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MICRO-DETERMINATION OF CLONAZEPAM IN PLASMA OR SERUM BY ELECTRON-CAPTURE GAS—LIQUID CHROMATOGRAPHY

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SUMMARY

A rapid method is described for the electron-capture gas chromatographic determination of clonazepam in plasma or serum using methyl-clonazepam as an internal standard. The analysis is performed isothermally on the silicone stationary phase SP-2510DA (Supelco). With this liquid phase, gas chromatographic properties are comparable to methods involving acid hydrolysis or derivatisation. A short pre-column containing another phase is added to enhance resolution. The method involves a single extraction, requires 100 μl of sample and has a detection limit of 3 nmol/l. Response is linear at concentrations from 5—900 nmol/l and thus clonazepam analysis both during therapy and after overdosage is possible. Plasma and serum clonazepam levels are interchangeable.

INTRODUCTION

Clonazepam is an established anticonvulsant agent widely used in the treatment of epilepsy [1—6]. Because of the narrow therapeutic range [7—11] and the risk of increased seizure frequency in overdosage [10], regular monitoring of plasma clonazepam levels during management with the drug is not only useful but necessary [10]. Whilst thin-layer [12] and high-performance liquid chromatographic methods [13,14] have been described, the low therapeutic concentration generally limits routine analysis of clonazepam on capillary samples to gas chromatography (GC) with electron-capture detection [8,9, 15—31].

GC of most benzodiazepines can be satisfactorily performed on phenyl silicone phases such as OV-1, OV-17 and OV-225 [32,33]. Because of difficulty in completely deactivating these liquid phases and the support material, clonazepam usually elutes with prolonged tailing of the peak on these stationary liquid phases, making quantitation difficult [9,15,19,21,26,28].

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Considerably better chromatographic peaks are obtained following modification of the drug, and despite many disadvantages including multiple extractions, long reaction times and loss of specificity, the most widely accepted GC assays for clonazepam are based on indirect determination of clonazepam following either acid hydrolysis to its benzophenone [9,15–19,34] or derivatisation [20—23].

Assays that measure the unchanged drug are the most likely candidates for a reference method in that they are simple and unambiguous [28]. Apart from methods for quantitating clonazepam on a standard OV-17 column [26—31], which, with the poor chromatographic properties of clonazepam, invariably require large sample volumes, column loading and peak quantitation by digital integration, three other GC methods remain for determining clonazepam as the intact moiety. The mass fragmentographic techniques of Min and co-workers [25,35] even when available, are rather expensive for routine analysis. The method of De Boer et al. [24], which uses a support coated open tubular column, suffers the disadvantage of requiring 1 ml of plasma for a single analysis, has nitrazepam as an internal standard and requires a two-step extraction. An expensive argon—methane mixture is required as carrier gas together with a solid injection system. The authors also examined a limited number of drugs for possible interference. Despite the injection and capillary column system used in the micro method described by Edelbroek and De Wolff [8], repeated injections of blank plasma extracts are still required before analysis to obtain peaks that are more gaussian. The organic infranatant is injected without aspiration of the aqueous phase resulting in some carryover of aqueous material in the syringe. Moreover, in our hands, the extraction mixture often formed an emulsion, making the method inconvenient. A four-fold attenuation change is required between the internal standard peak and clonazepam. A typical response curve with linearity to 475 nmol/l is reported.

By using two recently described GC column packings [36,37], 100 μl plasma and only one extraction step, we have developed a rapid, sensitive and selective assay for clonazepam, equally amenable to either routine clinical use, or emergency screening applications. With these phases, chemical modification of clonazepam is not required to obtain the sensitivity and peak symmetry characteristic of methods involving derivatisation or degradation.

EXPERIMENTAL

Reagents and glassware

All reagents are analytical grade. Clonazepam, 5-(*o*-chlorophenyl)-1,3-dihydro-7-nitro-2H-1,4-benzodiazepin-2-one was obtained from Roche Products (Sydney, Australia). The stock clonazepam solution, 250 μmol/l (78.9 mg/l) in absolute ethanol, is stable for at least six months at 4°C. The working clonazepam solution, 250 nmol/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 clonazepam), rapidly stir 100 ml of serum and slowly add about 3.2 mg of clonazepam; continue stirring the mixture for 1 h. Dilute 100 μl to 100 ml with serum and store in 0.2-ml aliquots at −15°C; this is stable for ten weeks.

Methyl-clonazepam was also obtained from Roche Products. The stock methyl-clonazepam internal standard solution, 100 μmol/l (33.0 mg/l) in ethyl acetate, is stable for at least six months at 4°C. The working methyl-clonazepam internal standard solution, 20 nmol/l in ethyl acetate—cyclohexane (4:1, v/v) is prepared fresh on each day of analysis.

Glass vials, 1 ml (Pierce Reactivials, Pierce, Rockford, IL, U.S.A.) were obtained complete with screw caps and PTFE-faced discs.

To a 1-ml Reactivial add 100 μl of plasma or serum. At the same time prepare reagent blank, control (QC) and standard vials. In the standard vials, place 10, 20, 40, 80 and 120 μl clonazepam working solution and evaporate to dryness at 40°C in a stream of dry nitrogen. To the blank and standard add 100 μl drug-free pooled human serum; to the control add 100 μl QC serum.

To each vial add 500 μl of working internal standard solution, cap securely, and vortex-mix for 60 sec, centrifuge at 2000 *g* for 1 min. Transfer the supernatant to another 1-ml Reactivial and evaporate the organic phase to approximately 50 μl at 40°C under a stream of nitrogen. Vortex each vial for 10 sec.

Gas—liquid chromatography

Gas—liquid chromatographic (GLC) analysis is performed using a Varian Aerograph Series 1440 gas chromatograph equipped with a ^{63}Ni electron-capture detector (8.5 mCi) and a 0.9 m × 2 mm I.D. glass column packed with GP 2% SP-2510DA on 100—120 mesh Supelcoport, with a 5-cm pre-column of 3% SP-2250DA on 100—120 mesh Supelcoport as previously described [36]. The instrument is operated isothermally with the oven, detector and injection port temperatures at 260°C, 300°C and 280°C, respectively. The carrier gas (nitrogen) flow-rate is 40 ml/min. Gas lines are fitted with filters containing molecular sieves (15 Å) 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 3.20 min and for clonazepam 8.30 min. The ratio of peak heights of clonazepam standard to methyl-clonazepam 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 co-medication was studied. Phenobarbital, pentothal, carbamazepine, 5,5-diphenylhydantoin, di-*n*-propylacetate, ethosuximide, primidone, diazepam, nitrazepam, sulthiame, paracetamol, pseudoephedrine, chlorpheniramine, sodium cromoglycate, theophylline, salbutamol, declamethasone, 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 sulthiame was found to interfere with the analysis of clonazepam. It elutes as a broad overlapping peak with a retention time of 8.8 min and makes quantitation difficult. However, of

TABLE I

RETENTION TIMES FOR BENZODIAZEPINES

Benzodiazepine	Retention time (min)
Oxazepam	0.75
Diazepam	1.35
Flunitrazepam	1.80
Prazepam	2.25
Flurazepam	2.80
Nordiazepam	2.95
Methyl-clonazepam	3.20
Nitrazepam 7-amino metabolite	5.20
Nitrazepam	6.05
Clonazepam 7-amino metabolite	7.10
Clonazepam	8.30
Chlordiazepoxide	–
Nitrazepam 7-acetamido metabolite	15.75
Clonazepam 7-acetamido metabolite	20.50

the 350 patient samples analysed using the above procedure, only one has contained sulthiame as a co-medication. The pharmacologically inactive metabolites of clonazepam [9] and other benzodiazepines were all satisfactorily resolved (Table I). Plasma components do not interfere and neither the selectivity of a nitrogen–phosphorus detector [34] nor an extensive clean-up procedure is required [15]. A typical chromatogram of an extract of plasma is illustrated in Fig. 1.

The lower limit of detection is approximately 3 nmol/l. Over a period of 24 months, the between-run coefficient of variation of the assay for samples (n = 98) having a concentration of 100 nmol/l was 5.9%. Within-run precision was determined by analysis of ten extractions of drug-free plasma that had been supplemented to a concentration of 75, 125 and 200 nmol/l; coefficients of variation were 4.9%, 4.2% and 3.0%, respectively.

Comparison of column packings

Clonazepam, nitrazepam and desmethyldiazepam give very broad and poorly defined peaks by electron-capture GC on the phenyl silicone phases OV-1, OV-17 and OV-225 and special treatment (loading) of the column is required to deactivate sites before and during analyses. Peak area rather than peak height measurement is necessary to compensate for variability in peak shape. To evaluate the SP-2510DA phase and compare its performance with the above phases, 5 μl of a solution containing 20 nmol/l of the three benzodiazepines were injected into each of the four columns. With the phase SP-2510DA, electron-capture detector response, determined from peak area, and peak symmetry were substantially improved and more consistent. The more symmetrical peaks improve quantitation by peak height and peak area and with the improved detector response, from lower column adsorption, the sensitivity for analysing subtherapeutic concentrations is increased, particularly in capillary samples. Priming the column is not required. Clonazepam standards carried through the extraction procedure gave a linear response curve on the

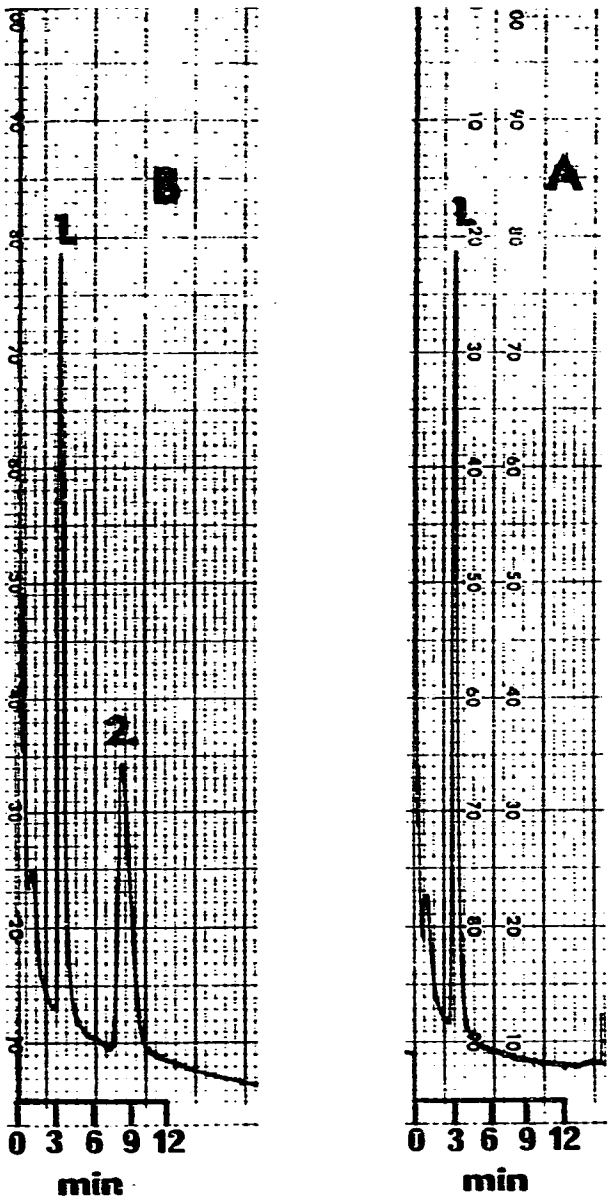


Fig. 1. Gas chromatograms of (A) extract of drug-free plasma, (B) extract of plasma sample containing clonazepam 100 nmol/l. Peaks: 1 = methyl-clonazepam (internal standard); 2 = clonazepam.

SP-2510DA phase in the range 5—900 nmol/l. The same standards showed linearity over a much smaller concentration 40—350 nmol/l on the other three phases. Calibration curves on liquid phases SP-2510DA and OV-17 are shown in Fig. 2.

Whilst the liquid phase SP-2510DA is not capable of adequately resolving all the benzodiazepines listed in Table I, when a short pre-column of SP-2250DA is added, elution of benzodiazepines which have similar retention times on the

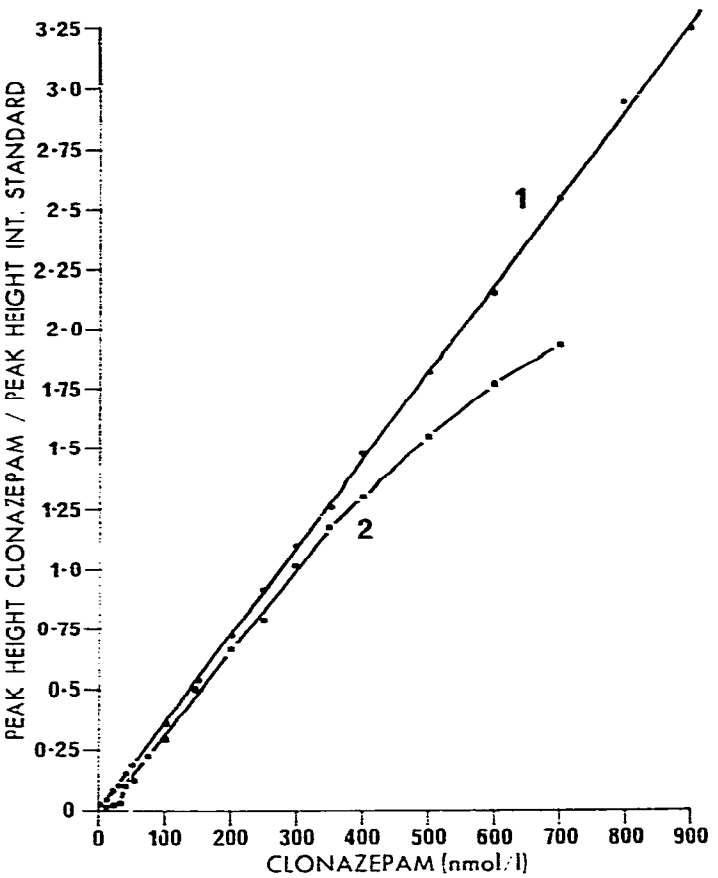


Fig. 2. Calibration curves of clonazepam relative to methyl-clonazepam, after extraction from plasma. Standards injected (1) onto SP-2510 DA-column with SP-2250DA pre-column and (2) onto OV-17 column.

single SP-2510DA phase is sufficiently retarded to give either complete separation, or separation with minimal overlap. 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 nitrazepam, diazepam and desmethyldiazepam levels.

The column packing deteriorates after approximately 350 injections of plasma extracts, resulting in loss of sensitivity and alteration of peak shape. Original performance is restored by repacking the 5-cm pre-column; the glass wool barrier between the two phases enables rapid and consistent replacement.

Extraction conditions

Various solvents were examined, 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, had poor electron-capture response and dissolved clonazepam. They included diethyl ether, ethyl acetate, *n*-butyl acetate, ethyl acetate—cyclohexane (4:1, v/v) and methyl acetate—cyclohexane (7:3, v/v). All solvents extracted

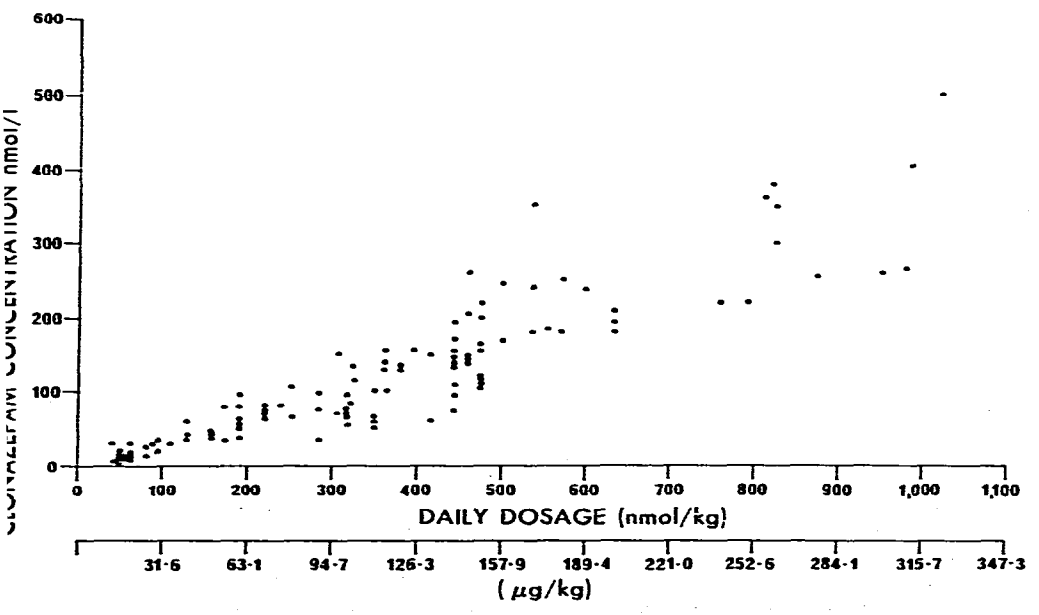
clonazepam with greater than 95% efficiency at a plasma to solvent ratio of 1:5 and at the normal pH of plasma, and no significant variations in the recovery of clonazepam occurred if mixing was continued for 60 sec or longer. Ethyl acetate—cyclohexane (4:1, v/v) gave least interference of plasma components and emulsion free extracts. Similar recoveries were obtained with and without buffer (pH 3—9). 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).

Practical applications

Fig. 3 shows the wide range in drug concentrations in plasma derived from patients of all ages who have been treated with clonazepam for the control of seizures, and the variability in plasma levels in different people taking comparable amounts of the drug. Because of a large number of variables such as patient compliance and sampling times not being specifically controlled, no attempt has been made to define the linear regression correlation between the daily dosage and the clonazepam plasma concentration.

Clonazepam levels obtained from paired serum and plasma samples are shown in Fig. 4. Correlation based on a least-squares linear regression formula gives a line of best fit with a slope of 1.042 and a Y-intercept of −1.419 nmol/l. The correlation coefficient is 0.990 (n = 19), thus clonazepam analysis on plasma and serum samples would appear to be interchangeable.

The small amount of plasma required (50—100 μl) allows clonazepam measurement in neonates and children using capillary blood samples. Adsorption losses, a factor regarded sufficiently serious by Parry and Ferry [22] to warrant the silanisation of glassware, are minimised by eliminating evaporation



ig. 3. Relationship between clonazepam plasma levels and the dose administered in 110 atients on chronic oral medication with clonazepam.

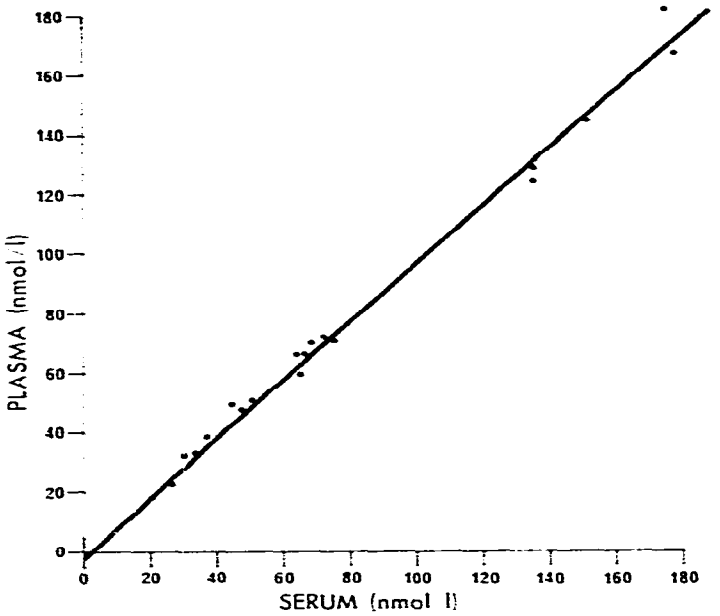


Fig. 4. Relationship between plasma and serum levels of clonazepam.

to dryness of the final extract and by conducting all manipulations quickly and conveniently in small vials. The internal standard, methyl-clonazepam is employed as it is a homologue of clonazepam and is not used as a drug.

In conclusion we believe that the procedure described offers significant improvement in peak shape, electron-capture detector response and linearity over other methods which measure the unchanged drug and in this regard is comparable to methods involving either derivatisation or acid hydrolysis. The selectivity of the column system enables other benzodiazepines to be well resolved and quantitated. With its simplicity, rapidity, sensitivity and small volume of plasma or serum required, clonazepam levels can be routinely monitored as frequently as other antiepileptic drugs.

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