam, oxazepam and temazepam were purchased from Orion Corporation (Espoo, Finland). Bromazepam, medazepam, midazolam and nordazepam were obtained from Roche (Mannheim, Germany), Lorazepam and zaleplon from Wyeth-Ayerst Laboratories (Pearl River, NY, USA) and zolpidem hemitartrate from Sanofi-Synthelabo (Paris, France). Alfa-OH-alprazolam was acquired from Pharmacia & Upjohn (Kalamazoo, MI, USA). Alprazolam was donated by the United Nations Narcotics Laboratory (Vienna, Austria), flurazepam was a donation from National Agency for Medicines (Helsinki, Finland), and phenazepam from the Republican Centre of Forensic Medicine (Moscow, Russia). An ampoule of alfa-OH-midazolam (100 $\mu g\,ml^{-1}$) was purchased from Radian Corporation (Austin, TX, USA). The chemical structures of all the studied analytes are illustrated in Fig. 1.

Silylating reagents, MTBSTFA and MSTFA were purchased from Sigma–Aldrich (St. Louis, MO, USA). BSTFA + 1% TMCS was from Supelco (Bellefonte, PA, USA). Acetonitrile, methanol, *n*-butyl acetate and Na_2HPO_4 of analytical grade, were supplied by Merck (Darmstadt, Germany). Sheep whole blood was acquired from the Internal Services of National Public Health Institute (Helsinki, Finland).

2.2. GC–MS parameters and instrumentation

The analysis was performed with an Agilent Technologies GC–MS 6890/5973 (Palo Alto, CA, USA) instrument

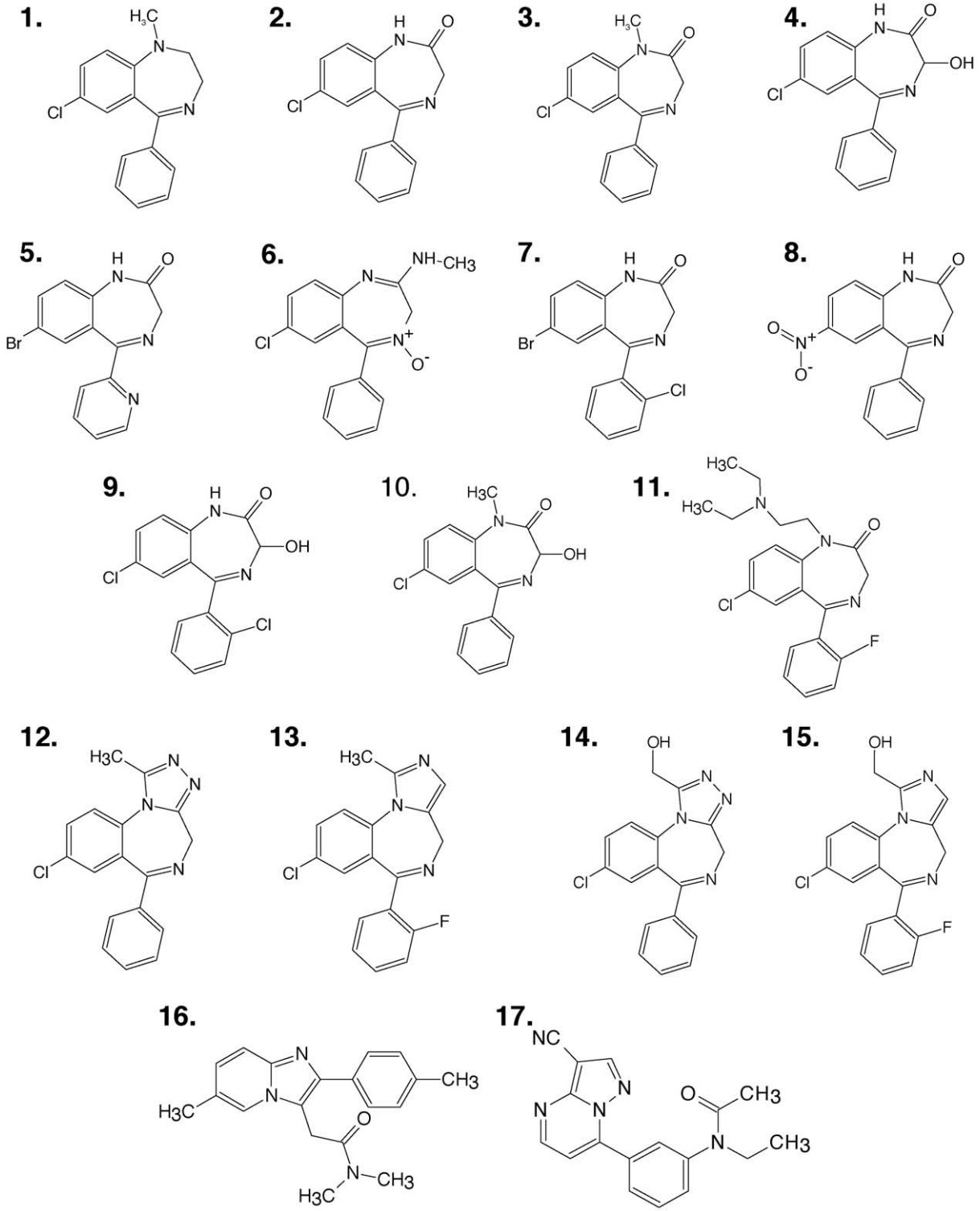

Fig. 1. Structures of different benzodiazepines, hydroxy metabolites and non-benzodiazepine hypnotic agents. 1,4-benzodiazepines: (1) medazepam, (2) nordazepam, (3) diazepam, (4) oxazepam, (5) bromazepam, (6) chlordiazepoxide, (7) phenazepam, (8) nitrazepam, (9) lorazepam, (10) temazepam and (11) flurazepam, ISTD; imidazobenzodiazepines: (12) alprazolam and (13) midazolam; OH-metabolites: (14) alfa-OH-alprazolam and (15) alfa-OH-midazolam; non-benzodiazepine hypnotic agents: (16) zolpidem and (17) zaleplon.

equipped with 7683 series autosampler. Gas chromatographic separations were carried out using a cross-linked 30 m DB-35 ms (0.32 mm inner diameter i.d., 0.25 μm film thickness) silica capillary column from J&W scientific (Folsom, CA, USA) and recessed double gooseneck liners (4.0 mm i.d.) from Restek (Bellefonte, PA, USA) were used. The initial temperature of the analytical column was 120 °C for one min, which was then increased at a rate of 15 °C per min to 330 °C which temperature was held constant for 2.80 min. Helium 5.6 (99.9996%) was used as the carrier gas at a constant flow rate of 1.5 ml min^{-1} after pulsed flow injection in splitless mode (2.0 μl) at an injection pressure of 90.5 kPa for 1.0 min. The split vent was opened 1.0 min after the injection. The injector port, transfer line, quadrupole and ion source temperature were set at 250, 300, 150 and 230 °C, respectively. In the mass spectrometric measurements, the EI mode was used at low resolution, applying an ionization energy of 70 eV. Manually adjusted target tuning was used instead of auto-tuning macro. The following values were applied (*m*/*z* 69 = 100%): *m*/*z* 50 (0.3–5.0%), 1.0%; *m*/*z* 131 (20–120%), 55%; *m*/*z* 219 (20–120%), 120%; *m*/*z* 414 (0.3–10%), 10%; *m*/*z* 502 (0.3–10%), 10%. Three characteristic ions, relative ion abundance of qualifier ions in respect to the target ion, and retention time were used for identification of each analyte. For quantitation, the peak height ratios of the analytes relative to the internal standard (ISTD) were compared to the standard straight line. In the scanning mode experiments, the mass range of 50–600 amu (2.67 scans/s) was applied. Data handling and system operations were controlled by HP Chemstation Software (B.01.00).

2.3. Sample treatment

0.5 ml of 0.5 M Na_2HPO_4 buffer was added to whole blood sample of 0.5 ml. All analyzed substances were extracted with 5 ml of butyl acetate (flurazepam 200 ng ml^{-1} in extraction solvent as internal standard) in a disposable 15 ml glass test tube. The mixture was vigorously pulse-shaken in a multitube vortexer for 30 s followed by the centrifugation (1700 × *g*, 5 min). The organic layer was transferred to a similar test tube and evaporated to dryness under a stream of air in a water bed at 75 °C (15–20 min). After the evaporation of the extraction solvent, 100 μl of the freshly prepared mixture of acetonitrile-MTBSTFA (80:20, v/v) was quickly added to the extraction residue. The sample was heated in capped test tubes to complete the derivatization reaction (80 °C, 30 min). After the heating procedure the samples were allowed to cool down to the ambient room temperature (10 min), transferred to vials containing 200 μl inserts, and analyzed by GC–MS.

2.4. Preparation of standard solutions

The concentrations of different substances varied according to the expected therapeutic levels that were based on previous experience in our laboratory and on the literature [41]. Because of the varying concentrations of each compound, the concentration of diazepam will be used as a reference value to illustrate the concentrations of other analytes in this manuscript. The concentrations of other compounds are always relatively comparable to the acetonitrilic stock solution concentration of diazepam in the performed validation experiments.

Acetonitrilic stock solution was prepared by weighing a defined mass of pure substances to volumetric flask (50 ml), excluding alfa-OH-midazolam that was available only in ampoules (100 μg ml^{-1} in methanol), and adding acetonitrile until a final volume was reached. The concentrations were: 400.0 μg ml^{-1} of diazepam, nordazepam, oxazepam and temazepam, 800.0 μg ml^{-1} of chlordiazepoxide, 80.0 μg ml^{-1} of medazepam, bromazepam, phenazepam, midazolam, nitrazepam, zaleplon and alfa-OH-alprazolam, 120.0 μg ml^{-1} of zolpidem and alprazolam, and 40.0 μg ml^{-1} of lorazepam. Alfa-OH-midazolam was added directly to a separate aqueous working solution (40.0 μg ml^{-1} of diazepam, 2.0 μg ml^{-1} of alfa-OH-midazolam) that was prepared by diluting the stock solution prior to each analysis or validation experiment. Aqueous analytical standard solutions were made from the working solution. Quality control (QC) samples were prepared to three different concentration levels for each compound in whole blood from a working solution. The corresponding concentrations in homogenized QC samples were: 1.0 (HIGH), 0.5 (MED) and 0.1 μg ml^{-1} (LOW) of diazepam. Stock solutions as well as QC samples were stored at −20 °C and alfa-OH-midazolam at +4 °C.

2.5. Validation

In linearity tests, blank whole blood (0.5 ml) was spiked with 50 μl of aqueous analytical standard solution. The measured concentrations (1 replicate) covered a range from subtherapeutic to toxic concentrations: 6.0, 4.0, 2.0, 1.0, 0.5, 0.2, 0.1, 0.05 and 0.025 μg ml^{-1} of diazepam, others relative to stock solution concentrations of diazepam, with the exception of alfa-OH-midazolam concentrations which were 1.2, 0.8, 0.4, 0.2, 0.1, 0.04, 0.02, 0.01 and 0.005 μg ml^{-1}. In addition, the concentrations of 0.01, 0.005 and 0.002 μg ml^{-1} of diazepam (i.e. 0.002, 0.001 and 0.0004 μg ml^{-1} of alfa-OH-midazolam) were included for determining the limit of detection (LOD) by using the signal-to-noise ratio (S/N) equal to 3. The least squares regression model was applied to calculate the regression line with a weighting factor of 1/concentration. Regression lines were accepted as linear if the determination coefficient of linearity (R^2) exceeded 0.980 and back-calculated concentrations of each calibration sample deviated less than ±20% from the respective nominal value.

Four-point calibration (1.0, 0.5, 0.2 and 0.1 μg ml^{-1} of diazepam) was used for daily calibration curves in accuracy, precision and extraction efficiency testing. Accuracy as

well as intra- and interday precision ($n = 8$ each) were determined at three different (LOW, MED and HIGH) concentration levels by adding 50 μl of aqueous analytical standard solution in whole blood (0.5 ml). To meet the internationally established quantitation criteria for determination analysis, accuracy and precision should be within ±15 and 15% relative standard deviation (RSD), respectively, and ±20 and 20% RSD on LOQ [42]. Extraction efficiency was defined by adding 45 μl of aqueous analytical standard solution (MED, $n = 5$) to the extraction solvent of blank whole blood samples after the separation of organic solvent (4.5 ml) corresponding to full recovery. The results were compared to normally analyzed spiked samples (MED, $n = 8$). In the selectivity experiments, two zero samples and 10 authentic blood samples that were confirmed negative by immunological screening and GC–MS were checked for background interference.

2.6. Derivatization

The most common commercially available silylating reagents, MTBSTFA, MSTFA and BSTFA including 1% TMCS as a catalyzing agent were compared to each other in terms of (1) sensitivity, (2) repeatability and (3) stability of the derivatives. In addition, (4) effect of solvent and (5) specificity of mass fragmentation in EI ionization were evaluated, and (6) the retention times of both TBDMS and TMS derivatives were documented.

In the experiment, whole blood (0.5 ml) was spiked with 50 μl of aqueous analytical standard solution (prepared from 1.0 mg ml^{-1} MeOH solutions, with the exception of alfa-OH-midazolam 0.1 mg ml^{-1}) containing the same amount of each benzodiazepine (5.0 μg ml^{-1}). Each test tube was extracted and derivatized (80 °C, 30 min) in normal pre-treatment conditions so that the only variable was the used reaction mixture. 80 μl of acetonitrile and 20 μl of each silylating reagents were added ($n = 5$ each) to derivatize the compounds after evaporation of the extraction solvent to dryness. Furthermore, one test series ($n = 5$) was prepared by adding only 100 μl of MTBSTFA to each test tube without using acetonitrile. The most intensive ions and relative responses versus ISTD of each benzodiazepine were ones that were included in the method and have active hydrogen-containing functional groups in their molecular structure vulnerable to derivatization reactions, i.e. nordazepam, oxazepam, bromazepam, phenazepam, lorazepam, nitrazepam, temazepam, alfa-OH-midazolam and alfa-OH-alprazolam were recorded. Flurazepam (ISTD) does not have active protons and is therefore suitable for comparison experiments.

To evaluate the stability of the derivatives, the experiment was repeated by analyzing the same samples after 100 h. The storage conditions between the chromatographic runs were as follows: no septa was changed (i.e. there was a hole from the injection needle after the first injection), the lights were on in the daytime and the sample vials were simply kept at on an autosampler tray between the analyses at room temperature. All mass spectrometric measurements were performed in scanning mode.

3. Results and discussion

3.1. Extraction

In spite of the continuous improvement of other alternative extraction techniques, solid-phase extraction (SPE) and liquid–liquid extraction (LLE) are still the most efficient techniques for the routinely performed analysis of various drug molecules. SPE was initially considered to replace LLE, but problems such as reproducibility, sorption capacity and interfering impurities reduce the attractiveness of SPE [43]. For example, the bleeding of sorbent material from the extraction cartridge might lead to higher background interference and possible selectivity problems. Furthermore, SPE is not directly appropriate for the analysis of whole blood samples without additional specimen preparation, e.g. precipitation of red blood cells or sonification, due to the clogging of the SPE cartridge. These problems can nevertheless be avoided by LLE. On the other hand, it has been frequently proposed that traditional LLE techniques are laborious, need large volumes of organic solvents, and the avoidance of emulsion formation is problematic. However, there is usually no need for long and/or multistage procedures, which would only increase unnecessarily the costs and pre-treatment time of the analysis, consumption of organic solvents and the extraction of interfering background matrix. In general, fast and practical solutions for routinely performed LLE, as well as the recovery of high magnitude of the various drug molecules are obtained by simple and vigorous pulsed mixing (30–60 s) with a multi-tube vortexer. Moreover, emulsion formation can be avoided by careful selection of the LLE solvent used.

Various benzodiazepines have both acidic hydroxyl and/or basic amine groups in their molecular structure which present at extreme pH values in ionic forms. However, maximum neutrality and thus overall pH optimum can be obtained in slightly basic conditions, which allow the simultaneous LLE of the compounds of interest in spite of both acidic and basic functional groups. LLE and a medium polarity solvent, *n*-butyl acetate, allows the necessary selectivity as well as more rapid (30 s) and efficient simultaneous extraction of the analytes than do the available SPE procedures. The emulsion formation, which is generally considered to be the major shortcoming of LLE, was completely avoided. Furthermore, *n*-butyl acetate is also comparatively safe for health, especially compared to other alternatives, and has a strong and typical odor, which make the presence of the solvent clearly recognizable and suitable for routine use. Only the relatively high boiling point of *n*-butyl acetate (126 °C) is its minor shortcoming, but is not a problem in the case of benzodiazepines and other hypnotics, which evaporate at significantly higher temperatures.

3.2. Comparison of silylation reagents

3.2.1. Retention times and mass fragmentation of the derivatives

Silyl derivatives are formed by the displacement reaction of active protons as a nucleophilic attack of the more electronegative heteroatom upon the silicon atom of the silylating reagent. Each active proton replaced by the TMS or TBDMS alkylsilyl group adds the molecular weight and correspondingly the mass-to-charge ratio (*m*/*z*) of the analyte by 72 or 114, respectively. TBDMS derivatives have therefore generally longer retention times in the analytical GC column than TMS derivatives, but generally also higher mass fragments and more specific mass fragmentation in the EI spectra, which both increase the selectivity of the method. Both the TMS and TBDMS derivatives are easily recognizable due to the intensive low mass ions. The *m*/*z* 73 corresponds to the TMS moiety and *m*/*z* 57 *tert*-butyl group that is fragmented in EI ionization from TBDMS derivatives. Neither of these ions cannot, however, be recommend for SIM identification, due to the unspecificity and high level of the background noise of the ions of low *m*/*z*.

Various benzodiazepines have polar functional hydroxyl and/or amine groups including active protons (for active groups, see Table 1), which can be silylated in displacement reactions. As could be expected due to the increased molecular weight, benzodiazepine TBMDS derivatives have longer retention times than the corresponding TMS derivatives. The difference in retention times between TMS and TBDMS derivatives was roughly 1 min per each replaced active proton. That is, lorazepam and oxazepam, having both a secondary amine and a hydroxyl group, had an approximately 2-min difference in retention times, while all the other studied analytes had a difference of about 1 min, excluding alfa-OH-alprazolam (1.54 min) due to the elution in the isothermal region. Nevertheless, all the analytes, regardless of the size of the alkylsilyl group, were sufficiently volatile to GC analysis, and the faster retention times of TMS derivatives therefore cannot be considered to be a significant advantage compared to TBDMS derivatives.

TMS and TBDMS benzodiazepine derivatives are both fragmented to predominant high mass ions in EI spectra; these ions are nicely separated from low mass ions originating from the matrix impurities and column bleed. As previously discussed, benzodiazepine TBDMS derivatives are also dominated by intensive $[M-57]^+$ ions [44], regardless of whether one or two (i.e. oxazepam and lorazepam) active protons were derivatized. On the contrary, TMS derivatives do not follow any standard pattern in mass fragmentation. For both of the derivatives, three diagnostic SIM ions can be selected, but the *m*/*z* values close to each other, decreasing the specificity of selected ions, have to be used for certain analytes. Furthermore, oxazepam and lorazepam 2TMS derivatives, i.e. two active protons both replaced by the TMS group, have the same most abundant ions of *m*/*z* 429, 430 and 431. This is not, however, a major selectivity problem, as they are clearly separated from each other in the chromatogram. In addition, one could also use more specific, but less intensive qualifier ions, which would improve the specificity, but decrease the sensitivity of the assay. On the contrary, oxazepam and lorazepam 2TBDMS derivatives, i.e. two active protons both replaced by the TBDMS group, have more specific mass fragmenta-

Table 1
Active groups of compounds and SIM parameters

No.	Compound	Active groups[a]	Retention time (min)	Time window (min)	Dwell time (ms)	SIM ions[b] (*m*/*z*)
1	Medazepam	–	11.56	8.00–13.20	20	242, 244 (36.1), 270 (20.4)
2	Nordazepam–TBDMS	–NH–	12.30			327, 329 (40.0), 328 (27.6)
3	Diazepam	–	12.93			284, 256 (132.8), 258 (54.8)
4	Oxazepam–2TBDMS	–NH–, –OH	13.03			457, 513 (35.5), 514 (30.7)
5	Bromazepam–TBDMS	–NH–	13.56	13.20–15.40	10	374, 372 (97.5), 346 (57.6)
6	Chlordiazepoxide[c]	–NH–	13.58			282, 283 (90.3), 284 (58.3)
7	Phenazepam–TBDMS	–NH–	13.70			407, 405 (74.8), 409 (30.3)
8	Midazolam	–	13.75			310, 312 (39.7), 325 (29.8)
9	Lorazepam–2TBDMS	–NH–, –OH	13.78			491, 515 (46.2), 493 (79.0)
10	Nitrazepam–TBDMS	–NH–	13.89			338, 292 (18.3), 394 (6.4)
11	Temazepam–TBDMS	–OH	14.22			357, 283 (55.7), 359 (39.7)
12	Alfa-OH-midazolam–TBDMS	–OH	15.08			398, 400 (40.0), 399 (33.9)
13	Zolpidem	–	15.12			235, 236 (24.4), 307 (14.9)
14	Alprazolam	–	16.16	15.40	30	279, 204 (75.7), 308 (54.7)
15	Zaleplon	–	16.47			305, 248 (323.7), 263 (105.9)
16	Alfa-OH-alprazolam–TBDMS	–OH	17.33			381, 383 (35.4), 382 (28.7)
Flurazepam, ISTD		–	14.28	13.20–15.40	10	86

[a] Active functional groups include free hydrogen atoms in the molecular structure of the compound. These hydrogen atoms can be – at least in theory-derivatized.

[b] Values in parentheses are the relative abundances of qualifier ions in respect to the quantitation ion.

[c] Chlordiazepoxide has a free hydrogen atom in the secondary amine group, which is not derivatized in the developed method, likely due to steric hindrance. Consequently, the analyzed form is underivatized.

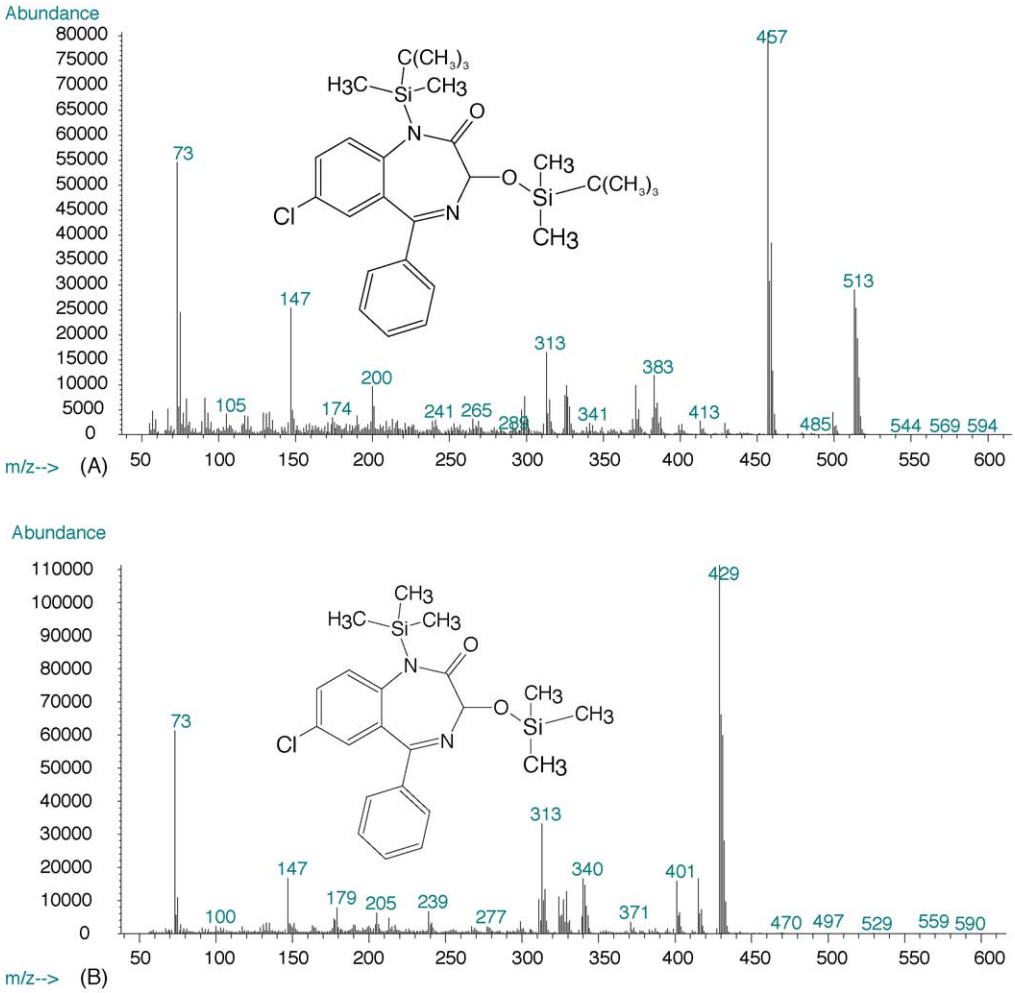

Fig. 2. Full-scan El mass spectra (50–600 amu) of oxazepam (A) TBDMS and (B) TMS derivatives.

tion and completely different higher mass diagnostic ions of *m*/*z* 457, 513, 514 and 491, 515, 492, respectively. The mass fragmentation of oxazepam TBDMS and TMS derivatives is shown in Fig. 2. The other studied analytes had unique fragmentation patterns, and no major differences in specificity between the TMS and TBDMS benzodiazepine derivatives in EI–MS spectra were observed. The retention times, most abundant ions and their relative ion abundances, in respect to the target ion for both derivatives are shown in Table 2.

3.2.2. *Sensitivity and repeatability of the derivatives*

The TBDMS derivatives showed superior properties in terms of sensitivity, repeatability and ease of derivative formation, compared to the TMS derivatives. The relative

Table 2
Retention times (R_t), the most abundant ions and relative ion abundances of TBDMS and TMS benzodiazepine derivatives

Compound	TBDMS derivatives		TMS derivatives	
	R_t (min)	Ions (*m*/*z*)[a]	R_t (min)	Ions (*m*/*z*)[a]
Nordazepam	12.30	327, 329 (40.0), 328 (27.6)	11.13	341, 342 (59.7), 343 (46.8)
Oxazepam	13.03	457, 513 (35.5), 514 (30.7)	11.22	429, 430 (59.1), 431 (55.1)
Bromazepam	13.56	374, 372 (97.5), 346 (57.6)	12.39	388, 386 (87.8), 389 (78.9)
Phenazepam	13.70	407, 405 (74.8), 409 (30.3)	12.56	387, 385 (99.7), 422 (98.7)
Lorazepam	13.78	491, 515 (46.2), 493 (79.0)	11.88	429, 431 (44.6), 430 (35.8)
Nitrazepam	13.89	338, 292 (18.3), 394 (6.4)	12.78	352, 306 (36.0), 254 (17.9)
Temazepam	14.22	357, 283 (55.7), 359 (39.7)	13.31	343, 257 (48.4), 345 (38.2)
Alfa-OH-midazolam	15.08	398, 400 (40.0), 399 (33.9)	14.00	310, 312 (34.5), 398 (41.7)
Alfa-OH-alprazolam	17.33	381, 383 (35.4), 382 (28.7)	15.79	381, 383 (39.7), 396 (37.2)

For instrumental information and GC–EI–MS conditions, see Section 2.2.

[a] Values in parentheses are the relative abundances of other ions (%) in respect to the predominant ion.

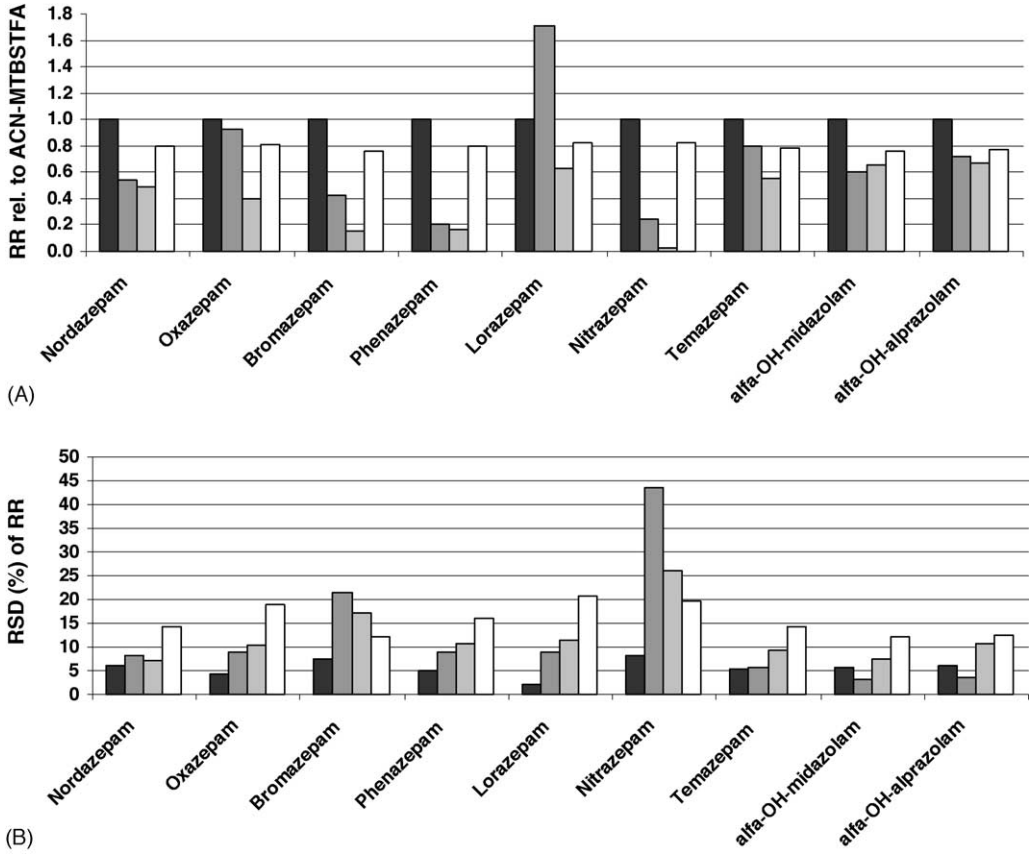

Fig. 3. (A) Average RR factors of various benzodiazepines (0.5 μg ml^{-1} each) vs. ISTD using the most common silylating reagents relative to TBDMS derivatives ($n = 5$ each) formed by the mixture of ACN–MTBSTFA (80/20, v/v). B: Repeatability of the derivatives using different derivatization mixtures. Black: TBDMS derivatives, ACN–MTBSTFA (80/20, v/v); grey: TMS derivatives, ACN–BSTFA + 1% TMCS (80/20, v/v); light grey: TMS derivatives, ACN–MSTFA (80/20, v/v); white: TBDMS derivatives, 100% MTBSTFA. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

responses (RR) between the benzodiazepine-TBDMS derivatives and ISTD ranged between 0.133–0.716 and 0.108–0.569 to the reaction mixtures of ACN–MTBSTFA (80/20, v/v) and MTBSTFA (100%), respectively. The corresponding values for benzodiazepine-TMS derivatives were 0.006–0.345 using ACN–MSTFA (80/20, v/v) and 0.056–0.387 for ACN–BSTFA + 1% TMCS (80/20, v/v). The repeatability of the derivatives ($n = 5$ each) measured by RSD were 2.20–8.16% (ACN–MTBSTFA), 12.2–20.7% (MTBSTFA), 7.64–26.0% (ACN–MSTFA) and 3.28–43.6% (ACN–BSTFA + 1% TMCS). The sensitivity and repeatability of the derivatives are graphically illustrated in Fig. 3. In addition, the RR factors of both TBDMS and TMS derivatives for each tested analyte are illustrated as a comparison to nordazepam derivative in Fig. 4.

A part of the differences in RR of the same analyte derivatives can be explained by the characteristic mass fragmentation in EI ionization. For example, lorazepam–2TBDMS has more diagnostic ions and specific mass fragments than lorazepam–2TMS. On the other hand, TMS derivatives of hydroxy metabolites have more characteristic ions than the corresponding TBDMS derivatives. This increases the specificity of mass fragmentation, but decreases the sensitivity of the most abundant ions. However, only slight variations in RR can be explained by the compound-specific mass fragmentation pattern, but not multiple response differences that are observed by using different reagents for certain analytes (Fig. 3). Therefore, it can be concluded that there are signicant variations in the ease of derivative formation and derivatization efficacy of the tested reagents to benzodiazepines.

All the TMS derivatives with low RR values shared one factor: the derivatization reaction takes place in the secondary amine group. Especially phenazepam, bromazepam and nitrazepam TMS derivatives have much weaker intensities than the corresponding TBDMS derivatives. Only the RR of lorazepam–2TMS, having both the secondary amine and hydroxyl groups in its molecular structure (Fig. 1), has a higher RR formed by BSTFA + 1% TMCS than the comparable TBDMS derivative. It should be noted, however, that the unspecific mass fragmentation of lorazepam–2TMS leads to the highly predominant target ion of m/z 429, which increases the sensitivity. In addition, MSTFA has still the lowest sensitivity, which further indicates that the derivatization of secondary amine groups of benzodiazepines with TMS reagents is problematic, whereas TBDMS derivatives are formed more easily. This is also supported by the repeatability data, which

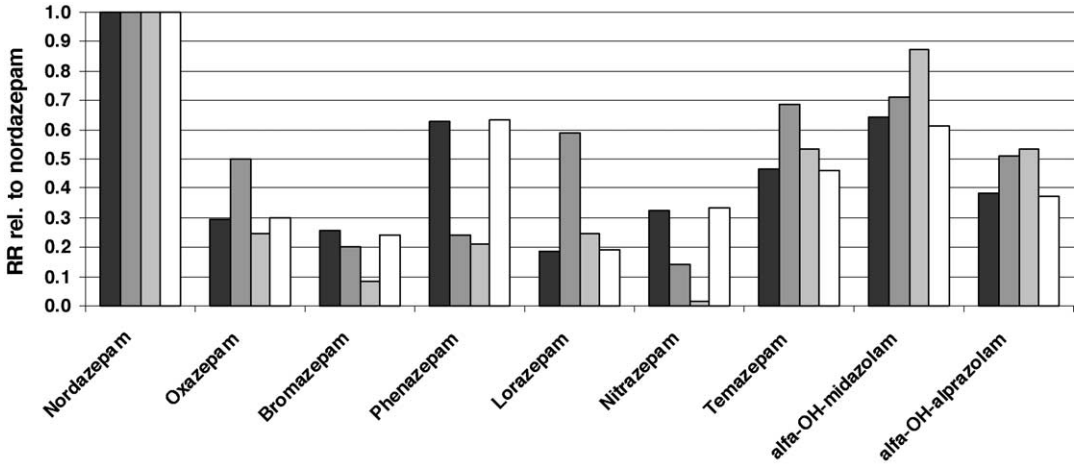

Fig. 4. (A) Average RR factors of each benzodiazepine derivative (0.5 μg ml^{-1} each) vs. ISTD compared to corresponding nordazepam derivative ($n = 5$ each). Black: TBDMS derivatives, ACN–MTBSTFA (80/20, v/v); grey: TMS derivatives, ACN–BSTFA + 1% TMCS (80/20, v/v); light grey: TMS derivatives, ACN–MSTFA (80/20, v/v); white: TBDMS derivatives, 100% MTBSTFA. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

confirms that by using the mixture of ACN–MTBSTFA, the most reproducible derivatization reaction is achieved for all the tested benzodiazepine analytes having a secondary amine group. On the contrary, OH groups of benzodiazepines were derivatized with ease by all the tested reagents and even slightly better repeatability was achieved for OH-metabolites by using ACN–BSTFA + 1% TMCS.

In conclusion, active protons in secondary amine groups of benzodiazepines are not stably derivatized with TMS reagents, although the silylation power of BSTFA + 1% TMCS was higher than the efficiency of MSTFA to derivatize secondary amine groups. This is in line with the generally accepted theory of the ease of derivative formation of different functional groups by silylating reagents: alcohol < phenol < carboxylic acid < amine < amide [34,45]. However, the TBDMS derivatives are formed more easily and have thus higher sensitivity as well as repeatability than the corresponding TMS derivatives.

3.2.3. Stability of the derivatives

The TBDMS derivatives had high overall stability in spite of the functional group(s) of the replaced active proton(s). For example, the percentual stabilities after 100 h varied from 78.8% (bromazepam) to 92.7% (temazepam and alfa-OH-alprazolam), and the reproducibilities from 3.04% (oxazepam) to 9.19% (bromazepam) when using ACN–MTBSTFA. The derivatives can therefore be stored for long periods waiting for the analysis. This is an advantage in routine use, but also suggests that stable, reproducible derivative formation correlates with the final stability of the derivatives. On the contrary, TMS derivatives do not have chemical stability if the active protons are replaced by TMS in secondary amine groups and this further confirms the difficulties to derivatize secondary amines by MSTFA or BSTFA. Furthermore, the stability of the OH group containing benzodiazepines derivatized by BSTFA–1% TMCS were 97.7–102.7% indicating that stable derivatives are formed in a case of the OH group, but not if the compound has a secondary amine group in its molecular structure culminating in low stability of nitrazepam–TMS after MSTFA or BSTFA–1% TMCS derivatization (4.13 and 10.2%, respectively). Fig. 5 gives the sensitivity, repeatability and percentual stability after 100 h.

3.2.4. Other considerations

The choice of a suitable derivatization reagent is only part of the development process to form sensitive, reproducible and stable silylated derivatives. The reaction conditions, such as solvent, heating time and temperature, volume of the reagent, and the place where the reaction takes place must be carefully considered.

Silylation reactions are sensitive to moisture, and anhydrous reaction conditions are thus needed. TMS derivatives are generally more vulnerable to hydrolysis, due to the less crowded alkyl substituent around the silicon atom, whereas TBDMS derivatives have been found to tolerate up to 1% water [46]. From the practical point of view, one should take care that there are no traces of water after the evaporation of the extraction solvent, and that the vials are tightly capped after derivatization prior to GC–MS analysis. In our laboratory, problems with TMS derivatives have been encountered in analysing drugs of abuse, even when the vials are tightly capped, due to the increased air humidity in the summer time. Therefore, if the laboratory is located in a region where high air humidity prevails or there have been problems with the stability of the silylated derivatives, we recommend performing the silylation reaction directly in vials under a nitrogen atmosphere, even though we have not experienced moisture problems with TBDMS formation of benzodiazepines.

The choice of solvent also drastically influences the derivatization reaction conditions. In our experience, acetonitrile has the best overall performance in the analysis of drugs

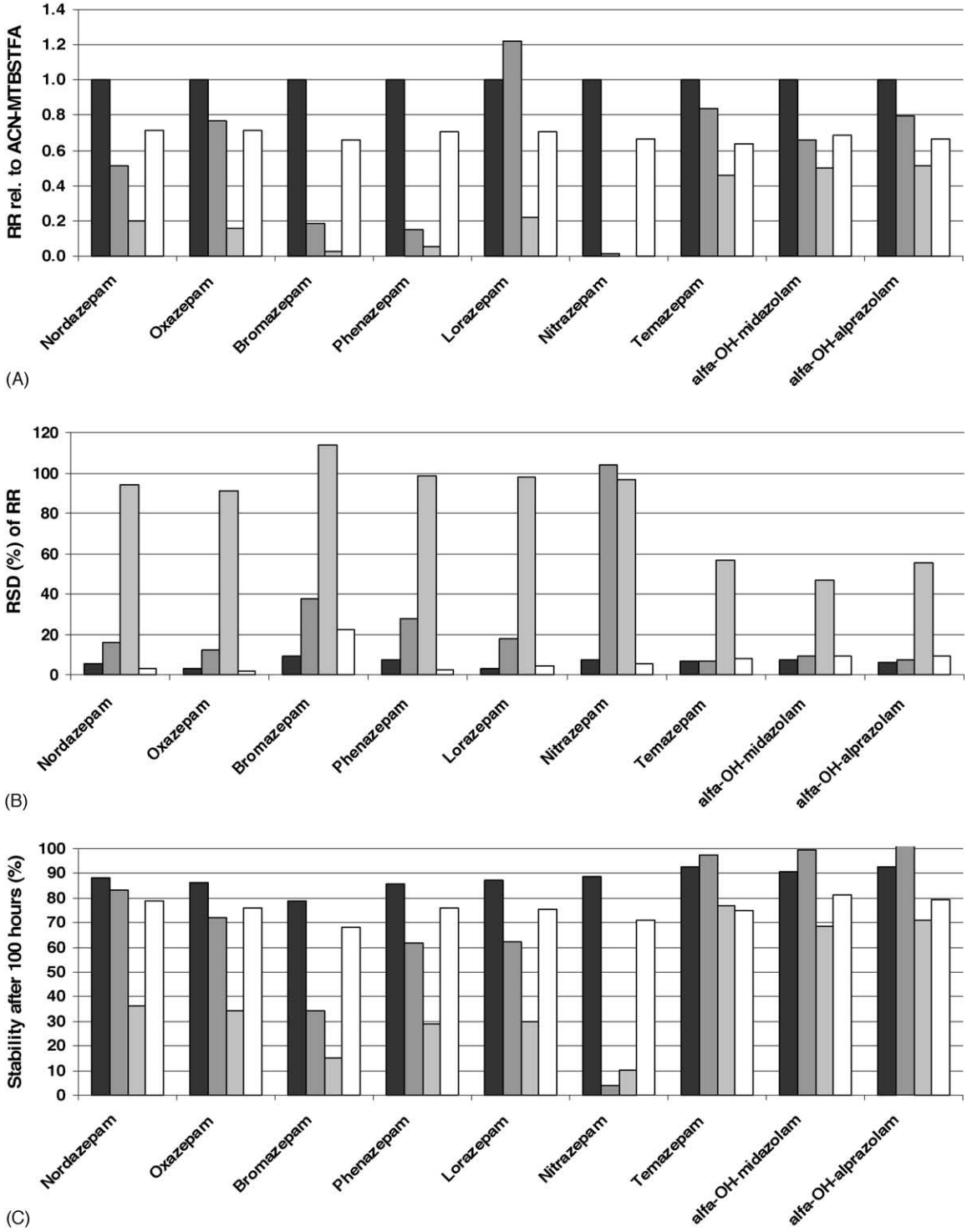

Fig. 5. Various parameters after 100 h of storage: (A) average RR factors of various benzodiazepines (0.5 μg ml^{-1} each) vs. ISTD using the most commonly used silylating reagents relative to TBDMS derivatives ($n = 5$ each) formed by the mixture of ACN–MTBSTFA (80/20, v/v); (B) repeatability of the derivatives using different derivatization mixtures; (C) percentual stability after 100 h of storage compared to initial response. Black: TBDMS derivatives, ACN–MTBSTFA (80/20, v/v); grey: TMS derivatives, ACN–BSTFA + 1% TMCS (80/20, v/v); light grey: TMS derivatives, ACN–MSTFA (80/20, v/v); white: TBDMS derivatives, 100% MTBSTFA. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

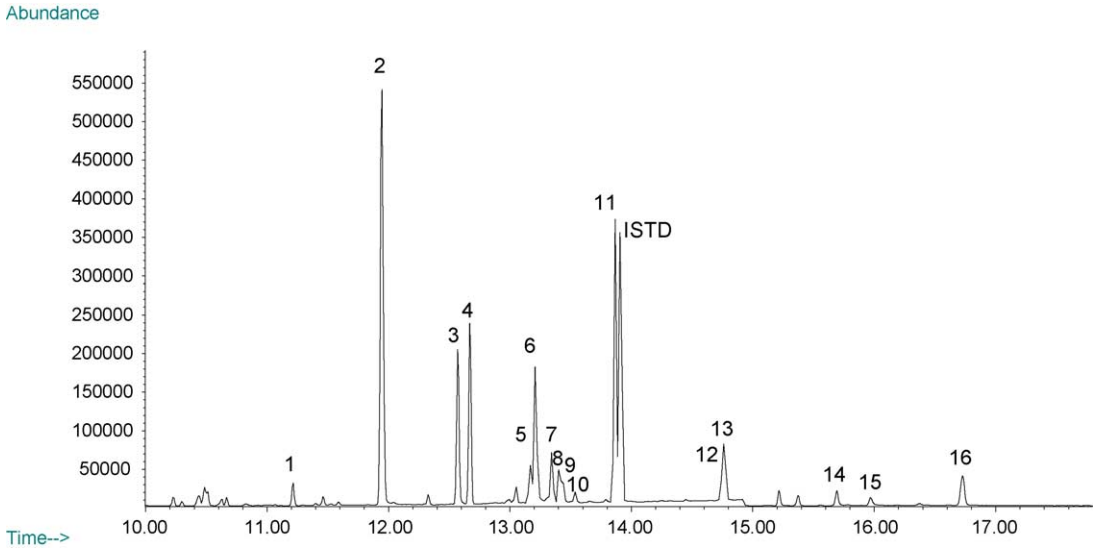

Fig. 6. Selected ion chromatogram of standard solution containing each analyte equal to the medium standard concentration (i.e. five times LOQ). The peak numbering refers to Tables 3 and 4. For instrumental information and chromatographic conditions, see Section 2.2.

of abuse, but comparable results have been observed with ethyl acetate to derivatize endocrine disruptors [36]. In addition, in our experiment the use of acetonitrile in the reaction mixture with MTBSTFA (80/20, v/v) improved the derivative formation, resulting in approximately 25% higher sensitivity as well as more reproducible and stable derivatives than with pure MTBSTFA. Silylating reagents are also relatively expensive, carcinogenic chemicals and unnecessary injecting of them into a GC–MS system should be avoided. The use of suitable solvent, such as acetonitrile, and possibly ethyl or butyl acetate is therefore recommended.

MTBSTFA (20 μl) were added to acetonitrile to completely derivatize, not only the analytes of interest, but also the matrix components. The excess use of derivatization reagent is of utmost importance, but more than this would be a waste of the reagent and is therefore not recommended. Benzodiazepines have maximally only of one or two functional groups capable of being derivatized. In the absence of polyfunctionality and steric hindrance, the reactions generally take place under mild conditions by MTBSTFA in secondary amine and hydroxyl groups as in the case of the studied benzodiazepines. Only the secondary amine group of chlordiazepoxide is not derivatized by either the TBMDS or TMS silylating reagents, but the chromatography and identification of the underivatized form is not problematic.

3.3. GC–MS analysis

In GC analyses, an adequate inner diameter of the analytical column was a necessity to achieve optimum

Table 3
Linearity, limit of detection (LOD), limit of quantitation (LOQ) and extraction efficiency

No.	Compound	Tested range (ng ml^{-1})	Linearity range (ng ml^{-1})	(r^2)[a]	LOD (ng ml^{-1})	LOQ (ng ml^{-1})	Extraction efficiency (%)
1	Medazepam	5–1200	5–400	0.996	0.4	20	96.0
2	Nordazepam-TBDMS	25–6000	50–2000	0.997	<2	100	95.4
3	Diazepam	25–6000	25–2000	0.995	2	100	96.3
4	Oxazepam-2TBDMS	25–6000	50–2000	0.997	<2	100	93.3
5	Bromazepam-TBDMS	5–1200	10–400	0.996	10	20	93.7
6	Chlordiazepoxide	50–12000	50–2000	0.996	4	200	107.0
7	Phenazepam-TBDMS	5–1200	5–800	1.000	2	20	101.9
8	Midazolam	5–1200	5–1200	0.999	2	20	103.6
9	Lorazepam-2TBDMS	2.5–600	2.5–400	1.000	2	10	100.1
10	Nitrazepam-TBDMS	5–1200	10–200	0.982	5	20	91.9
11	Temazepam-TBDMS	25–6000	25–4000	0.995	2	100	109.9
12	Alfa-OH-midazolam-TBDMS	5–1200	5–100	0.999	0.4	5	88.2
13	Zolpidem	7.5–1800	7.5–1800	0.995	1.5	30	100.1
14	Alprazolam	7.5–1800	15–1800	0.998	5	30	90.4
15	Zaleplon	5–1200	10–1200	0.990	10	20	88.1
16	Alfa-OH-alprazolam-TBDMS	5–1200	5–1200	0.998	2	20	97.0

[a] r^2, Square of correlation coefficient with a weighting factor of 1/concentration.

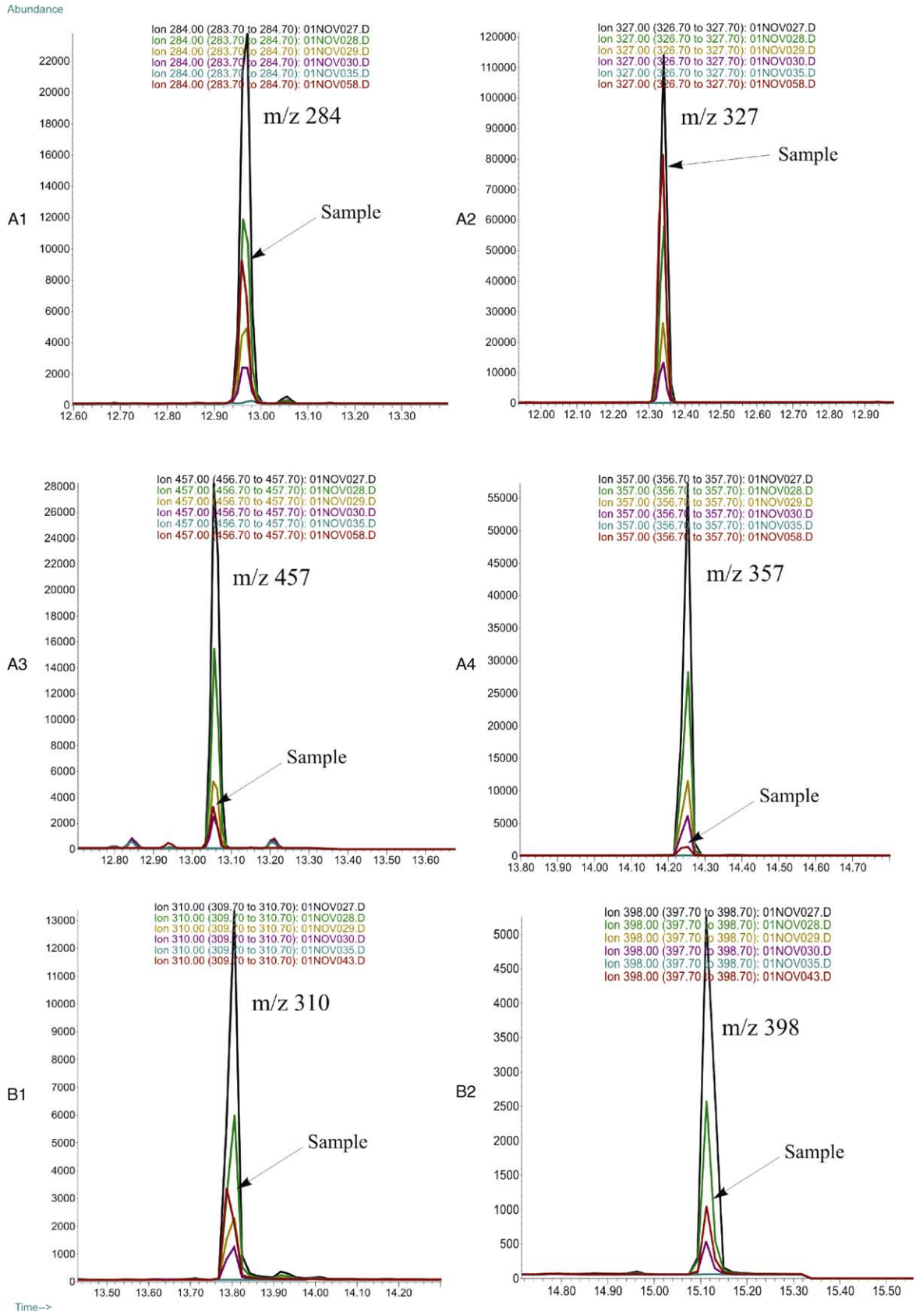
Abundance
A1
Ion 284.00 (283.70 to 284.70): 01NOV027.D
Ion 284.00 (283.70 to 284.70): 01NOV028.D
Ion 284.00 (283.70 to 284.70): 01NOV029.D
Ion 284.00 (283.70 to 284.70): 01NOV030.D
Ion 284.00 (283.70 to 284.70): 01NOV035.D
Ion 284.00 (283.70 to 284.70): 01NOV058.D
m/z 284
Sample
A2
Ion 327.00 (326.70 to 327.70): 01NOV027.D
Ion 327.00 (326.70 to 327.70): 01NOV028.D
Ion 327.00 (326.70 to 327.70): 01NOV029.D
Ion 327.00 (326.70 to 327.70): 01NOV030.D
Ion 327.00 (326.70 to 327.70): 01NOV035.D
Ion 327.00 (326.70 to 327.70): 01NOV058.D
m/z 327
Sample
A3
Ion 457.00 (456.70 to 457.70): 01NOV027.D
Ion 457.00 (456.70 to 457.70): 01NOV028.D
Ion 457.00 (456.70 to 457.70): 01NOV029.D
Ion 457.00 (456.70 to 457.70): 01NOV030.D
Ion 457.00 (456.70 to 457.70): 01NOV035.D
Ion 457.00 (456.70 to 457.70): 01NOV058.D
m/z 457
Sample
A4
Ion 357.00 (356.70 to 357.70): 01NOV027.D
Ion 357.00 (356.70 to 357.70): 01NOV028.D
Ion 357.00 (356.70 to 357.70): 01NOV029.D
Ion 357.00 (356.70 to 357.70): 01NOV030.D
Ion 357.00 (356.70 to 357.70): 01NOV035.D
Ion 357.00 (356.70 to 357.70): 01NOV058.D
m/z 357
Sample
B1
Ion 310.00 (309.70 to 310.70): 01NOV027.D
Ion 310.00 (309.70 to 310.70): 01NOV028.D
Ion 310.00 (309.70 to 310.70): 01NOV029.D
Ion 310.00 (309.70 to 310.70): 01NOV030.D
Ion 310.00 (309.70 to 310.70): 01NOV035.D
Ion 310.00 (309.70 to 310.70): 01NOV043.D
m/z 310
Sample
B2
Ion 398.00 (397.70 to 398.70): 01NOV027.D
Ion 398.00 (397.70 to 398.70): 01NOV028.D
Ion 398.00 (397.70 to 398.70): 01NOV029.D
Ion 398.00 (397.70 to 398.70): 01NOV030.D
Ion 398.00 (397.70 to 398.70): 01NOV035.D
Ion 398.00 (397.70 to 398.70): 01NOV043.D
m/z 398
Sample
Time-->

Table 4
Accuracy as well as intra- and interday precision

No.	Compound	Nominal concentration (ng ml^{-1})	Accuracy (%)	Intraday precision (%)	Interday precision (%)
1	Medazepam	20/100/200	6.6/−6.5/6.4	2.4/6.6/5.2	12.7/8.4/9.9
2	Nordazepam–TBDMS	100/500/1000	16.4/−2.5/-4.1	4.6/3.3/4.9	6.4/7.4/5.8
3	Diazepam	100/500/1000	−3.3/−2.1/−2.4	4.2/3.7/5.2	7.0/6.1/5.3
4	Oxazepam–2TBDMS	100/500/1000	17.7/−6.6/−0.2	4.4/2.8/5.6	9.4/8.3/4.6
5	Bromazepam–TBDMS	20/100/200	0.3/−2.1/−8.9	7.9/4.1/7.6	6.9/10.2/9.9
6	Chlordiazepoxide	200/1000/2000	15.4/−3.2/−8.2	8.1/5.3/4.9	9.8/13.8/12.1
7	Phenazepam–TBDMS	20/100/200	6.7/−3.3/−1.0	5.9/1.9/3.5	7.6/8.6/7.4
8	Midazolam	20/100/200	8.4/4.8/3.8	4.3/5.2/5.0	4.8/7.5/12.7
9	Lorazepam–2TBDMS	10/50/100	3.6/1.8/−9.1	6.8/4.6/6.1	6.5/8.5/10.5
10	Nitrazepam–TBDMS	20/100/200	17.8/−16.3/11.8	4.5/9.5/3.7	3.8/13.8/15.9
11	Temazepam–TBDMS	100/500/1000	3.7/6.0/1.1	6.5/5.3/4.5	4.5/11.6/10.5
12	Alfa-OH-midazolam–TBDMS	5/25/50	8.4/−2.3/−5.7	4.2/9.3/4.9	9.5/4.9/8.9
13	Zolpidem	30/150/300	1.5/14.6/5.0	10.7/3.0/7.2	13.6/8.2/9.0
14	Alprazolam	30/150/300	0.1/−5.1/−3.3	4.8/3.4/6.3	8.2/9.4/11.3
15	Zaleplon	20/100/200	4.0/−11.7/−6.5	3.0/8.0/8.2	12.5/10.1/12.9
16	Alfa-OH-alprazolam–TBDMS	20/100/200	−0.1/0.2/−8.6	3.4/4.7/4.5	8.0/6.8/9.2

chromatographic conditions for benzodiazepines (data not shown). 0.32 mm i.d. columns offered the best tradeoff for intensive, symmetric and sharp peak shapes, while still maintaining the acceptable GC–MS conditions. The cross-linked mid-polar DB-35ms (35% phenyl–65% methyl polysiloxane) analytical column offered high chemical and thermal stability, which permitted raising the GC temperature high enough without significant column bleed. The loss of analyte response was noted when the columns of 0.25 mm i.d. or less were used for the separation, even when the benzodiazepines were properly derivatized. On the other hand, benzodiazepines having polar functional groups can be analyzed even without derivatization if the columns of 0.53 mm i.d. are used, as we have shown elsewhere with electron capture detection (ECD) [10]. However, columns with an i.d. as high as 0.53 mm are problematic with MS detection to attain a sufficient carrier gas inlet pressure while maintaining appropriate chromatographic conditions with sufficiently short column length (<30 m).

Three characteristic ions, one target ion and two qualifiers, were selected from full-scan MS spectra for mass spectrometric detection using EI ionization. The sensitivity was enhanced by performing the detection in the selected ion-monitoring (SIM) mode. The choice of ions was based on signal-to-noise ratio (S/N) and carefully checking for possible background interference. All ions with possible interference were omitted. The sensitivity of the method was further improved by modification of the instrumental parameters. First, the compounds were divided to three different SIM time windows. The number of windows was kept relatively small for ease of operation in day-to-day analyses. Second, a pulsed-flow injection model was used during the injection to ensure that a maximum amount of the analyte was introduced to the analytical column. The carrier gas (helium) flow was temporarily raised up to 3 ml min^{-1} up to 1 min, and lowered for the rest of the analysis to the typical 1.5 ml min^{-1}. Approximately twofold sensitivity increments were proportionately obtained. And finally, a target-tuning macro was used instead of autotuning to enhance the sensitivities of high *m*/*z*. The standard tuning of mass spectrometry proposed by the manufacturer generally emphasizes the intensities of low *m*/*z* at the expense of high *m*/*z*, which are more characteristic of the studied analytes. Fig. 6 shows the GC–MS selected ion chromatogram of the spiked standard solution, and Table 1 gives the SIM parameters.

3.4. Validation

All validation data are summarised in Tables 3 and 4. No interfering peaks originating from the biological background matrix, the used chemical reagents, column bleed or other commonly abused drugs in authentic routine samples, were observed with the selected SIM ions and retention times of the analytes in the selectivity experiments. In Fig. 7, two routinely performed, authentic whole blood samples obtained from different sources are illustrated with

Fig. 7. Selected (target) ion chromatograms of two authentic whole blood samples (A and B) illustrated with the spiked calibration standards and blank whole blood sample. Sample 1: (Al) diazepam (0.354 μg ml^{-1}); (A2) nordazepam (0.728 μg ml^{-1}); (A3) oxazepam (0.106 μg ml^{-1})[a] and (A4) temazepam (below LOQ)[a]. Sample 2: (Bl) midazolam (0.056 μg ml^{-1}) and (B2) alfa-OH-midazolam (0.010 μg ml^{-1}). [a]Temazepam and oxazepam are largely abused by themselves, but are also the metabolites of various benzodiazepines. For example, temazepam and oxazepam are both metabolites of diazepam, as well as oxazepam is a metabolite of chlordiazepoxide and temazepam. Thus, if the metabolic profiling is of prime importance, lower LOQ values should be used for temazepam and oxazepam.

a blank whole blood extract. Both persons were suspected by the police of driving a car while under the influence of drugs.

The weighted linear calibration model ($1/c$) offered a wide linear response across the typical concentrations of the compounds of interest (Table 3). The sensitivity of the method was improved by optimizing the various instrumental parameters (see Section 3.3) and derivatization chemistry, as well as obtaining reproducible recoveries of high magnitude (Table 3). Therefore, even sub-therapeutic concentrations of various analytes can be detected if necessary. The LOD values are given in Table 3.

Accuracy as well as intra- and interday precision values were within required limits for all tested compounds at each tested concentration level (Table 4), except that the accuracy in medium concentration level for nitrazepam was slightly above the criterion (−16.3%). To ensure also the quantitation of the low concentrations, the lowest validated concentration for each compound meeting the quantitation criteria was chosen as LOQ, although several compounds had high S/N ratios below LOQ (Table 3).

Nitrazepam displayed a slightly different chromatographic properties than the other determined analytes, possibly due to the nitro-group containing molecular structure. Therefore the use of its deuterated analogue should be considered in order to achieve fully quantitative identification. Moreover, in countries where flurazepam is abused the deuterated benzodiazepine analogues would be a safer choice as ISTD, although flurazepam is usually present in human whole blood in minute quantities, even if used [47,48]. The concentrations equal to a few ng ml^{-1} can be considered negligible, if one takes into account the concentration of ISTD per sample, i.e. 2000 ng ml^{-1}.

4. Conclusions

This is the first presented scientific paper illustrating the major differences as regards the suitability of the most commonly used silylating reagents in the analysis of benzodiazepines. MTBSTFA forms sensitive, reproducible and stable derivatives, regardless of whether the benzodiazepine molecule has active hydrogen atoms in the secondary amine group and/or the hydroxyl group. On the contrary, reagents forming trimethyl silyl derivatives seem to react stably only with hydroxyl groups; this is a major shortcoming, as a majority of active hydrogen(s) containing benzodiazepine molecules do have secondary amine groups. On the basis of the derivatization experiment, an analytical GC–EI–MS procedure was developed for simultaneous determination of various benzodiazepines, hydroxy metabolites and non-benzodiazepine hypnotics in whole blood. Moreover, this procedure could be easily expanded to the other benzodiazepine molecules as well. It is a rapid, sensitive, reliable and non-laborious procedure primarily developed for routine clinical and forensic toxicological applications, but could also be useful in other fields concerning analytical solutions of the determined analytes.

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