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Short communication

Determination of lorazepam in plasma and urine as trimethylsilyl derivative using gas chromatography–tandem mass spectrometry

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Abstract

A procedure based on gas chromatography–tandem mass spectrometry for identification and quantitation of lorazepam in plasma and urine is presented. The analyte was extracted from biological fluids under alkaline conditions using solid-phase extraction with an Extrelut-1 column in the presence of oxazepam-d_5 as the internal standard. Both compounds were then converted to their trimethylsilyl derivatives and the reaction products were identified and quantitated by gas chromatography–tandem mass spectrometry using the product ions of the two compounds (*m/z* 341, 306 and 267 for lorazepam derivative and *m/z* 346, 309 and 271 for oxazepam-d_5 derivative) formed from the parent ions by collision-induced dissociation in the ion trap spectrometer. Limit of quantitation was 0.1 ng/ml. This method was validated for urine and plasma samples of individuals in treatment with the drug.

Keywords: Derivatization, GC; Lorazepam

1. Introduction

Lorazepam [7-chloro-5-(2-chlorophenyl)-1,3-dihydro-3-hydroxy-2H-1,4-benzodiazepin-2-one] (Fig. 1) is one of the 1,4-benzodiazepine derivatives widely used and misused as anxiolytic and sedative agent [1,2]. Hence, the identification and quantitation of this drug in biological fluids can be of use in clinical toxicology and forensic medicine. Lorazepam is extensively metabolized to its inactive glucuronide conjugate, therefore only negligible amounts of free lorazepam are present in blood and excreted in urine [3]. For this reason, a specific and sensitive analytical method is required especially in case of blood examinations, because of the low drug concentrations present in this matrix, unless an hydrolysis step is included.

Gas chromatography–mass spectrometry (GC–

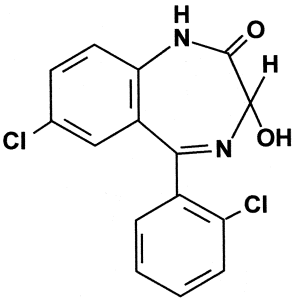

Fig. 1. Structure of lorazepam.

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MS) is the method of choice for drug testing in legal medicine, due to its high sensitivity and selectivity, and has been reported for several benzodiazepines [4–6] and specifically for lorazepam [7–13] in biological fluids. The employment of tandem mass spectrometry (MS–MS) can extraordinarily increase sensitivity and selectivity of GC–MS, suppressing the background noise due to interferents coming from biological matrices and thus overcoming the problem of determination of drugs in low concentrations [14].

The present paper describes a GC–MS–MS assay for the detection and quantitation of lorazepam in plasma and urine samples, using oxazepam-d_5 as the internal standard (I.S.). Quantitation of the analyte was performed using characteristic product ions formed from the parent ions by collision-induced dissociation (CID) inside the ion trap of the mass spectrometer.

2. Experimental

2.1. Chemicals and material

Benzodiazepines standards, β-glucuronidase type H-1 from *Helix pomatia* (348 100 units/g solid), bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS), control blank plasma (lithium heparin) and urine controls were supplied from Sigma (Milan, Italy). Oxazepam-d_5 was a gift from Professor J. Segura (IMIM, Barcelona, Spain). Extrelut-1 and Extrelut-3 extraction columns were from Merck (Bracco, Milan, Italy). All other reagents were of analytical grade from Farmitalia–Carlo Erba.

2.2. Gas chromatography–mass spectrometry

GC–MS analyses were performed using a Varian 3400 gas chromatograph coupled to a Saturn II ion trap detector (Varian, Turin, Italy). Chromatographic separation was achieved using a Restek Rtx-5 MS (30 m×0.25 mm I.D., 0.25 μm film thickness, Varian) under the following conditions: the initial temperature was 60°C, held for 1 min, then increased by 30°C/min to 295°C and held for 5 min for a total of 13.83 min per run. Purified helium (99.99%) was used as a carrier gas at a linear velocity of 1 ml/min. The injection port contained a 0.5 mm I.D. silanized glass liner. Injections were made in the splitless mode. Between two subsequent injections, the glass liner was washed with 1 μl ethyl acetate. The injection port temperature was 250°C, and the transfer line temperature was 280°C. Ion trap temperature was maintained at 200°C. Firstly, full scan analysis of reference standards was performed by scanning over a mass range of 80–500 amu with the MS system operated in the electronic impact mode with an emission current at 20 μA for the filament. Then, for each compound, a parent peak was completely isolated to form product ions by CID. The ion m/z 429.2 was isolated for lorazepam–TMS and the ion m/z 434.2 for oxazepam-d_5–TMS. Then, for each parent ion the CID excitation amplitude was selected to obtain maximum product ions current. The CID excitation amplitudes selected for lorazepam–TMS and oxazepam-d_5–TMS were 1.2 and 1.0 V, respectively with an excitation time of 20 ms.

2.3. Standards and controls

Solutions of stock reference standards (1 mg/ml, 10 μg/ml, 1 μg/ml) were prepared in methanol and stored below 0°C. Aqueous dilutions were made fresh daily for each analysis. Aliquots of these standards were spiked into blank plasma or urine and used throughout the entire sample preparation to create calibration curves and to determine solid-phase extraction (SPE) recoveries, intra- and inter-day variabilities.

2.4. Sample collection protocol

Serum and urine samples were obtained from individuals admitted at the emergency room of two different nearby hospitals, who claimed the use or the misuse of Tavor, a preparation widely prescribed in Italy as tranquilizer or sleep-inducing drug with lorazepam as the active principle. No information could be obtained regarding the daily dose, the last dose administered and the time elapsed from the last dose and blood or urine collection. A toxicological drug screening of those samples was previously executed at the local hospital by immunoassay and disclosed the presence of benzodiazepines. Sub-

sequently, the samples were screened with general high-performance liquid chromatography methods, and only in a few cases could lorazepam be identified or quantified. Furthermore, none of the samples underwent a process of hydrolysis. Serum and urine samples were stored at −20°C until analysis.

2.5. Sample preparation

A first aliquot of plasma or urine (1 ml) with 20 μl of oxazepam-d_5 (1 μg/ml) added was mixed with 0.2 ml of 50 m*M* $NaHCO_3$ and transferred to an Extrelut-1 glass column. After 5 min, the analytes were eluted under gravity with 3 ml dichloromethane–isopropanol (9:1, v/v). The organic phase was evaporated to dryness under nitrogen.

In order to verify data reported on extensive metabolism of lorazepam to its glucuronide conjugate, a second aliquot of plasma or urine underwent enzymatic hydrolysis, as suggested by the literature [6,9,11]. In brief, 1 ml of biological fluid, with 20 μl of oxazepam-d_5 (1 μg/ml) added, was mixed with 0.5 ml of β-glucuronidase solution (5000 units/ml in 0.1 *M* acetate buffer, pH 5) and heated at 65°C for 3 h. After hydrolysis, the samples were allowed to cool at room temperature and then were extracted as reported above.

Dry extracts were derivatized by silylation with 20 μl BSTFA containing 1% TMCS for 20 min at 70°C and 1 μl of specimen was injected into the GC–MS system.

2.6. Stability studies

Stability studies were conducted in blank plasma and urine spiked with 50 ng/ml and 500 ng/ml lorazepam, respectively, stored identically to patients samples at −20°C. These two concentrations were selected as commonly encountered in non-hydrolyzed plasma and urine samples we analyzed. Five samples were analyzed on the day of samples preparation (control samples) and then every seven days for a three-month period. Furthermore, two plasma and urine samples from patients treated with lorazepam were reanalyzed once a month for a three-month period.

3. Results and discussion

Fig. 2 shows the total product ion current chromatogram of a standard mixture of lorazepam–TMS and oxazepam-d_5–TMS at a concentration of 2 ng/μl and the product ion mass spectra obtained under our working conditions. For identification and quantitation the following product ions masses were selected: lorazepam–TMS, m/z 341, 306 and 267; oxazepam-d_5–TMS, m/z 346, 309 and 271.

The linearity of the method was evaluated on two different calibration curves of the peak-area ratios of lorazepam–TMS (y, arbitrary units) to the internal standard versus the concentration of the analyte (x, ng/ml) which were prepared and checked daily from blank plasma and urine spiked with nine different concentrations of the drug (0.1, 0.5, 1, 5, 10, 50, 100, 200 and 500 ng/ml) and carried through the entire procedure. Each point of the curve was the mean of three different preparations. The calibration curves were linear over the range 0.1 to 500 ng/ml for plasma and urine samples (slope 1.23, intercept: −0.06, r=0.991; slope 1.49, intercept: −0.08, r=0.995, respectively). The analytical recoveries of lorazepam in plasma and urine samples and the intra- and inter-day variabilities were evaluated at three different concentrations on six days and are summarized in Table 1. The limit of quantitation (LOQ), defined as the lowest measurable concentration at a signal-to-noise ratio of 6 and relative standard deviation (RSD) <15% was 0.1 ng/ml.

No indication of drug instability was found in blank plasma and blank urine spiked with the analyte as the amount of the analyte recovered in samples analyzed during a three-month period did not statistically differ from control samples. The same results were obtained on reanalyzing patients samples.

Some other benzodiazepines commonly used and misused were examined for their possible interference with the determination of lorazepam. Diazepam, nordiazepam, oxazepam, flunitrazepam and triazolam standard solutions (10 ng/μl) were treated with BSTFA containing 1% TMCS and injected into the GC–MS system. None of the drugs interfered with the assay.

Using the methodology described above, plasma and urine samples obtained from the emergency departments of two different nearby hospitals were

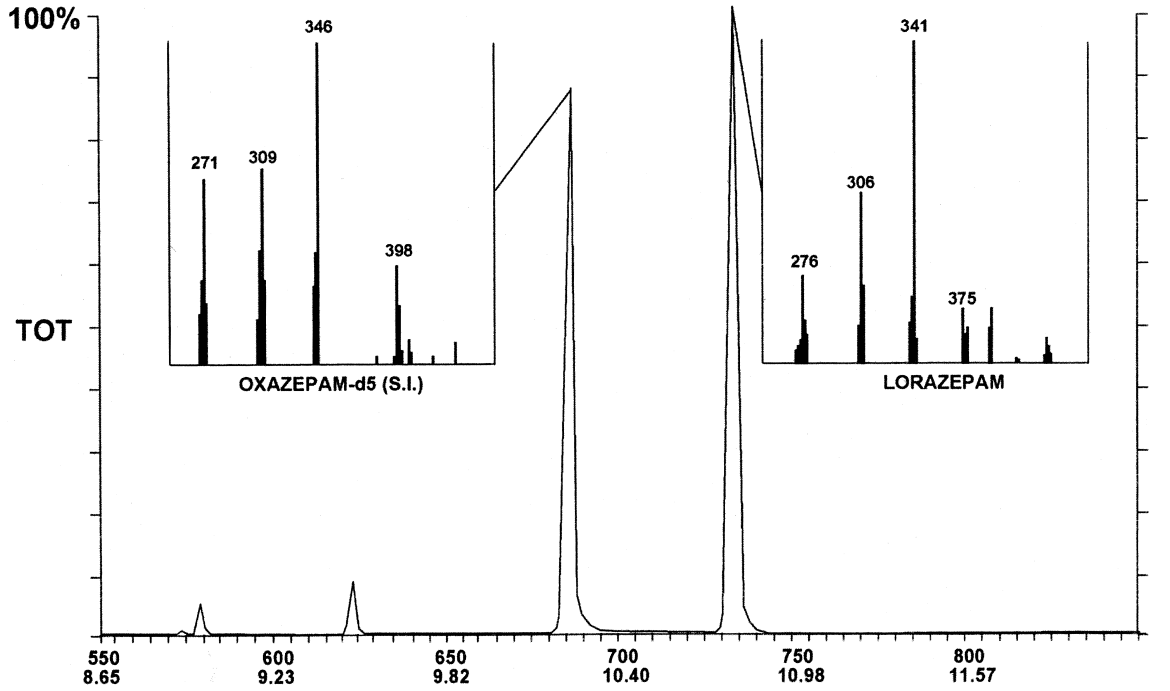

Fig. 2. Total product ion current chromatogram of a standard mixture of lorazepam–TMS and oxazepam-d_5–TMS at a concentration of 2 ng/μl and the product ion mass spectra obtained under our working conditions.

analyzed. The results obtained are summarized in Table 2 and Fig. 3 depicts the chromatogram of the extract of a plasma sample G containing 0.3 ng/ml lorazepam.

Lorazepam could be identified and quantitated in all plasma and urine samples with a concentration range of 0.3 to 54.5 ng/ml and of 473.2 to 668.4 ng/ml, respectively. Furthermore, when the samples were hydrolyzed and reanalyzed, the concentration of drug greatly increased in both biological fluids. These results are in agreement with the international literature which reports negligible amounts of free lorazepam in blood and an extensive metabolism to its glucuronide conjugate [3,5,8,12].

Table 1
Analytical recovery and precision of the method (n=5)

Concentration of lorazepam (ng/ml)	Recovery (%) (mean±SD)	RSD (%)	
		Intra-day	Inter-day
Plasma samples			
0.2	86.1±3.7	4.3	5.1
1	87.5±3.3	3.8	4.5
100	88.1±2.9	3.3	3.9
Urine samples			
0.2	85.7±3.3	3.8	4.4
1	87.9±3.1	3.5	4.0
100	88.0±3.1	3.5	3.8

Table 2
Concentration of lorazepam in plasma and urine samples

Subject	Unconjugated lorazepam (ng/ml)		Total lorazepam (ng/ml)	
	Plasma	Urine	Plasma	Urine
A	21.4	559.0	568.2	1345.1
B	35.4	473.3	240.0	1226.3
C	31.3	N.A.[a]	668.6	N.A.
D	54.5	N.A.	657.4	N.A.
E	25.0	668.4	646.1	1046.2
F	0.8	590.2	6.3	998.7
G	0.3	N.A.	1.5	N.A.
H	9.4	N.A.	34.0	N.A.
I	31.4	N.A.	400.0	N.A.
L	45.2	N.A.	672.3	N.A.
M	1.9	N.A.	72.6	N.A.

[a] N.A.=Not available.

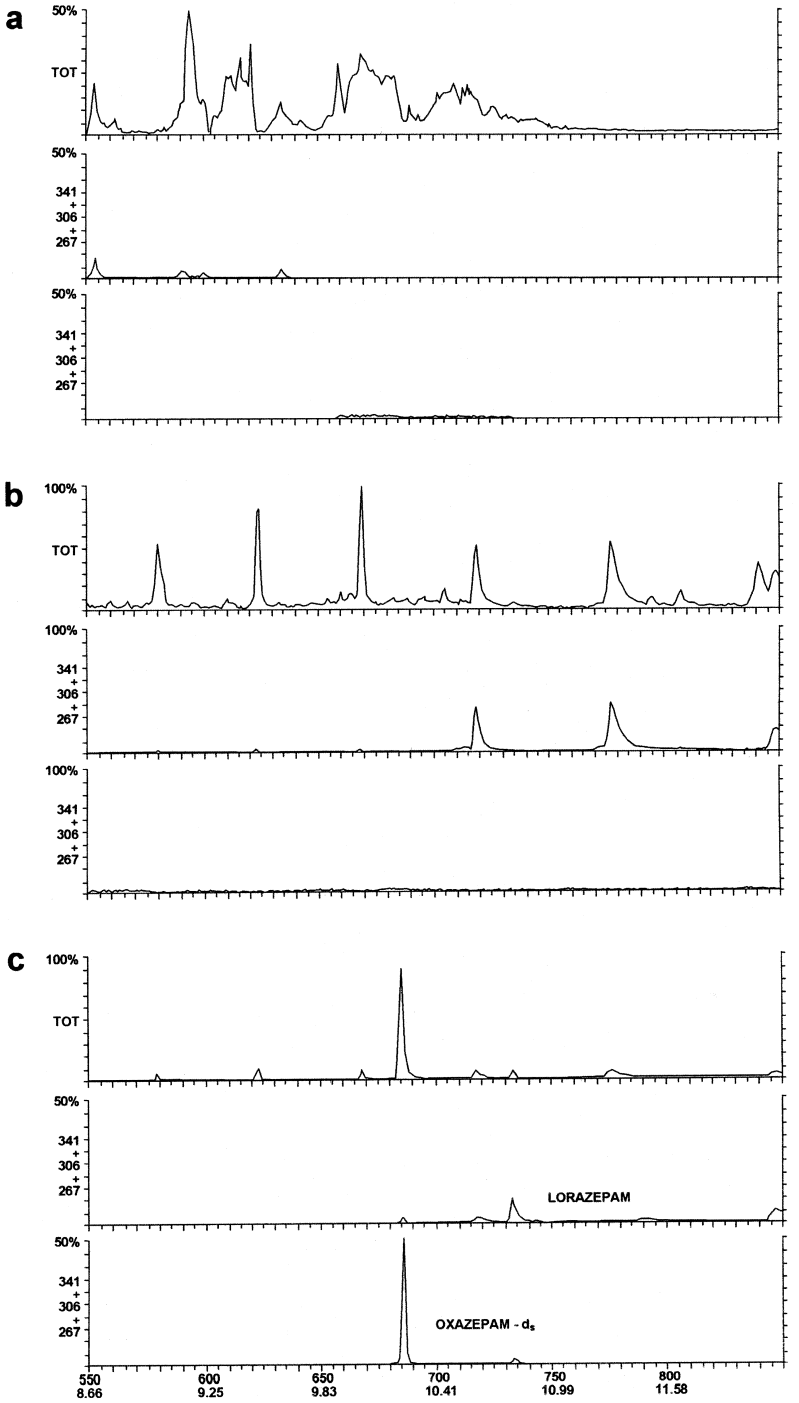

Fig. 3. (a) Chromatogram of an extract of blank plasma; (b) chromatogram of an extract of a plasma containing 0.3 ng/ml lorazepam and oxazepam-d_5 as I.S.

4. Conclusions

In conclusion, a new method employing GC–MS–MS proposed for the quantitative determination of lorazepam in plasma and urine samples. The main advantages are that the SPE procedure employing Extrelut columns is a reliable and quick procedure, and the assay is highly selective and sensitive and particularly suitable when low drug concentrations are expected and an hydrolysis of the biological sample cannot be performed.

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