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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF CARBAMAZEPINE AND CARBAMAZEPINE 10,11-EPOXIDE IN PLASMA AND SALIVA FOLLOWING SOLID-PHASE SAMPLE EXTRACTION

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SUMMARY

A rapid, sensitive and simple to operate high-performance liquid chromatographic method for the simultaneous determination of carbamazepine (CBZ) and carbamazepine 10,11-epoxide (CBZ-EP) in plasma and saliva is described. The drug and its metabolite are extracted from both plasma and saliva using commercially available reversed-phase octadecylsilane bonded silica columns (Bond-Elut C_{18}, 2.8 ml capacity). Separation of CBZ and CBZ-EP was achieved by reversed-phase chromatography, using a mobile phase consisting of acetonitrile—methanol—water (19:37:44) at a flow-rate of 1.8 ml/min in conjunction with a Nova-Pak C_{18} column. The analytical column, in Radial-Pak cartridge form, was used in combination with a Z-module RCSS and protected by a Guard-Pak precolumn module containing a Guard-Pak μBondapak C_{18} insert. Using ultraviolet detection at 214 nm, levels in the region of 50—100 ng/ml for CBZ and CBZ-EP can be measured with only 250 and 500 μl of plasma and saliva, respectively. The method, which has been used to determine steady-state concentrations of the drug and its metabolite in paediatric patients receiving CBZ monotherapy, is also suitable for pharmacokinetic studies.

INTRODUCTION

The anticonvulsant drug, carbamazepine (5-carbamoyl-5*H*-dibenz[*b*,*f*]-azepine), is widely used in the treatment of epilepsy in children [1]. It is generally accepted that seizure control is most effective when plasma carbamazepine (CBZ) concentrations are kept within the range 4–12 μg/ml, whilst levels exceeding the upper limit have been associated with toxic side-

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effects [2]. However, the relationship between CBZ concentration and therapeutic effect is complicated by the formation of a potentially active metabolite, carbamazepine 10,11-epoxide (CBZ-EP) [3]. In studies using rats, CBZ-EP displayed anticonvulsant properties similar to those of the drug itself [4]. Although this activity has yet to be confirmed in man, CBZ-EP-related side-effects have recently been reported in children treated with CBZ [5]. In view of this, and the narrow therapeutic index of the parent drug, simultaneous monitoring of both CBZ and CBZ-EP in plasma or saliva from epileptic patients is indicated.

A variety of methods have been applied to the determination of CBZ in biological fluids including UV spectrophotometry [6], gas chromatography (GC) [7—12], enzyme-multiplied immunoassay technique (EMIT) [13, 14] and high-performance liquid chromatography (HPLC) [15—28]. However, simultaneous measurement of CBZ and CBZ-EP is only possible using chromatographic techniques such as GC [7—12] or HPLC [15–28]. Unfortunately, GC methods tend to produce variable results since CBZ is unstable when subjected to the elevated temperatures used in these assays and may be partially converted to iminostilbene and acridine derivatives [29]. Furthermore, with the exception of the method of Elyas et al. [22], published HPLC procedures [15–28] possess a number of apparent disadvantages which include large sample requirement [15–17, 21, 26, 27], long retention times [18–20, 24, 25], tedious extraction procedures [15, 17, 19, 28] and lack of sensitivity towards CBZ-EP resulting from selection of sub-optimal detector characteristics [15—17, 19, 25–27]. However, like the majority of these HPLC procedures, the method of Elyas et al. [22] was not applied to the determination of CBZ and CBZ-EP in saliva. Only two studies examined salivary levels in addition to plasma or serum measurements of CBZ and its epoxide metabolite [19, 20].

In order to overcome these apparent disadvantages, the method presented here differs from previous attempts in two respects. First, in common with the approach used by Kumps [25], a ternary solvent mixture (acetonitrile—methanol—water) was adopted for the mobile phase. This was used in combination with the radially compressed Nova-Pak C_{18} column to provide rapid separation of CBZ and CBZ-EP. Secondly, solid-phase extraction of the drug and its major metabolite from plasma and saliva achieved economies in sample handling prior to analysis compared with traditional liquid—liquid extraction procedures.

EXPERIMENTAL

Reagents

Ethanol (absolute alcohol AR quality) was purchased from James Burrough (London, U.K.). Acetonitrile (HPLC S grade) and methanol (HPLC grade) were obtained from Rathburn Chemicals (Walkerburn, U.K.). Carbamazepine, carbamazepine 10,11-epoxide, 10-methoxycarbamazepine and 10,11-dihydro-10,11-*trans*-dihydroxycarbamazepine (CBZ-DIOL) were gifts from Ciba-Geigy (Horsham, U.K.). Nitrazepam was donated by Roche Products (Welwyn Garden City, U.K.).

Extraction columns and vacuum apparatus

Rapid sample processing was achieved using Bond-Elut C_{18} columns, 2.8 ml capacity, in conjunction with a Vac-Elut vacuum apparatus. These are both manufactured by Analytichem International (Harbor City, CA, U.S.A.) and supplied by Jones Chromatography (Llanbradach, U.K.).

Equipment

The high-performance liquid chromatograph consisted of a Waters Assoc. Model 510 constant-volume pump, U6K injector, Lambda-Max Model 481 variable-wavelength LC spectrophotometer and a Model 730 data module.

Chromatography

A Waters Assoc. Z-Module RCSS was used in conjunction with a Nova-Pak C_{18} Radial-Pak cartridge (10 cm × 8 mm I.D., 4-μm spherical, fully capped, C_{18} bonded silica) and protected by a Waters Assoc. Guard-Pak precolumn module containing a Guard-Pak μBondapak C_{18} insert. Freshly prepared mobile phase consisting of acetonitrile—methanol—water (19:37:44) was filtered through a 0.22-μm Millipore filter (Durapore type GVWP) and degassed prior to use. Chromatography was performed at ambient temperature using a flow-rate of 1.8 ml/min which produced a back-pressure in the region of 6.82 MPa (1000 p.s.i.). A variable-wavelength LC spectrophotometer (Lambda-Max Model 481) operating at 214 nm with a sensitivity setting of 0.05 a.u.f.s. was used to monitor the eluent. This was linked to a Model 730 data module programmed to provide a chart-speed of 198 mm/h.

Preparation of internal standard solution

The internal standard, nitrazepam, was dissolved in ethanol (10 mg in 100 ml) to provide the working concentration of 100 μg/ml. This solution was incorporated into the sample by adding a 25-μl aliquot to 250 μl of plasma or a 12.5-μl aliquot to 500 μl of saliva, as described in the Extraction procedure.

Extraction procedure

Plasma. CBZ and CBZ-EP were extracted from plasma using reversed-phase octadecylsilane bonded silica columns (Bond-Elut C_{18}, 2.8 ml capacity). These were conditioned immediately prior to use by drawing two column volumes of acetonitrile (2 × 2.8 ml) followed by a similar volume of water through the column under vacuum. On releasing the vacuum, 250 μl of the plasma sample followed by 25 μl of the internal standard solution (nitrazepam, 100 μg/ml in ethanol) were loaded onto the column. The sample containing internal standard was allowed to equilibrate for 1 min before reapplying the vacuum. Following loading, the vacuum was again released and a further equilibration period of 2 min preceded the washing stage. Washing was achieved by drawing one column volume of water followed by a similar volume of water—acetonitrile (80:20) through the column under vacuum. Finally, CBZ and CBZ-EP together with the internal standard were eluted from the Bond-Elut C_{18} column with 750 ml of ethanol. This ethanolic extract was evaporated to dryness under nitrogen at 55°C and the residue was reconstituted in 250 μl of mobile phase. The reconstituted samples were injected, using 10—25 μl aliquots, directly into the chromatograph.

Saliva. Saliva samples were frozen at −20°C, thawed and centrifuged (600 *g* for approx. 10 min) prior to use. This procedure was essential to overcome difficulties resulting from the high viscosity of freshly obtained saliva. To compensate for the lower concentrations of CBZ and CBZ-EP found in saliva, the sample volume was increased to 500 μl, the amount of internal standard was reduced (12.5 μl nitrazepam, 100 μg/ml in ethanol) and the injection volume increased (25 μl); other conditions were as described for the extraction of plasma samples.

Preparation of calibration standards

Stock solutions of CBZ (1 mg/ml and 100 μg/ml) and CBZ-EP (100 μg/ml) were made up in acetonitrile. Calibration standards containing both components were prepared from these by adding appropriate aliquots to plasma to produce concentrations of 0.5, 1, 2, 4, 10 and 20 μg/ml in the case of CBZ and 0.2, 0.5, 1, 1.5, 2 and 4 μg/ml for CBZ-EP. In the case of saliva, additions of standard solutions were made to provide calibration standards containing 0.5, 1, 2.5, 5 and 7.5 μg/ml for CBZ and 0.25, 0.5, 0.75, 1.5 and 2 μg/ml for CBZ-EP.

Extraction recovery experiment

Samples were prepared in plasma by adding authentic CBZ and CBZ-EP to provide plasma concentrations of 1 and 10 μg/ml and 0.5 and 2 μg/ml for CBZ and CBZ-EP, respectively. Saliva samples containing 1 and 5 μg/ml of CBZ and 0.5 and 1.5 μg/ml of CBZ-EP were produced in a similar manner. These concentration levels were chosen as being representative of values approaching the upper and lower limits of the calibration range for each component. Following extraction by the previously described procedure, 10–25 μl of the reconstituted samples in the case of the plasma and 25-μl aliquots for saliva were injected directly into the chromatograph. The peak heights obtained were compared with those from injections of standard solutions and the percentage recovery determined.

Comparative assay

Twenty-one plasma samples and nineteen saliva specimens were obtained randomly from children receiving CBZ therapy. These were analysed for CBZ and CBZ-EP using the HPLC method described and for CBZ by the EMIT procedure which was used in accordance with the manufacturer's instructions. Unfortunately, the latter does not permit the simultaneous determination of CBZ and its epoxide so comparisons were limited to the parent drug.

RESULTS

The chromatogram illustrated in Fig. 1A is that obtained following injection of a solution of authentic components in mobile phase. CBZ-EP, CBZ and the internal standard, nitrazepam, are well separated with retention times of 3.02, 4.80 and 5.43 min, respectively. These values, together with the retention times of a number of commonly prescribed drugs and preparations used in paediatric medicine, are listed in Table I. Fig. 1B represents a typical chromatogram

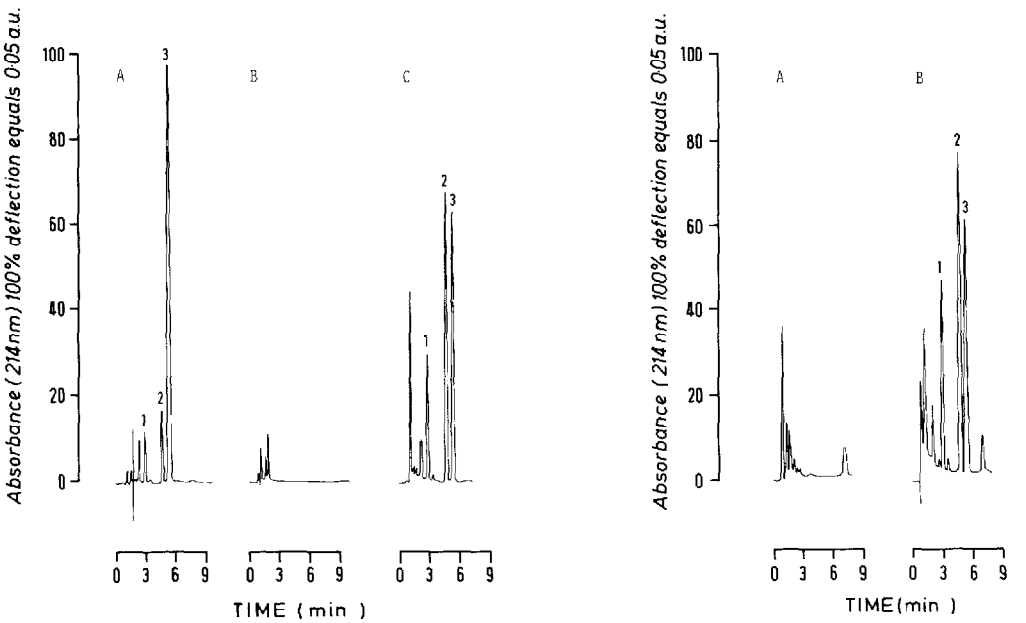

Fig. 1. (A) Chromatogram of authentic components in mobile phase. (B) Chromatogram of extracted drug-free plasma obtained from a patient who was not receiving carbamazepine. (C) Chromatogram of a typical extracted plasma sample from a patient receiving long-term carbamazepine monotherapy. Concentrations of carbamazepine and carbamazepine 10,11-epoxide determined (using a 15-μl injection) were 7.13 and 2.10 μg/ml, respectively. Peaks: 1 = carbamazepine 10,11-epoxide; 2 = carbamazepine; 3 = nitrazepam (internal standard). The peak immediately preceding that of carbamazepine 10,11-epoxide, with a retention time of 2.40 min, represents an additional carbamazepine metabolite, 10,11-dihydro-10,11-*trans*-dihydroxycarbamazepine.

Fig. 2. (A) Chromatogram of extracted drug-free saliva obtained from a patient who was not taking carbamazepine. (B) Chromatogram of a typical extracted saliva sample obtained from a patient receiving chronic carbamazepine therapy. Carbamazepine and carbamazepine 10,11-epoxide levels determined (using a 25-μl injection) were 1.98 and 0.86 μg/ml, respectively. Peaks: 1 = carbamazepine 10,11-epoxide; 2 = carbamazepine; 3 = nitrazepam (internal standard).

obtained from extracted plasma containing no CBZ or CBZ-EP. This trace clearly illustrates the absence of endogenous components which might interfere with the quantitation of the drug and its metabolite in plasma. The chromatogram shown in Fig. 1C is representative of extracted plasma samples obtained from children following prolonged CBZ therapy. Consequently, levels of CBZ in these patients are considered to be at steady state. Fig. 2A illustrates a typical trace of extracted saliva from a patient not receiving CBZ whilst Fig. 2B represents that from a patient taking the drug. Comparison of Fig. 2A and B clearly shows the absence of interferences which might prevent the accurate determination of CBZ and CBZ-EP in saliva.

It would appear, from Table II, that the recovery of CBZ (in the region of 90 ± 5%) and CBZ-EP (approximately 93 ± 2%) from both plasma and saliva is independent of concentration at the levels investigated. In addition, the precision of the extraction from both plasma and saliva [coefficient of varia-

TABLE I

RETENTION DATA OF SOME DRUGS AND RELATED COMPOUNDS USED IN PAEDIATRIC MEDICINE

Compound	Retention time (min)	Compound	Retention time (min)
Acetazolamide	<2	Prednisone	3.95
Theobromine	<2	Ethoxyzolamide	4.07
Theophylline	<2	Chlormethiazole edisylate	4.32
1,7-Dimethylxanthine	<2	Phenytoin	4.34
Caffeine	<2	Prednisolone	4.75
Cefuroxime	<2	Carbamazepine	4.80
Chlorothiazide	<2	Clonazepam	5.37
Acetaminophen	<2	Nitrazepam	5.43
10,11-Dihydro-10,11-*trans*-dihydroxycarbamazepine	2.40	10-Methoxycarbamazepine	6.25
Ampicillin	2.75	Diazepam	13.35
Konakion (vitamin $K_{1(20)}$)	2.80	Netilmicin	N.D.*
Phenobarbitone	2.92	Acetylsalicylic acid	N D.
Carbamazepine 10,11-epoxide	3.02	D,L-α-Tocopherol	N.D.

*N.D. = Peak not observed up to 20 min.

TABLE II

EXTRACTION RECOVERIES OF CARBAMAZEPINE AND ITS EPOXIDE METABOLITE FROM PLASMA AND SALIVA

Component	Concentration (μg/ml)	Recovery (%)	Coefficient of variation (%)
Plasma			
Carbamazepine	1.0	89.42	7.88
	10.0	88.00	3.31
Carbamazepine 10,11-epoxide	0.5	94.48	3.55
	2.0	91.58	2.82
Nitrazepam (internal standard)	10.0	94.81	3.13
Saliva			
Carbamazepine	1.0	85.57	9.65
	5.0	94.63	4.13
Carbamazepine 10,11-epoxide	0.5	91.05	2.16
	1.5	94.13	9.09
Nitrazepam (internal standard)	2.5	86.62	6.44

tion (C.V.) < 10%] also appears to be unaffected by concentration. Furthermore, the recovery values (in the region of 90.5 ± 4.5%) and C.V. values (< 10%) for the internal standard (nitrazepam) are of the same order as those for CBZ and CBZ-EP from both plasma and saliva.

Calibration curves were obtained by comparing the peak-height ratio (CBZ or CBZ-EP/internal standard) with the actual concentration of CBZ or CBZ-EP in plasma and saliva. The graphs were linear for plasma samples over the range 0.5—20 μg/ml and 0.2—4 μg/ml for CBZ and CBZ-EP, respectively. In the case

of saliva samples the relationship was linear over the range 0.5—7.5 μg/ml and 0.25—2 μg/ml for CBZ and CBZ-EP, respectively. The correlation coefficients (r) and slope values obtained from extracted plasma samples were 0.997 and 0.151 for CBZ and 0.993 and 0.197 for CBZ-EP. For extracted saliva samples, the respective correlation coefficients (r) and slopes were 0.999 and 0.598 and 0.986 and 0.809 for CBZ and CBZ-EP.

The effect of sample storage on the precision of the method was examined by analysing replicate plasma and saliva samples which had been stored at −20°C for up to twelve weeks. These were prepared with CBZ concentrations of 1 and 10 μg/ml and CBZ-EP concentrations of 0.5 and 2 μg/ml for plasma and, in the case of saliva, 1 and 5 μg/ml and 0.5 and 1.5 μg/ml for CBZ and CBZ-EP, respectively. In all cases the inter-batch coefficients of variation were less than 10% (see Table III) and the range of recovery values was 104 ± 6%. Clearly, storage of plasma and saliva samples at −20°C for up to twelve weeks did not produce any deterioration in either precision or extraction efficiency.

TABLE III

EFFECT OF STORAGE AT −20°C ON REPRODUCIBILITY OF MEASUREMENTS OBTAINED FROM SAMPLES PREPARED IN PLASMA AND SALIVA

Component	Concentration added (μg/ml)	Mean concentration determined (n = 27) (μg/ml)	Coefficient of variation (%)	Recovery (%)
Plasma				
Carbamazepine	1	1.10	7.77	110.0
	10	9.90	5.32	99.0
Carbamazepine 10,11-epoxide	0.5	0.54	9.79	108.0
	2.0	2.02	6.53	101.0
Saliva				
Carbamazepine	1	1.05	6.48	105.0
	5	5.00	4.00	100.0
Carbamazepine 10,11-epoxide	0.5	0.52	6.88	104.0
	1.5	1.48	6.64	98.67

The HPLC method was compared with the EMIT technique by analysing 21 plasma samples from paediatric patients receiving CBZ. As the EMIT procedure did not permit measurements of CBZ-EP it was only possible to compare CBZ values which ranged from 2.4 to 10.9 μg/ml as measured by EMIT (mean ± S.D., 5.93 ± 1.91 μg/ml). The correlation between results obtained using the two methods was excellent (r = 0.963, slope = 1.09) and a paired "t" test showed no significant difference between the two sets of results ($p > 0.8$). A similar comparison was carried out on nineteen saliva specimens. Saliva CBZ values ranged from 1.00 to 5.60 μg/ml as measured by EMIT (mean ± S.D., 2.20 ± 1.08 μg/ml). The correlation coefficient (r) and slope values were 0.934 and 1.005, respectively. This strong correlation was confirmed by a paired "t" test which showed no significant difference between the two sets of results ($p > 0.6$).

DISCUSSION

Rapid separation of CBZ and its epoxide metabolite was achieved by selecting a short, efficient column (10 cm × 8 mm I.D. Waters Assoc. Nova-Pak C_{18}, 4-μm spherical particles) in Radial-Pak cartridge form which was used in conjunction with a Z-Module RCSS. The composition of the ternary mobile phase was evaluated to provide short retention times for CBZ and CBZ-EP (4.80 and 3.02 min, respectively) whilst maintaining excellent separation. The selectivity of the procedure can be appreciated by referring to Table I which contains the retention data of a variety of drugs and medications commonly used in paediatric medicine. With the exception of phenobarbitone and prednisolone, the components listed do not interfere with the determination of CBZ and CBZ-EP using this approach. In addition, it is also clear from Table I that a further metabolite of CBZ, CBZ-DIOL, is well resolved from both CBZ and CBZ-EP. In a recent paper, Wad [24] has indicated that this additional metabolite reaches significant levels in plasma following CBZ therapy. Indeed a peak with a retention time of 2.40 min corresponding with that of CBZ-DIOL (see Table I) is clearly visible in extracted plasma from epileptic patients receiving CBZ (see Fig. 1C). Although CBZ-DIOL does not appear to possess significant anticonvulsant properties [30] Wad [24] points out that it could present problems with the quantitation of other components particularly in rapid, multi-drug applications such as that of Kabra et al. [31]. This aspect seems to have been overlooked until recently and only the methods of Wad [24] and Kumps [25] take account of it.

A number of earlier methods using UV detection at 254 nm have demonstrated poor sensitivity towards CBZ-EP [15—17, 19, 25]. However, by using a variable-wavelength UV detector and selecting a wavelength where the relative responses of CBZ and CBZ-EP are more equal, it is possible to achieve excellent sensitivity for both components [18, 20, 22—24, 28]. In this paper, UV detection at 214 nm also permitted quantitation of both CBZ and CBZ-EP at high and low concentrations without resorting to the double internal standard approach [19]. The internal standard we have used, nitrazepam, may not be suitable for some applications; in cases where this drug is co-administered 10-methoxycarbamazepine, which has a slightly longer retention time of 6.25 min (see Table I), may be a suitable alternative. The sensitivity obtained using this approach allows quantitation of plasma and saliva concentrations in the region of 100 and 50 ng/ml for CBZ and CBZ-EP, respectively. This is adequate for both routine therapeutic drug monitoring applications and pharmacokinetic studies.

Improved sample preparation was achieved by using solid-phase extraction columns (Bond-Elut C_{18}, 2.8 ml capacity) in preference to the more commonly used liquid—liquid extraction procedures [15—19, 22—24, 27, 28]. A similar approach had been used successfully by Kabra et al. [31] for the simultaneous extraction of a number of anticonvulsants from serum. However, although CBZ was one of the drugs evaluated using the above method [31], the epoxide metabolite was not included. In the present method, investigation of salivary levels of CBZ and CBZ-EP necessitated the use of larger solid-phase extraction columns (2.8 ml capacity) since the highly viscose saliva rapidly blocked the

smaller capacity Bond-Elut columns recommended by Kabra et al. [31]. Solid-phase extraction was also employed by Kinberger et al. [20] for the analysis of CBZ, CBZ-EP and other anticonvulsant drugs in serum. These drugs were adsorbed onto activated charcoal, eluted with a small volume of dichloromethane and, following evaporation and reconstitution in mobile phase, analysed by HPLC. Unfortunately, details of extraction efficiencies were not included in the above publication [20]. Recovery values for CBZ and CBZ-EP, obtained using the present method (see Table II), are comparable with those previously reported using both liquid—liquid [15—19, 22—24, 27, 28] and solid-phase [31] extraction techniques. The Bond-Elut columns were used in combination with the previously mentioned Vac-Elut system to facilitate rapid extraction of up to ten samples at a time. Furthermore, in order to minimise costs, extraction columns can be regenerated for repetitive use by washing with two column volumes of methanol as suggested by Kabra et al. [31].

Clinically, the method is being used to determine steady-state plasma and saliva levels of the parent drug and its epoxide metabolite in long-term paediatric patients receiving CBZ monotherapy and preliminary results obtained from four such patients are included. The mean CBZ dose received by these patients was 8.24 mg/kg (range, 7.69—8.82 mg/kg) and this was administered at 12-h intervals (8.00 a.m. and 8.00 p.m.). Blood and saliva specimens were taken prior to each dose and at 4 h post-dose in all cases. The mean minimum concentrations ($\overline{C}_{min}$) determined in pre-dose samples were 6.33 and 1.24 μg/ml for plasma CBZ and CBZ-EP and 1.69 and 0.59 μg/ml for CBZ and CBZ-EP in saliva. The mean maximum concentrations ($\overline{C}_{max}$) obtained from post-dose samples were 7.79 and 1.27 μg/ml and 2.19 and 0.65 μg/ml for plasma and saliva CBZ and CBZ-EP values, respectively.

The strong relationship between plasma and saliva levels of both CBZ and CBZ-EP is reflected by the correlation coefficients (r) of 0.693 ($p < 0.01$) and 0.857 ($p < 0.001$) for CBZ and CBZ-EP, respectively. The mean saliva/plasma ratio for CBZ is 0.266 (S.D., 0.08) whilst the value for CBZ-EP is 0.568 (S.D., 0.12). These values reflect the different degree of protein binding (75% in the case of CBZ and 45—50% for CBZ-EP [32] and are in good agreement with previously published values [33—35]. The relative concentrations of CBZ and its epoxide in plasma and saliva, expressed as a ratio (CBZ-EP/CBZ), were 0.20 (S.D., 0.06) and 0.34 (S.D., 0.09) for plasma and saliva, respectively. These results, which again reflect differences in protein binding between the two components, are comparable with CBZ-EP/CBZ ratios obtained by other workers [33, 36].

The method continues to be used in ongoing studies of the disposition of CBZ and CBZ-EP in plasma and saliva obtained from paediatric patients and the full clinical results will be reported upon completion.

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