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Monitoring of benzodiazepines (clobazam, diazepam and their main active metabolites) in human plasma by column-switching high-performance liquid chromatography

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ABSTRACT

A column-switching high-performance liquid chromatographic method for the simultaneous determination of clobazam, diazepam and their main metabolites in human plasma is described. A 200-μl plasma sample was directly injected into a precolumn filled with TSK-gel PW. After a washing step with potassium phosphate buffer, the retained substances were backflushed into a reversed-phase column with a mobile phase of acetonitrile–phosphate buffer–diethylamine. Various drugs frequently co-administered with clobazam or diazepam do not interfere with the determination.

INTRODUCTION

Clobazam and diazepam are two benzodiazepines (1,5- and 1,4-benzodiazepine, respectively) used as muscle relaxants and anxiolytic agents. Clobazam is often prescribed as an anticonvulsant drug in the treatment of minor seizure disorders in epilepsy, in conjugation with other major anticonvulsant drugs [1]. Diazepam is also the recommended treatment for controlling status epilepticus [2] or febrile convulsions in children [3]. There are two major metabolic transformations of diazepam and clobazam in humans [4,5]: N-demethylation to N-desmethyldiazepam (DMD) and N-desmethylclobazam (DMC) (active metabolites) and hydroxylation to 3-hydroxydiazepam and 4′-hydroxyclobazam, which are then demethylated to 3-hydroxy-N-desmethyldiazepam (oxazepam) and 4′-hydroxy-N-desmethylclobazam (minor metabolites). As a result of the relationships between clobazam [6] and diazepam [7] concentrations in plasma and seizure control, which have already been demonstrated, clinical monitoring of plasma diazepam or clobazam concentrations would be useful to establish better dosage guidelines for these drugs. In addition, the large variations reported in steady-state concentrations among patients suggest the need for accurate monitoring of both the drugs and their active metabolite [8,9].

Several quantitative analytical procedures for determining clobazam, diazepam and their metabolites in plasma have been developed using gas chromatography (GC)–mass spectrometry [10,11], GC with flame ionization, thermoionic or electron-capture detection [10,12–17] and high-performance liquid chromatography (HPLC)

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[5,18–23]. Reported procedures for sample preparation have been based on solvent extraction from alkaline media, solid-phase extraction or an automated sample processor [24]. These extraction steps for sample cleaning are very time-consuming and sometimes decrease the recovery. On-line extraction ensures a clean chromatographic trace, and allows a small sample size.

We have developed a column-switching HPLC method for the automated determination of clobazam, diazepam, DMC, and DMD in plasma. This method can be adapted to other benzodiazepines (BZDs) by using gradient elution.

EXPERIMENTAL

Reagents and chemicals

Clobazam and desmethylclobazam were gifts from Diamant (Puteaux, France); diazepam, desmethyldiazepam and flunitrazepam were from Roche (Neuilly, France). Monobasic and dibasic potassium phosphates and phosphoric acid were analytical grade (Merck, Darmstadt, Germany). All solvents used (water, acetonitrile and methanol) were HPLC grade (Merck).

Preparation of standards

Stock standard solutions were prepared by dissolving 100 mg of each compound in 100 ml of methanol and were stored in amber-coloured glass bottles at 4°C. Working standard solutions were prepared by dilution of the stock solutions with water or human plasma to give concentrations ranging from 20 to 5000 ng/ml.

Apparatus and chromatographic conditions

A Beckman high-performance liquid chromatograph (Gold system, Beckman, Gagny, France), equipped with two pumps (Models 116 and 126), a variable-wavelength UV detector (Model 166), a six-port automatic switching valve and a system controller (Tandon, Colombes, France), was used. Peaks were detected at 230 nm. The precolumn (45 × 4.0 mm I.D.) was filled with TSK-gel PW (G 3 PW, 12 μm, Tosohass, Touzard et Matignon, Vitry, France), and the separation was performed on a C_{18} column (Ultrasphere ODS, 3 μm, 75 × 4.6 mm I.D., Beckman).

Plasma samples (100 or 200 μl) were injected into the TSK precolumn and washed with a dibasic potassium phosphate buffer (50 m*M*, pH 7.5) for 5 min (1.0 ml/min). The precolumn eluent passed through the switching valve to waste. The BZDs retained in the precolumn were eluted in backflush mode into the analytical column with a acetonitrile–monobasic potassium phosphate buffer (65 m*M* with 1% of diethylamine, v/v, pH 5.4 with phosphoric acid) (33:67, v/v) at a flow-rate of 1.0 ml/min for 5 min (isocratic mode). The precolumn was washed with acetonitrile–water (60:40, v/v, 1.0 ml/min) for 6 min and re-equilibrated for 10 min with the dibasic potassium phosphate buffer (50 m*M*, pH 7.5, 1 ml/min) before injection of the next sample. The total cycle time was 25 min. The elution can equally be carried out in the gradient mode. A slightly different programme enables the separation and quantification of other BZDs (Table I).

Extraction efficiency

The recoveries of the extraction step were determined by comparing standards dissolved in the phosphate buffer and directly injected into the analytical column with plasma standards injected into the two-column HPLC system. Each standard was injected in triplicate. The recoveries of BZDs were calculated at each concentration by dividing mean peak areas from plasma standards by the mean peak areas obtained from the direct standards.

TABLE I

GRADIENT PROGRAMME FOR DMC, DMD, CLOBAZAM AND DIAZEPAM (OTHER BZDs)

Step	Acetonitrile (%, v/v)	Phosphate buffer (%, v/v)	Time (min)
1	25 (22)	75 (78)	0 (0)
2	25 (22)	75 (78)	5 (5)
3	28 (25)	22 (75)	15 (15)
4	45 (60)	55 (40)	25 (30)

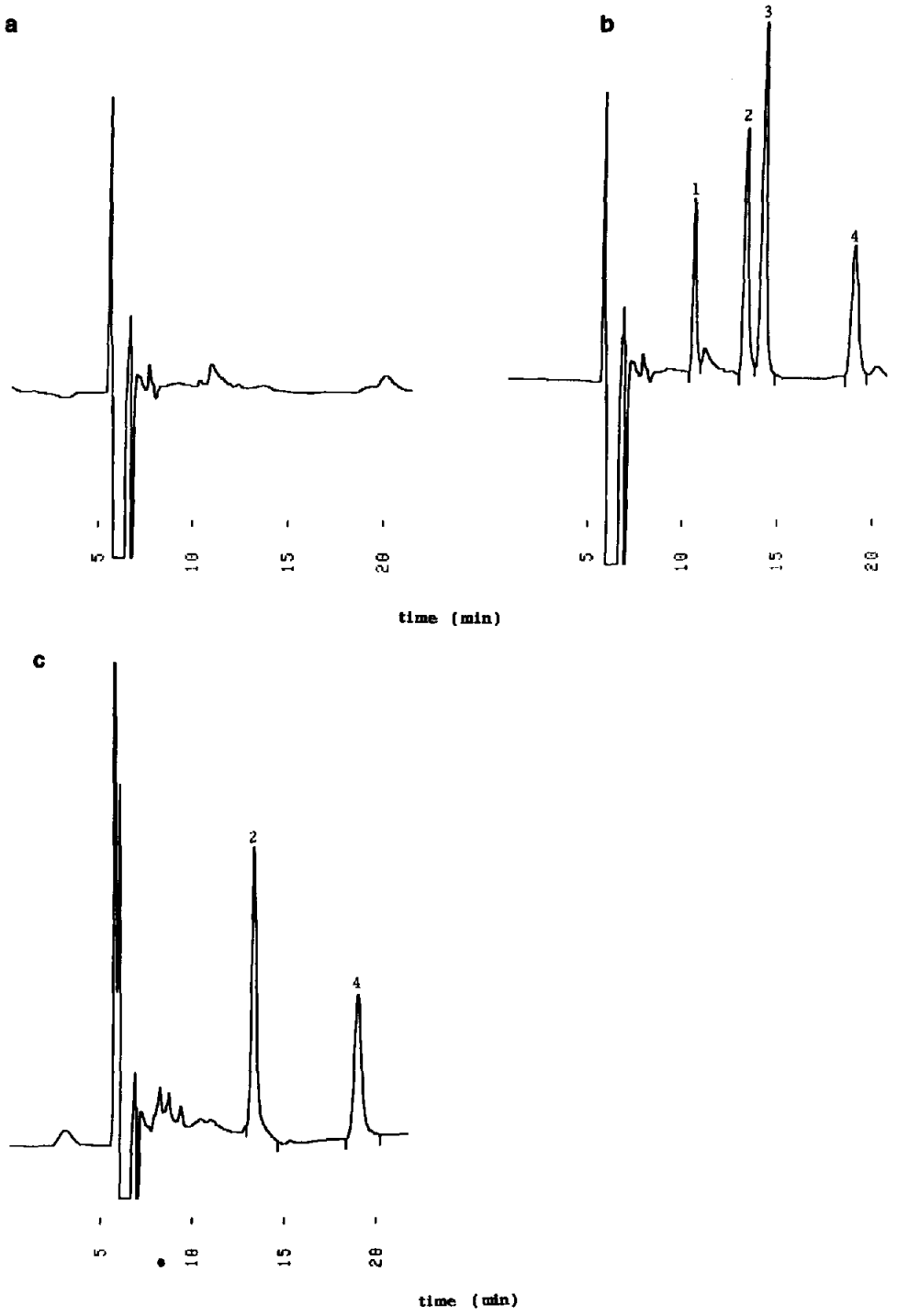

Fig. 1. Chromatograms of (a) blank plasma, (b) standard mixture of benzodiazepines in human plasma (each component *ca.* 250 ng/ml), (c) plasma sample from patient after oral administration of diazepam (DMD 322 ng/ml; diazepam 295 ng/ml). Isocratic elution. Peaks: 1 = desmethylclobazam; 2 = desmethyldiazepam; 3 = clobazam; 4 = diazepam.

RESULTS AND DISCUSSION

Chromatography and specificity

Fig. 1 shows chromatograms from (a) blank human plasma, (b) human plasma spiked with DMC, DMD, clobazam and diazepam, and (c) plasma of a patient after oral administration of diazepam (DMD 322 ng/ml; diazepam 295 ng/ml). The retention times of BZDs were 10.4, 13.1, 14.1 and 18.9 min, respectively (isocratic mode), and 17.6, 22.2, 22.8 and 25.8 min, respectively (gradient mode). The most common antiepileptic drugs (*i.e.* carbamazepine, diphenylhydantoin, ethosuximide, phenobarbital, primidone and valproic acid) do not interfere with this assay.

Calibration curve, reproducibility and recovery

The calibration curves of the four BZDs were linear from 50 to 1000 ng/ml with a correlation coefficient of 0.999 (Table II). The detection limits at a signal-to-noise ratio of 2 were 20 ng/ml for DMC and DMD, 15 ng/ml for clobazam and 25 ng/ml for diazepam.

Within-day and day-to-day coefficients of variation (C.V.) and recoveries are shown in Table III. The C.V. of peak areas are not statistically

TABLE II

LINEARITY OF THE ASSAY BY SPIKING A PLASMA POOL WITH SCALED AMOUNTS OF DMC, DMD, CLOBAZAM AND DIAZEPAM

Regression equations and correlation coefficients (r). y: peak areas; x: concentrations (ng/ml).

Drug	Regression equation	r
DMC	$y = 0.0120x - 0.1172$	0.999
DMD	$y = 0.0213x - 0.0602$	0.999
Clobazam	$y = 0.0204x - 0.0251$	0.999
Diazepam	$y = 0.0181x - 0.0604$	0.999

TABLE III

WITHIN-DAY, DAY-TO-DAY COEFFICIENTS OF VARIATION (C.V.s) AND RECOVERIES OF THE ASSAYS FOR DMC, DMD, CLOBAZAM AND DIAZEPAM

Drug	Level (ng/ml)	C.V. (%)		Recovery (%)
		Within-day ($n = 5$)	Day-to-day ($n = 7$)	
DMC	50	7.3	–	96.2
	100	2.5	7.0	93.8
	500	2.6	4.1	94.8
	1000	2.3	4.4	95.2
DMD	50	2.6	–	97.7
	100	3.0	6.7	97.5
	500	2.6	2.8	99.8
	1000	2.4	3.3	97.8
Clobazam	50	1.8	–	98.9
	100	2.6	9.0	98.9
	500	4.0	4.8	96.3
	1000	2.4	3.7	99.3
Diazepam	50	7.7	–	101.1
	100	3.7	12.0	97.6
	500	2.1	4.4	99.2
	1000	0.9	3.7	97.8

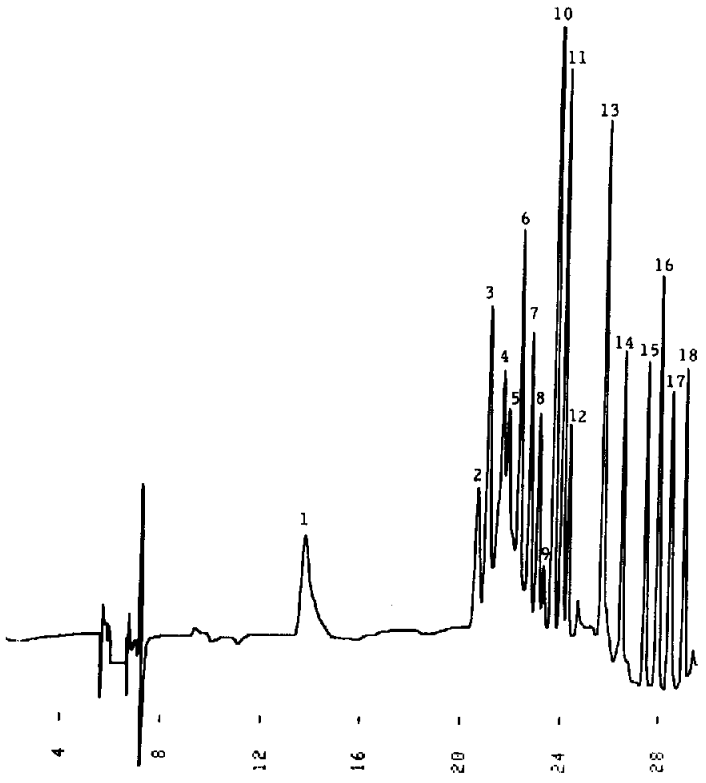

Fig. 2. Chromatogram of a standard mixture of benzodiazepines in human plasma (each component *ca.* 250 ng/ml). Peaks: 1 = bromazepam; 2 = nitrazepam; 3 = oxazepam, estazolam; 4 = desmethylclobazam; 5 = lorazepam, clonazepam; 6 = chlordiazepoxide; 7 = alprazolam; 8 = flunitrazepam, triazolam; 9 = temazepam; 10 = desmethyldiazepam, clorazepate; 11 = clobazam; 12 = tofisopam; 13 = diazepam; 14 = loflazepate; 15 = clotiazepam; 16 = tetrazepam; 17 = medazepam; 18 = prazepam.

different (paired Student's test, $p < 0.02$) from the C.V. of peak-area ratios (internal standard flunitrazepam). It is for this reason that the addition of an internal standard to the sample is not necessary.

The chromatographic theory and method development behind on-line pre-concentration or column-switching techniques have been well reviewed and described [25,26]. For the assay of tricyclic antidepressants, Matsumoto *et al.* [27] used a TSK precolumn PW that they considered superior to a bovine serum albumin-coated reversed-phase column used in their previous studies [28,29]. This precolumn proved to be equally satisfactory for the BZDs, and so a precolumn suitable for several different classes of drugs can be envisaged.

The technique presented is not necessarily better than methods already published. It is more adapted to the internal way of working in our laboratory where we have to determine, daily, a limited amount of different drugs (therapeutic drug monitoring or toxicology). The column-switching run is a constant one, but the analytical mobile phase has to be adapted for other drugs (other BZDs, tricyclic antidepressants, antifungal agents, antibiotics).

By using the technique presented here, the handling of the biological samples is avoided. It is also a technique that can be adapted perfectly to the therapeutic ranges of DMC, DMD, clobazam and diazepam in repeated administration of the parent drugs, but it might be insufficient after application of a single therapeutic dose. This

technique is easily automated, and a slightly different programme in the gradient mode (Table I) enables the separation and quantification of other BZDs. A representative chromatogram is shown in Fig. 2. Further investigations are in progress.

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