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Note

Micro-determination of clobazam and N-desmethylclobazam in plasma or serum by electron-capture gas chromatography

N.R. BADCOCK* and G.D. ZOANETTI

Department of Chemical Pathology, Adelaide Children's Hospital, King William Road, North Adelaide, S.A. 5006 (Australia)

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Clobazam is a 1,5-benzodiazepine believed to have a high therapeutic index and a low incidence of side effects [1–2]. Metabolism [3] involves both dealkylation, to the pharmacologically similar N-desmethylclobazam (DMC) [4], and hydroxylation, principally to 4′-hydroxyclobazam and 4′-hydroxy-N-desmethylclobazam: information about the pharmacological efficacy of the hydroxylation products has not been published. Because of large inter-subject variability [5,6] and the demonstrated relationships in adjunctive therapy between clobazam [7,8] and DMC [9] concentrations and seizure control, clinical monitoring of plasma levels would be helpful to establish better dosage guidelines for this drug. This is particularly important in children and the elderly, who commonly demonstrate drug disposition characteristics that differ from those of adults [10]. Whilst several methods for quantitating clobazam and its active metabolite have been described involving gas chromatography (GC)–mass spectrometry [11,12] and GC with flame-ionization [13] or thermionic detection [11,14,15–17], the low therapeutic concentration of the parent drug generally limits routine analysis and pharmacokinetic studies of clobazam on capillary samples to GC with electron-capture detection [18–21].

Previous GC assays with electron-capture detection, however, have problems and disadvantages. All but one method, that of Riva et al. [21], employ other widely prescribed benzodiazepines as internal standards. In all but one, the authors examined a limited number [18], or no drugs at all [19,20], for possible interference. None of these methods examined possible interference from the two major hydroxy metabolites. Most employ phenyl silicone stationary phases such as OV-101 [20], OV-17 [18] or OV-25 [19] which, because of difficulty in achieving

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complete deactivation and despite column priming [20], elute DMC with prolonged tailing of the peak. This makes quantitation difficult and reduces sensitivity. Riva et al. [21] attempt to overcome the problem by employing the column packing SP-2510 DA, which incorporates a special deactivating process to prevent tailing: however, DMC (a basic compound) shows indications of irregular adsorption to this stationary phase, and cannot be measured concomitantly. This is clearly unsatisfactory as it is likely that the anti-epileptic effect of clobazam on chronic administration can be attributed more to DMC than to the parent compound [9,22]. In addition, sensitivity is poor since clobazam is eluted high on the down front of the solvent peak. In all of these procedures, the sample volume requirement is relatively large (0.5–1.0 ml), which is a decided disadvantage in cases of severe sample limitation, for example in young children on whom frequent other tests may also be required. Another disadvantage is the use of highly toxic extractants such as benzene [19–21] and toluene [18]. All of these factors limit the usefulness of the procedures for clinical laboratories.

By using a recently described GC column packing [23], 100 μl plasma, and a much less toxic solvent than used previously, we have developed a rapid, sensitive and selective assay for clobazam and its active metabolite which is equally amenable to either routine clinical use or pharmacokinetic studies. We have used this method to investigate the stability of the drug in plasma and have also examined, for the first time, the relationship between paired serum and plasma samples and interference to the assay from the hydroxylation products.

EXPERIMENTAL

Reagents and glassware

All reagents are analytical grade. Glass centrifuge tubes, 10 ml (Quick-fit® BC24/C14T) complete with glass stoppers, were obtained from J. Bibby Science Products (Stone, Staffordshire, U.K.). Clobazam (7-chloro-1-methyl-5-phenyl-1H-1,5-benzodiazepine-2,4(3H,5H)-dione), DMC, 4′-hydroxyclobazam and 4′-hydroxy-N-desmethylclobazam were obtained from Hoechst Australia. The stock clobazam solution, 200 μmol/l (60.1 mg/l) in absolute ethanol, is stable for at least six months at 4°C. The working clobazam solution, 1.0 μmol/l in absolute ethanol, is prepared fresh on the day of analysis. The stock DMC, 200 μmol/l (57.3 mg/l) in absolute ethanol, is stable for at least six months at 4°C. The working DMC, 5.0 μmol/l in absolute ethanol, is prepared fresh on the day of analysis.

To prepare the quality control (QC) serum (drug-free pooled human serum containing added clobazam and DMC) rapidly stir 100 ml of serum and slowly add about 6.0 mg and 28.7 mg of clobazam and DMC, respectively; continue stirring the mixture for 1 h. Dilute 500 μl to 100 ml with serum and store in 0.2 ml aliquots at −20°C; this is stable for at least five months.

Methylclonazepam was obtained from Roche Products (Sydney, Australia). The stock methylclonazepam internal standard solution, 100 μmol/l (33.0 mg/l) in absolute ethanol, is stable when stored in amber bottles at 4°C for at least

twelve months. The working methylclonazepam internal standard solution, 200 nmol/l in ethyl acetate is prepared fresh on the day of analysis.

To a 10-ml glass Quickfit centrifuge tube add 100 μl of plasma or serum. At the same time prepare reagent blank, control (QC) and standard tubes. In the standard tubes, place 10, 50, 100, 200 and 300 μl of clobazam and DMC working solutions and evaporate to dryness at 40°C in a stream of dry nitrogen. To the blank and standards add 100 μl drug-free pooled human serum; to the control add 100 μl QC serum.

To each tube add 1 ml of working internal standard solution, stopper and vortex-mix for 60 s; centrifuge at 2000 *g* for 1 min. Transfer the supernatant to another 10 ml Quickfit centrifuge tube and evaporate to dryness as described above. Dissolve the residue in 50 μl of ethyl acetate and vortex each tube for 10 s.

Gas chromatography

GC analysis is performed using a Varian Aerograph Series 1440 gas chromatograph equipped with ^{63}Ni electron-capture detector (8.5 mCi) and a 0.9 m×2 mm I.D. silanised glass column packed with 3% CP-Sil 34 on Chromosorb G HP, 100–120 mesh, with a 5-cm pre-column of 1% CP-Sil 5 on Chromosorb G HP, 100–120 mesh (Chrompack, Middelburg, The Netherlands). Silane-treated glasswool plugs are of minimal size and length . The instrument is operated isothermally with the oven, detector and injection port temperatures at 255, 300 and 280°C, respectively. The carrier gas (nitrogen) flow-rate is 60 ml/min. Gas lines are fitted with filters containing molecular sieves (15A) and the electrometer range is 10^{-10} A/mV with attenuation of 4. The chromatogram is recorded on a Linear 361 recorder set at 1 mV full scale.

Inject 5 μl of each resulting solution into the chromatograph. Under the above conditions, the retention time for the internal standard is 7.8 min, for clobazam 4.2 min and for DMC 9.7 min. The ratio of peak heights of clobazam and DMC to methylclonazepam is calculated and the value of QC and unknown specimens calculated by direct proportion.

RESULTS AND DISCUSSION

Selectivity and precision

Interference from drugs encountered as comedication was studied. Phenobarbital, pentothal, carbamazepine, 5,5-diphenylhydantoin, di-*n*-propylacetate, ethosuximide, primidone, diazepam, nitrazepam, lorazepam, temazepam, prazepam, chlordiazepoxide, flurazepam, flunitrazepam, alprazolam, sulthiame, lamotrigine, paracetamol, pseudoephedrine, chlorpheniramine, sodium cromoglycate, theophylline, salbutamol, dexamethasone, prednisone, erythromycin, ampicillin, benzylpenicillin, gentamycin, amoxycillin and bactrim were dissolved in water at concentrations above their upper therapeutic limits. Extractions and GC determinations were performed as described. Only lamotrigine was found to interfere with the analysis. It is eluted as a symmetrical peak with a retention time of 7.4 min and makes quantitation difficult. However, lamotrigine is an investigational anticonvulsant and, of the 320 patient samples analysed by the procedure described

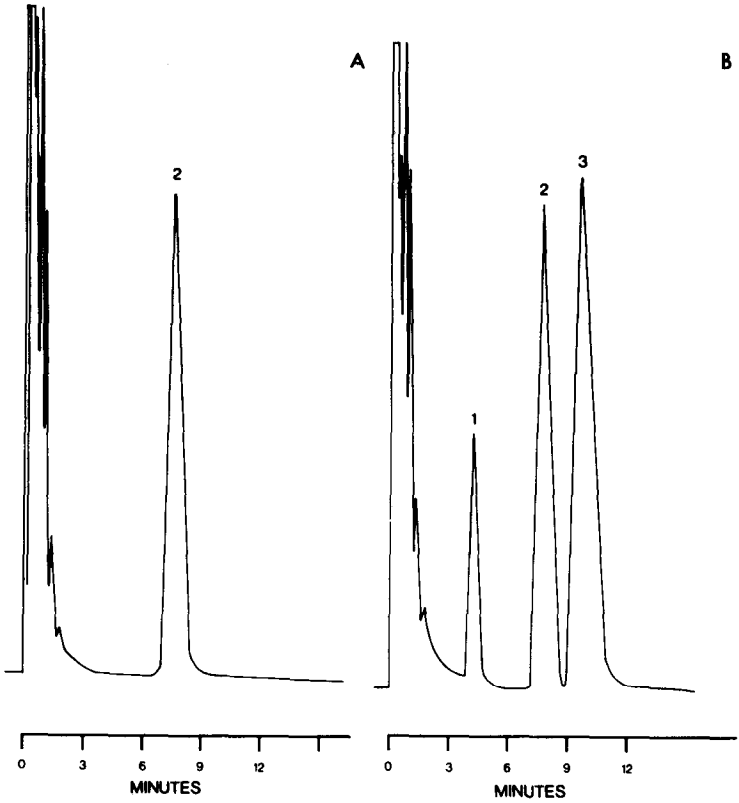

Fig. 1. Gas chromatograms of (A) extract of drug free plasma, (B) extract of plasma sample containing clobazam 0.7 μmol/l and DMC 3.3 μmol/l. Peaks: 1 = clobazam; 2 = methylclonazepam; 3 = DMC.

above, only one contained lamotrigine as a comedication. Both major hydroxylated metabolites had not been eluted after 30 min. Extracts of plasma samples from patients on diazepam, clonazepam, nitrazepam, phenobarbital, carbamazepine, 5,5-diphenylhydantoin, ethosuximide, primidone, di-*n*-propylacetate and sulthiame showed no interfering peaks. Lignocaine (sometimes found in samples as an artifact following its use as a local anaesthetic during multiple blood collections, especially during pharmacokinetic studies) also did not interfere in the determinations. Plasma components do not interfere and neither plasma adjusted to a particular pH [1,18,19,21], nor multiple extractions [18,19] are required.

Fig. 1 shows a chromatogram of blank plasma and a chromatogram of plasma from a patient being treated with clobazam. Detection limit, defined as a signal twice the height of the noise level, was approximately 7 nmol/l for clobazam and 15 nmol/l for DMC. Concentration and peak height were linearly related throughout the concentration range investigated; 0.05–3.0 μmol/l for clobazam and 0.5–20.0 μmol/l for DMC. This adequately covers the therapeutic range of the drug in adjunctive therapy [0.15–1.0 μmol/l (0.05–0.3 μg/ml) for clobazam, 2.8–14.0 μmol/l (0.8–4.0 μg/ml) for DMC [24]]. The regression equations obtained from data on the five standards in plasma were, for clobazam

TABLE I

PRECISION OF ASSAY OF CLOBAZAM AND DMC

	Clobazam (mean ± S.D.) (μmol/l)*	C.V. (%)	DMC (mean ± S.D.) (μmol/l)**	C.V. (%)
Within-day ($n=10$)	0.098 ± 0.005	4.8	2.502 ± 0.105	4.2
	1.013 ± 0.031	3.1	10.007 ± 0.290	2.9
Between-day ($n=21$)	0.990 ± 0.041	4.1	4.997 ± 0.185	3.7

*To convert μmol/l to μg/ml multiply by 0.3007.
**To convert μmol/l to μg/ml multiply by 0.2867.

$y=0.7304x-0.0049$ ($r=0.9999$); for DMC $y=0.3199x-0.0085$ ($r=0.9995$). Absolute recoveries of clobazam and DMC at the minimum and maximum of the concentration range investigated varied from 98 to 102%; the correlation coefficient of 0.990 ($n=15$) for clobazam and of 0.993 ($n=16$) for DMC determinations on paired plasma and serum samples shows that the results are interchangeable. The within-day reproducibility of the method was obtained with replicate plasma standards containing 0.1 and 1.0 μmol/l clobazam and 2.5 and 10.0 μmol/l DMC. The between-run reproducibility of the assay was calculated over a period of five months on aliquots of plasma having a concentration of 1.0 and 5.0 μmol/l clobazam and DMC, respectively. The results are summarised in Table I.

Column operation

Difficulties we encountered in preliminary studies, when using recommended phenyl silicone phases OV-101 [20], OV-17 [18] and OV-25 [19], were due to the low concentrations of the drug in plasma and adsorption onto the chromatographic column packing despite priming of the column before and during analysis to deactivate sites. Tailing of the metabolite peak, similar to that apparent in previously published chromatograms, was also a problem and peak area rather than peak height measurement was necessary to compensate variability in peak shape.

These problems were overcome by using a new deactivated column packing 3% CP-Sil 34, a phenyl silicone/polyethylene glycol copolymer especially developed and tested for anti-epileptic drugs, in combination with a pre-column and minimal silane-treated glass wool plugs. With this system, peak symmetry, especially for DMC, is substantially improved. The more symmetrical peaks allow quantitation by peak height and with the improved detector response, from lower column adsorption, the sensitivity for analysing subtherapeutic concentrations is increased, particularly in capillary samples. The solvent and clobazam peaks are well separated. This is in contrast to that indicated by Riva et al. [21], and is achieved without using high (10%) liquid phase loadings, which, when combined with a column temperature of 265 °C as recommended by Greenblatt [20], reduces

electron-capture detector sensitivity. No priming is required [20] and there are no artifactual peaks to interfere with quantitation [17].

Whilst the liquid phase CP-Sil 34 adequately resolves all three peaks of interest, its low temperature limit (265°C) restricts injector temperature. Adding a short pre-column of the more thermally stable liquid phase CP-Sil 5 (temperature limit 325°C) overcomes this problem. The pre-column packing deteriorates after approximately 500 injections of plasma extracts, resulting in loss of sensitivity and alteration of peak shape. Original performance is easily restored by repacking the 5-cm pre-column; the glass wool barrier between the two sections enables rapid and consistent replacement.

Oxazepam, lorazepam, temazepam, flunitrazepam, diazepam and N-desmethyldiazepam, traditionally early eluted 1,4-benzodiazepines, produce symmetrical peaks which are completely resolved both from the solvent front and from one another when injected onto the clobazam column. In principle then, our assay procedure can be used to quantitate other benzodiazepines and we have, performing extractions and GC analysis as described above, also determined oxazepam, lorazepam, diazepam and N-desmethyldiazepam levels; late eluted benzodiazepines such as alprazolam, nitrazepam and clonazepam have retention times on this column that are too long for their analysis to be practicable.

Extraction conditions

Many previously published assay methods have used benzene to extract clobazam from biological fluids. Benzene, however, is very toxic and its use should be prohibited and a substitute found whenever possible. Isopentyl alcohol has been added to benzene to minimise adherence to glassware [20]. Another extractant commonly chosen has been diethyl ether [17,22,25] which is also extremely dangerous because of its explosive and flammable nature. Chloroform [26] and dichloromethane [27] have also been used but require removal of the top (aqueous) layer before transfer and care is required to avoid contamination; emulsions can also cause problems.

We examined various solvents, together with extraction time and pH. Extractants chosen for investigation were immiscible with and less dense than plasma to provide for efficient removal of the solvent after extraction, were relatively non-hazardous and dissolved clobazam and DMC. They included ethyl acetate, ethyl acetate–isopentyl alcohol (98.5:1.5, v/v), ethyl acetate–cyclohexane (4:1, v/v), ethyl acetate–dichloromethane (4:1, v/v), *n*-butyl acetate, xylene and xylene–isopentyl alcohol (98.5:1.5, v/v). All solvents extracted parent drug and metabolite with greater than 95% efficiency at a plasma to solvent ratio of 1:10 and at the normal pH of plasma and no significant variation or improvement in analytical recovery occurred when isopentyl alcohol was included or mixing was continued for longer than 60 s. All extractants showed negligible interference from plasma components and gave emulsion-free extracts: the single solvent ethyl acetate was chosen because of its universal availability and relatively low boiling point.

Similar recoveries of clobazam were obtained with and without buffer (pH 3–9); recoveries of DMC were pH dependent and maximal at pH >6. For this

reason a buffer solution was not included in the extraction, but may be necessary if the pH of the plasma sample were to greatly exceed normal values (e.g. in some post mortem specimens).

Stability in plasma

The photochemical decomposition and temperature decay of 1,4-benzodiazepines in biological fluids has been reported previously [28,29]; Greenblatt [20], however, found plasma samples from patients receiving clobazam were stable at room temperature for at least 24 h and not sensitive to ambient light or to temperature $< 40\,^{\circ}C$. In order to test whether clobazam and its active metabolite were stable under conditions previously shown to cause decomposition of 1,4-benzodiazepines, we exposed five plasma and five serum samples (which had been collected from patients receiving clobazam on a chronic basis) to light at room temperature for 72 h. When replicate analyses on fresh and stored samples were performed and the resultant data subjected to statistical analysis as outlined by Timm et al. [30], no significant difference in clobazam or DMC concentration was found between samples. The presence of the more stable carboxamide group in place of the readily hydrolysed imine group found in the 1,4-benzodiazepines would be an appropriate explanation for these stability differences and would be consistent with the observation that open-ringed molecules have been detected among the metabolites of some 1,4-benzodiazepines [31], whereas the metabolites of clobazam all retain the diazepine ring [3].

Practical applications

Using the above procedure, we have determined clobazam and DMC levels in a number of children and adults who have been treated with clobazam for the control of seizures. Concentrations in the epileptic child at steady state have been similar to those in adult patients and have ranged from 0.1 to 2.5 μmol/l for clobazam and from 0.9 to 15.7 μmol/l for DMC. These concentrations are of the same order as those reported previously in studies of adult patients [3,6,8]; this suggests agreement between methods and independence of age on levels. However, we failed to find, particularly in children at steady state, the same apparent consistency in the ratios between parent drug and metabolite as has been reported previously in adults [8,14,17,27], with ratios from our quantitations varying from 1:1 up to 1:150. The fact that increasing control has been shown to be associated with more rapid metabolism of the drug [8] might suggest a therapeutic significance for the parent drug to metabolite ratio; we are currently engaged upon the assessment of the relevance of this relationship.

In conclusion, we believe that the procedure described offers significant improvement in peak shape, peak resolution, selectivity and choice of both extractant and internal standard over other GC assays which employ electron-capture detection. With its simplicity, rapidity, sensitivity and small volume of plasma or serum required, clobazam and DMC levels can be routinely monitored as frequently as other anti-epileptic drugs.

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