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Simultaneous determination of clobazam and its major metabolite in human plasma by a rapid HPLC method

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Abstract

A rapid and specific HPLC method has been developed and validated for simultaneous determination of clobazam, the anticonvulsant agent, and its major metabolite in human plasma. The sample preparation was a liquid–liquid extraction with tuloene yielding almost near 100% recoveries of two compounds. Chromatographic separation was achieved with a Chromolith™ Performance RP-18e 100 mm × 4.6 mm column, using a mixture of a phosphate buffer (pH 3.5; 10 mM)–acetonitrile (70:30, v/v), in isocratic mode at 2 ml/min at a detection wave-length of 228 nm. The calibration curves were linear ($r^2 > 0.998$) in the concentration range of 5–450 ng ml^{-1}. The lower limit of quantification was 5 ng ml^{-1} for two compounds studied. The within- and between-day precisions in the measurement of QC samples at four tested concentrations were in the range of 0.89–9.1% and 2.1–10.1% R.S.D., respectively. The developed procedure was applied to assess the pharmacokinetics of clobazam and its major metabolite following administration of a single 10 mg oral dose of clobazam to healthy volunteers.

Keywords: Clobazam; *N*-Desmethylclobazam; Plasma; Monolithic; Pharmacokinetics

1. Introduction

Clobazam (CLB) is a 1,5-benzodiazepine with anxiolytic and anticonvulsant properties and is used for sedation and as an antiepileptic drug, presenting some advantages over 1,4-benzodiazepines [1–4]. In other applications clobazam is used as a covering drug when there is a change in therapy. The drug's action is very quick (usually effective within a couple of hours but no longer than a few days) and the monitoring of the drug's haematic levels is of great clinical interest in order to determine its correct use. It is extensively metabolized in the liver and *N*-desmethylclobazam (NDCLB) is the major metabolite also possessing pharmacological profile similar to the parent drug (Fig. 1) [1]. Moreover, NDCLB is accumulated during long-term treatment achieving concentration levels up to 10-times greater than clobazam and therefore it may be an important factor in both therapeutic and toxic responses [1,5,6].

Several techniques have been reported for CLB and NDCLB quantification in biological fluids using gas chromatography (GC) [7–15] which is not routinely used in analysis of drugs, and high-performance liquid chromatography (HPLC) [16–28]. HPLC methods for CLB and NDCLB as for other benzodiazepines can determine low concentrations by optimizing variables such as sample volume, injection volume, detector sensitivity, detection wavelengths and other chromatographic conditions as well as application requirements [16–26]. Most all of the methods were based on the preliminary extraction of CLB from the sample using organic solvents. However, large (1 ml) sample volume and no internal standard [18], relatively high limit of detection (10–20 ng/ml) with interference problems [19] and no metabolite determination [26] are of those disadvan-

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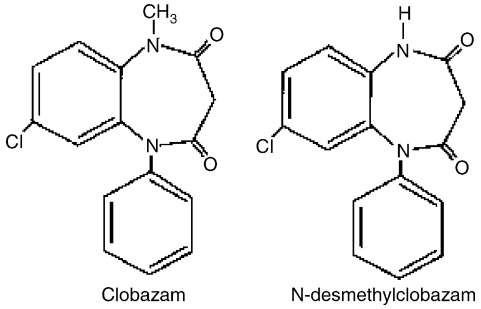

Fig. 1. Chemical structures of CLB and NDCLB.

tages. Direct injection HPLC reported method suffers from very high LOD of 500 ng/ml [27]. Bolner et al. reported a solid–liquid extraction method [28]. However no metabolite detection and relatively long run time is of their disadvantages. The limited flow rate applied to routine HPLC columns may result in long run time in most of reported methods. This may be resolved by using newly developed monolithic HPLC columns.

The Chromolith^{TM} column, as a monolithic HPLC column has silica rod composing the Chromolith^{TM} column with a biporous structure made up of macropores and mesopores, which offers 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 macropores and mesopores constitute a three-dimensional network such as coral for 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. In a recent study, Bugey and Staub [29] used a rapid HPLC method using a monolithic column for analysis of benzodiazepins but the procedure of extraction is still sophisticated and limit of detection is about 20 ng/ml.

Therefore, we have developed an HPLC method using a Chromolith^{TM} column for the quantitative determination of CLB and NDCLB. 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.

2. Experimental

2.1. Materials

The pure substances of clobazam and *N*-desmethylclobazam and alprazolam as internal standard were kindly supplied by Dr. Abidi pharmaceutical Co. (Tehran, Iran). HPLC-grade acetonitrile and toluene, and analytical grade monobasic potassium phosphate were supplied by Merck (Darmstadt, Germany).

2.2. Preparation of standard solutions

Stock solutions of all the compounds (1 mg/ml) were prepared in acetonitrile and were stable for 3 months when stored at 4 °C. Working solutions were prepared daily from these stock solutions by dilution with distilled water.

2.3. Apparatus and chromatographic condition

The chromatographic apparatus consisted of a low-pressure gradient HPLC pump, a UV variable-wavelength detector and an online degasser, all from Knauer (Berlin, Germany). A Rheodyne model 7725i injector with a 100 μl loop was used. The data was acquired and processed by means of ChromGate chromatography software (Knauer, Berlin, Germany).

Chromatographic separation was achieved by a Chromolith^{TM} Performance RP-18e 100 mm × 4.6 mm column (Merck, Darmastadt, Germany) protected by a Chromolith^{TM} Guard Cartridge RP-18e 5 mm × 4.6 mm.

For the mobile phase, a mixture of a phosphate buffer (pH 3.5; 10 mM)–acetonitrile (70:30, v/v), was delivered in isocratic mode at 2 ml/min at a detection wave-length of 228 nm.

2.4. Sample preparation

The preparation of plasma samples was by liquid–liquid extraction (LLE). The conditions consisted of mixing 0.5 ml of plasma with 20 μl alprazolam as internal standard (4 μg/ml) in a 2 ml Eppendorf polypropylene tube and then extracting with 1.5 ml of toluene. After vertical agitation (1 min) and centrifugation (10,000 rpm, 1 min), the upper organic layer was transferred into a conical tube and evaporated under a gentle stream of air. The dried extract was reconstituted in 150 μl of a mobile phase and a 100 μl aliquot was injected on to the HPLC system.

2.5. Preparation of calibration standards

Starting from pooled stock solution of CLB and NDCLB 1 mg/ml in acetonitrile, standards were prepared using pooled human drug free plasma obtained from healthy volunteers as diluent. The calibration curve was performed with standards of the final concentrations of 5, 10, 15, 25, 50, 100, 150, 200, 300 and 450 ng/ml in human plasma. Working solution of alprazolam (4 μg/ml in water) was prepared daily by dilution of stock solution (1 mg/ml in acetonitrile).

2.6. Accuracy, precision, limit of quantification (LOQ) and recovery

Accuracy, between- and within-day precisions of the method were determined for each compound according to FDA guidance for bioanalytical method validation [30]. Three replicate spiked serum samples were assayed between-

and within-day at four different concentrations (15, 100, 300 and 450 ng/ml) for each analyte. The concentrations were calculated using calibration curves prepared and analyzed in the same run. Accuracy was calculated as deviation of the mean from the nominal concentration. Between- and within-day precision were expressed as the relative standard deviation of each calculated concentration. For the concentration to be accepted as LOQ the percent deviation from the nominal concentration (accuracy) and the relative standard deviation has to be ±20% and less than 20%, respectively, considering at least five times the response compared to blank response. Average recovery of each compound was determined by comparing AUC obtained after injection of the processed QC samples with those achieved by direct injection of the same amount of drug in distilled water at different concentrations (three samples for each concentration level).

2.7. Application of the method

Fourteen volunteers were included in this study. The study protocol was approved by the Ethics Committee of Tehran University of Medical Sciences and written informed consent was obtained from the volunteers. Volunteers were not allowed to take any other medication for 2 weeks before and throughout the study. The volunteers received 10 mg CLB tablets as test formulation (formulated by Abidi Pharmaceutical Co.) or a 10 mg Frisium tablets as reference product (Aventis, UK) after an overnight fast in a double blind cross-over bioequivalence study. Intake of food was delayed for 3 h after medication. Peripheral venous blood samples were taken from each volunteer at predetermined intervals and plasma samples were stored at −20 °C until analysis.

2.8. Calculation of pharmacokinetic parameters

Plasma concentration–time curves of CLB and its metabolite were evaluated by non-compartmental analysis. Maximum plasma concentration C_{max} and the time to C_{max} (T_{max}) were obtained directly from the individual plasma concentration versus time curves. The terminal half-life, $t_{1/2}$ was obtained from log–linear regression analysis of the plasma concentration time curves in the terminal phase. The area under plasma concentration–time curve up to last quantifiable plasma concentration ($AUC_{lqc)}$ was determined according to the linear trapezoidal method.

3. Results

3.1. Selectivity and chromatography

The separation achieved using the experimental conditions of the present assay for CLB and its main metabolite are presented in Fig. 2. Selectivity was indicated by absence of any endogenous interference at retention times of peak of interest as evaluated by chromatograms of control human plasma and plasma spiked with two compounds. Retention times for CLB, NDCLB and IS were 2.4, 1.6 and 1.9 min, and their relative R.S.D.s (%) calculated from 25 consecutive injections were 5.2, 4.3 and 4.9, respectively. No change in column efficiency and back pressure was observed over whole study time.

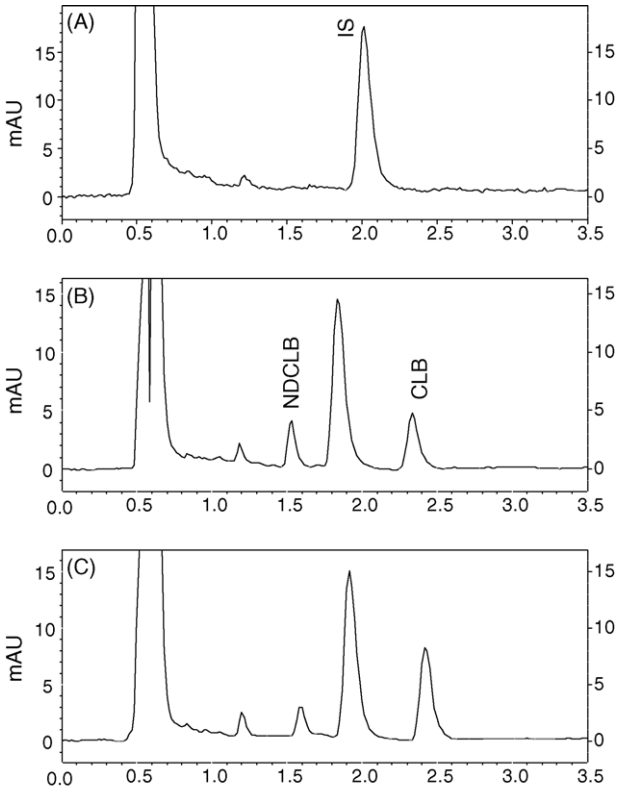

Fig. 2. Chromatograms of (A) spiked human plasma with IS, (B) plasma spiked with 75 ng/ml of clobazam and *N*-desmethylclobazam, and (C) plasma of the same person 10 h after oral administration of 10 mg clobazam (CLB = 125 ng/ml and NDCLB = 55 ng/ml).

3.2. Linearity

Ten point calibration curves for CLB and its metabolite on separate days were linear over the concentration range of 5–450 ng ml^{-1}. The equations for means ($n = 3$) of three standard curves are: for CLB, $y = 0.0004x - 0.0135$ ($r^2 = 0.998$); NDCLB, $y = 0.0076x + 0.0297$ ($r^2 = 0.998$). R.S.D. (%) val-

Table 1
Limit of quantitation (LOQ) for clobazam and *N*-desmethylclobazam ($n = 3$)

	Concentration (ng ml^{-1})	Between-day R.S.D. (%)	Accuracy (%)
Clobazam	5	5.8	88.1
N-Desmethylclobazam	5	2.9	92.3

Table 2
Between- and within-day variability, accuracy, and recovery for determination of clobazam and *N*-desmethylclobazam

Concentration (ng ml^{-1})	Between-day variability (n = 3)		Within-day variability (n = 3)		Recovery (n = 3)	
	R.S.D. (%)	Accuracy (%)	R.S.D. (%)	Accuracy (%)	%	R.S.D. (%)
Clobazam						
15	9.9	83.4	9.1	81.9	102.3	4.0
100	8.8	97.5	5.7	94.6	93.6	2.9
300	10.1	98.3	3.3	94.8	99.8	0.9
450	8.8	93.2	0.4	91.4	93.8	3.3
N-Desmethylclobazam						
15	4.5	92.3	12.6	103.8	93.5	6.3
100	2.1	102.3	7.6	100.2	95.0	3.4
300	8.6	100.7	0. 9	99.5	98.0	2.2
450	5.5	94.4	1.5	93.8	100.2	1.9

Table 3
Pharmacokinetic data (mean ± S.D.) obtained from healthy volunteers following oral administration of 10 mg clobazam (n = 14)

	T_{max} (h)	C_{max} (ng ml^{-1})	AUC_{lqc} (ng h ml^{-1})	$t_{1/2}$ (h)
Clobazam	1.4 ± 0.6	173 ± 33.3	340.7 ± 984.9	39.6
N-Desmethylclobazam	56.6 ± 17.8	26.8 ± 11.9	1362.2 ± 441.6	–

ues (slopes, intercepts) were (3.44, 13.5), (2.08, 12.5) for CLB and NDCLB, respectively.

3.3. Limit of quantification

LOQs as defined previously were 5 ng ml^{-1} for each compound. The LOQ values for two analytes are reported in Table 1.

3.4. Recovery, accuracy and precision

The results from the validation of the method in human plasma are listed in Table 2. The method proved to be accurate and precise: accuracy at four concentration levels ranged from 83.4 to 100.7% for all compounds. The within- and between-day precision ranged from 0.89 to 9.1% and 2.1 to 10.1%, respectively for all analytes. The absolute recoveries ranged from 93.5 to 102.3%.

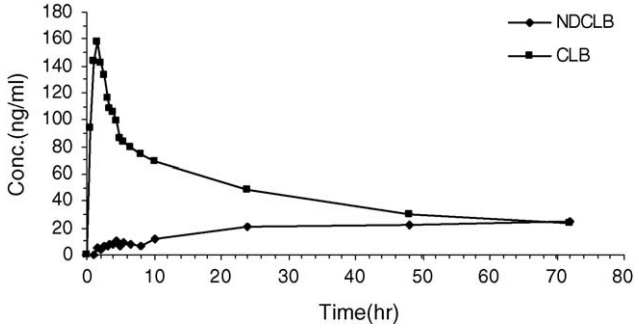

Fig. 3. Mean concentration–time profile of clobazam and *N*-desmethylclobazam after administration of a single 10 mg oral dose of clobazam to 14 volunteers.

3.5. Application of the method

To apply the developed and validated method, the pharmacokinetics of CLB and its metabolite was assessed in 14 volunteers. Plot of CLB and NDCLB mean plasma concentrations as a function of time following oral dosing is shown in Fig. 3.

The pharmacokinetic parameters of clobazam and its metabolite derived by non-compartmental analysis are summarized in Table 3.

4. Discussion

The described method was established as a rapid analytical tool in a pharmacokinetic study requiring short retention time, high precision, sensitivity and small volumes of plasma for analyses. The parameters of the assay obtained in the course of validation processes presented above in the results section were considered satisfactory for its clinical application. A simple analytical procedure based on one-step extraction and a total run time of 3.0 min allows the possibility of determination some 50 samples a day.

The procedure originally developed for CLB and its metabolite was found to be effective for benzodiazepine derivatives in general and it seems that the method may be easily adapted for such drug determinations.

5. Conclusion

This isocratic HPLC–UV method for clobazam and *N*-desmethylclobazam in human plasma may be fully recom-

mended for pharmacokinetic studies as well as for therapeutic drug monitoring.

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