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Determination of zonisamide (3-sulphamoylmethyl-1,2-benzisoxazole) in plasma at therapeutic concentrations by high-performance liquid chromatography

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ABSTRACT

A selective and sensitive liquid chromatographic method for the determination of zonisamide in small (0 1 ml) plasma samples is described. After adding internal standard, a direct solvent extract of the sample is examined by reversed-phase liquid chromatography with ultra-violet spectrophotometric detection The method is rapid, simple and capable of determining plasma levels after therapeutic ingestion of zonisamide Some results from a dose-ranging clinical trial are presented

INTRODUCTION

Zonisamide is an antiepileptic drug of novel chemical structure (Fig. 1) which has a spectrum of activity in experimental animals resembling that of phenytoin. Its metabolism and disposition have been elucidated [1] and its pharmacokinetics investigated using a range of doses in healthy human volunteers [2]. The latter study demonstrated that clearance and apparent volume of distribution decreased at higher doses as a result of the non-linear binding of zonisamide in erythrocytes.

When a single 400-mg dose of zonisamide was administered to epileptic patients who were optimised on monotherapy with either phenytoin or carbamazepine its clearance was significantly increased and half-life decreased compared with healthy volunteers [3,4].

The pharmacokinetics of both single and multiple doses of zonisamide have also been studied in epileptic patients who were maintained on multi-drug therapy. The mean plasma elimination half-life was similar to that obtained in pa-

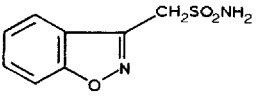

Fig 1 Structure of zonisamide.

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tients on monotherapy, but there were differences between steady-state and single-dose plasma clearance which indicate a non-linear relationship [5,6].

Two isocratic reversed phase high-performance liquid chromatographic (HPLC) assays have been reported for the determination of zonisamide in plasma. The initial methods required large (1.0 ml) volumes of plasma and a laborious extraction procedure [5,7]. Furthermore the assays were only calibrated over a low concentration range and possible interference from other drugs, their metabolites and endogenous substances was not thoroughly investigated.

Juergens [8] considered that isocratic separations were not suitable for studying patients undergoing multi-drug treatment in Western Europe and developed a procedure which was designed to separate and quantitate zonisamide together with all of the commonly prescribed anticonvulsant drugs and their metabolites in 12 min. The method utilised solvent extraction of 0.5 ml plasma without the addition of buffer and examination of the residue by gradient elution reversed-phase HPLC with detection at 207 nm.

The present assay was developed to measure plasma concentrations of zonisamide in a British multi-centre dose-ranging trial where the drug was added to on-going medication and plasma levels of all co-prescribed anticonvulsants were determined within 24 h of sample receipt. When carbamazepine and clobazam constituted part of the treatment, the concentrations of their active metabolites were also measured, with all routine monitoring being performed by established techniques [9–13].

The zonisamide assay reported here is a rapid, isocratic, reversed-phase HPLC procedure which requires only 0.1 ml plasma and has been calibrated throughout the range of plasma concentrations likely to be encountered in the multi-centered trial. It has been fully validated with regard to precision, accuracy and selectivity, and some dose *versus* plasma concentration data are reported from the studies.

EXPERIMENTAL

Column liquid chromatography

An M45 constant-flow reciprocating pump (Waters Assoc., Harrow, U.K.) was used in conjunction with an LC3 UV detector (Pye Unicam, Cambridge, U.K.) and a Model 28000 recorder (Bryans Southern, Mitcham, U.K.). The analytical column was a 25 cm × 4.4 mm I.D. stainless-steel tube which was packed with Hypersil 5 MOS (Technicol, Stockport, U.K.). This was protected by a 10 cm × 4.4 mm I.D. precolumn which was dry-packed with CO:Pell ODS (30–38 μm) (Whatman, Maidstone, U.K.). The mobile phase was prepared by mixing 50 ml of 1 *M* sodium hydroxide with 58 ml of 1 *M* acetic acid and diluting to 1.0 l with glass-distilled water prior to adding 580 ml of spectroscopic grade (S) acetonitrile. The system was operated at ambient temperature and a flow-rate of 1.5 ml/min. The detector wavelength was set at 280 nm and the sensitivity at 0.16 a.u.f.s. Injections were made via a Rheodyne 7125 valve fitted with a 20-μl loop (Magnus Scientific, Sandbach, U.K.).

Reagents

Dichloroethane (HPLC grade) and acetonitrile (S grade) were obtained from Rathburn (Walkerburn, U.K.). A phosphate buffer (pH 4.5) comprising 0.5 *M* sodium dihydrogen orthophosphate in distilled water was used to adjust the pH of the plasma prior to extraction and the internal standard solution consisted of 3-sulphamoylmethyl-6-fluoro-1,2-benzisoxazole (40 mg/ml in methanol). The internal standard together with pure zonisamide were supplied by Parke-Davis (Eastleigh, U.K.).

Extraction procedure

All samples were extracted in duplicate.

Plasma (100 μl), phosphate buffer (250 μl) and internal standard solution (50 μl) were added to a 10-ml conical tube fitted with a ground-glass stopper. An Eppendorf pipette was used to measure the plasma, the buffer was aliquoted by means of an Oxford dispenser and the internal standard was added with a repeating Hamilton syringe. Dichloroethane (7.0 ml) was then dispensed into the tube which was stoppered and vortex-mixed for 1 min. After standing for a few minutes to allow the phases to separate the plasma layer was aspirated to waste and the organic phase passed through a Whatman No. 1 filter paper into a clean conical tube. The solvent was evaporated to dryness by using a stream of air and placing the tube in a warm water bath After reconstituting the residue in 50 μl of mobile phase, a 20-μl aliquot was injected onto the HPLC system.

Measurement

Zonisamide standards which were prepared by spiking the drug at a range of concentrations into plasma were carried through the extraction procedure with each batch of samples. These calibration standards were prepared in bovine plasma, which had first been tested to ensure that it was free from background interference Using a Hamilton repeating syringe, 50–600 μl of a stock zonisamide solution (1.0 mg/ml in methanol) were dispensed into 20-ml screw-cap vials. After carefully evaporating the solvent under a stream of air, 10 ml of the bovine plasma were added to each vial. An additional standard was prepared at a concentration of 3 mg/l by further diluting the 60 mg/l calibrator twenty-fold with tested plasma.

Quantification was achieved by injecting an extract from each calibrator onto the column, measuring the peak heights of drug and internal standard to an extrapolated baseline and performing multi-linear regression of the ratio of the peak heights against zonisamide concentration. The drug concentration in a sample was calculated by comparing the peak-height ratio of the sample with the calibration graph.

Quality control

Freeze-dried quality control sera were prepared for reconstitution to 1.5-ml

volumes at zonisamide concentrations of 5 and 15 mg/l. These were analysed in duplicate with each batch of samples.

RESULTS AND DISCUSSION

Under the conditions described both drug and internal standard gave sharp symmetrical peaks with baseline separation. No interfering peaks in the same region as zonisamide or the internal standard have been encountered either from the constituents of normal human plasma (see Fig. 2) or from patients prescribed a range of other drugs (Table I). In addition haemolysis of the specimen did not produce interference in the chromatogram, thus enabling whole blood samples to be analysed if required. Most anticonvulsants are extracted by this method and elute from the column, but few are detected at 280 nm when present at ther-

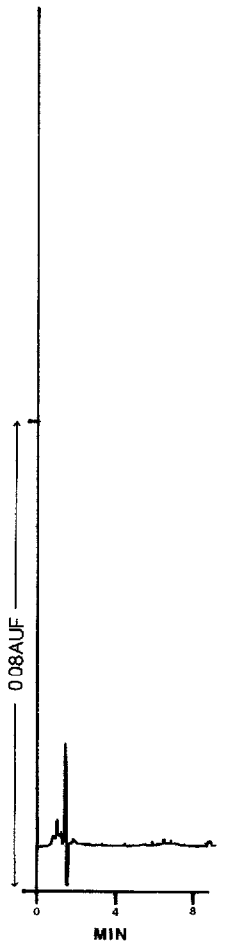


Fig 2. Chromatogram of an extract of blank human plasma (internal standard omitted)

TABLE I

DRUGS TESTED AND SHOWN NOT TO INTERFERE WITH THE ASSAY

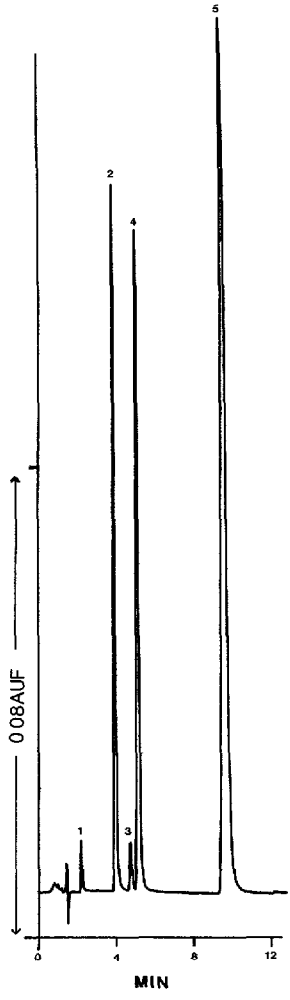
Acetazolamide	Metronidazole
N-Acetylprocainamide	Naproxen
Ampicillin	Paracetamol
Caffeine	Paraxanthine
Carbamazepine	Pentobarbitone
Carbamazepine-10,11-diol	Phenobarbitone
Carbamazepine-10,11-epoxide	Phenytoin
Cefuroxime	Primidone
Cimetidine	Procainamide
Chloramphenicol	Salicylic acid
Clobazam	Sulphadimidine
Desmethylclobazam	Sulphamethoxazole
Desmethylmethsuximide	Sulthiame
Ethosuximide	Theophylline
Mefanamic acid	Thiopentone

apeutic plasma concentrations. Carbamazepine and its two metabolites, the 10,11-epoxide and 10,11-diol, might be detected at 280 nm, but are resolved from zonisamide and the internal standard (Fig. 3). At 280 nm the absorbance values of these metabolites are much lower than that of either carbamazepine or zonisamide (approximately 10%); therefore, whilst carbamazepine itself might be evident in patients co-prescribed this drug, its plasma metabolite concentrations are normally so low that they are not detected (Fig. 4); however, the chromatographic conditions were established to ensure that the epoxide and diol metabolites of carbamazepine could be resolved from zonisamide in order to preclude the possibility of a positive interference with the assay should an interaction with this new drug cause abnormally elevated metabolite concentrations.

Reproducibility

The within- and between-run precision of the method was determined using the bi-level quality control samples and the results are summarised in Table II. It was found during the course of these studies that zonisamide stability in both the calibration standards and reconstituted quality control sera was poor when they were stored at 4°C with drug loss occurring particularly in sera where microbial growth was obvious. Since antimicrobial agents had not been added to the serum this problem was avoided by storing all calibrators, controls and samples at −20°C.

A further precision study was performed by blind analysis of three patient samples which were supplied by Parke-Davis (Ann Arbor, MI, U.S.A.) and analysed in duplicate on seven separate occassions. Table III summaries these results.



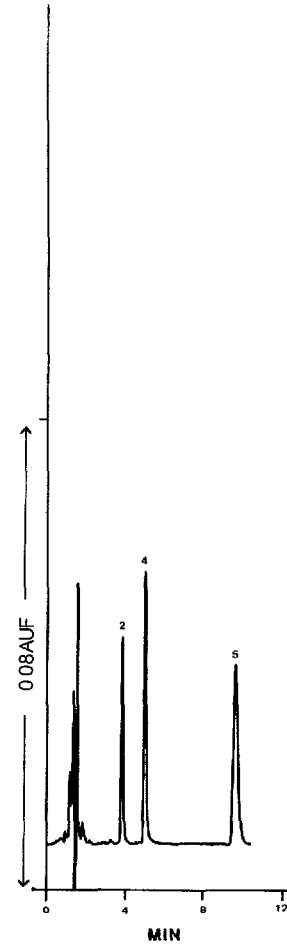


Fig 3. Separation of carbamazepine and its metabolites from zonisamide and internal standard under the conditions described. Peaks 1 = carbamazepine-10,11-diol, 2 = zonisamide, 3 = carbamazepine-10,11-epoxide, 4 = internal standard; 5 = carbamazepine.

Fig 4 Chromatogram of a plasma extract from a patient prescribed zonisamide. Peaks· 2 = zonisamide (12.1 mg/l); 4 = internal standard, 5 = carbamazepine (8 2 mg/l), carbamazepine-10,11-epoxide was also present at a concentration of 1 6 mg/l

Accuracy

The lower limit of accurate measurement, defined as 10 × S.D. of the lowest standard, was 0.9 mg/l. Accuracy was determined by exchanging patient samples with another laboratory which was operating a properly validated assay (Dr. U. Juergens, Department of Biochemistry, Gesellschaft für Epilepsieforschung, Bielefeld, F.R.G.). Eighteen samples containing from 2–35 mg/l zonisamide were tested and the results of this comparison are shown in Fig. 5. In addition, accu-

TABLE II

PRECISION FOR ANALYSIS OF THE BI-LEVEL QUALITY CONTROL SAMPLES.

	Mean concentration (mg/l)	*n*	Standard deviation (mg/l)	Coefficient of variation (%)
Within-run	4 92	13	0 192	3 9
	14 80	13	0 714	4 8
Between-run	4 84	22	0 295	6 1
	13 79	22	0 461	3.3

racy was evaluated by reference to the values later assigned to the three patient samples supplied by Parke-Davis (see Table III).

CONCLUSION

The present method is selective, precise, accurate and requires only a short solvent extraction and subsequent concentration prior to chromatography thus making possible the determination of therapeutic zonisamide levels using only a small plasma sample. The procedure easily dovetails with routine GC methodology to enable multi-drug monitoring of clinical trials to be completed within 24 h. The sensitivity of the assay would also be adequate to enable zonisamide concentrations to be measured in plasma at 60 h and whole blood for 96 h following a single 400-mg dose [3,4]. Quantification is by reference to similarly treated calibrators; therefore, it is not necessary to determine the absolute extraction efficiency or apply recovery factors to each analytical run.

Since developing the method 58 samples have been analysed from patients who were participating in the increasing dosage, multi-centred trial of zonisamide and Fig. 6 is a scattergram relating dose of drug prescribed to plasma concentration in these patients. Pilot clinical studies indicated that 7–40 mg/l was a useful plasma level range when zonisamide was added to other anticonvulsant treat-

TABLE III

PRECISION OF ANALYSIS FOR THREE PATIENT SAMPLES

	Target value (mg/l)	Mean value (mg/l)	*n*	Standard deviation (mg/l)	Coefficient of variation (%)
C_1	26 0	27.4	14	0.95	3.5
C_2	11 9	12.2	14	0.72	5 9
C_3	38 7	37 4	14	1.48	4 0

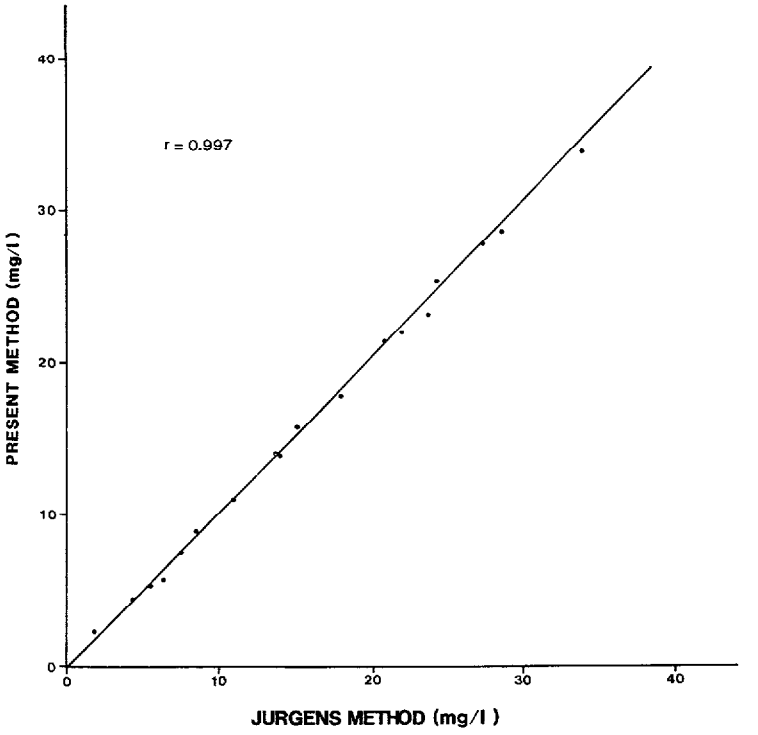


Fig 5 Comparison of zonisamide plasma concentrations determined by reversed-phase gradient HPLC and the present isocratic method· r = 0 997, range = 2–35 mg/l

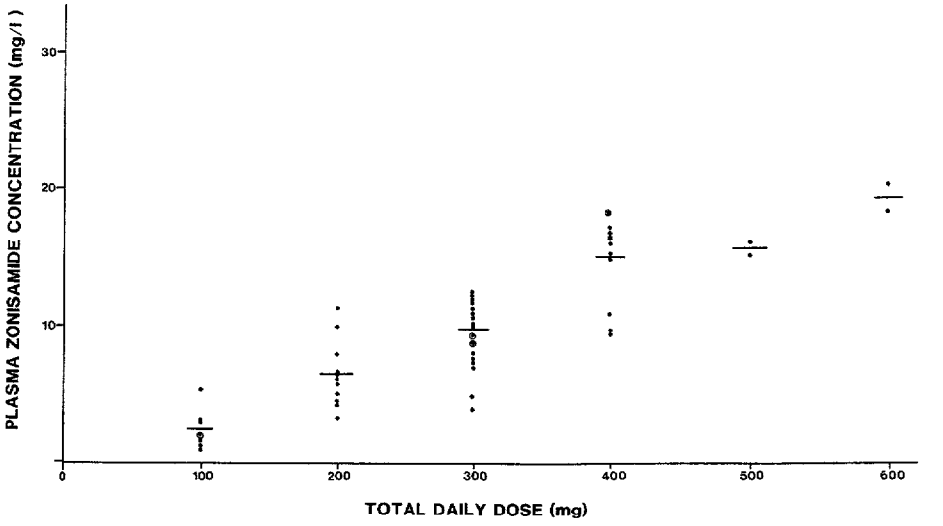


Fig. 6. Scattergram relating dose of drug prescribed to plasma zonisamide concentration in 58 samples.

ment, although most patients showed signs of toxicity with concentrations greater than 30 mg/l [2]. This information has been used as the basis upon which to suggest 20 mg/l as the target steady-state concentration for zonisamide in plasma but since the drug is taken up into erythrocytes by a temperature-dependent and saturable transport process, any future requirements for therapeutic drug monitoring will be limited by our ability not only to handle samples consistently, but also to interpret the significance of plasma levels when red cell uptake is concentration-dependent.

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