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Simultaneous quantification of four benzodiazepines from whole blood by high-performance liquid chromatography in forensic toxicological analysis

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Abstract A rapid high performance liquid chromatography method, using a monolithic column, was developed for quantitative determinations of benzodiazepines (diazepam, clonazepam, lorazepam, midazolam) in whole blood. A liquid-liquid extraction step with n-chlorobutane isolates the drugs from alkalinized blood. The separation was carried out in reversed phase conditions using a Chromolith Performance (RP-18 100x4.6 mm) column. For the mobile phase, a mixture of a phosphate buffer (pH= 2.5)/acetonitrile (65/35 v/v), in isocratic mode at 2 mL/min. An ultraviolet spectrophotometer was used as the detector at the wavelength of 220 nm. The total run time of the analytical method is less than 4-6 minutes. The calibration curves showed linearity and the correlation coefficient of each individual curve was greater than 0.995. The method was linear over a concentration range of 0.03-0.6 µg/mL for clonazepam, lorazepam and midazolam. For diazepam of linearity was over the range 0.04-5.0µg/mL. Quantification limits ranged from 0.03-0.04µg/mL and the accuracy were from 80% to 105% for the recovery test.
The results indicate that this analytical method is simple, specific, accuracy, sensitive, demonstrating from the validation data and a higher robustness. The proposed method is applied routinely in forensic toxicological analysis involving blood.

Keywords: benzodiazepines, forensic toxicological analysis, HPLC, monolithic column

1. Introduction

Benzodiazepines (BDZ) are a class of drugs commonly prescribed for the anxiolytic, hypnotic, anticonvulsivant and muscle relaxant activity. Their frequent use can lead to overdose or voluntary or involuntary intoxication, toxic effects were potentiated by co administration with alcohol, opioid analgesics, antidepressants, etc. Their presence in biological samples (blood, urine, gastric content, etc.) is a marker of the severity of overdosage or intoxication, is directly proportional to the amount of benzodiazepines into the human body. Therefore toxicological screening laboratories must have modern equipment and apply advanced quantitative methods [1,2].

Literature reported the simultaneous determination of benzodiazepines in various biological samples. Gas chromatography coupled with ECD (electron capture detector), NPD (nitrogen phosphorus detector) and MS (mass spectrometry) has been described in the analysis of these drugs. Phase derivatization of benzodiazepines can cause problems to representatives thermolabile [3-6].

These inconveniences have been removed by using liquid chromatography coupled with UV or MS detector [7-15].

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The LC (liquid chromatography) methods using conventional chromatographic columns, which lead to low flow rates and increase the time of analysis, which is an inconvenience for forensic and clinical toxicology laboratories. It was recently produced a new chromatographic column, monolith, allowing the use of large volumes of mobile phase and short analysis time. This type of column produced a good separation carried out in a mixture of the components. Monolithic columns provides a faster separation than conventional columns [16-18].

In this study was used and validated HPLC-UV method for simultaneous determination of four benzodiazepines in whole blood available in the domestic market, using a monolithic column Chromolith Performance (RP-18 100 x 4.6 mm). Biological samples are subjected to liquid-liquid extraction, as alkalinisation of whole blood, using as a solvent n-chlorobutane.

2. Experimental

2.1. Instruments and equipment

Centrifuge, digital pH meter, vortex, analytical balance, automatic pipettes; HPLC Agilent Technologies 1200 series with degasser, quaternary pump, autosampler, column oven, detector DAD (Agilent Technologies, USA); Column Chromolith Performance RP-18 100 x 4.6 mm (Merck AG, Germany).

2.2. Materials and methods

Clonazepam, lorazepam, midazolam, diazepam (LGC Standards, Germany); n-chlorobutane, methanol, acetonitrile, phosphate buffer pH 2.5, ammonia solution 25% (Merck AG, Germany)

Chromatographic conditions:

The mobile phase consisted of phosphate buffer (pH 2.5)/acetonitrile (65/35, v/v). The detector wavelength was 220 nm. The flow rate was maintained at 2 mL/min. The injection volume was 20µL and run-time 5-6 minutes.

Working standard solutions were prepared from solutions of concentration 1mg/mL in methanol benzodiazepine. Blood samples were subjected to liquid-liquid extraction with n-butyl chloride and treated according to the method of Staub Bugey 2004.

Method for simultaneous determination of four benzodiazepines has been validated according to guidelines. The parameters for this validation include the following: selectivity/specificity, linearity domain, LOD/LOQ, precision (repeatability and interim precision), accuracy (recovery) [19, 20].

Table 1. Results of method validation for the determination of whole blood for BDZs

BDZ (µg/mL)	Linearity	R^2	Recovery (%)	LOQ (µg/mL)	Repetability (n = 6) RSD (%)	Interim precision (n = 6) RSD (%)
Midazolam (0.03-0.6)	y=0.0038x-0.0498	0.9988	84	0.03	4.1	4.5
Lorazepam (0.03-0.6)	y=0.0031x-0.0485	0.9975	80	0.03	2.5	4.6
Clonazepam (0.03-0.6)	y=0.0028x+0.0473	0.9961	89	0.03	3.4	4.5
Diazepam (0.04-5.0)	y=0.0022x+0.0956	0.9981	105	0.04	2.9	4.1

BDZ (benzodiapines); R^2 (correlation coefficient); LOQ (quantification limit); RSD (relative standard deviation),

3. Results and Discussions

3.1. Specificity/selectivity

Specificity determination method was investigated by analyzing six blank matrix samples. No interference was observed with the analyte of endogenous compounds present in the matrix. The chromatograms showed no peaks at retention times of benzodiazepines. (**Fig. 1**)

3.2. Linearity (Calibration Curve)

To check the linearity concentration range was chosen based on expected concentrations in blood samples as they have been published in the literature. To establish the calibration curves, standard solutions were used with the following concentrations: 0.03, 0.05, 0.10, 0.20, 0.30, 0.60 µg/mL clonazepam, lorazepam, and midazolam, or 0.04, 0.15, 0.250, 0.50, 1.00, 5.00 µg/mL for diazepam. Calibration curves were plotted for each compound, and the correlation coefficient was greater than 0,995. (**Table 1**)

3.3. Detection limit (LOD) and quantification limit (LOQ)

In order to determine the limit of quantification, successive dilution of the reconstituted samples containing 0.10 µg/mL were performed. The experimental results showed that the diluted solution of 0.03 µg/mL (midazolam, lorazepam, clonazepam) and 0.04 µg/mL (diazepam) and RSD (relative standard deviation) values were obtained (n = 6) of less than 10%. The quantification limit values for determining these benzodiazepines at therapeutic doses are presented in Table 1.

3.4. Precision (repeatability and interim precision)

The method was evaluated by calculating the repeatability and interim precision.

Repeatability was determined by repeated analysis (n =6) of three different concentrations of each substance (0.05, 0.20, 0.50 µg/mL for clonazepam, lorazepam, midazolam and 0.50, 2.00, 5.00 µg/mL for diazepam). RSD for repeatability was below 5%. Interim precision was determined by repeated analysis (n = 6) of the same concentrations of substance over a period of three days. RSD interim precision was below 5%. (Table 1)

3.5. Accuracy(recovery)

In order to establish this parameter reconstituted samples were prepared containing the blood sample and added to solutions of known concentrations of benzodiazepines. The results showed recovery of four benzodiazepines (80-105%) (**Table 1**)

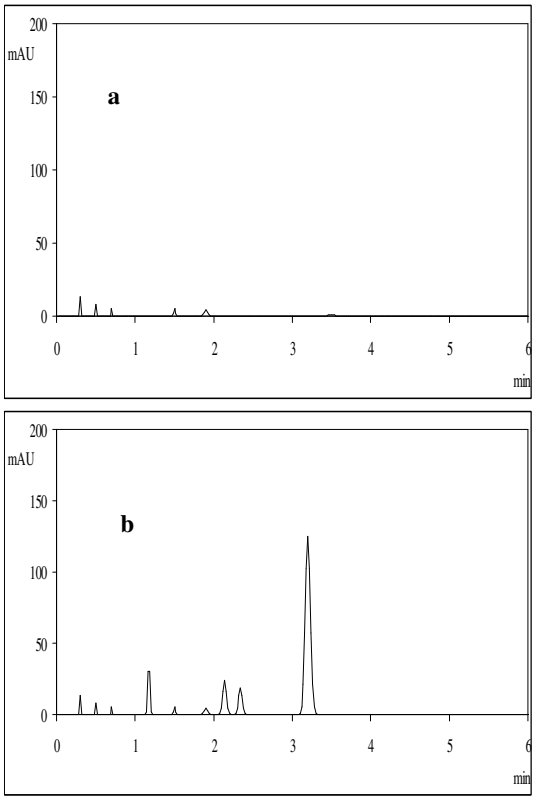

Fig. 1. Chromatogram of blood sample containing the blank (a) and the four benzodiazepines (b). Chromolith Performance (RP-18 100 x 4.6 mm). The mobile phase consisted of phosphate buffer (pH 2.5)/acetonitrile (65/35, v/v). The detector wavelength was 220 nm. The flow rate was maintained at 2 mL/min. The injection volume was 20µL and run-time 5-6 minutes.

3.6. Analysis times

Screening provided for each benzodiazepine was less than 4 minutes. Working relatively high flow

and quick permeability of the column makes the analysis time to be short. The retention times of the four analyzed benzodiazepines were 1.17 minutes for midazolam, lorazepam for 2.14 minutes, 2.34 minutes to 3.12 minutes for diazepam and clonazepam (Fig. 1).

4. Conclusions

In this study was used and validated LC-UV method for simultaneous determination of whole blood of four benzodiazepines (clonazepam, lorazepam, midazolam, diazepam), using a monolithic column Chromolith Performance (RP-18 100 x 4.6 mm). The method was validated by the following parameters: selectivity/specificity, linearity domain, LOD/LOQ, precision (repeatability and interim precision), accuracy (recovery).

A fast and simple LC-UV method was developed that allows simultaneous analysis of four benzodiazepines in whole blood.

The method allows screening of four benzodiazepines, separately or together, at therapeutic concentrations and in case of overdose or poisoning. Finally, the procedure has been proven suitable for the determination of benzodiazepines in postmortem blood samples.

5. References

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