Ann Toxicol Anal. 2008; 20(3): 113-116
DOI: 10.1051/ata/2009011

Available online at:
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ANNALES DE TOXICOLOGIE ANALYTIQUE

Short paper / Article court

Determination of four benzodiazepines with direct injection of whole blood by a column switching technique

Dosage de quatre benzodiazépines par injection directe de sang total et extraction en ligne de l'échantillon

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Abstract – Introduction: A simple, sensitive and reproducible method was developed for determination of four benzodiazepines (Clonazepam, Flunitrazepam, Triazolam and Diazepam) with direct injection of whole blood by column switching and UV detection. **Methods**: Whole blood samples were diluted 1:1 with water and then directly injected onto an Onyx Monolithic C18 column for a preliminary clean-up and then separated with a C18 150 × 4.0 mm I.D. reversed-phase column at room temperature. The mobile phase of the monolithic column consisted of pH 2.5 phosphate-acetonitrile buffer (85:15 v/v) at flow rate of 2.5 mL/min. The mobile phase of the analytical column consisted of pH 2.5 phosphate-acetonitrile buffer (60:40, v/v) at flow rate of 1.0 mL/min. The detection was carried out at 240 nm. No internal standard was necessary. **Results**: The method was linear over a concentration range of 0.1–10 μg/mL for Flunitrazepam and Triazolam. For Clonazepam and Diazepam of linearity was over the range 0.3–10 μg/mL. Quantification limits ranged from 0.1 to 0.3 μg/mL and detection limits from 0.05 to 0.1 μg/mL. Recovery ranged from 95% to 98%. Within-day and between-day coefficients of variation ranged from 2.1% to 6.2%. The present method has been applied successfully to the determination of toxic concentration of benzodiazepines in forensic toxicology. **Conclusion**: Monolithic columns have been demonstrated to be suitable for on-line extraction of benzodiazepines in whole blood. Precision, accuracy and sensitivity are similar to those of more time-consuming conventional assays, as manual off-line solid-phase or liquid-liquid extraction, thus offering a more simple and versatile analytical tool. This new method is particularly suitable for the analysis of benzodiazepines in *post mortem* whole blood, a situation very common in the forensic.

Key words: Benzodiazepines, monolithic column, column switching

Résumé – Introduction : Pour doser quatre benzodiazépines (clonazepam, flunitrazepam, triazolam et diazepam), une méthode simple et reproductible a été mise au point, par injection directe de sang total avec extraction en ligne et détection UV. **Méthodes :** Les échantillons de sang total ont été dilués dans de l'eau (1:1), puis injectés directement sur une colonne monolithique Onyx C18 pour le traitement préliminaire. Ils ont ensuite été séparés au moyen d'une colonne en phase inverse C18 150 × 4,0 mm de diamètre intérieur. La phase mobile de la colonne monolithique était constituée d'un tampon phosphate-acétonitryle à pH 2,5 (85:15 v/v) délivrée à un débit de 2,5 mL/minute. La détection a été réalisée à 240 nm. Il n'a pas été nécessaire d'utiliser un étalon interne. **Résultats :** La méthode s'est révélée linéaire dans l'intervalle de concentrations 0,1–10 μg/mL pour le flunitrazepam et le triazolam. Pour le clonazepam et diazepam, la méthode s'est révélée linéaire dans l'intervalle 0,3–10 μg/mL. Les limites de quantification étaient situées entre 0,1 et 0,3 μg/mL tandis que les limites de détection étaient comprises entre 0,05 et 0,1 μg/mL, pour un pourcentage d'extraction entre 95 et 98 %. Les coefficients de variation intra-jour et inter-jour variaient de 2,1 % à 6,2 %. Cette méthode a été appliquée avec succès pour déterminer les concentrations toxiques de benzodiazépines en toxicologie médico-légale. **Conclusion :** Les colonnes monolithiques se sont révélées adaptées pour l'extraction en ligne de benzodiazepines dans le sang total. La précision, l'exactitude et la sensibilité se sont avérées similaires à celles de méthodes conventionnelles, telle l'extraction manuelle hors ligne en phase solide ou liquide-liquide. Elles constituent ainsi un outil analytique plus simple et plus souple. Cette nouvelle méthode est particulièrement adaptée pour la détection *post mortem* des benzodiazépines dans le sang total, situation fréquente en médecine légale.

Mots clés : Benzodiazepines, colonne monolithique, extraction en ligne

Received 6 June 2008, accepted after revision 19 January 2009
Published online 19 March 2009

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1 Introduction

In this paper we describe a rapid and effective method for the determination of four benzodiazepines in a biological fluid difficult to treat such as whole blood and with a minimum of sample manipulation. In a previous work we used extraction columns filled with C18 silica of 40 μm particle size [1]. This approach produces good results in terms of reproducibility and sensitivity but the main drawback is that their frits get clogged on the top of the extraction column with the need to replace them often. Recently, monolithic columns have been developed. These are special chromatography columns that do not contain packed silica but a single cylinder material. The monolithic columns are widely used for the determination of substances by directly injecting biological fluids [2, 3]. The main advantage they have over conventional columns is that they do not get easily clogged because they do not contain any type of frit. Moreover, monolithic columns are not as efficient as conventional columns and are available in significantly less variety. In this paper we propose a non conventional use of these columns as extraction columns rather than as analytical columns. This use of monolithic columns is rather unusual although they have been used in the past [4]. In this work we used this approach for the analysis of four benzodiazepines in post mortem whole blood, a very common situation in the forensic field.

2 Materials and methods

Chemicals and reagents

Clonazepam, Flunitrazepam, Triazolam, Diazepam, Acetonitrile, HPLC grade (CHROMASOLV® Plus, P.N. 34998-2.5L), Sodium phosphate monobasic dihydrate (BioChemika Ultra, P.N. 71502-1KG) and phosphoric acid (BioChemika Ultra, P.N. 438081-500ML) were of analytical grade and purchased from Sigma Aldrich (Sigma-Aldrich, St. Louis, MO, USA). The water was reagent grade (18.2 MΩ cm at 25 °C of resistivity) obtained from a Milli-Q system (Millipore, Billerica, Massachusetts, USA).

HPLC instrument

The HPLC system consisted of a Varian Vista 5500 Solvent Delivery System (Pump A) from Varian (Varian Inc., Walnut Creek, CA, USA), a Beckman System Gold 126 Programmable HPLC pump (Pump B) from Beckman (Beckman Coulter Inc., Fullerton, CA, USA) and a Shimadzu SPD-20AV UV/Vis detector (Shimadzu corporation, Kyoto, Japan) set at 240 nm. The injector was a Rheodyne Model 7125 manual injection valve equipped with a 200 μL sample loop. The coupled-column system was operated by a six-port, automated, switching valve (Valco, Schencon, Switzerland) controlled by the Vista 5500 HPLC pump (Fig. 1).

The analytical column was a C18 5.0 μm reversed-phase column (150 × 4.6 mm) from Supelco (Sigma-Aldrich, St. Louis, MO, USA). The extraction column was Onyx C18 25×4.6 mm I.D. Monolithic column. The chromatograms were integrated with a Star 5.5 software from Varian.

Extraction-analysis switching procedure

The extraction was performed with an Onyx Monolithic C18 column (A) using a mobile phase constituted of phosphate buffer of pH 2.5 - acetonitrile (85:15 v/v) (mobile phase 1), at a flow rate of 2.5 mL/min. Separation was performed on a C18 reversed-phase column (150×4.6 mm) (column B) using a mobile phase constituted of 40% acetonitrile in phosphate buffer of pH 2.5 (mobile phase 2), at a flow rate of 1.0 mL/min. The coupled-column system was operated by a six-port, automated, switching valve controlled by pump A (Fig. 1). Extraction and analysis proceeded as follows:

1. After sample dilution (1:1, with water), 200 μL of the solution were injected onto the extraction column (A) where the analytes were retained while the matrix, passing through the column, was directed to waste, at a flow rate of 2.5 mL/min. At the same time, the analytical column (B) was conditioned with mobile phase 2 (switching valve at the initial position: Fig. 1A).
2. After a period of 1.0 min, the valve was switched and mobile phase 2 from pump B eluted the analytes trapped on the extraction column (column A) to the analytical column (column B) (Fig. 1B).
3. After a period of 1.0 min, the valve was switched to the initial position and extraction column (column A) was conditioned again with mobile phase 1 in order to prepare it for the next sample. Simultaneously, pump B maintained the flow of mobile phase 2 through the analytical column where the analytes were separated and detected.
4. The complete cycle time (extraction, elution, injection, analysis) was 15.0 min.

Method validation

The analytical procedure was validated in terms of recovery, linearity, precision, accuracy, and sensitivity. The recovery was determined by comparing the peak areas of standard solutions extracted with the procedure just described with the peak areas resulting from the same solutions injected directly onto the analytical column, at three different concentration levels. Linearity was examined at concentrations within the range 0.1–10.0 μg/mL (0.3–10 μg/mL for Clonazepam and Diazepam). Seven aliquots of whole blood were spiked with a stock solution of benzodiazepines to obtain seven calibration samples containing 0.1 (0.3), 0.5, 1.25, 2.5, 5.0, 7.5, 10.0, μg/mL of benzodiazepines. The samples were analysed and the peak areas of the benzodiazepines fitted *versus* the sample concentration to obtain a calibration curve. Precision and accuracy, shown in (Table I), were determined by injecting the same sample six times, and then calculating the mean and the standard deviation of peak areas. Quantification limits, defined as lower concentrations with %RSD < 20% (n = 5), ranged from 0.1 to 0.3 μg/L and detection limits, defined as concentrations with S/N = 3, ranged from 0.05 to 0.1 mg/L.

3 Results and discussion

The analytical methods that make use of a solid phase extraction step have many advantages over those using liquid

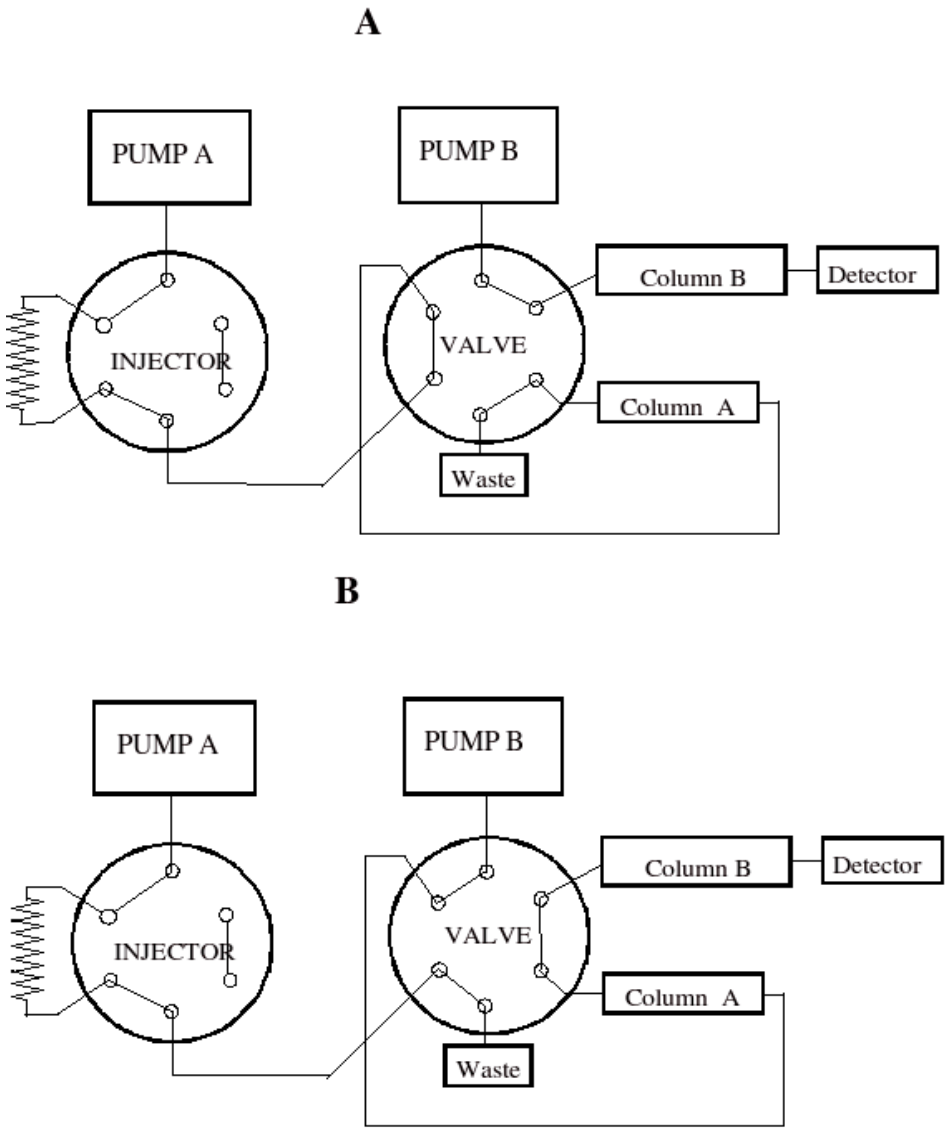

Fig. 1. The column-switching system.

Table I. Within-day precision, accuracy, quantification limit, detection limit, and linearity for benzodiazepines in whole blood.

Nominal	Actual value	Precision	Accuracy	LOQ	LOD	Linearity
µg/mL	(mean ± S.D., n = 6)	%	%	µg/mL	µg/mL	R^2
Clonazepam				0.3	0.1	(0.3–10.0 µg/mL) R^2 = 0.996
1.0	1.05 ± 0.04	3.5	105.2			
5.0	4.85 ± 0.15	3.1	0.97			
Flunitrazepam				0.1	0.05	(0.1–10.0 µg/mL) R^2 = 0.998
1.0	0.97 ± 0.05	4.8	97.3			
5.0	5.23 ± 0.11	2.1	104.6			
Triazolam				0.1	0.05	(0.1–10.0 µg/mL) R^2 = 0.997
1.0	0.98 ± 0.05	5.1	98.4			
5.0	4.79 ± 0.17	3.5	95.8			
Diazepam				0.3	0.1	(0.3–10.0 µg/mL) R^2 = 0.995
1.0	1.06 ± 0.07	6.2	105.8			
5.0	5.14 ± 0.13	2.6	102.8			

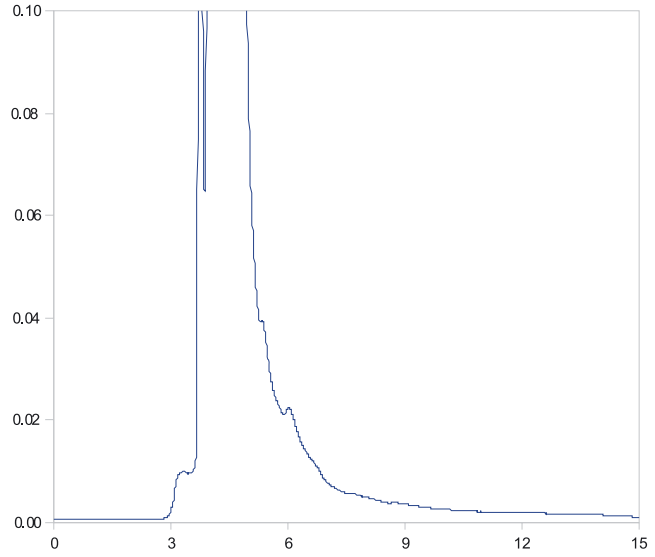

Fig. 2. HPLC chromatogram of whole blood blank sample with no benzodiazepines.

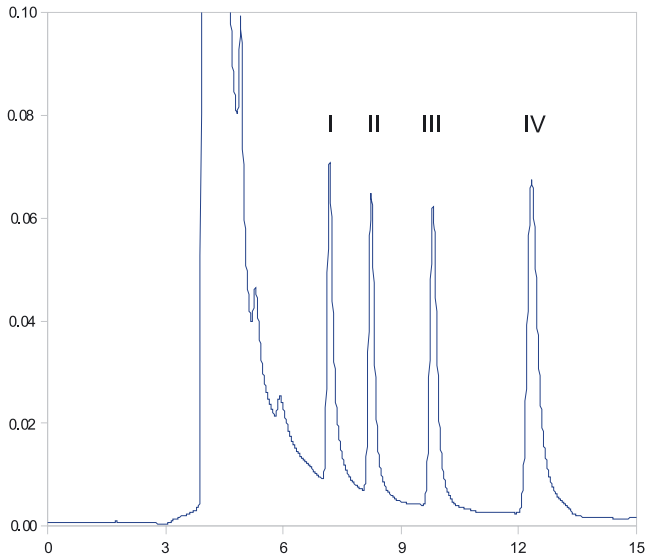

Fig. 3. HPLC chromatogram of whole blood blank spiked with 5 μg/mL of benzodiazepines. R.T.: triazolam (I) 7.5, clonazepam (II) 8.6, flunitrazepam (III) 10.2, diazepam (IV) 12.8 min.

phase extraction or simple deproteinization. The main advantages are that they are rapid, effective and easy to use. The use of monolithic columns as extraction columns is rather unusual, but in this paper we have proved that it is suitable for determining four benzodiazepines in a difficult biological fluid such as whole blood. The monolithic column is used for an initial clean-up, while the real analysis is performed through a conventional analytical column.

Figure 2 shows the chromatogram of a whole blood sample with no drugs while Figure 3 shows the same sample of whole blood to which the four benzodiazepines at the concentration of 5 μg/mL were added. Figure 2 shows that whole blood without drugs does not have interfering peaks.

Table I shows the results of our determinations in terms of reproducibility and accuracy. The method was linear over a concentration range of 0.1–10 μg/mL for Flunitrazepam and Triazolam. For Clonazepam and Diazepam of linearity was over the range 0.3–10 μg/mL. Quantification limits ranged from 0.1 to 0.3 μg/mL and detection limits from 0.05 to 0.1 μg/mL. Due to direct injection of blood and the absence of a preconcentration step they are not very low, but acceptable in forensic toxicology. Recovery ranged from 95% to 98%. Within-day and between-day coefficients of variation ranged from 2.1% to 6.2%. Due to good reproducibility of the method, the high recovery, the automated processing of samples that eliminates any manipulation, internal standard have not been necessary. Internal standards are useful in LC mainly to correct accuracy problems caused by changes in the volume of injections or loss of sample during a work-up, otherwise they does not provide any benefit (and may indeed contribute additional error).

The monolithic column was regenerated after each working day or after not more than 50 analyses by back-flushing with pH 2.0 50% buffer and 50% Acetonitrile.

In this way both extraction and analytical columns maintained a consistently high performance over time.

4 Conclusion

Monolithic columns have been demonstrated to be suitable for on-line extraction of benzodiazepines in whole blood. Precision, accuracy and sensitivity are similar to those of more time-consuming conventional assays, as manual off-line solid-phase or liquid-liquid extraction, thus offering a more simple and versatile analytical tool. This new method is particularly suitable for the analysis of benzodiazepines in post mortem whole blood, a situation very common in the forensic.

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