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SIMULTANEOUS QUANTITATION OF SALIVARY CARBAMAZEPINE, CARBAMAZEPINE-10,11-EPOXIDE, PHENYTOIN AND PHENOBARBITONE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A rapid, sensitive and accurate high-performance liquid chromatographic method for the simultaneous quantitation of phenobarbitone, phenytoin, carbamazepine and carbamazepine-10,11-epoxide in saliva is described Only small volumes of saliva (100 μl) are required Separation of the drugs is achieved by reversed-phase chromatography on a Nova-Pak C_{18} column, with a mobile phase of acetonitrile–phosphate buffer at a flow-rate of 2 0 ml/min Detection is effected by ultraviolet absorption at 215 nm The total run time is under 12 5 min per assay A precipitation but no extraction step is involved, simplifying the assay method Salivary concentrations in the range 0 25–25 μg/ml for carbamazepine, 0 5–20 μg/ml for phenytoin and phenobarbitone and 0 4–20 μg/ml for carbamazepine-10,11-epoxide can be measured Recovery varies from 94 to 108% The method has been used for routine measurements of anticonvulsants in saliva collected daily from patients with intractable epilepsy

INTRODUCTION

Saliva is a useful fluid for measuring drug levels in patients taking phenobarbitone (PB), phenytoin (PHT) and carbamazepine (CBZ) The fluid is easy and painless to collect, and drug concentrations in it reflect the free (i.e unbound) portion of these drugs in plasma [1–3]. Chronic epileptic patients often take several anticonvulsants, and an assay for routine use should ideally measure all agents taken in a single run. CBZ, an iminostilbene derivative, is

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partly converted in the body to carbamazepine-10,11-epoxide (CBZ-EP) which displays anticonvulsant properties similar to those of the parent compound [4] and has been reported to produce clinical side-effects in children [5] In view of this, and the relatively narrow therapeutic index of carbamazepine, an ideal assay of anticonvulsants in saliva should also measure this metabolite.

Several high-performance liquid chromatographic (HPLC) methods for the determination of antiepileptic drugs in biological fluids have been described [6–16] but only three have been traced which measured the salivary concentrations of both CBZ and CBZ-EP [6,14,15] in addition to their serum or plasma concentrations. Although many of the published HPLC methods could be modified for saliva drug assays, they possess disadvantages, e.g. large volume ($>500\,\mu l$) of sample required [7–9,12], long retention times [6,11,13,15] and/or tedious extraction procedures [6,7,9,10]. The methods which have been described for assay of the drugs in saliva involved either solid-phase [14] or liquid–liquid extraction procedures [6,15] or used a double internal standard approach [6]. Each assay method provided excellent results for subjects on anticonvulsant monotherapy, but some encountered difficulties if multiple anticonvulsants were involved. At least one assay involving only a precipitation step has been described [16], but his method could not resolve PHT from CBZ, and was applicable only to plasma.

We have devised a rapid, non-extractive HPLC assay to measure CBZ, CBZ-EP, PHT and PB in saliva. This assay requires only 100 μl of saliva – desirable because many patients, especially children or those in institutions, cannot supply much larger volumes even with stimulation of salivation The method involves a precipitation but not an extraction step, but can still adequately resolve each anticonvulsant with sufficient sensitivity for routine measurement.

EXPERIMENTAL

Apparatus

The liquid chromatograph used comprised a Waters M510 pump, a Kortec K65B autoinjector, and a Waters Lamda-Max Model 481 LC variable-wavelength UV spectrophotometer, with a Shimadzu Chromatopac C-R3A integrator. Analysis was performed on a reversed-phase C_{18} column (Waters, 4-μm Novapak in a Waters RCM-100 radial compression system) preceded by a guard column of C_{18} Corasil Bondapak (Waters) The mobile phase, acetonitrile–phosphate buffer (pH 6.0, 0.067 *M* (30·70, v/v) was run at 2 ml/min at ambient temperature ($22 \pm 2°C$) and the UV detector set at a wavelength of 215 nm

Reagents

Chemicals used were CBZ, CBZ-EP and 10-methoxycarbamazepine (internal standard) (Ciba Geigy Australia), PHT (Parke-Davis Australia) and PB

(Queensland Chemicals). Acetonitrile and methanol were HPLC grade (Mallinckrodt, ChromAR). Potassium dihydrogen orthophosphate was obtained from BDH Australia.

Preparation of internal standard solution

The internal standard, 10-methoxycarbamazepine, was dissolved in acetonitrile (5 mg in 100 ml) A 1 10 dilution with acetonitrile gave the working internal standard solution (5 mg/l). This solution was incorporated into each sample by adding 200 μl to 100 μl of saliva.

Preparation of mobile phase

Acetonitrile (300 ml) was diluted to 1 l with phosphate buffer (0.067 *M* KH_2PO_4, with pH then adjusted to 6.0 by the addition of 2 *M* NaOH) and filtered through a 0.22-μm Millipore filter prior to use

Preparation of standards

Standard solutions of CBZ (1 μg/ml), CBZ-EP (0.4 μg/ml), PHT (2 μg/ml) and PB (2 μg/ml) were prepared in methanol. The solutions were sealed and refrigerated until use (stable for at least six months at 4°C). Aliquots of standard solutions were dispensed into screw-capped 10-ml tapered polypropylene tubes to give concentrations of 0, 0.25, 0.5, 1 0, 2.5, 5 0, 10 0 and 25.0 μg/ml for CBZ, 0, 0 5, 1.0, 2.0, 5 0, 10.0 and 20 0 μg/ml for PB and PHT and 0, 0.4, 1.0, 2 0, 4.0, 10.0 and 20 0 μg/ml for CBZ-EP. The tubes were warmed (30°C) and all solvent evaporated under a gentle stream of air Drug-free human saliva (100 μl) and internal standard (200 μl) were then added to each tube Standard curves were prepared for each drug separately and when the four drugs were present simultaneously The curves for each drug singly, and in combination, were very similar.

Saliva samples

Saliva samples were frozen at −20°C, until thawed and centrifuged (2000 *g* for 10 min) prior to assay. This procedure was necessary to overcome difficulties resulting from the high viscosity of freshly obtained saliva.

Assay procedure

Internal standard in acetonitrile (200 μl) was added to 100 μl of thawed saliva or saliva standards in 10-ml polypropylene tubes, and the mixture vortex-mixed for 30 s. The precipitate was sedimented by centrifugation at 2000 *g* for 10 min. Aliquots of 50 μl of the clear supernatant were chromatographed The ratio of area under the anticonvulsant peak to that under the 10-methoxycarbamazepine (internal standard) peak was determined by the Shimadzu C-R3A integrator.

Quantitation

Calibration graphs were constructed by plotting the peak-area ratio of drug to internal standard against the known concentration of anticonvulsant added to drug-free saliva to cover the concentration range 0–25 μg/ml for CBZ, and 0–20 μg/ml for PB, PHT and CBZ-EP

Recovery

As the procedure involved a protein precipitation step, but not an extraction step, recovery was assessed in terms of the amount of drug heterogeneously trapped in the precipitated protein pellet Chromatographic peak areas for drugs and internal standard in saliva were measured in triplicate for three concentrations and compared with the areas obtained for samples of similar concentrations prepared in water.

Selectivity

Samples of drug-free saliva were spiked with a variety of marketed antiepileptic drugs and analysed to ascertain any interference with the chromatography of PB, PHT, CBZ, CBZ-EP or 10-methoxycarbamazepine

Precision and accuracy

Precision and accuracy were assessed on three separate occasions using six replicate samples at concentrations of 1, 2 and 5 μg/ml for PB and PHT, 0 5, 1, 2 5 and 10 μg/ml for CBZ and 0 4, 2 and 4 μg/ml for CBZ-EP. Measured concentrations were determined by application of the standard curve. Precision was assessed by considering the standard deviation of the replicate sets and accuracy was assessed in terms of the deviation of the measured concentrations from the nominal 'true' concentration

Minimum quantifiable concentrations

The minimum quantifiable concentration was regarded as three times the value of the standard deviation of the set of measured estimates of the lowest drug concentration that was measured in constructing the standard curve.

RESULTS

Performance of the HPLC system

The chromatogram illustrated in Fig 1A was obtained following an injection of a solution of authentic compounds in mobile phase. PB, CBZ, PHT and CBZ-EP are well separated with retention times of 4 4, 7.7, 8.7 and 3 9 min, respectively. 10-Methoxycarbamazepine, the internal standard, elutes at 11.1 min. Fig. 1B is a chromatogram of drug-free human saliva and Fig. 1C is a chromatogram of saliva from a patient receiving carbamazepine.

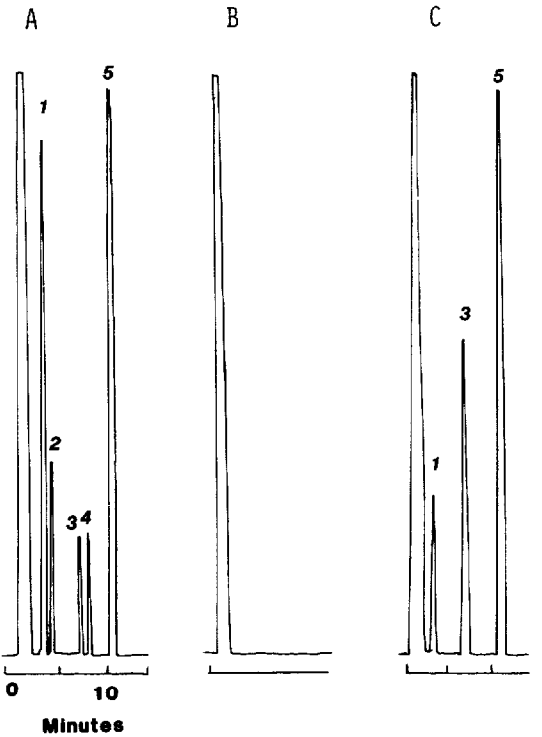

Fig 1 (A) Chromatogram of authentic compounds in mobile phase Peaks 1=carbamazepine-10,11-epoxide (2 μg/ml), 2=phenobarbitone (2 μg/ml), 3=carbamazepine (1 μg/ml), 4=phenytoin (2 μg/ml), 5=10-methoxycarbamazepine (internal standard) (B) Chromatogram of drug-free human saliva (C) Chromatogram of saliva from a patient stabilized on carbamazepine The concentrations of carbamazepine (3) and carbamazepine-10,11-epoxide (1) were determined as 2 26 and 0 79 μg/ml, respectively

Reproducibility and linearity

Standard curves for CBZ, CBZ-EP, PB and PHT were linear in each case, with correlation coefficients of 0.9997, 0.9999, 0 9995 and 0.9999, respectively. The assay was precise, with coefficients of variation for eighteen samples analysed at PB concentrations of 1, 2 and 5 μg/ml being 7.6, 7 0 and 2 8%, respectively. The corresponding figures for CBZ at 0.5, 1.0, 2.5 and 10 μg/ml were 5 5, 5.4, 2.5 and 4.3%, respectively, while for PHT at 1, 2 and 5 μg/ml they were 6.9, 5 1 and 5.5%, respectively, and for CBZ-EP at 0.4, 2 and 4 μg/ml they were 4.3, 7.2 and 2.6%. The assay was accurate over the ranges studied, with deviation from the nominal value ranging from ±1.4 to 8.5%.

Recovery

The recovery ranged from 95.4 to 99% for PB, from 94 5 to 102.7% for CBZ, from 97 3 to 108.6% for CBZ-EP and from 96.4 to 102.2% for PHT The in-

crease in recovery from saliva over water may reflect a small concentrating effect of removing salivary proteins from solution and does not affect quantitation to any significant degree.

Interference

No endogenous compounds have interfered chromatographically with any of the drugs assayed in saliva from over twenty subjects. Methylphenobarbitone, oxcarbazepine and clonazepam can also be resolved chromatographically in this assay, with retention times of 10.6, 4.8 and 14.2 min, respectively Primidone elutes at 3.1 min and is very close to the injection peak. Sodium valproate, ethosuximide, methsuximide and sulthiame are not detected.

Minimum quantifiable concentration

The minimal quantifiable concentration, defined as three times the standard deviation of the lowest point on the calibration curves, was 0.13 μg/ml for PHT, 0.17 μg/ml for PB, 0.08 μg/ml for CBZ and 0.06 μg/ml for CBZ-EP.

DISCUSSION

The assay technique described above is fast, easy to perform, inexpensive and particularly useful for analysing saliva from patients taking multiple anticonvulsants. Small samples of saliva (100 μl) can be used. Saliva is a useful fluid for measuring most anticonvulsants [1–3], with the ratio of saliva to whole plasma PB concentration being approximately 0.33 [1,17], PHT 0.1 [2,17] and CBZ 0.26 [1,18]). However, a correction for the effects on ionization due to pH differences between saliva and plasma is desirable in the case of PB [19]. Although a 'therapeutic' range for the drugs in saliva has not been as well defined as for plasma, calculated ranges would be 3–8 μg/ml for PB, 1–2.5 μg/ml for PHT and 1–4 μg/ml for CBZ. CBZ-EP, which occurs in saliva at approximately 43% of its plasma concentration [20], also has an anticonvulsant effect; from the clinical point of view the sum of the concentrations of parent drug and epoxide may be of greater interest than CBZ concentrations alone [21].

The assay can measure accurately concentrations of the major anticonvulsants over the ranges 0.25–25 μg/ml for CBZ, 0.5–20 μg/ml for PB and PHT and 0.4–20 μg/ml for CBZ-EP. The minimum quantifiable concentrations are 0.08, 0.17, 0.13 and 0.06 μg/ml, respectively These values would correspond to whole plasma levels of 0.31 μg/ml for CBZ, 0.51 μg/ml for PB and 1.3 μg/ml for PHT As these plasma concentration values are well below the lower levels of the 'therapeutic' plasma range for each drug, the assay is sensitive enough to be used in routine monitoring of these agents. It has been used in pharmacokinetic studies following a single dose of CBZ, though traces of drug retained in the mouth confounded the measurements for a few hours after drug intake [22].

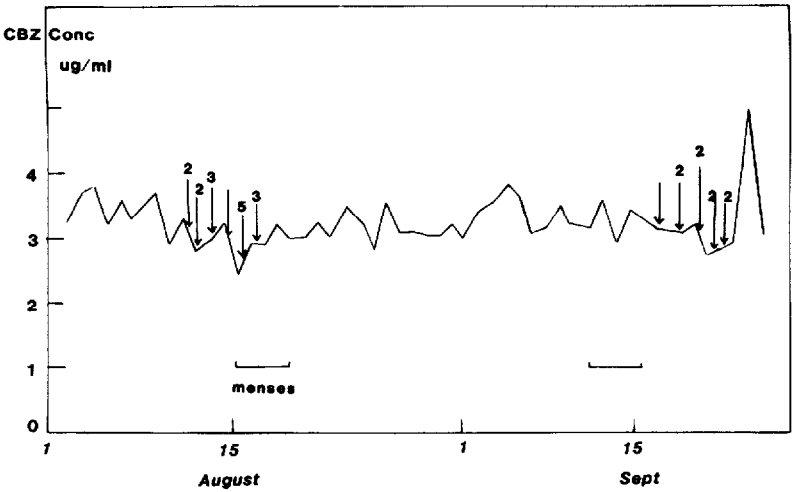

Fig 2 Time course of salivary carbamazepine levels in a patient receiving 200 mg of the drug twice daily Arrows indicate days of seizure activity

We are using this assay to follow daily predose morning salivary anticonvulsant levels in intractable epileptics over periods of several weeks Anticonvulsant drug levels can be correlated with seizure frequency and the effect of the menstrual cycle on drug levels noted An example of the daily time course of CBZ levels in the saliva of a 32-year-old female patient with complex partial seizures is illustrated in Fig. 2 Some of the subjects studied have been institutionalized, and the need for smaller volumes of saliva than in certain commonly used assays avoids the problems with acceptance that may arise if larger volumes of saliva, which requires some form of salivary flow stimulation, are sought. The ability to rapidly separate and measure accurately three commonly used anticonvulsants also makes the method useful for analysing large batches of salivary samples

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