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Rapid analysis of benzodiazepines in whole blood by high-performance liquid chromatography: use of a monolithic column

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Abstract

In a previous work [J. Pharm. Biomed. Anal. 23 (2000) 447] a rapid high-performance liquid chromatography (HPLC) method, using a monolithic column in HPLC coupled with a diode-array detector, was developed for the quantitative determination of benzodiazepines in whole blood. The present method has been applied to the assay of eight benzodiazepines amongst the most frequently encountered in forensic toxicology: clonazepam, desalkylflurazepam, diazepam, flunitrazepam, lorazepam, midazolam, nordiazepam and oxazepam. The sample pre-treatment involved a liquid–liquid extraction of blood samples by *n*-butyl chloride. The separation was carried out in reversed-phase conditions using a Chromolith™ Performance (RP-18e 100 × 4.6 mm) column. The mobile phase was composed of a phosphate buffer (35 mM, pH 2.1) and acetonitrile (70:30, v/v) and the flow-rate was 2 ml/min. The duration of the analysis was less than 4 min and the results of validation, including linearity, precision, recovery, limit of quantification, were satisfactory. The therapeutic and toxic concentrations usually encountered for these substances could be measured. The compounds were separated by a monolithic column which, on account of its particular structure, could bear higher flow-rates than usually found for this kind of analysis. The present method has been applied to two real cases and was tested with about 30 compounds.

Keywords: Benzodiazepines; High-performance liquid chromatography; Monolithic column

1. Introduction

Benzodiazepines (BZD) belong to a group of substances known for their sedative, hypnotic and anticonvulsant properties and are prescribed worldwide for the therapy of anxiety, sleep disorders and convulsive attacks. However, on account of their excessive utilisation and their implication in many cases of multi-drug abuse, BZD are often found in fatal cases of drug intoxication. Indeed, apart from their therapeutic applications, BZD are often used abusively with other classes of compounds such as opiates, antidepressants and alcohol by drug addicts. Moreover, because of their drug abuse potential, BZD are frequently present in the blood of drivers involved in traffic accidents [2,3]. Finally, some BZD such as flunitrazepam are

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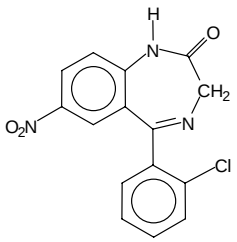
deliberately misused in cases of chemical submission during sexual assault [4]. It was therefore essential for a forensic laboratory to develop a rapid method for this class of compounds.

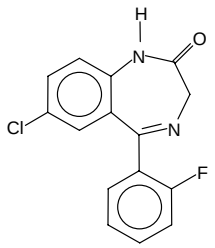
Several chromatographic methods for the analysis of BZD have been reported. Amongst them are gas chromatography (GC) using electron capture detection (GC/ECD) [5], nitrogen–phosphorus detection (GC/NPD) [6,7], and more frequently, mass spectrometry detection (GC/MS) [8–12].

Nevertheless, GC needs a derivatization step [10] to increase the volatility of BZD on account of their thermolability. For these reasons, high-performance liquid chromatography has carved out a place of choice in the analysis of these drugs.

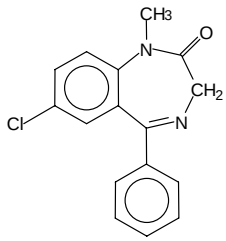
The choice of detectors offers several possibilities among which are the coupling of HPLC/UV or HPLC/DAD [13–27], and more recently the coupling of HPLC/MS which offers interesting selectivity and specificity [28].



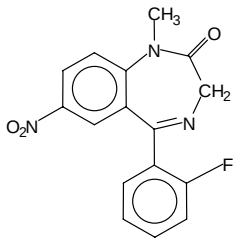
Clonazepam



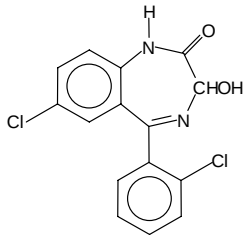
Desalkylflurazepam



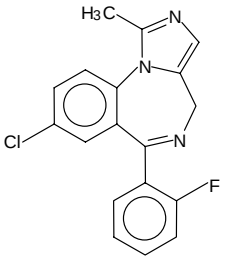
Diazepam



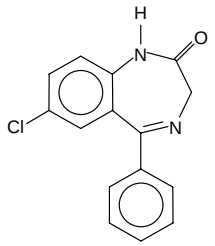
Flunitrazepam



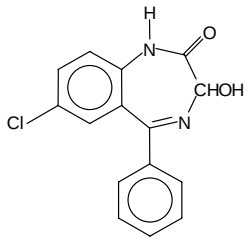
Lorazepam



Midazolam



Nordiazepam



Oxazepam

Fig. 1. Chemical structure of the eight benzodiazepines studied.

The topic has been revisited in a recent retrospective review of methods for BZD analysis [29].

Unfortunately, most of these methods are time consuming because of the low flow-rates employed. Thus the use of a monolithic column, such as the Chromolith^{TM} column, may be an alternative to this problem. Indeed, the silica rod composing the Chromolith^{TM} column has a biporous structure made up of macropores and mesopores, which offer a high porosity compared to usual columns. Macropores (i.d. 2 μm) form a dense network through which the mobile phase can rapidly flow and mesopores (i.d. 13 nm) form a fine internal structure and create a large specific surface area (http://www.chromolith.com). Both constitute a three-dimensional network such as coral for example. This type of column has greater permeability and can therefore be employed with high flow-rates without loss of performance or limitations due to increased pressure. The monolithic columns therefore achieve faster separation than that of conventional columns.

Referring to the previous methodological developments in our laboratory [1], we have developed an HPLC/DAD method using a Chromolith^{TM} column for the quantitative determination of benzodiazepines. The objective of this study was to considerably reduce the duration of analysis while maintaining the sensitivity required for the detection of these compounds in their therapeutic range.

The present method has been applied to the rapid screening of eight benzodiazepines frequently prescribed in Switzerland. For practical reasons, the

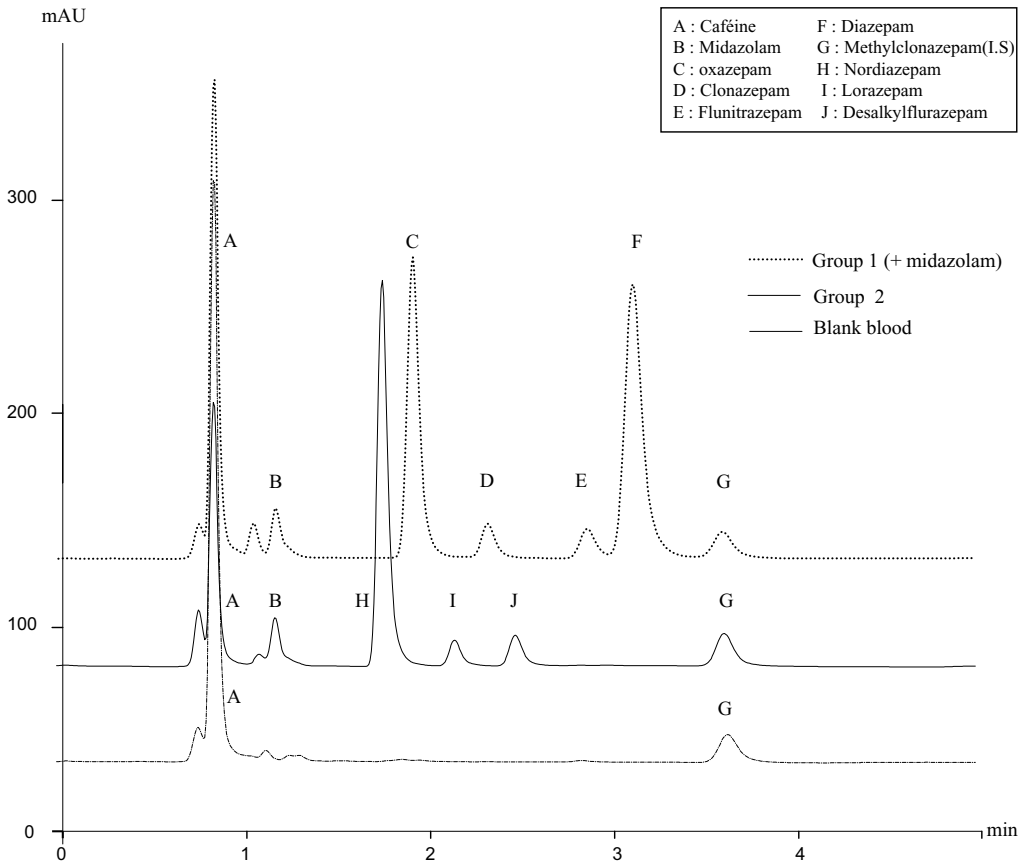


Fig. 2. Chromatograms corresponding to the blood extracts of different groups of benzodiazepines studied. Chromatographic conditions: mobile phase phosphate buffer–acetonitrile (65/35), Chromolith^{TM} Performance column RP-18 (12.5 × 4.6 mm), 20 μl injection, flow-rate 2 ml/min.

compounds have been divided into two groups: clonazepam, diazepam, flunitrazepam and oxazepam on the one hand, and lorazepam, desalkylflurazepam, midazolam and nordazepam on the other.

The structures of these compounds are given in Fig. 1.

2. Experimental

2.1. Chemicals and reagents

HPLC-grade methanol and acetonitrile were respectively purchased from Merck (Darmstadt, Germany) and Romil (Cambridge, England). The internal standard was kindly supplied by Roche Laboratories (Basel, Switzerland). Human blood was obtained from the University Hospital of Geneva (Switzerland). Monobasic phosphate potassium and phosphoric acid were supplied by Merck (Darmstadt, Germany).

2.2. Instruments and chromatographic conditions

BZD were analysed with the a High-Performance Liquid Chromatography/Diode Array Detection HP Series 1100 system (Hewlett Packard, Palo Alto, USA) and the separation was achieved with a Chromolith™ Performance RP-18e 100 × 4.6 mm column (Merck, Darmastadt, Germany) protected by a Chromolith™ Guard Cartridge RP-18e 5 × 4.6 mm. Methylclonazepam was used as internal standard (I.S.). For the mobile phase, a mixture of a phosphate buffer (pH 2.1; 35 mM)–acetonitrile (70:30, v/v), was delivered in isocratic mode at 2 ml/min at a detection wavelength of 220 nm.

2.3. Stock solutions

Stock standard solutions were prepared by dissolving each BZD in methanol to obtain a concentration of 1 mg/ml.

The blood standards were prepared by dilution of stock solutions with drug free blood.

2.4. Sample preparation

The preparation of blood samples was by liquid–liquid extraction (LLE). The conditions consisted of basifying 1 ml of blood, (spiked with 30 μl of internal standard at 10 mg/l) with 50 μl of ammonia solution at 25% (pH 11.5) and then in extracting lot with 5 ml of *n*-butylchloride. After vertical agitation (2 min)

Table 1
Results of validation in whole blood for the eight benzodiazepines tested

Compounds (μg/l)	Linearity	LOQ (μg/l)	Mean recovery (%)	Intraday R.S.D. % (n = 6)			Interday R.S.D. % (n = 18)
				Low concentration	Average concentration	High concentration	Average concentration
Clonazepam (20–500)	$y = 0.003x + 0.0404$, $R^2 = 0.9940$	20	88	3.7	3.8	3.3	5.2
Desalkylflurazepam (30–500)	$y = 0.0038x + 0.0031$, $R^2 = 0.9953$	20	75.5	2.7	1.1	2.4	4.5
Diazepam (30–5000)	$y = 0.0026x + 0.1935$, $R^2 = 0.9964$	30	88	2.3	3.7	1.7	4.3
Flunitrazepam (20–500)	$y = 0.0028x + 0.0418$, $R^2 = 0.9902$	20	110	5.5	3.2	3.4	3.6
Lorazepam (30–500)	$y = 0.0033 - 0.0410$, $R^2 = 0.9954$	20	47	1.2	3.4	2	6.9
Midazolam (30–500)	$y = 0.0045x - 0.0593$, $R^2 = 0.9982$	20	73	4.4	3.9	1.7	6.6
Nordiazepam (30–5000)	$y = 0.0042x + 0.0387$, $R^2 = 9942$	30	80	2.2	2	2.6	4
Oxazepam (30–5000)	$y = 0.0022x + 0.0459$, $R^2 = 0.9986$	30	60	4.3	4.1	2.7	5.8

Low, average, high concentrations are respectively 50, 200, 500 μg/l for clonazepam, desalkylflurazepam, flunitrazepam, lorazepam, midazolam and 500, 2000, 5000 μg/l for diazepam, nordiazepam, oxazepam.

and centrifugation (5000 rpm, 10 min), the tubes were stored in a freezer until the water phase froze. Then, the upper organic layer was transferred into a conical tube and evaporated under a gentle stream of nitrogen.

The dried extract was reconstituted in 50 μl of a mixture of buffer–acetonitrile (70:30; v/v) and a 20 μl aliquot was injected on to the HPLC system.

3. Results and discussion

3.1. Validation of the method

All BZD tested were well separated by the chromatographic system and the complete screening of each group of compounds was achieved in less than 4 min. The chromatograms obtained are given in Fig. 2 and results of the validation are presented in Table 1.

3.1.1. Extraction recovery

The average recovery of BZD was estimated by comparing the analyte peak areas obtained from spiked blood samples before extraction with those in methanolic solutions.

3.1.2. Limit of quantification (LOQ)

The limit of quantification of each compound was determined by repeated injections ($n = 6$) of a spiked blood with BZD in decreasing concentrations. Then, the LOQ was evaluated as the lowest concentration that could be measured with acceptable accuracy (R.S.D. $\leq$ 10%). The determined limits of quantification were 20 ng/ml for clonazepam, desalkylflurazepam, flunitrazepam and lorazepam and 30 ng/ml for the other BZD, allowing the measurement of therapeutic concentrations for the eight compounds except for flunitrazepam, where the concentrations in the higher range of the therapeutic zone could be measured.

3.1.3. Linearity

The linearity of the method was validated by preparing triplicate blood standards at the following

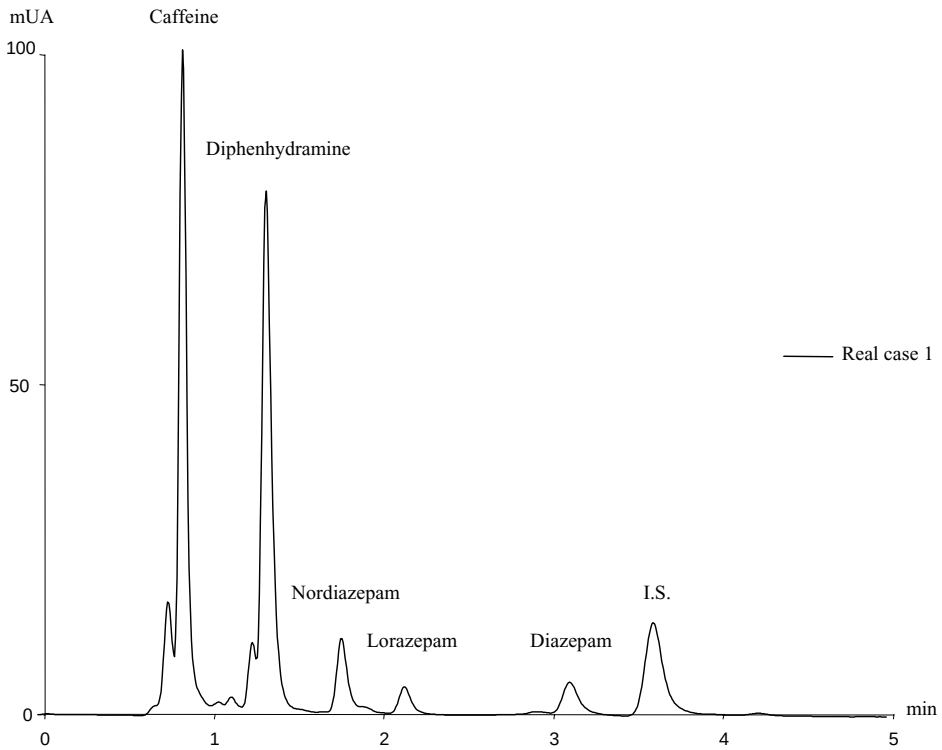


Fig. 3. Chromatogram of an autopsy case.

concentrations: clonazepam, desalkylflurazepam, flunitrazepam, lorazepam and midazolam: 20, 30, 50, 100, 200, 300 and 500 μg/l; oxazepam, diazepam and nordiazepam: 30, 150, 250, 500, 1000, 3000 and 5000 μg/l; all compounds gave linear relationships over the whole range tested with coefficients of correlation greater than 0.99 (Table 1).

3.1.4. Precision

The precision of the method has been evaluated by calculating the intraday repeatability and the interday reproducibility. The intraday repeatability was determined, over 1 day, with repeated analysis ($n = 6$) at three different concentrations of each drug (low, average and high) and the intraday relative standard deviations (R.S.D.) obtained were lower than 5% (Table 1). The interday reproducibility was determined with repeated analysis ($n = 6$) at the same concentration, over a period of 3 days. The interday R.S.D. obtained were approximately 5% (Table 1).

3.1.5. Time of analysis

The complete screening of each group of compounds was achieved in less than 4 min. As previously mentioned, the use of an elevated flow-rate due to the permeability of this type of column enabled the analysis time per sample to be reduced by a factor of up to 5 compared with assays based on packed HPLC columns [1].

3.2. Applications to real cases

The applicability of the method on human whole blood extracts was demonstrated on two real human cases implying not only BZD, but also other classes of compounds.

3.2.1. Autopsy case

The sample was obtained from a 46-year-old man found dead with a bottle of alcohol and a box of sleeping pills by his side.

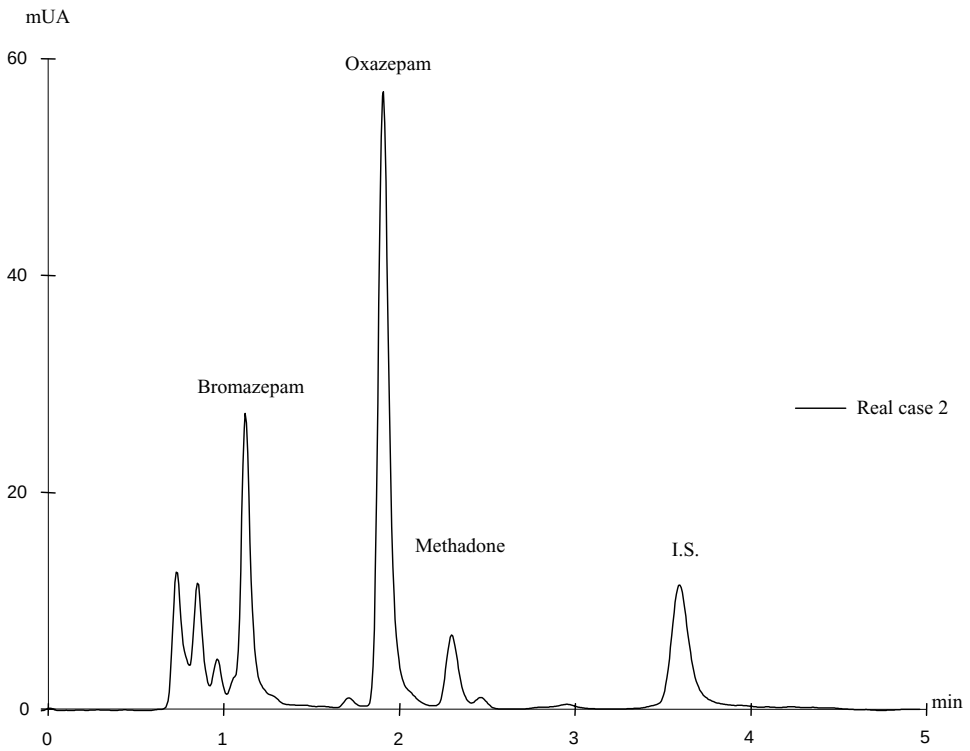


Fig. 4. Chromatogram of a road traffic case.

The blood alcohol level was 3.1‰. The chromatogram obtained by HPLC/DAD (Fig. 3) showed the presence of diphenhydramine (DNPH), diazepam, nordiazepam and lorazepam. The concentrations measured by the present method, were the following: 0.09 mg/l for diazepam and 0.1 mg/l for nordiazepam. The assay of DPNH by GC/NPD revealed a toxic concentration. Accordingly, it was concluded that death was due to a mixed intoxication by ethyl alcohol and DPHD. BZD, found in therapeutic concentrations, could have potentiated the action of the other drugs.

3.2.2. Road traffic case

The sample was obtained from a 40-year-old man involved in a road accident. The screening of blood by the proposed method revealed the presence of bromazepam, oxazepam and methadone (Fig. 4). Cocaine and benzoylecgonine were also found and the concentrations measured by GC/MS were the following: 0.1 mg/l for cocaine and 0.9 mg/l for benzoylecgonine. The measured concentrations by our method were the following: 1.22 mg/l for oxazepam, 0.69 mg/l for bromazepam and 0.23 mg/l for methadone. These results confirmed the suspicion of driving under the influence of drugs.

Table 2
List of benzodiazepines detected by the Chromolith™ column

Benzodiazepines	RT	RRT	Maximum wavelengths (nm)
7-Aminoclonazepam	0.83	0.2299	220
7-Acetamidoflunitrazepam	0.85	0.2355	249
Desmethylchlordiazepoxide	0.89	0.2465	246, 310*
7-Aminoflunitrazepam	0.88	0.2438	246
Chlordiazepoxide	0.91	0.2521	246, 310*
Bromazepam	1.13	0.3130	235
Midazolam	1.15	0.3186	220
Flurazepam	1.17	0.3241	231
Demoxepam	1.48	0.4100	237, 308*
Clorazepate	1.77	0.4903	235, 282*
Nordiazepam	1.74	0.4820	235, 283*
Oxazepam	1.9	0.5263	232, 315*
Alprazolam	2.07	0.5734	222
Lorazepam	2.12	0.5873	231
Clonazepam	2.31	0.6399	220, 310
Triazolam	2.33	0.6454	223
Desalkylflurazepam	2.47	0.6842	231
Flunitrazepam	2.86	0.7922	220, 254*, 310*
Temazepam	2.9	0.8033	233
Diazepam	3.14	0.8698	234, 282*
Lormetazepam	3.44	0.9529	232
Metylclonazepam (I.S.)	3.61	1	220, 250*, 313*

* Maximum wavelengths of less intensity.

Table 3
List of other compounds detected by the Chromolith™ column

Other compounds	RT	RRT	Maximum wavelengths (nm)
Nicotine	0.68	0.1884	260
Quinine	0.73	0.2022	209, 251, 316*, 342*
Codéine	0.74	0.2050	214, 286*
Cafeine	0.81	0.2244	207, 274*
Propanolol	0.82	0.2271	215, 293
Tramadol	0.87	0.2410	220, 272*
Desmethylvenlafaxine	0.93	0.2576	227
Venlafaxine	0.97	0.2687	227
Diphenhydramine	1.27	0.3518	220
Carbamazepine	1.67	0.4626	213, 286*
Nortryptiline	1.97	0.5457	207, 241*
Amitryptiline	2.12	0.5873	207, 241*
Methadone	2.27	0.6288	220
Fluoxetine	2.59	0.7175	238

* Maximum wavelengths of less intensity.

3.3. Applications to other compounds

Screening was carried out for approximately 20 compounds, amongst them BZD and varied compounds frequently encountered in forensic toxicology. A library has been constituted with the spectra of the different drugs.

Tables 2 and 3 list their retention times (RT), their relative retention times (RRT), as well as the maxima wavelengths observed.

4. Conclusion

The HPLC procedure proposed is a rapid technique for the quantitative determination of BZD in whole blood. With this method, one can analyse approximately 20 benzodiazepine compounds and quantify routinely eight benzodiazepines including to those most used in Switzerland. Furthermore, the limits of quantification allow the measurement of therapeutic concentrations for all BZD, except for flunitrazepam. For this compound, only concentrations in the higher range of the therapeutic zone could be measured. Finally, the proposed method was not only suitable for the assay of benzodiazepines but could also be applied to other compounds such as methadone.

Moreover, it will be interesting to test the applicability of this method to antidepressants, in view of their increasing importance in forensic toxicology.

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