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A fast LC-APCI/MS method for analyzing benzodiazepines in whole blood using monolithic support

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Abstract

A simple and fast procedure was developed for the simultaneous determination of eight benzodiazepines (BZDs) in whole blood using liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry (LC-APCI-MS). Sample pretreatment was carried out using a simple liquid–liquid extraction (LLE) with *n*-butylchloride, and chromatographic separation was performed using a monolithic silica column to speed up the analytical process. APCI and electrospray ionization (ESI) were compared. Whereas both ionization techniques appeared suitable for BZDs, APCI was found to be slightly more sensitive, especially for the determination of frequently low-dosed compounds. The method was validated according to the guidelines of the "Société Française des Sciences et Techniques Pharmaceutiques" (SFSTP) in the concentration range of 2.5–500 μg/L. The limit of quantification (LOQ) was 2.5 μg/L for all the compounds. Validation data including linearity, precision, and trueness were obtained, allowing subtherapeutic quantification of frequently low-dosed BZDs. The high selectivity of the mass spectrometer, along with the properties of the monolithic support, allowed unequivocal analysis of the eight compounds in less than 5 min. To demonstrate the potential of the method, it was used for the analysis of benzodiazepines in postmortem blood samples.
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Keywords: APCI; Benzodiazepines; LC–MS; Monolithic column; Validation

1. Introduction

Benzodiazepines (BZDs) are a large group of substances with a wide range of potency and physicochemical properties [1] and are frequently prescribed for anxiety, sleep disorders, or convulsive attacks.

Besides the problem of dependence, resulting from overprescription and/or inappropriate prescription, in low concentrations these compounds are often misused in drug-facilitated assault or in road traffic offences.

Several methods have been reported for the simultaneous determination of BZDs in biological fluids. Gas chromatography coupled with electron capture detection (ECD) [2], nitrogen phosphorus detection (NPD) [3], or, more frequently, mass spectrometry detection (MS) [4–7] has been described for the analysis of these drugs. However, although most of these methods are sufficiently sensitive, they are known to be labour intensive and time consuming because of the derivatization step needed before analysis. Furthermore, some BZDs are thermolabile.

The most widely used method is by far liquid chromatography (LC) with ultraviolet [8–10] or MS detection, with APCI [11–13] as well as electrospray ionization (ESI) [14–17]. However, most of these methods do not simultaneously offer the advantages of fast separation, sufficient sensitivity and simple implementation.

The development of high-throughput analytical methods is required in forensic or clinical toxicology nowadays, because the number of samples investigated in laboratories, is continuously increasing. Furthermore, there should also be as short a delay as possible in providing results of analyses. The most obvious approach to speed up the analytical process is to increase the velocity of the mobile phase. Unfortunately, the high back pressure generated has limited this approach when using conventional columns and HPLC systems. Recently introduced, continuous media have overcome this problem by providing an accessible flow path within the column. Amongst these supports, silica monolithic columns are in the forefront because of their properties, which allow fast separations at elevated flow rates

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without generating a high back pressure and without any loss of chromatographic properties [18].

These supports consist of a continuous porous silica rod with large pores, of 2 μm i.d., and small diffusive pores, of 13 nm i.d., namely macropores and mesopores, respectively. The macropores are connected in a dense network, enabling fast transport at the active sites, whereas the mesopores provide an extended surface area.

In contrast with conventional stationary phases, a monolithic material exhibits a large porosity (80%) and allows the use of high flow rates by overcoming mass transfer limitations [18].

Consequently, monolithic supports have found widespread applicability in reversed-phase HPLC and have become popular for performing very fast and highly efficient separations [14,15,19,20].

We developed a rapid method [8] for determining benzodiazepines (BZDs) in whole blood using high performance liquid chromatography-diode array detector (HPLC-DAD) with a silica monolithic support.

However, the specificity and sensitivity of the UV detection method can be improved with the use of mass spectrometry.

Therefore, the objective of this study was to develop and to validate a simple and fast LC–MS procedure for determining subtherapeutic concentrations of eight commonly prescribed BZDs in whole blood.

2. Experimental

2.1. Chemicals and reagents

HPLC grade methanol and ammonium hydroxide solution (25%) were purchased from Merck (Darmstadt, Germany). HPLC grade acetonitrile and *n*-butylchloride were supplied by Romil (Cambridge, UK).

Ammonium formate and formic acid were obtained from Fluka (Buchs, Switzerland).

Methanolic solutions of all BZDs used (1000 mg/L), namely clonazepam (Clz), diazepam (Dzp), flunitrazepam (Flz), lorazepam (Lrz), midazolam (Miz), *N*-desalkylflurazepam (Des), nordiazepam (Ndz), and oxazepam (Oxz), were purchased from Cambridge Isotope Laboratories Inc. (Andover, MA, USA), and the internal standard (IS), methylclonazepam, was kindly donated by Roche Laboratories (Basel, Switzerland).

Working standard solutions were obtained by dilution of stock solutions with methanol to reach concentrations ranging from 0.1 to 10 μg/L.

A stock solution of methylclonazepam (1000 mg/L) was prepared by dissolving 10 mg in 10 mL of methanol. This solution was then diluted with methanol to obtain a final concentration of 5 mg/L.

Human blood was obtained from the University Hospital of Geneva (Geneva, Switzerland).

The structure of the studied compounds is given in Fig. 1.

2.2. Sample preparation

After spiking with 50 μL of IS solution (5 mg/L methylclonazepam) and adding 50 μL of 25% ammonia solution, 1 mL of blood sample was handled by liquid–liquid extraction (LLE) with 5 mL of *n*-butylchloride. After vertical shaking for 2 min and centrifugation at 5000 rpm for 10 min, the tubes were stored in a freezer (−20 °C) for 20 min. The upper organic phase was then transferred into a conical vial and evaporated to dryness under a gentle stream of nitrogen. The residue was dissolved in 50 μL of mobile phase and 10 μL of this solution was injected into the chromatographic system.

2.3. Liquid chromatography–mass spectrometry

All experiments were performed on an Agilent Series 1100 system (Agilent, Waldbronn, Germany) equipped with an autosampler, degasser, and quaternary pump. MS detection was conducted using an Agilent Series 1100 MSD single quadrupole (Agilent, Waldbronn, Germany), VL version, equipped either with an ESI or with an APCI interface. The Chemstation software suite (Agilent, Waldbronn, Germany), version A.10.02, was used for data processing and instrument control.

A Chromolith^{TM} Performance RP-18e column (100 mm × 4.6 mm i.d.) protected by a Chromolith^{TM} Guard from Merck (Darmstadt, Germany), was used for the chromatographic separation.

The isocratic mobile phase consisted of 5 mM aqueous ammonium formate adjusted to pH 3 with formic acid–acetonitrile (65:35, v/v), and the flow rate was 1.5 mL/min.

The mass spectrometer was operated in the positive ion mode for both APCI and ESI, but final experimentations were conducted using the APCI probe.

The following APCI inlet conditions were used: Nitrogen was used both as a nebulizing gas at a pressure of 55 psi, and as a drying gas at a temperature of 300 °C with a flow rate of 5 L/min, the capillary voltage was set at 4000 V, the vaporizer temperature was 350 °C, and the corona current was 4 μA.

The fragmentor voltage (skimmer) was set at 80 V for the nine compounds.

For quantification, molecular target ions of the nine compounds were used in the positive selected ion monitoring (SIM) mode, and the *m/z* values are given in Table 1.

2.4. Preparation of calibration standards and quality control samples

Calibration samples of the eight BZDs were prepared on each day, in triplicate, at seven concentration levels (CAL = 2.5, 5, 10, 50, 100, 250 and 500 μg/L) by adequately spiking blank blood with appropriate amounts of standard solutions and analysed to establish the daily calibration curve of each compound.

Quality control samples were prepared independently in the same way, in quadruplicate, at four concentration levels, representing the entire range of calibration.

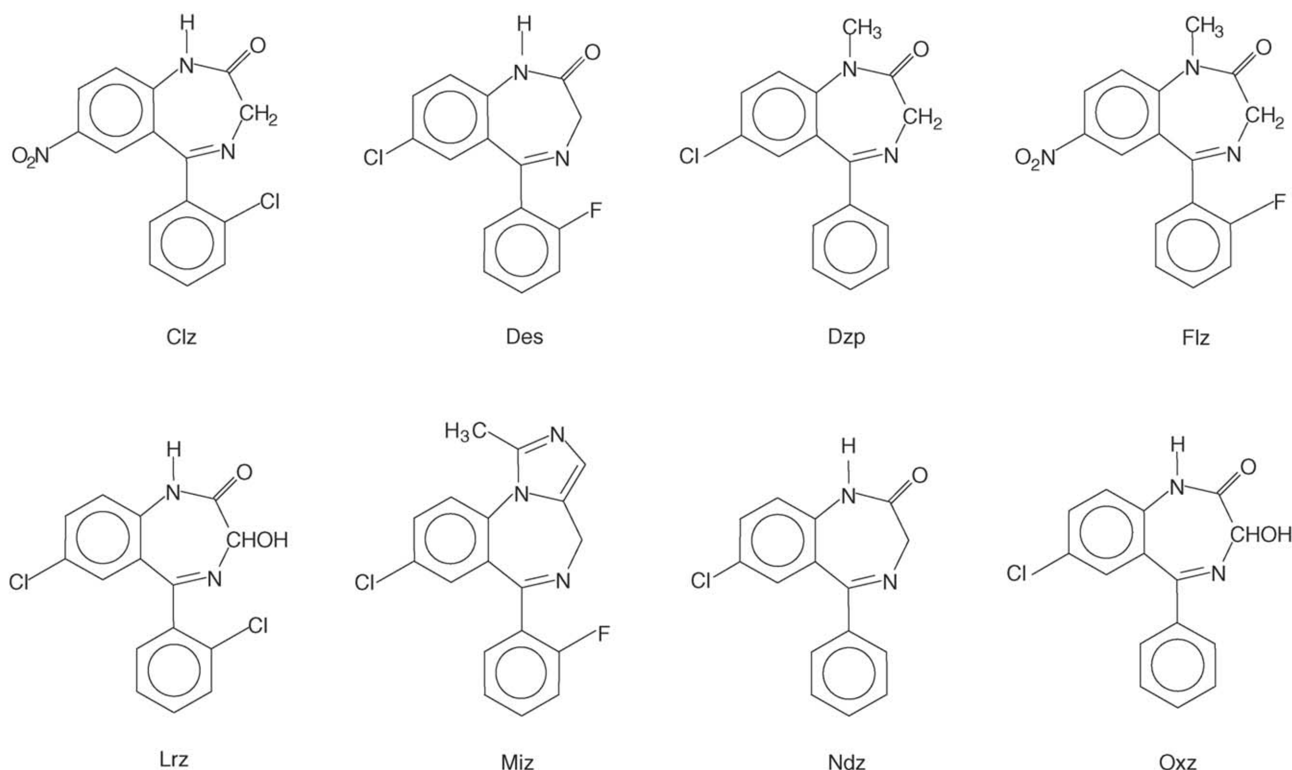

Fig. 1. Chemical structure of the BZDs studied.

Table 1
APCI-MS ions used for the quantification of BZDs

Compounds	Protonated molecular ions (*m*/*z*)
Nordiazepam	271
Diazepam	285
Oxazepam	287
N-Desalkylflurazepam	289
Flunitrazepam	314
Clonazepam	316
Lorazepam	321
Midazolam	326
Methylclonazepam (IS)	330

The spiked samples were then treated following the sample preparation procedure described in Section 2.2.

3. Results and discussion

3.1. LC–MS analysis

The responses of some BZDs were studied by flow injection analysis (FIA) using two different ionization techniques, atmospheric pressure chemical ionization and ESI. Though both were found to be suitable for the analysis of BZDs, APCI was found to be slightly more sensitive, especially for the assay of frequently low-dosed compounds like Flz or Lrz, and was used for further investigations. These results are in agreement with the work of Smink et al. [12]. As an example, the sensitivities obtained with the two sources is given in Fig. 2 for lorazepam.

Furthermore, APCI is widely accepted to be less susceptible to cause signal suppression [21–23] and is therefore more likely to provide a robust procedure [13].

Final optimization of the APCI conditions (corona discharge, nebulizer and auxiliary gas flow, temperature of auxiliary gas and fragmentor voltage) was investigated by direct introduction of each component in the MS detector (data not shown). APCI creates gas-phase ions at atmospheric pressure with a Corona discharge and induces mainly protonated molecular ions for all the compounds tested.

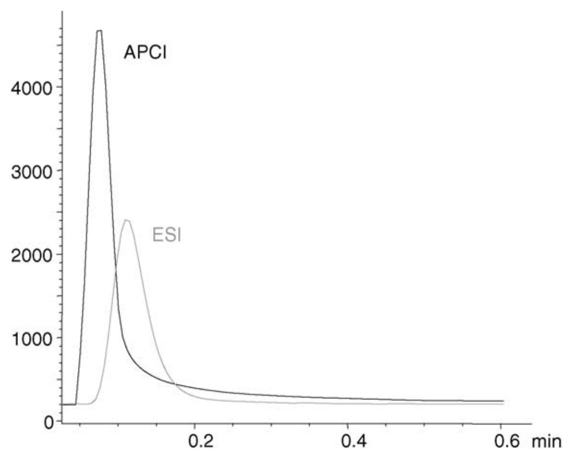

Fig. 2. Comparison of sensitivity obtained for lorazepam with APCI and ESI interfaces in FIA mode. Conditions: lorazepam (1000 μg/L) in mobile phase at 0.4 mL/min for ESI and 1.5 mL/min for APCI.

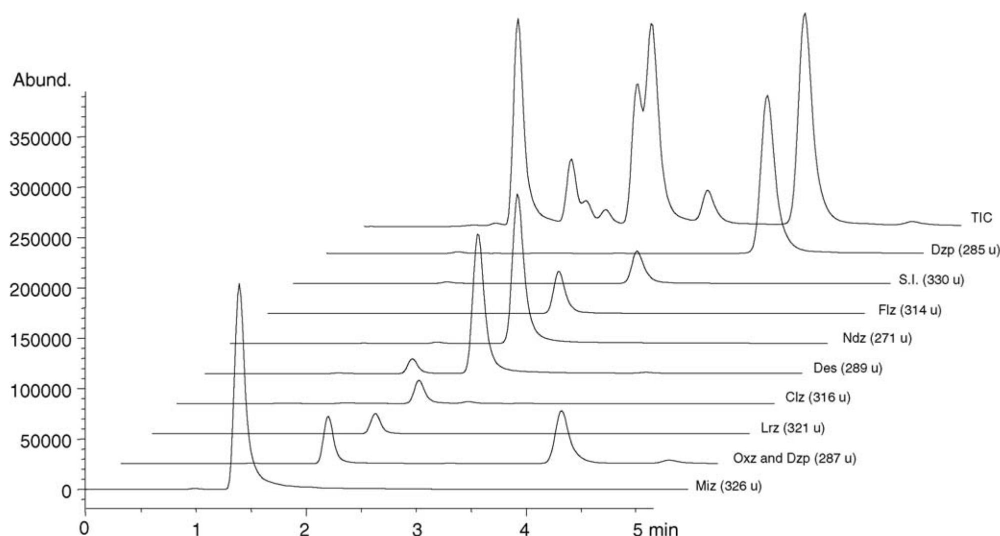

Fig. 3. Extracted ions from whole blood spiked with 250 μg/L of each BZD (for experimental conditions, see text).

With regard to the monolithic support employed, the mobile phase flow rate must be higher than that with particulate columns to reach optimal chromatographic conditions.

Indeed, the porosity and permeability of the stationary phase allow high flow rates, and high efficiencies because of the better mass transfer and low column backpressures.

It must be pointed out that the mobile phase split did not decrease signal intensity with the ESI interface. On the other hand, a significant loss in sensitivity was nobserved with the APCI interface as expected for this mass-flow-dependant device. To mitigate this problem, the APCI source was used with the most elevated flow rate suitable for this interface while keeping in mind that such conditions are a compromise between an optimal flow rate for the ionization mode and for the column used.

APCI ionization can be used between 0.4 and 2 mL/min. After evaluation, a value of 1.5 mL/min was found to provide simultaneously a short run time and good stability of the MS signal.

A typical chromatogram is given in Fig. 3. Separation was achieved in less than 5 min.

3.2. Validation procedure

The developed analytical method needed to be validated according to specific criteria [24] for its overall suitability to be evaluated in terms of precision and trueness.

The strategy of validation was based on the guidelines of the "Société Française des Sciences et Techniques Pharmaceutiques" (SFSTP) [25–27] and was adapted to our specific requirements for forensic toxicology. The general approach was based on variance analysis (ANOVA) to determine the precision and trueness of the data over 3 nonconsecutive days.

To validate all these criteria, two kinds of samples were prepared.

Calibration (Cal) samples and validation samples corresponding to quality control (QC) samples used in routine analysis.

In preliminary assays, several regression models were tested to select the most suitable and simple response function. Calibration curves were based on the drug to internal standard peak area ratio of each compound and the validation range was between 2.5 and 500 μg/L for all the BZDs tested. Because the hypothesis of variance homogeneity was rejected, the simplest regression model without data transformation, based on the least squares method, could not be retain. Therefore, the best weighting factor was chosen taking into account the relationship between the logarithm of the natural variance and the concentration as described elsewhere [25] and determined to be $1/x$. Further evaluations were conducted to check the model's adequacy (residual plot examination, lack of fit test, data not shown).

The precision and trueness of the method were determined with independent QC samples at four concentrations ($k = 4$) over three non consecutive days, representing the entire calibration range chosen for each compound. Each QC sample was extracted and analyzed four times ($n = 4$).

The precision of the method was determined by computing the relative standard deviations for the repeatability ($R_{\mathrm{R.S.D.}}$) and for the between days variability expressed as the intermediate precision ($\mathrm{IP}_{\mathrm{R.S.D.}}$), at each concentration level of the QC samples.

The linearity was calculated by plotting the obtained concentrations of the QC samples versus the theoretical concentrations introduced.

The results obtained are presented in Tables 2 and 3.

3.2.1. Extraction recovery

As demonstrated by Souverain et al. [22], LLE appeared to be the most efficient sample preparation technique for obtaining clean extracts with no MS signal suppression. The average recovery of all BZDs tested previously was determined by HPLC-DAD using the same extraction procedure and by

Table 2
Linearity data of the validation procedure

Compound	Equation	Coefficient of determination
Nordazepam	$y = 0.9307x - 0.2802$	$R^2 > 0.999$
Diazepam	$y = 0.9457x - 0.7356$	$R^2 > 0.999$
Oxazepam	$y = 0.9413x - 2.5974$	$R^2 = 0.997$
N-Desalkylflurazepam	$y = 0.938x + 0.3775$	$R^2 > 0.999$
Flunitrazepam	$y = 0.9857x - 0.5789$	$R^2 > 0.999$
Clonazepam	$y = 0.9661x - 0.072$	$R^2 > 0.999$
Lorazepam	$y = 0.9586x - 2.6052$	$R^2 = 0.997$
Midazolam	$y = 0.9563x - 0.1665$	$R^2 > 0.999$

comparing the analyte peak areas obtained from spiked blood samples before extraction with those in mobile phase solutions. Thus, the recoveries varied from 47 to 110% [8].

3.2.2. Limit of quantification (LOQ)

The LOQ was first estimated with a signal-to-noise ratio (S/N) of 10 and then confirmed with the QC samples. The final LOQ was chosen as the lowest concentration analyzed with the trueness equal to $100 \pm 20\%$ and the repeatability as well as the intermediate precision less than 20%.

The LOQs determined were set at 2.5 μg/L for the eight BZDs tested, allowing the measurement of subtherapeutic concentrations of these compounds.

From this experiment, it appeared that the LOQs achieved with MS detection (2.5 μg/L) are lower than those previously obtained with DAD (20 or 30 μg/L, depending of the BZD analysed) [8].

3.2.3. Precision

As mentioned above, the precision of the method was assessed by calculating the repeatability and intermediate precision. As shown in Table 3, the repeatability and the intermediate precision were less than 20% for all concentrations tested.

3.2.4. Trueness

Trueness refers to the closeness of agreement between a conventionally accepted value and a mean experimental one [25,26,28]. Thus, the trueness was determined by calculating the percentage difference between measured and theoretical concentration values. For the eight compounds, the trueness values varied from 83.3 to 99.1%, which represents a range of 100% (±20%).

3.2.5. Linearity

Linearity was calculated by fitting the back-calculated concentrations of the QCs as a function of the concentrations introduced and by applying the linear regression model on the basis of the least square method [29].

All the compounds gave linear relationships over the whole range tested and a good closeness, R^2, above 0.997, was observed for all the analytes.

The equations and coefficients of correlation obtained are given in Table 2.

3.3. Selectivity and matrix effect

The selectivity of the method was assessed by analyzing six different blood extracts. As exemplified in Fig. 4, No interfering peaks were observed at the retention time and/or at the specific detection window of each analyte.

The matrix effect (ME), resulting in suppression or enhancement of the signal, could generally be attributed to co-eluting components, which interact with the target compound during the ionization step in the interface. The ME with the APCI source is clearly less investigated than with the ESI source, but it is generally reported that APCI is less susceptible to this phenomenon because ionization takes place in the gas phase. Indeed, the use of an APCI source instead of an ESI source allows to reduce matrix effect for LC–MS analysis of pharmaceutical compounds in plasma [22]. According to Matuszewski et al. [21], the eventual ME was determined by comparing the areas of neat standard solutions in the mobile phase (*A*) and six different blood extracts supplemented with the same amount of standards (*B*) using the following formula:

$$\mathrm{ME}\,\% = \left(\frac{B}{A}\right) \times 100.$$

Table 3
Precision (repeatability *R*/intermediate precision IP) and trueness data for QC samples

QC (μg/L)	Compounds							
	Ndz	Dzp	Oxz	Des	Flz	Clz	Loz	Miz
Precision $n = 4$, *R* (R.S.D. %)/IP (R.S.D. %)								
2.5	6.9/12.8	10.5/10.6	10.5/20.8	8.6/11.2	7.0/9.7	9.1/11.2	6.8/18.2	14.1/19.3
5	6.5/7.9	8.4/8.3	5.5/17	7.8/8.4	11.1/10.9	11.2/12.6	6.6/19.1	7.2/8.0
250	6.2/6.2	5.8/8	8.3/12.6	11.6/11.1	8.3/7.6	7.4/8.3	7.0/14.0	7.6/8.8
400	8.9/10.4	9.1/9.0	10.2/15.9	9.6/10.3	10.1/9.8	14.8/16.9	13.2/17.3	10.8/12.3
Trueness ($n = 4$, %)								
2.5	95.0	96.4	83.3	91.1	98.2	98.8	93.5	98.2
5	91.0	91.8	84.5	91.9	94.6	94.2	86.6	90.2
250	92.3	92.3	86.8	95.2	96.7	96.5	97.9	95.8
400	93.2	95.2	95.9	93.4	99.1	96.6	97.9	95.5

The calibration range is 2.5–500 μg/L for all BZDs tested.

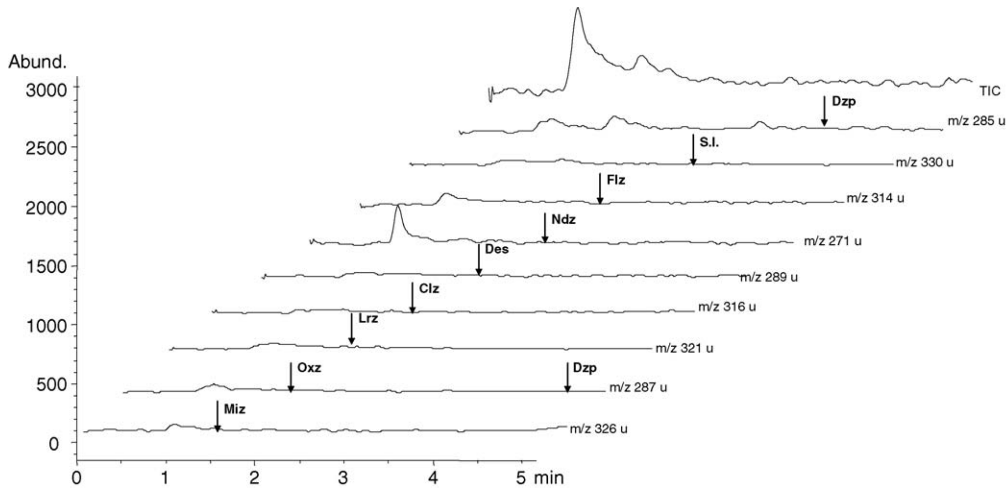

Fig. 4. A representative blank blood sample extracted without addition of the internal standard.

It appeared that from 5 to 100 μg/L, the ME was insignificant, but for higher concentrations (400 μg/L), a slight enhancement of the signal (about 15%) was observed for all compounds, including the IS. Therefore, no significant effect was observed on the quantitative results.

3.4. Applications to real cases

The applicability of the method to human whole blood extracts was demonstrated routinely in our laboratory. Moreover, blood analysis was applied to several real criminal cases. One of them is presented below.

The sample was obtained from a 55-year-old woman found dead in a bathroom with two pink pills by her side. Several empty boxes of sleeping pills were found in her residence.

The blood alcohol level determined by gas chromatography-flame ionization detection (GC-FID) was 1.7 g/kg.

Different immunoassays revealed the presence of BZDs both in urine and blood samples.

Nordiazepam and zolpidem were detected in the blood by GC-NPD, and an extract was analyzed using our LC-APCI-MS method.

Ndz and zolpidem were also identified using LC–MS, but another BZD, lorazepam, was found as well using this technique.

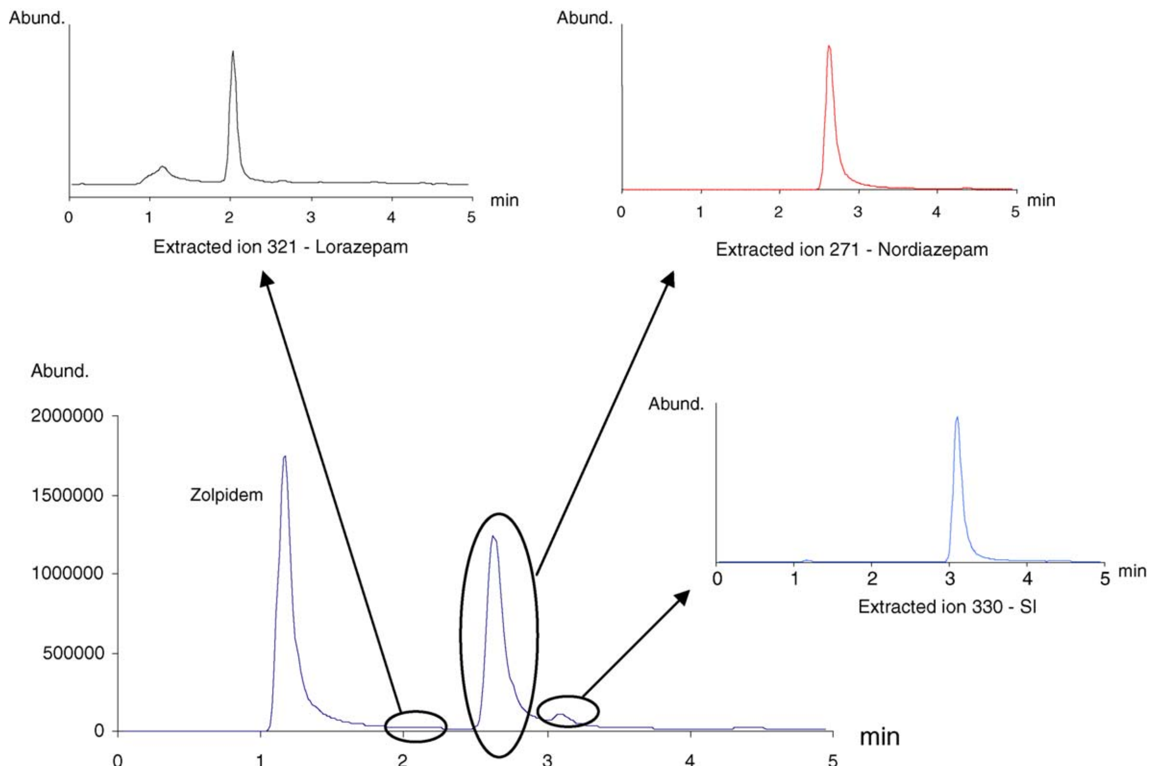

Fig. 5. Chromatogram of an autopsy case.

Because of the high content of Ndz, the blood extract was diluted by a factor of 5 to obtain a value in the calibration range. The Ndz concentration found was 1900 μg/L, which was confirmed that obtained by GC-NPD analysis (1850 μg/L).

The concentration of Lrz found was 20 μg/L.

The concentrations measured for each compound (except for Lrz) were in the toxic range, and the pharmacological interactions between the drugs and alcohol could explain the cause of death.

The present method has allowed us to rapidly detect and quantify two BZDs, amongst them a low-dosed compound, Lrz, which has not been identified previously using other analytical methods.

A typical chromatogram for this real case is shown in Fig. 5.

4. Conclusion

A fast and simple LC-APCI-MS method was developed that allows simultaneous analysis of eight BZDs in whole blood. Thanks to the use of a silica monolithic stationary phase, the total cycle time was less than 5 min.

The method was fully validated in terms of linearity, precision, and trueness. It also appeared suitable for determining low-dosed compounds like flunitrazepam or lorazepam. For these compounds, the LOQs have been reduced from 20 μg/L with HPLC-DAD to 2.5 μg/L with the present method.

Finally, the procedure has been proven suitable for the determination of BZDs in postmortem blood samples.

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