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Determination of 1,4-benzodiazepines by high-performance liquid chromatography-electrospray tandem mass spectrometry

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Abstract

A sensitive and selective method to determine diazepam, N-desmethyldiazepam, nitrazepam, flunitrazepam and medazepam in human serum and urine was established employing solid-phase extraction and high-performance liquid chromatography–electrospray tandem mass spectrometry (HPLC–ESI-MS–MS) with the selected reaction monitoring (SRM) mode. Using diazepam-d_5 and N-desmethyldiazepam-d_5 as internal standards, recoveries between 90.4 and 109.7% were determined. The routine quantification limit was set at 2 ng/ml for serum and urine at a signal-to-noise ratio of 10:1.

Keywords: 1,4-Benzodiazepines; Diazepam; N-Desmethyldiazepam; Nitrazepam; Flunitrazepam; Medazepam

1. Introduction

Besides chromatographic methods like thin-layer chromatography (TLC) [1], high-performance liquid chromatography (HPLC) [2,3], micellar electrokinetic chromatography [4] and gas chromatography (HRGC) [5–7], immunoassays [8], radioreceptor assays [9] as well as mass spectrometry (MS) [10,11] are used in the analysis of benzodiazepines. For most selective and sensitive analysis, HRGC–MS with electron impact (EI) or chemical ionization (CI) in the positive and negative modes plays an outstanding role [12–17]. However, this method suffers from the thermal instability and low volatility of benzodiazepines, requiring derivatization, e.g. by trimethylsilylation, prior to HRGC–MS analysis.

This drawback can be overcome by LC–MS coupling, which ideally combines the advantages of a gentle separation method with the sensitivity and selectivity of mass spectrometry, especially by application of the recently developed soft ionization interfaces like electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI) [18,19]. Whereas the LC–APCI-MS analysis of triazolam and its metabolites has been reported recently [20], the application of HPLC–ESI-MS–MS for qualitative and quantitative analysis of benzodiazepines has not been described to date.

This paper reports an ultrasensitive method for the quantification of five 1,4-benzodiazepines (**1–5**) from human serum and urine, using HPLC–ESI-MS–MS after sample preparation by solid-phase extraction.

2. Experimental

2.1. Chemicals

Diazepam (**1**), N-desmethyldiazepam (**2**),

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medazepam (**3**), nitrazepam (**4**), flunitrazepam (**5**), diazepam-d_5, N-desmethyldiazepam-d_5 and monobasic potassium phosphate (purity 99%) were from Sigma–Aldrich (Deisenhofen, Germany). Water, methanol, acetonitrile and dichloromethane, all of HPLC-gradient grade, were from Merck (Darmstadt, Germany). HPLC-grade acetic acid was purchased from Baker (Deventer, Netherlands). Hexane and ammonium hydroxide were provided from a local supplier in reagent-grade purity. Hexane was distilled before use. The C_{18} Bond Elut (3 ml, 200 mg) and the narc-2 Bakerbond (6 ml) solid-phase extraction columns were from Varian (Harbor City, CA, USA) and Baker, respectively.

2.2. Analytical procedure

Analysis was performed on a triple stage quadrupole TSQ 7000 LC–MS–MS system with ESI interface (Finnigan MAT). Data acquisition, mass spectrometric determination and calculation (integration of the peak areas) were carried out on a Personal DECstation 5000/33 (Digital Equipment, Unterföhring, Germany) and ICIS 8.1 software (Finnigan MAT). For HPLC, a Knauer HPLC pump 64 equipped with micro pump heads and a LiChrospher 60-RP select B column (100×2.0 mm I.D., 5 μm; Knauer, Berlin, Germany) were used. The loop injection volume was 5 μl and the solvent flow of methanol–water–acetonitrile (1:1:1, v/v) (pH 6) was set to 100 μl/min. The addition of traces of acid, e.g. 0.05% trifluoroacetic acid, did not enhance ion yield (data not shown).

The mass spectrometer was operated in the selected reaction monitoring (SRM) mode with argon at a pressure of 0.27 Pa as collision gas. Positive ions were detected with a total scan time of 1.0 s for all SRM experiments and a dwell time of 2 ms. The temperature of the heating capillary, serving simultaneously as repeller electrode (20 V), was 250°C; and the electrospray capillary voltage and the electron multiplier voltage were set to 6 kV and 2.3 kV, respectively. Nitrogen served as sheath (344.7 kPa) and auxiliary gas (5 l/min). For disintegration of solvent clusters by APCI, the octapole offset was set to 5 eV. Selected ion pairs for the SRM experiment were (offset voltages in brackets) *m/z* 285/257 (−30 eV), *m/z* 271/140 (−30 eV), *m/z* 271/242 (−30 eV), *m/z* 282/236 (−25 eV), *m/z* 314/268 (−25 eV), *m/z* 290/262 (−30 eV) and *m/z* 276/213 (−30 eV) for **1–5** as well as diazepam-d_5 and N-desmethyldiazepam-d_5, respectively. These ion pairs represent the protonated molecule ion $[M+H]^+$ and the most abundant product ion for each of the 1,4-benzodiazepines under study.

2.3. Sample preparation

A 1-ml volume of human serum (urine) was spiked with concentrations between 50 and 650 ng/ml of **1–5** and 50 ng/ml of the internal standards (diazepam-d_5, N-desmethyldiazepam-d_5).

2.4. Extraction procedure

A Supelco VISIPREP system using vacuum at 2 kPa, provided by a water-jet pump, was employed.

2.4.1. C_{18} solid-phase extraction columns

After conditioning the column with 2 ml of methanol followed by 2 ml of water, the spiked serum (urine) samples were applied to the column, which was washed subsequently with 2 ml of distilled water. The elution was performed with 500 μl of methanol. The eluate was evaporated under a gentle stream of nitrogen at 60°C to dryness and the residue was redissolved in 100 μl of methanol–water–acetonitrile (1:1:1, v/v), from which 5 μl was injected into the HPLC–MS–MS system.

2.4.2. Narc-2 solid-phase extraction columns

To a 1-ml volume of spiked serum (urine), 400 μl of a 0.1 *M* KH_2PO_4 buffer (pH 6.0) was added. After rigorous mixing the pH was checked and, if required, adjusted to 4–6. After conditioning the column with 2 ml of methanol followed by 2 ml of 0.1 *M* KH_2PO_4 buffer (pH 6.0), the sample was applied to the column (1 ml/min). Subsequently, the column was washed with 1 ml of 0.1 *M* KH_2PO_4 buffer (pH 6.0)–methanol (8:2, v/v), followed by 1 ml of 1 *M* acetic acid. After drying under vacuum for 5 min, the column was rinsed with 1 ml of hexane and dried again for 1 min. The elution was carried out by using twice 2 ml of dichloromethane (containing 4% ammonium hydroxide, pH 11). The eluate was evaporated under a gentle stream of

nitrogen at 50°C and, after redissolving in 100 μl of methanol–water–acetonitrile (1:1:1, v/v), 5 μl was injected into the HPLC–MS–MS system.

2.5. Quantitative analysis

Quantification was based on the ratios of the peak areas of the 1,4-benzodiazepines (**1–5**) to that of the isotopically labelled species. Accuracy and reproducibility of the method were demonstrated by the results of duplicate analyses of serum (urine) spiked with **1–5** at concentrations between 50 and 650 ng/ml.

2.5.1. Calibration graphs

The dynamic ranges for the calibration graphs were 1–1000 ng/ml. The solvent was methanol–water–acetonitrile (1:1:1, v/v). The final concentrations of **1–5** for each point on the calibration graph were 1, 5, 20, 50, 100, 200, and 1000 ng/ml. The concentrations of the internal standards diazepam-d_5 and N-desmethyldiazepam-d_5 were 50 ng/ml in all samples.

2.5.2. Calculations

The peak areas of **1–5** were measured and their ratios to the peak areas of diazepam-d_5 and N-desmethyldiazepam-d_5, respectively, were calculated. The calibration graphs were generated by non-weighted linear regression. The concentration of **1–5** in the spiked samples was calculated by using the equation

$$Y = A + BX$$

where Y=the concentration of the 1,4-benzodiazepine in ng/ml and X=the peak area ratio of this 1,4-benzodiazepine to that of the corresponding internal standard (cf. Table 1). A and B are constants generated by the linear regression.

Table 1
Control sample results for different 1,4-benzodiazepines in human serum and urine (n=2)

Benzodiazepine	Theoretical concentration (ng/ml)	Concentration (mean±S.D.) (ng/ml)	Recovery (%)
(a) Matrix: serum; bonded extraction phase: C_{18}			
Flunitrazepam[a] **5**	50.0	51.2±1.1	102.4
Nitrazepam[b] **4**	50.0	45.5±0.3	91.0
Diazepam[b] **1**	100.0	109.7±5.6	109.7
N-Desmethyldiazepam[a] **2**	100.0	104.9±0.7	104.9
Medazepam[a] **3**	100.0	98.1±0.5	98.1
(b) Matrix: serum; bonded extraction phase: narc 2			
Flunitrazepam[a] **5**	650	663.0±2.2	102.0
Nitrazepam[b] **4**	140	143.3±15.1	102.4
Diazepam[b] **1**	140	137.4±5.1	98.1
N-Desmethyldiazepam[a] **2**	120	121.6±4.2	101.3
(c) Matrix: urine; bonded extraction phase: C_{18}			
Flunitrazepam[a] **5**	60	58.0±1.6	96.7
Nitrazepam[b] **4**	50	45.2±1.3	90.4
Diazepam[b] **1**	100	103.9±1.7	103.9
N-Desmethyldiazepam[a] **2**	120	115.2±1.1	96.0
Medazepam[a] **3**	100	93.7±6.4	93.7
(d) Matrix: urine; bonded extraction phase: narc 2			
Flunitrazepam[a] **5**	150	146.3±0.5	97.5
Nitrazepam[b] **4**	80	76.7±4.5	95.9
Diazepam[b] **1**	100	100.0±6.3	100.0
N-Desmethyldiazepam[a] **2**	100	97.8±1.6	97.8
Medazepam[a] **3**	80	77.1±1.0	96.4

[a] N-Desmethyldiazepam-d_5 used as internal standard for quantification.
[b] Diazepam-d_5 used as internal standard for quantification.

3. Results and discussion

Initial experiments revealed that the electrospray process was able to transform benzodiazepine molecules from solution into ions in the gas phase effectively. As a result of this ion evaporation process, an abundant protonated molecule ion $[M+H]^+$ was formed, sometimes accompanied by smaller solvent cluster peaks. The tandem MS technique enabled us to apply the multiple SRM mode, resulting in highest sensitivity and selectivity as well as significant reduction of background noise.

The general procedure for a SRM experiment is the following: quadrupole 1 is set to a defined m/z ratio; in quadrupole 2 ('collision cell'), this ion is fragmented by collision with argon gas molecules ('collision-induced dissociation' [CID]). Finally, only one of the product ions can pass through quadrupole 3, because it is also set to a defined m/z ratio. Besides the retention time of HPLC, the analyte is determined by its protonated molecule ion (selected in quadrupole 1) and its specific product ion (selected in quadrupole 3). This procedure guarantees the high selectivity of the SRM experiment; coeluting and therefore interfering matrix components are excluded from detection.

Fig. 1 shows a SRM chromatogram of blank human serum. Except for the signals of the deuterated 1,4-benzodiazepines, which were added to the blank sample, no endogenous interfering substances were observed within the retention times required for the 1,4-benzodiazepines (**1–5**) as well as the sensitivity of the entire analytical instrumental method (cf. Fig. 2).

As a representative example, Fig. 2 shows the result of a SRM experiment of a standard sample in methanol–water–acetonitrile (1:1:1, v/v). The concentrations of **1–5** and that of their deuterated internal standards amounted to 200 ng/ml and 50 ng/ml, respectively. Ions with m/z 140, 213, 236, 242, 262, and 268, each representing characteristic product ions, were monitored simultaneously. As expected, the retention times of the standards and the corresponding 1,4-benzodiazepines were identical. No H/D exchange with methanol, water or acetonitrile was observed.

As an additional representative example, in Fig. 3 the product ion spectrum of N-desmethyldiazepam-d_5 is given. The most abundant prod-

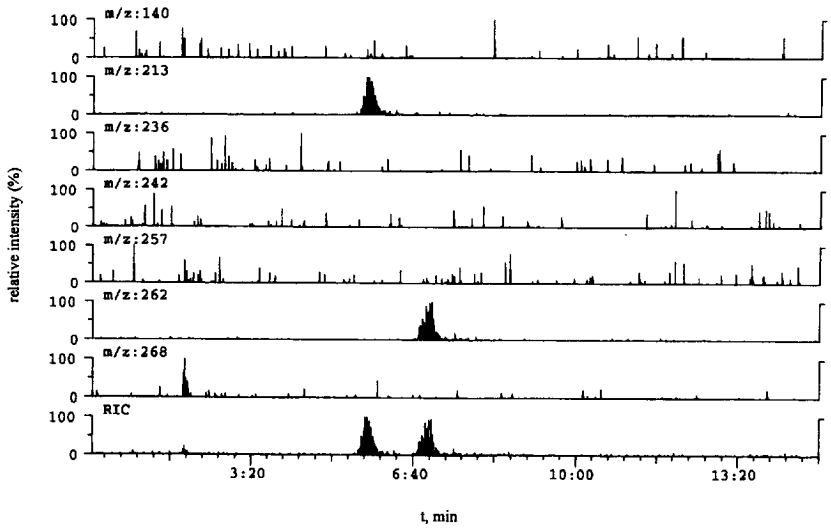

Fig. 1. SRM mass chromatogram of a blank serum sample. Monitored m/z ratios: 213, N-desmethyldiazepam-d_5; 262, diazepam-d_5; 140, 236, 242, 257, 268 (see Fig. 2). RIC: reconstructed ion chromatogram.

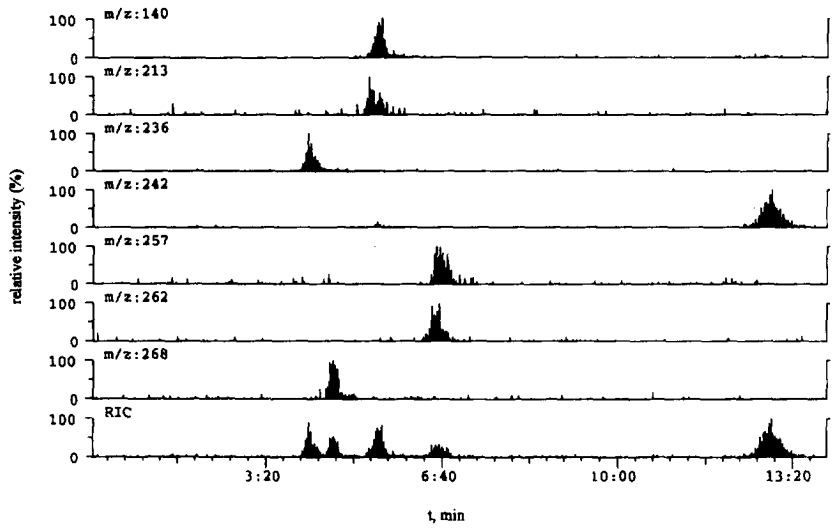

Fig. 2. SRM chromatogram of different 1,4-benzodiazepines. Monitored m/z-ratios: 140, N-desmethyldiazepam, 213, N-desmethyldiazepam-d_5; 236, nitrazepam; 242, medazepam; 257, diazepam; 262, diazepam-d_5; 268, flunitrazepam. RIC: reconstructed ion chromatogram.

uct ion peak at m/z 213, originating from the protonated molecule ion (m/z 276; m/z 278 is the ^{37}Cl isotope peak) by a loss of CO (−28 amu) and Cl (−35 amu), was selected for SRM detection. The same CID fragmentation pattern has been described previously for **2** in a quadrupole ion trap mass spectrometer [10].

A typical linear calibration graph for **2** is given

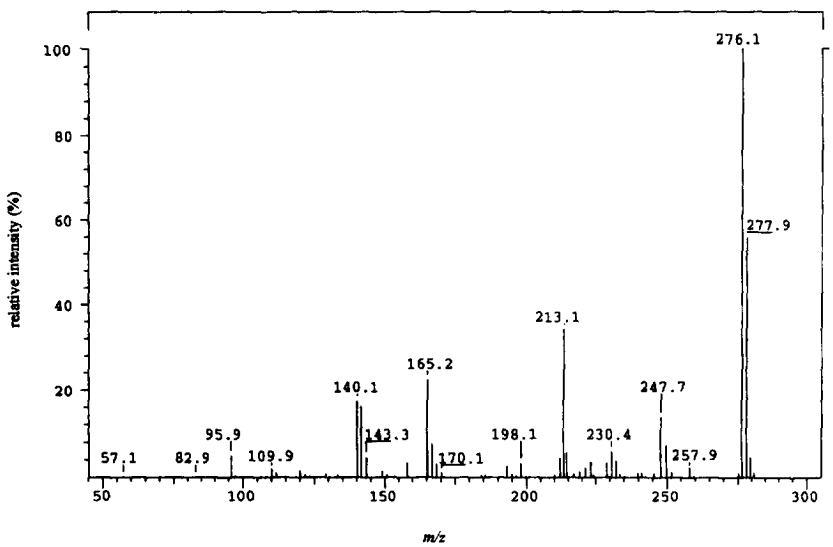

Fig. 3. Product ion mass spectrum of N-desmethyldiazepam-d_5 (argon pressure 0.27 Pa; offset voltage −27 eV).

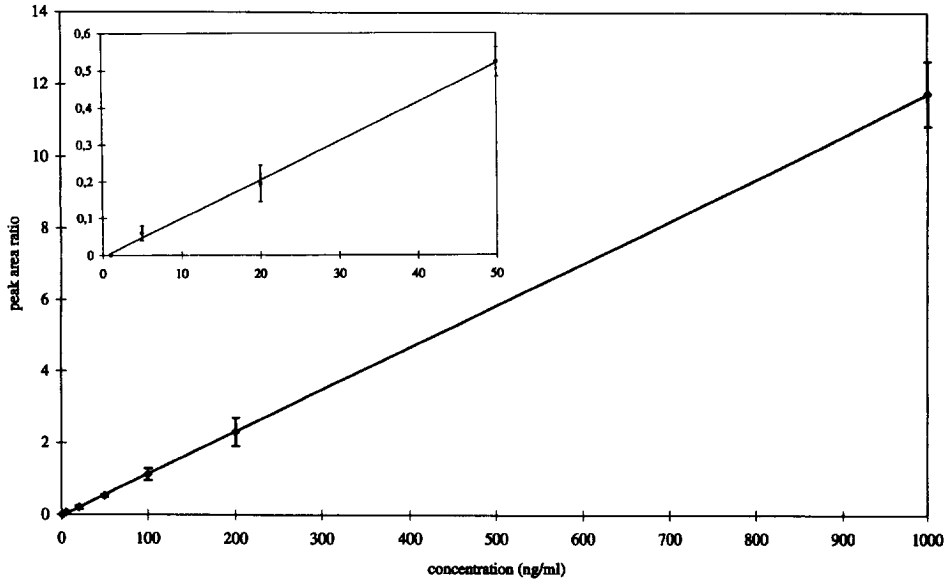

Fig. 4. Linear calibration graph for N-desmethyldiazepam (**2**) (r^2=0.9999).

in Fig. 4. The peak-area ratio of **2** and its deuterated internal standard is correlated to the concentration of **2**.

Fig. 5 shows the result of a typical SRM chromatogramm of human serum spiked with **1–5** and the internal standards diazepam-d_5 and N-desmethyldiazepam-d_5. The concentrations of **4** and **5** were found to be 45.5 ng/ml and 51.2 ng/ml, respectively. The concentrations of **1**, **2** and **3** were determined to be 109.7 ng/ml, 104.9 ng/ml and 98.1 ng/ml, respectively (cf. Table 1a).

Table 1 summarizes the determined concen-

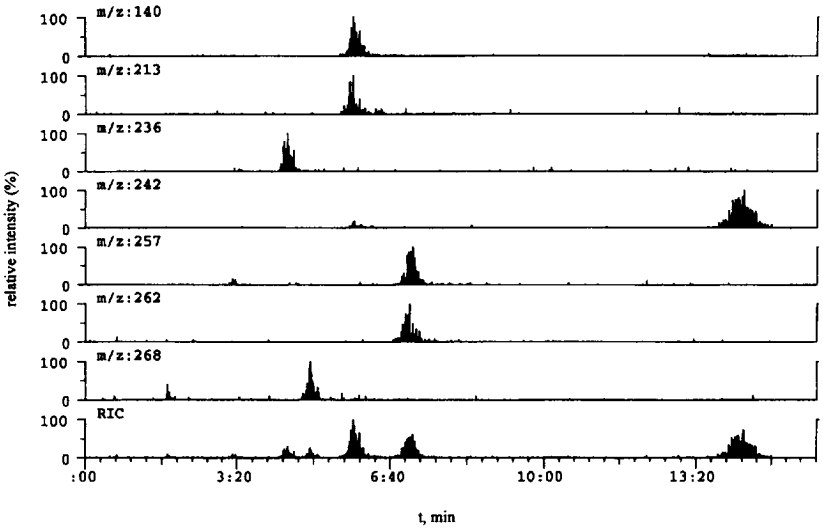

Fig. 5. SRM mass chromatogram of spiked human serum (sample preparation was performed with C_{18} solid-phase extraction columns). Monitored m/z-ratios: 140, N-desmethyldiazepam, 213, N-desmethyldiazepam-d_5; 236, nitrazepam; 242, medazepam; 257, diazepam; 262, diazepam-d_5; 268, flunitrazepam. RIC: reconstructed ion chromatogram.

trations, the standard deviations and the recovery rates for **1–5** in human serum (urine) after solid-phase extraction. The regression correlation coefficients were better than 0.999 for all the calibration graphs. The errors for all human serum and urine samples (n=30) were within 10% and the average values for all samples were in the range 90–110% of the theoretical value.

4. Conclusions

The SRM mode in HPLC–ESI-MS–MS analysis, which also provides structural information about the analytes, allows one to analyze large numbers of samples with high sensitivity and selectivity, especially when solid-phase extraction is used for rapid sample preparation. In contrast to HRGC analysis, the 1,4-benzodiazepines can be separated by HPLC without any further derivatization. Deuterated internal standards assure the accuracy of the quantitative analysis, because the isotopically labelled compounds are identical or closely related in structure and chemical properties with the analytes.

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