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Simple and sensitive high-performance liquid chromatographic method for the determination of 1,5-benzodiazepine clobazam and its active metabolite *N*-desmethylclobazam in human serum and urine with application to 1,4-benzodiazepines analysis

Paweł K. Kunicki*

Clinical Pharmacology Unit, Department of Clinical Biochemistry, National Institute of Cardiology, Alpejska 42, 04-628 Warsaw, Poland

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Abstract

A HPLC–UV determination of clobazam and *N*-desmethylclobazam in human serum and urine is presented. After simple liquid–liquid extraction with dichloromethane the compounds and an internal standard diazepam were separated on a Supelcosil LC-8-DB column at ambient temperature under isocratic conditions using the mobile phase: CH_3CN–water–0.5 *M* KH_2PO_4–H_3PO_4 (440:540:20:0.4, v/v and 360:580:60:0.4, v/v for serum and urine, respectively). The detection was performed at 228 nm with limits of quantification of 2 ng/ml for serum and 1 ng/ml for urine. Relative standard deviations for intra- and inter-assay precision were found below 8% for both compounds for all the tested concentrations. The described procedure may be easily adapted for several 1,4-benzodiazepines.

Keywords: Clobazam; Benzodiazepines

1. Introduction

Clobazam (CLB) is a 1,5-benzodiazepine with anxiolytic and anticonvulsant properties and thus is used for sedation and as an antiepileptic drug, presenting some advantages over 1,4-benzodiazepines [1–4]. 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]. In our clinics CLB has been administered to patients premedicated before cardiopulmonary bypass surgery and we were interested in the effect of such a procedure on CLB pharmacokinetics and pharmacodynamics. Thus a sensitive method for the determination of CLB with NDCLB in serum and urine was necessary.

Several techniques have been reported for CLB and NDCLB quantification, using gas chromatography (GC) [7–15] and high-performance liquid chromatography (HPLC) [16–25]. These techniques have been reviewed [26–29]. HPLC methods for CLB and NDCLB as for other benzodiazepines can

*Tel.: +48-22-8153-413; fax: +48-22-8154-003.
E-mail address: biblnauk@ikard.waw.pl (P.K. Kunicki).

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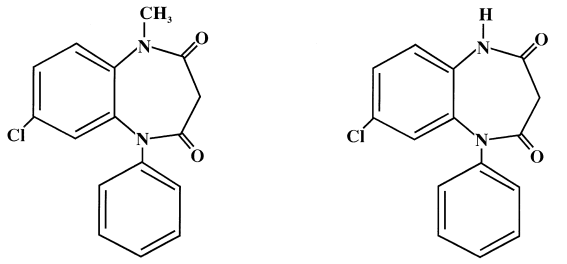

CLB **NDCLB**

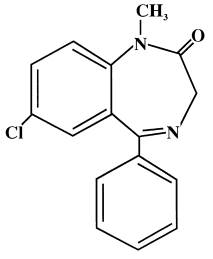

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Fig. 1. Chemical structures of clobazam (CLB), *N*-desmethylclobazam (NDCLB) and diazepam (I.S.).

determine low ng/ml concentrations by optimising variables such as sample volume, injection volume, detector sensitivity, detection wavelengths and other chromatographic conditions as well as application requirements [16–25,29–33]. A sensitive method for analysis of CLB and NDCLB in plasma and urine has been reported by Biehler et al. [18]. Unfortunately the extraction procedure involved large (1 ml) sample volume, no internal standard was used and there were no data describing precision and accuracy. Tomasini et al. [19] reported an assay for the determination of CLB, NDCLB and their hydroxy metabolites in plasma and urine but the limit of detection was set at 10–20 ng/ml for each compound with corresponding interference problems. A limit of detection of nearly 0.5 ng/ml for CLB was reported by Zilli and Nisi [30] in a method designed only for the parent drug in serum.

Therefore, despite the existence of some procedures for the analysis of CLB with NDCLB in plasma or serum no appropriate method for the sensitive determination in urine has been reported. This paper presents a simple and sensitive procedure for CLB and NDCLB determination in human serum and urine suitable for therapeutic drug monitoring, pharmacokinetic and bioavailability studies as well as for toxicological purposes.

2. Experimental

2.1. Chemicals

The pure substances of CLB and NDCLB were from Hoechst (Frankfurt, Germany). Diazepam, oxazepam, nitrazepam, lorazepam, medazepam, temazepam, clonazepam, flunitrazepam, estazolam, desmethyldiazepam, chlordesmethyldiazepam, lormetazepam were obtained from Polfa (Warsaw, Tarchomin, Poland).

Stock solutions of all the compounds (1 mg/ml) were prepared in acetonitrile and were stable for 3 months when stored at 4°C. HPLC-grade acetonitrile and dichloromethane were from Merck (Darmstadt, Germany), methanol, water, glycine and KH_2PO_4 were obtained from BDH (Poole, UK) and H_3PO_4 was from J.T. Baker (Deventer, The Netherlands). All other chemicals were of analytical grade. β-Glucuronidase type H-5 with sulfatase activity (EC 3.2.1.31) was from Sigma (St. Louis, MO, USA).

2.2. Chromatography

The HPLC isocratic system consisted of a pump (Model 2150, LKB, Bromma, Sweden), an injector with 50-μl loop (Model 7125, Rheodyne, Cotati, CA, USA), a UV detector (Spectra 100, Spectra-Physics, San Jose, CA, USA) and an integrator (Chrom Jet 4400, Spectra-Physics). The separation of compounds was made on a Supelcosil LC-8-DB (250×4.6 mm, 5 μm) column protected with Supelguard LC-8-DB (20×4.6 mm, 5 μm) pre-column (Supelco, Bellefonte, PA, USA) at ambient temperature. The mobile phase was CH_3CN–water–0.5 *M* KH_2PO_4–H_3PO_4 (440:540:20:0.4, v/v and 360:580:60:0.4, v/v for serum and urine, respectively) was pumped at a flow-rate of 1.5 ml/min. Detection was set at λ=228 nm.

2.3. Sample preparation

A 0.2-ml volume of serum or 0.4 ml of urine was transferred to a 15-ml Pyrex glass test tube, then mixed with 200 ng (20 μl) of diazepam methanolic solution (internal standard, I.S.) and with 50 μl (serum) or 200 μl (urine) of 0.5 *M* glycine solution. Next, dichloromethane (2 ml for serum or 4 ml for urine analysis) was added and the sample was vigorously shaken for 3 min. After centrifugation (3000 *g*) for 5 min and freezing at −20°C the organic layer was quantitatively transferred to a 10-ml Pyrex conical glass centrifuge tube and evaporated to dryness in a water bath at 37°C under a stream of argon. Then the dried extract was reconstituted in 100 μl of a mobile phase and a 50-μl aliquot was injected onto the column.

When enzymatic hydrolysis was carried out 0.4 ml of urine sample after addition of 0.2 ml of 0.5 *M* KH_2PO_4 was incubated with 100 μl (1000 units) of β-glucuronidase for 24 h in a water bath at 37°C and then treated as described above. These conditions guaranteed complete hydrolysis.

Working solutions for calibration and controls were prepared from the stock solutions by an adequate dilution in methanol. Working solutions were added to drug-free serum or urine to obtain the concentration levels of: 25, 100, 400, 1000, 3000 ng/ml for CLB and NDCLB in serum and: 20, 50, 200, 500, 1000 ng/ml for both compounds in urine. The following procedures were as described above for sample preparation. The method was fully validated.

3. Results

3.1. Separation

CLB, NDCLB and I.S. were well separated from the biological background under the described chromatographic conditions.

Analysis in serum: CLB, NDCLB and I.S. were separated at retention times of 6.6, 4.9 and 7.9 min, respectively (Fig. 2). The peaks were of good shape, completely resolved from one another. No interference with constituents from serum matrix was observed. The mobile phase used guaranteed good repeatability of retention times.

Analysis in urine: for urine analysis the modification of mobile phase composition was necessary because that established for serum did not allow to obtain satisfactory resolution of tested compounds from impurities extracted from urine. This was observed especially for NDCLB. By decreasing acetonitrile percentage simultaneously with the increase of buffer strength a new mobile phase was established. CLB, NDCLB and I.S. were separated at retention times of 11.7, 7.9 and 14.9 min, respectively (Fig. 3). The peaks were symmetrical and completely resolved.

Several 1,4-benzodiazepine derivatives were checked for possible interference with the method when both (serum and urine) mobile phases were in use. These drugs were eluted in retention times of 4.3–6.4 min (mobile phase for serum) and of 6.7–11.5 min (mobile phase for urine). The described analytical conditions for chromatographic separation ensured easy identification of CLB, NDCLB and diazepam in the tested benzodiazepines. Detailed information about retention data is given in Tables 1 and 2.

3.2. Wavelength optimisation

The criterion for analytical wavelength selection was maximal absorbance of CLB and NDCLB. That was checked experimentally with a series of injections of CLB, NDCLB and I.S. standards mixtures onto the column when other chromatographic conditions were settled. The detector response for CLB and NDCLB was the highest at 228 nm which is in good agreement with the findings of Hajdu et al. [8].

3.3. Calibration

The calibration curves were obtained by analysing four samples for each tested concentration. The curves were linear and described by following equations: $y=0.001219x+0.009153$ ($r=0.999987$) for CLB in serum, $y=0.001295x+0.006716$ ($r=0.999991$) for NDCLB in serum, $y=0.002406x-0.006256$ ($r=0.999969$) for CLB in urine, $y=0.002600x-0.007738$ ($r=0.999931$) for NDCLB in urine.

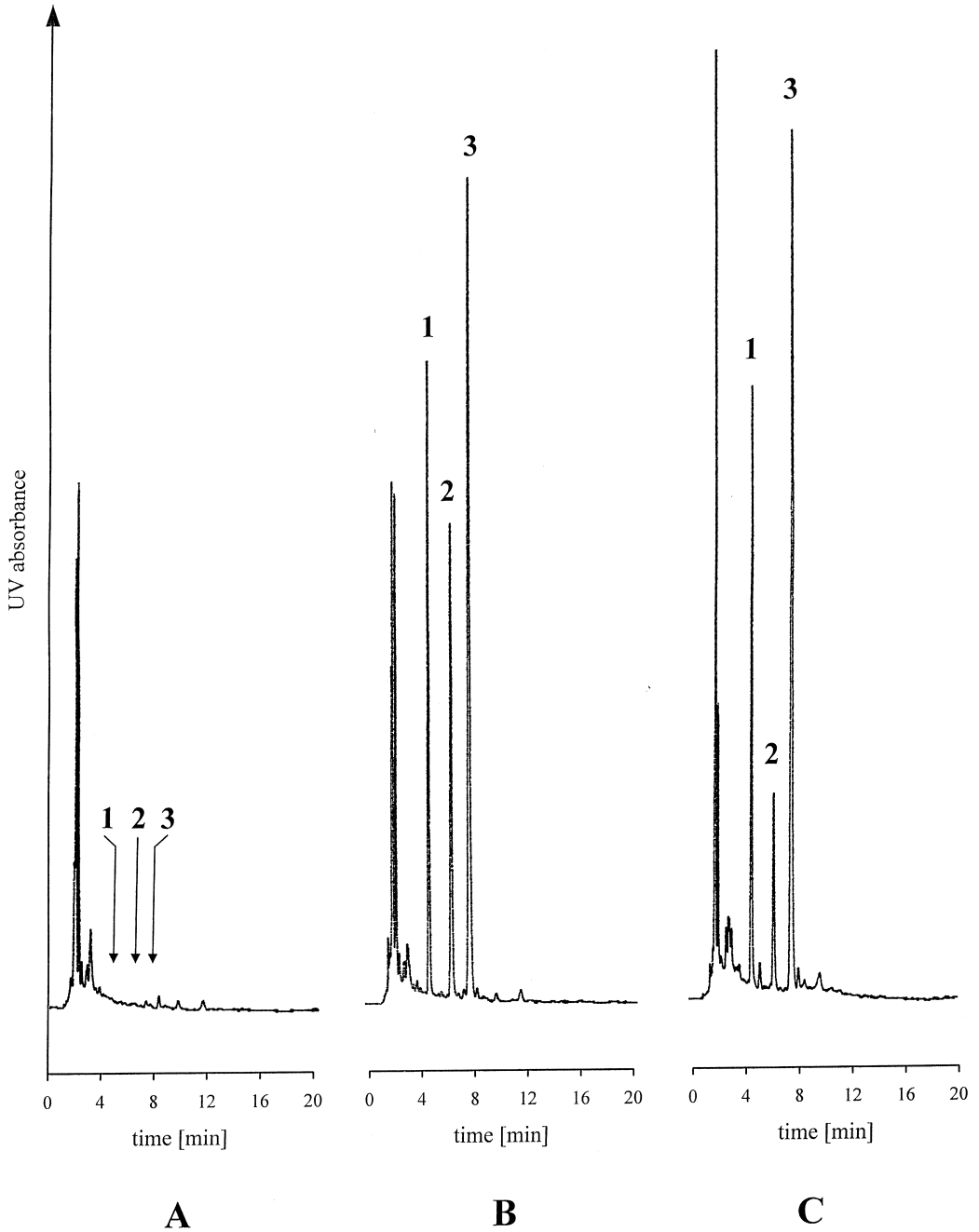

Fig. 2. Chromatograms of serum samples analysed as described in Experimental (attenuation 32): (A) drug-free serum, (B) drug-free serum spiked with CLB and NDCLB to obtain the concentrations of 400 ng/ml for both compounds, (C) serum sample taken from the patient treated with clobazam containing 150 ng/ml of CLB and 354 ng/ml of NDCLB. Peaks: CLB: 6.6 min (2), NDCLB: 4.9 min (1), I.S.: 7.9 min (3).

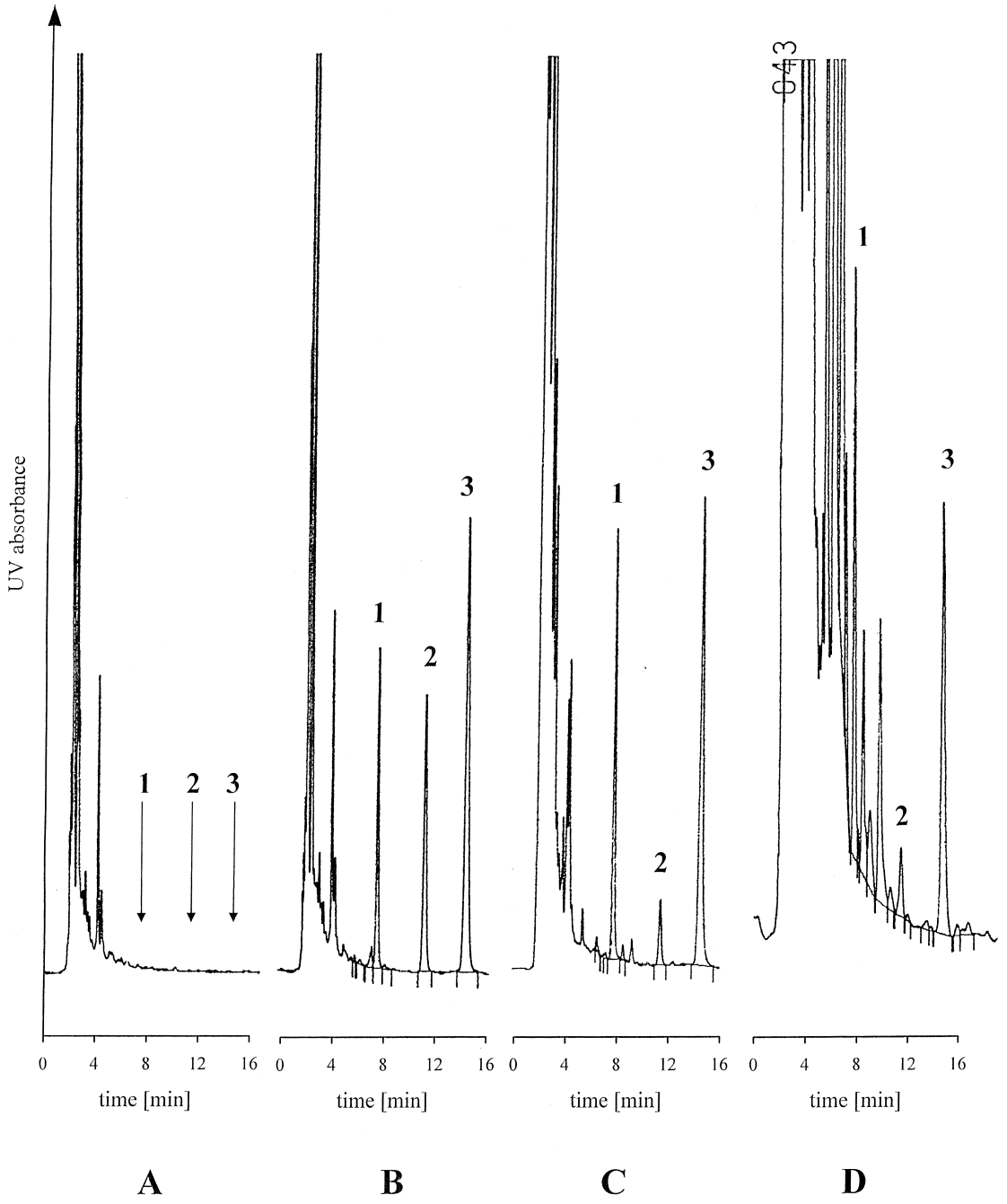

Fig. 3. Chromatograms of urine samples analysed as described in Experimental (attenuation 32): (A) drug-free urine, (B) drug-free urine spiked with CLB and NDCLB to obtain the concentrations of 200 ng/ml for CLB and 150 ng/ml for NDCLB, (C) urine sample taken from the patient treated with clobazam containing 49 ng/ml of CLB and 213 ng/ml of NDCLB, (D) urine sample after hydrolysis taken from the patient treated with clobazam containing 52 ng/ml of CLB and 292 ng/ml of NDCLB. Peaks: CLB: 11.7 min (2), NDCLB: 7.9 min (1), I.S.: 14.9 min (3).

Table 1
Extraction and retention data of 1,4-benzodiazepine derivatives from serum[a]

Compound	Yield of extraction (%)	Retention time (min)
Oxazepam	93.76±1.32	4.3
Nitrazepam	97.78±6.10	4.7
Lorazepam	86.02±3.36	4.7
Estazolam	91.16±0.82	5.1
Clonazepam	90.84±1.49	5.2
Desmethyldiazepam	95.33±1.46	5.5
Temazepam	87.85±2.96	5.9
Flunitrazepam	85.93±4.47	6.2
Chlordesmethyldiazepam	91.68±2.19	6.3
Lormetazepam	90.71±2.69	6.4
Medazepam	89.25±2.92	6.7

[a] Analytical conditions as described in Experimental. Yield of extraction is given as a mean±SD of three measures.

3.4. Extraction

Dichloromethane was chosen for simple liquid–liquid extraction because in preliminary findings it gave an extraction yield of about 100% for CLB, its metabolite and also for diazepam. The absolute extraction recovery was analysed by comparing the peak areas for extracted calibration standards with those obtained from direct injection of equivalent quantities of standards. Excellent recovery was achieved for all the compounds, i.e., serum: 101.23% (97.62–104.38) for CLB, 100.34% (97.32–103.47) for NDLCB; urine: 101.03% (99.10–102.02) for CLB, 102.77% (99.91–105.40) for NDLCB with stable recoveries for the concentrations covering the calibration ranges (data in parentheses). The internal standard diazepam was extracted in 96.26±4.53% (n=20) and in 100.44±3.56% (n=40) from serum and urine, respectively. A similar extraction yield has been reached by other authors using dichloromethane [17,21].

For checking the applicability of the extraction procedure for 1,4-benzodiazepine derivatives other than diazepam, the methanolic working solutions (2 μg/ml, corresponding to a drug serum concentration of 200 ng/ml and a drug urine concentration of 100 ng/ml) were prepared from standards of: oxazepam, nitrazepam, lorazepam, medazepam, temazepam, clonazepam, flunitrazepam, estazolam, desmethyldiazepam, chlordesmethyldiazepam, lormetazepam. These working solutions were added to drug-free serum and urine and the determinations were carried

Table 2
Extraction and retention data of 1,4-benzodiazepine derivatives from urine[a]

Compound	Yield of extraction (%)	Retention time (min)
Oxazepam	95.81±2.70	6.7
Nitrazepam	101.68±1.53	7.4
Lorazepam	91.68±2.47	7.5
Medazepam	88.15±0.91	7.6
Estazolam	102.01±4.51	8.2
Clonazepam	93.03±1.14	8.6
Desmethyldiazepam	100.48±6.36	9.4
Temazepam	97.46±3.79	9.9
Flunitrazepam	100.49±2.06	10.5
Chlordesmethyldiazepam	92.69±3.62	11.4
Lormetazepam	93.03±2.39	11.5

[a] Analytical conditions as described in Experimental. Yield of extraction is given as a mean±SD of three measures.

out in triplicate essentially as described above for sample preparation. The extraction was found to be efficient for all the compounds ranging from 85.93 to 97.78% when serum was used and even higher, i.e., 88.15 to 102.01% for urine analysis. All those drugs were slightly better extracted from urine (except medazepam 88.15% vs. 89.25%) than from serum. Detailed data are presented in Tables 1 and 2.

3.5. Validation

The precision of the method was examined using the data from calibration for intra-assay and analysing standard samples in duplicate on 4 different days for inter-assay precision. The relative standard deviation (RSD) for CLB and NDCLB determinations was always below 8%. The accuracy computed from intra-assay data as well as detailed information on precision is included in Tables 3 and 4.

The limit of detection (signal-to-noise ratio 3:1) was experimentally set at 0.1 ng of CLB and NDCLB standards injected onto the column, which corresponds to 1 ng/ml of serum (0.5 ng/ml of urine) sample. The limit of quantification was also taken experimentally and set at 2 ng/ml for serum analysis and at 1 ng/ml for the determinations in urine. The range was then 2–3000 ng/ml for CLB and NDCLB in serum and 1–1000 ng/ml for both compounds in urine.

3.6. Clinical application

The method was successfully used in testing clobazam pharmacokinetics in patients undergoing cardiopulmonary bypass surgery. Typical concentration–time courses of CLB and NDCLB are presented in Fig. 4.

4. Discussion

The described method was established as an analytical tool in a pharmacokinetic study requiring high precision, sensitivity and small volumes of blood 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 allows the possibility of determination some 30 samples a day even without an autosampler.

The assay of a urine sample certainly takes longer than that of a serum sample but only partially due to chromatographic separation. CLB and NDCLB are excreted via the kidney unchanged and also (mainly

Table 3
Precision and accuracy of CLB and NDCLB determinations in serum[a]

	Concentration added (ng/ml)	Intra-assay		Inter-assay			
		Factor determined[b]	RSD (%)	Factor determined[b]	RSD (%)	Concentration determined (ng/ml)	Accuracy (%)
CLB	25	0.0316±0.0017	5.23	0.0313±0.0025	8.00	25.00	100.00
	100	0.126±0.0029	2.34	0.123±0.0045	3.70	97.34	97.34
	400	0.507±0.0107	2.11	0.482±0.0176	3.65	387.85	96.96
	1000	1.235±0.0182	1.47	1.181±0.0187	1.58	961.17	96.12
	3000	3.664±0.0216	0.59	3.590±0.0467	1.30	2937.02	97.90
NDCLB	25	0.0330±0.0024	7.40	0.0304±0.0017	5.58	21.54	86.17
	100	0.135±0.0040	2.98	0.128±0.0074	5.78	96.30	96.30
	400	0.524±0.0096	1.83	0.507±0.0225	4.45	386.39	96.60
	1000	1.313±0.0100	0.76	1.240±0.0255	2.06	952.50	95.25
	3000	3.888±0.0189	0.49	3.752±0.0472	1.26	2892.55	96.42

[a] The data for precision are expressed as a mean±SD of four measures. Concentration determined was calculated using calibration curve data.

[b] Factor determined – the ratio of peak area of analysed compound to peak area of internal standard.

Table 4
Precision and accuracy of CLB and NDCLB determinations in urine[a]

	Concentration added (ng/ml)	Intra-assay		Inter-assay			
		Factor determined[b]	RSD (%)	Factor determined[b]	RSD (%)	Concentration determined (ng/ml)	Accuracy (%)
CLB	20	0.0495±0.0015	3.06	0.0486±0.0025	5.24	20.09	100.44
	50	0.117±0.0024	2.09	0.120±0.0046	3.82	50.73	101.46
	200	0.468±0.0143	3.06	0.482±0.0146	3.04	202.95	101.47
	500	1.187±0.0196	1.65	1.206±0.0155	1.29	503.85	100.77
	1000	2.406±0.0247	1.03	2.420±0.0534	2.21	1008.40	100.84
NDCLB	20	0.0562±0.0037	6.66	0.0549±0.0023	4.18	19.59	97.95
	50	0.129±0.0071	5.52	0.130±0.0034	2.64	50.27	100.55
	200	0.497±0.0147	2.96	0.517±0.0145	2.81	201.85	100.92
	500	1.281±0.0285	2.23	1.292±0.0139	1.07	499.89	99.98
	1000	2.600±0.0156	0.60	2.610±0.0662	2.54	1006.76	100.68

[a] The data for precision are expressed as a mean±SD of four measures. Concentration determined was calculated using calibration curve data.
[b] Factor determined – the ratio of peak area of analysed compound to peak area of internal standard.

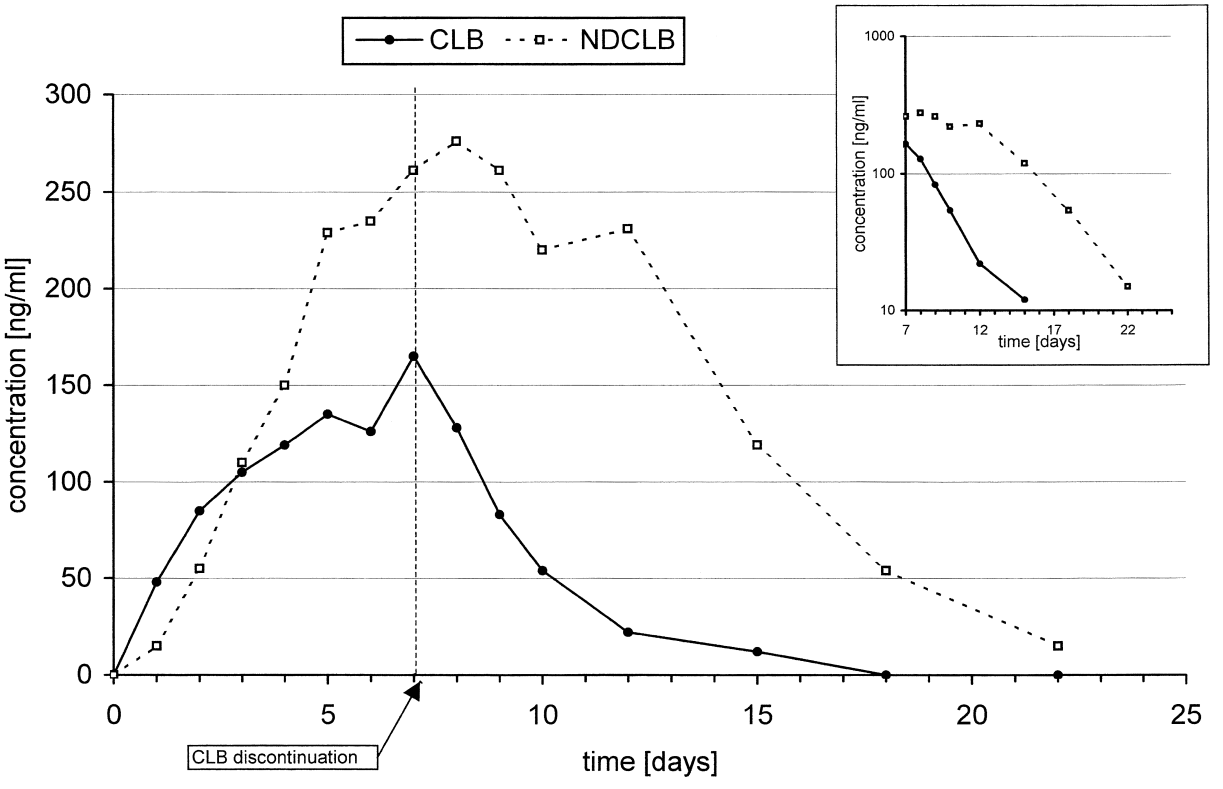

Fig. 4. An example of the concentration–time curve for clobazam elimination from serum taken from a patient (56 years, 68 kg, male) treated orally with CLB 5 mg b.i.d. for 7 days (the last two doses were doubled) as premedication before cardiopulmonary bypass surgery. Drug discontinuation was shown using dotted line marked by an arrow what also indicates the start of surgery.

metabolite) in conjugated forms, so urine enzymatic hydrolysis with β-glucuronidase sulfatase was necessary for complete description of elimination processes. Therefore the method was also applied to urine samples preincubated with the enzyme. Consecutive analytical steps were exactly the same as for untreated urine. The probes after hydrolysis were, as expected, much more ''dirty'' and in some of them peaks co-eluting especially with NDCLB and CLB were observed, nevertheless there was no problem with quantification (Fig. 3).

The procedure originally developed for CLB and NDCLB was found to be effective for benzodiazepine derivatives in general – it seems that the method may be easily adapted for such drug determinations. The limit of quantification for those compounds being under these conditions about 10 ng/ml may be improved by selection of the best analytical wavelength and elevation of sample volume. Undoubtedly, the mobile phase composition should be optimised for each particular drug but it does not seem to be a problem from an analytical point of view.

The very low threshold for CLB and NDCLB quantification is of great importance because in many patients parent drug and metabolite were completely eliminated from serum and urine during the sampling period. Thus the concentration values were often below 10 ng/ml.

In conclusion, this isocratic HPLC–UV method for clobazam and *N*-desmethylclobazam in human serum and urine may be fully recommended for pharmacokinetic studies as well as for therapeutic drug monitoring.

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