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SIMULTANEOUS LIQUID CHROMATOGRAPHIC ANALYSIS FOR CARBAMAZEPINE AND CARBAMAZEPINE 10,11-EPOXIDE IN PLASMA AND SALIVA BY USE OF DOUBLE INTERNAL STANDARDIZATION

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SUMMARY

High-performance liquid chromatography (HPLC) was used for simultaneous quantitation of carbamazepine (CBZ) and carbamazepine 10,11-epoxide (CBZ-EP) in plasma and saliva. Because concentrations of CBZ can greatly exceed those of CBZ-EP after single doses, two internal standards, lorazepam and N-desmethyldiazepam were added to all samples. Following extraction with chloroform, the components are separated on a μBondapak CN column with a mobile phase composed of 30% acetonitrile in water. Total chromatography time is 10 min. Concentrations of CBZ and CBZ-EP as low as 18 and 56 ng/ml, respectively, can be detected using 0.5 ml of plasma or saliva. The maximum within-day and day-to-day coefficients of variation for both compounds are 6.3 and 7.0%, respectively. Specificity of the method was supported by a significant correlation ($r = 0.99$) between assay results of the present method and those of a previously published HPLC assay. Application of the method to protein binding and salivary measurements in a single-dose CBZ disposition study is demonstrated.

INTRODUCTION

Carbamazepine (CBZ) is an anticonvulsant drug that has been shown to be as effective as phenytoin or phenobarbital in the treatment of grand mal and complex partial seizures [1]. The utility of plasma CBZ measurements for therapeutic monitoring of patients with convulsive disorders has been demonstrated [2]. Optimum seizure control usually occurs when plasma CBZ

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concentrations are maintained in the range of 4—12 μg/ml, while concentrations greater than 12 are associated with minor and major toxicities [3]. Adjustment of the dosage, based on the determination of plasma CBZ concentrations is therefore considered very important.

The relationship between CBZ plasma concentrations and therapeutic effect is complicated by the formation of a pharmacologically active metabolite, carbamazepine 10,11-epoxide (CBZ-EP). In rats, this metabolite has been found to display anticonvulsant properties comparable to those of the parent drug [3]. Although this activity has not been confirmed in man, it may be possible that measurement of both CBZ and CBZ-EP in plasma or saliva would be more useful in the clinical management of epileptic patients [4, 5].

Previously reported techniques for measuring CBZ and/or CBZ-EP in biological fluids include gas—liquid chromatography (GLC) [6, 7], enzyme-mediated immunoassay [8], and more recently high-performance liquid chromatography (HPLC) [9—11]. Because of its simplicity, specificity and ability to separate thermally labile compounds at room temperature, HPLC is often the assay method of choice in a clinical laboratory. This report describes a rapid and sensitive HPLC method for simultaneous analysis of CBZ and CBZ-EP in both plasma and saliva. Because concentrations of CBZ-EP can be much smaller than those of the parent drug following single doses of CBZ, two internal standards were employed to facilitate more reliable quantitation of both compounds simultaneously. Application of the method to a single-dose CBZ disposition study is demonstrated.

EXPERIMENTAL

Chemicals and reagents

CBZ and CBZ-EP were gifts from Geigy Pharmaceuticals (Ardsley, N.Y. U.S.A.). Lorazepam (LOR) was donated by Wyeth Laboratories (Philadelphia, Pa., U.S.A.) and N-desmethyldiazepam (ND) was obtained from Roche Laboratories (Nutley, N.J., U.S.A.). Acetonitrile and chloroform, ultraviolet grade, were purchased from Burdick and Jackson Labs. (Muskegon, Mich., U.S.A.) and reagent grade tribasic sodium phosphate was obtained from J.T. Baker (Phillipsburg, N.J., U.S.A.).

Drug standards

Stock solutions of CBZ and CBZ-EP were prepared in methanol at concentrations of 400 and 100 μg/ml, respectively. These solutions were further diluted with water to produce solutions of the desired concentration. Stock solutions of the internal standards, LOR and ND, were prepared in methanol at concentrations of 100 μg/ml and a working internal standard solution was prepared in water to contain 10 μg/ml of LOR and 30 μg/ml of ND. A chromatography drug reference mixture containing 25 μg/ml of CBZ and CBZ-EP, 5 μg/ml of LOR and 15 μg/ml of ND was prepared in methanol. All solutions were stored in amber-colored glass bottles at 4°.

Apparatus

A Model 841 high-pressure liquid chromatograph (DuPont Instruments,

Wilmington, Del., U.S.A.) equipped with a 254 nm ultraviolet detector and fitted with a Rheodyne Model 7120 sample injection valve (Perkin-Elmer, Norwalk, Conn., U.S.A.) was used. Analyses were performed on a μBondapak CN (particle size 10 μm) column from Waters Assoc. (Milford, Mass., U.S.A.). Detector output was recorded at 1 mV with an Omniscribe recorder (Houston Instruments, Austin, Texas, U.S.A.).

Other equipment included 13 × 150 mm PTFE-lined screw-capped culture tubes, 15-ml conical centrifuge tubes, a reciprocating shaker, bench-top vortex-type mixer, high-speed centrifuge, and an analytical evaporating bath with a nitrogen gas source.

Extraction procedure

Saliva, plasma or buffer solution (0.5 ml) was transferred to a glass culture tube containing 25—50 μl of the aqueous internal standard mixture and 0.5 ml of aqeuous saturated tribasic sodium phosphate solution. After gentle mixing, 7 ml of chloroform was added and the mixture shaken for 15 min. After centrifugation (1200 *g*, 10 min) the aqueous (top) layer was aspirated and discarded. The remaining chloroform layer was transferred to a conical glass tube and evaporated at 40° under a gentle stream of nitrogen. The sides of each tube were rinsed with an additional 1 ml of chloroform and evaporated again. The residue was dissolved in 100 μl of mobile phase, vortex-mixed for 30 sec, and 50 μl of the extract injected into the chromatograph.

Chromatography and quantitation

All chromatography was performed at room temperature. The mobile phase consisted of 30% acetonitrile in distilled deionized water and the flow-rate was 1.2 ml/min. Column effluent was monitored at 254 nm with detector sensitivity set at 0.04—0.16 a.u.f.s. Chart speed of the recorder was 20 cm/h.

Plasma standards were prepared by adding CBZ and CBZ-EP to drug-free pooled plasma to give final concentrations ranging from 0.5 to 30 μg/ml. The standards were processed according to the procedure, peak heights measured, and the peak height ratios of CBZ-EP:LOR and CBZ:ND were calculated. Standard curves relating peak height ratios to respective drug concentrations were used to calculate each drug concentration in the unknown samples.

Recovery

CBZ, CBZ-EP, LOR and ND were added to drug-free plasma, saliva and phosphate buffer and then analyzed according to the procedure but without any added internal standards. Carefully measured aliquots of the reconstituted extracts were injected and peak heights corresponding to each compound were measured. Absolute recovery was calculated by comparing these peak heights with peak heights obtained by direct injection of pure drug standards.

Precision

Precision of the method was evaluated by analysis of plasma standards containing both CBZ and CBZ-EP at concentrations of 0.5 and 30 μg/ml. Within-day precision was obtained by analyzing plasma standards 10 times in one day. The same plasma standards were stored in aliquots at —20° in glass

tubes and analyzed once daily for 11 days over 2 months to assess day-to-day precision.

Interferences

Seven anticonvulsant and sedative drugs, commonly used with CBZ, were tested for potential interferences with our procedure by comparing the retention times for methanolic standards of these drugs with those of CBZ, CBZ-EP, LOR and ND.

Comparative assays

Twenty saliva and plasma samples were randomly obtained from 15 epileptic patients and analyzed for CBZ and CBZ-EP by the HPLC method of Kabra and Marton [12] and by our method. According to the former method, components were extracted from acidified plasma and separated on a C_{18} μBondapak column (Waters Assoc.) with a mobile phase of 37% acetonitrile in water. Although this method was originally developed for quantitation of CBZ only, it was possible to quantitate the epoxide metabolite as well.

Clinical application

One volunteer took 400 mg of CBZ (Tegretol®, Geigy Pharmaceuticals) orally. Samples of plasma and saliva were obtained over a 72-h period and stored at −20° until analyzed. Protein binding of CBZ was determined by equilibrium dialysis of plasma against an equal volume of isotonic phosphate buffer, pH 7.4, in a water bath maintained at 37°. Analysis of buffer and plasma after 24 h allowed calculation of free CBZ concentrations.

RESULTS

Resolution and sensitivity of the chromatographic system were determined daily by injection of 25 μl of the standard drug reference mixture. A typical chromatogram of the mixture is shown in Fig. 1A. This same mixture was extracted from drug-free plasma according to the procedure and chromatographed; the resulting chromatogram is shown in Fig. 1C. Chromatographic peaks are sharp and symmetrical allowing use of peak heights rather than peak area to quantitate detector response. Extracts of drug-free plasma yielded no interference from endogenous plasma components with the exception of a small peak having a retention time slightly longer than that of CBZ-EP, as shown in Fig. 1B. Although complete separation of this component from CBZ-EP can be obtained by decreasing the flow-rate, it only represents 0.06 μg/ml of CBZ-EP, thus contributing minimal interference with plasma CBZ—EP concentrations during chronic dosing of CBZ. Retention times for CBZ-EP, CBZ, LOR and ND under our conditions are 4.0, 5.2, 6.1, and 9.5 min, respectively. With use of a mobile phase at pH 5, column-life is estimated to be at least 9 months with daily use.

Linearity of detector response was evaluated by injecting 50 μl of various methanolic standards containing amounts of CBZ and CBZ-EP ranging from 0.3 to 30 μg. Detector response (peak height) was linear over this range for both compounds, with both curves passing through the origin. Peak height ratios of

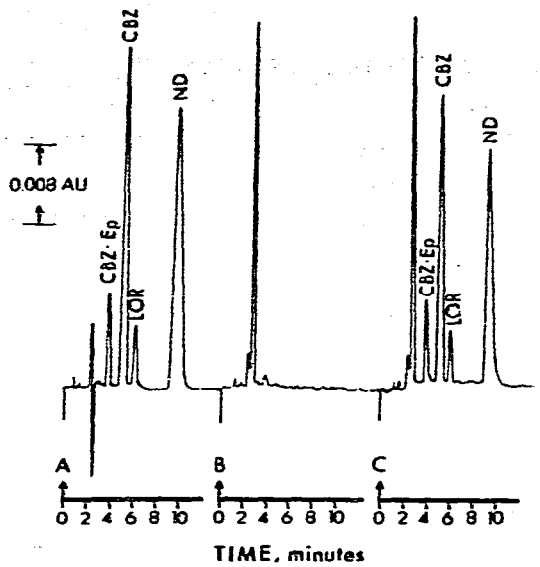

Fig. 1. Chromatograms of carbamazepine and its 10,11-epoxide: (A) standard mixture of compounds, CBZ and CBZ-EP, 0.625 μg; LOR, 0.125 μg; ND, 0.375 μg; (B) extract of drug-free plasma; (C) standard mixture extracted from plasma.

CBZ-EP:LOR and CBZ:ND from extracted samples were also linearly related to concentration over the range of 0.5—30 μg/ml.

The limits of detection, allowing a signal-to-noise ratio of 4, are 9 and 28 ng for CBZ and CBZ-EP, respectively. The sensitivity allows for quantitation of at least 0.02 μg/ml of CBZ using 0.5 ml of sample. Because of the plasma impurity that is partially eluted with CBZ-EP, the sensitivity of detection for this metabolite is 0.12 μg/ml using 0.5 ml of plasma and 0.06 μg/ml using buffer or saliva.

TABLE I

ANALYTICAL RECOVERY

Drug	Concn. (μg/ml)	*n*	Recovery (%)		
			Saliva	Buffer	Plasma
Carbamazepine 10,11-epoxide	1.0	5	103	99.8	109
	10	5	100	96.3	100
Carbamazepine	1.0	5	106	100	103
	10	5	100	97	99.6
Lorazepam	0.2	4	101	101	103
	2.0	4	97.6	109	97.6
N-desmethyldiazepam	0.6	4	104	100	104
	6.0	4	102	107	98.7

TABLE II

PRECISION OF ASSAY FOR CARBAMAZEPINE AND ITS 10,11-EPOXIDE

	Carbamazepine			10,11-Epoxide		
	Concn. (μg/ml)	*n*	C.V. (%)	Conc. (μg/ml)	*n*	C.V. (%)
Within-day	0.5	10	4.4	0.5	10	6.3
	30	10	2.4	30	10	5.0
Day-to-day	0.5	11	7.0	0.5	11	5.0
	30	11	3.1	30	11	4.6

TABLE III

RETENTION TIMES FOR SELECTED DRUGS

Drug	Retention time (min)
Primidone	3.2
Ethosuximide	3.4
Carbamazepine 10,11-epoxide	4.0
Phenobarbital	4.7
Carbamazepine	5.2
Oxazepam	5.5
Phenytoin	5.9
Lorazepam	6.2
Clorazepate	7.0
N-Desmethyldiazepam	9.5
Diazepam	10.6

Analytical recoveries at two concentrations of CBZ-EP, CBZ, LOR and ND are given in Table I. Recovery of all compounds ranged from 96 to 109% with no perceivable dependence on drug concentration or sample media. As a result, analytical standards of CBZ and CBZ-EP were prepared in plasma and used to quantitate unknown concentrations in plasma, buffer or saliva.

As shown in Table II, within-day precision of CBZ and CBZ-EP varied from 2.4 to 6.3% while day-to-day precision for these compounds in frozen plasma ranged from 3.1 to 7.0%. The stability of CBZ and CBZ-EP in plasma stored at 20° in glass tubes was thus determined to be at least 2 months.

Table III lists the retention times for methanolic standards of various anticonvulsant and sedative drugs detected by the chromatographic system. Of those drugs tested, oxazepam and phenytoin showed potential interference with analysis of CBZ and LOR, respectively. Although interference by phenytoin was eliminated by our basic extraction procedure, potential interference by oxazepam remains.

Fig. 2 shows the results of the two HPLC analyses for CBZ and CBZ-EP in 20 plasma and saliva samples from patients receiving CBZ maintenance therapy for convulsive disorders. Due to obvious interferences with quantitation of CBZ-EP using the method of Kabra and Marton [12] only 10 out of 20 patient samples were analyzed for this compound. No obvious interferences with either

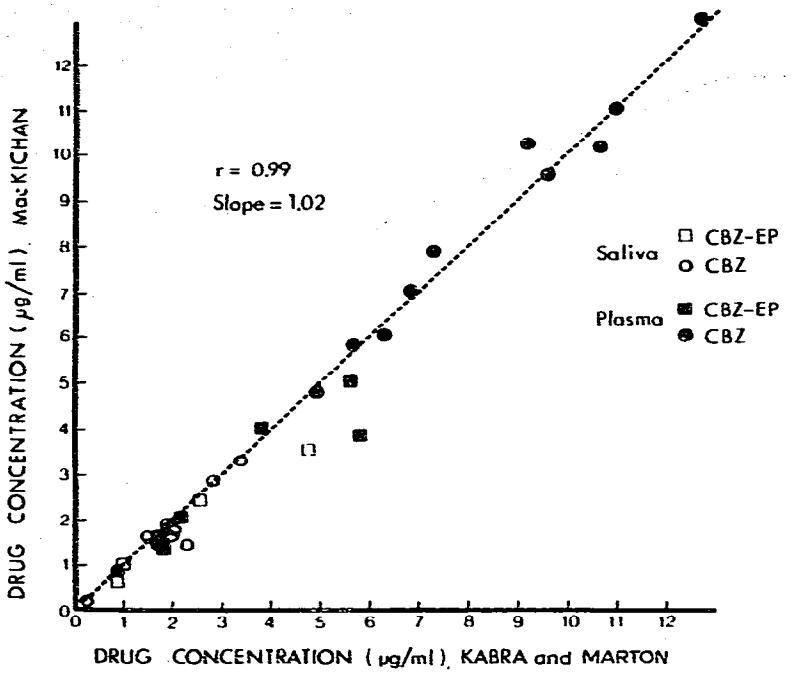

Fig. 2. Concentrations of CBZ and CBZ-EP in plasma and saliva of epileptic patients as measured by the present HPLC method and by the HPLC method of Kabra and Marton [12].

compound were noted with the use of the author's HPLC assay. Concentrations of CBZ and CBZ-EP ranged, respectively, from 0.2 to 3.3 and 0 to 2.4 μg/ml in saliva and from 4.8 to 13 and 0.4 to 4 μg/ml in plasma. The graph demonstrates an excellent correlation between concentrations of both compounds as determined by the two methods, with a correlation coefficient of 0.99 and a slope of unity.

Fig. 3 shows results of the analyses of CBZ and CBZ-EP in plasma and CBZ in saliva from a subject given a single 400-mg dose of CBZ. A peak CBZ concentration of 5 μg/ml in plasma was reached in approximately 8 h, while the peak CBZ-EP concentration was approximately 0.7 μg/ml. Saliva and free plasma concentrations of CBZ averaged 24% and 25% of total plasma CBZ concentrations, respectively. Although concentrations of CBZ-EP were detected in plasma following a single dose, sensitivity of the assay did not allow quantitation in saliva or protein binding determinations. The decline of CBZ and CBZ-EP in plasma and of CBZ in saliva were log-linear demonstrating similar half-lives of 30, 33 and 29 h, respectively.

DISCUSSION

The method presented here is rapid, reproducible, specific and sensitive enough to allow simultaneous quantitation of both CBZ and CBZ-EP in plasma and saliva for routine monitoring of CBZ therapy. In addition, the double internal standardization approach, similar to that used by Greenblatt [13] for

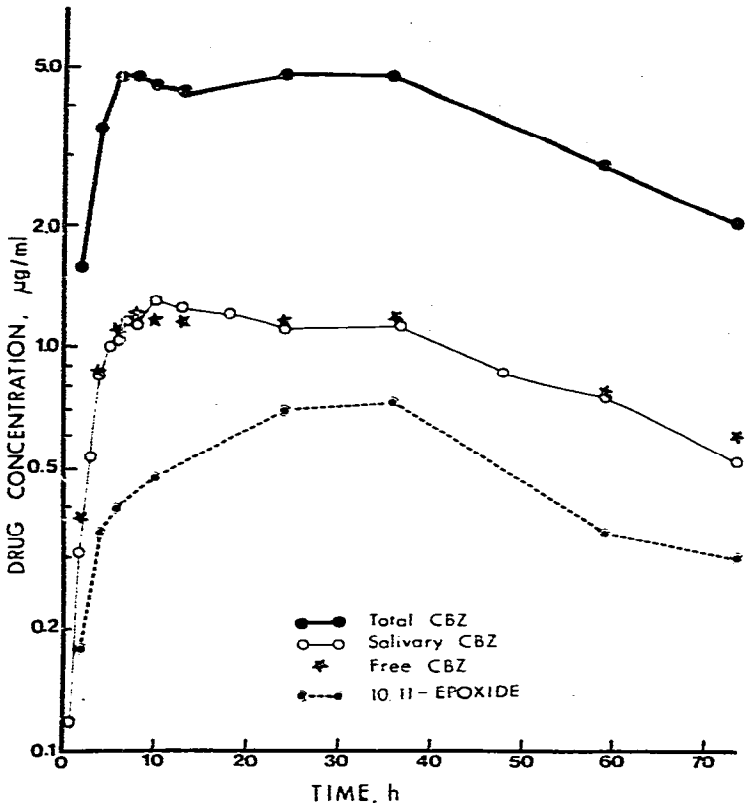

Fig. 3. Concentrations of CBZ and CBZ-EP in plasma and saliva during 72 h after a single 400-mg oral dose of CBZ given to a normal volunteer.

diazepam, greatly facilitates single-dose disposition studies of CBZ in humans. Relatively high concentrations of the parent compound, CBZ, are quantitated with use of a relatively large amount of one internal standard, ND, while the low concentrations of the epoxide metabolite are more accurately quantitated with use of a smaller amount of the second internal standard, LOR. In studies involving chronic dosing of CBZ, when concentrations of the metabolite approach those of the parent compound, both internal standards are not necessary. The potential problem of drug interference with one internal standard can be circumvented, however, by use of the other.

Due to its simplicity and rapidity, enzyme-mediated immunoassay may be the most common method of routine clinical CBZ analysis [8]. This method has the disadvantages of non-specificity and inability to quantitate the epoxide metabolite, making it unsuitable for most research studies of CBZ metabolism and disposition [8].

In addition to the capability for simultaneous analysis of CBZ and its active metabolite, our HPLC method shares with most GLC methods [6, 7] the advantages of high specificity and sensitivity. Both of these compounds, however, have been shown to decompose at GLC temperatures [14]. As a result, lengthy and tedious sample preparation procedures such as derivatization are often necessary. Liquid chromatography, in addition to

simplicity of instrumentation and sample preparation, has the advantage of allowing separation of thermally labile compounds at ambient temperature.

Early HPLC methods for simultaneous analysis of CBZ and CBZ-EP [9–11] have suffered from relative insensitivity to the epoxide metabolite. In order to reliably quantitate this compound, these methods require either extraction of large volumes of plasma and/or alteration of detector sensitivity during the chromatography step. In addition, some HPLC methods [9, 10] require the use of more lengthy extraction procedures to eliminate interferences from concomitantly administered drugs. With our method, the desired sensitivity is achieved for both CBZ and CBZ-EP using only 0.5 ml of plasma or saliva and a one-step extraction procedure, with minimal interference from other anticonvulsant drugs.

The results of our single-dose CBZ study are in excellent agreement with observations of other investigators. As compared to the peak CBZ concentration of 5 μg/ml attained in our study, Levy et al. [15] using a GLC technique, measured peak CBZ concentrations of 3.3—5.4 μg/ml in serum of normal subjects following a single 400-mg CBZ dose. Using HPLC, Eichelbaum et al. [4] observed average peak concentrations of 2 μg/ml and 0.1 μg/ml for CBZ and CBZ-EP, respectively, in the plasma of normal subjects following single 200-mg doses. The length of time (8 h) required to reach the peak plasma CBZ concentration in this study was within the range of 2—18 h reported by other investigators [15, 16] and has been explained by dissolution-rate limited absorption of the tablet secondary to the low water solubility of this drug [15].

As compared to the 30-h CBZ elimination half-life reported in this study, half-lives ranging from 24 to 55 h have been reported in single-dose studies by investigators using different analytical techniques [4, 15, 17]. In addition, the parallel decline of the plasma concentration–time profiles for CBZ and its epoxide metabolite observed in our study is also consistent with previous reports [4, 5]. The explanation for this lies in the fact that the half-life of CBZ-EP is much shorter than that of the parent drug [18]. Because the metabolite cannot be removed any faster than it is produced, an apparent half-life equal to that of the parent drug is observed.

Analysis of CBZ in saliva can provide a convenient and non-invasive alternative to the determination of CBZ in plasma. In this study, saliva CBZ concentrations in the volunteer subject paralleled plasma concentrations with a mean saliva:plasma ratio of 0.24. This is in good agreement with recent studies [19, 20] in which the saliva:plasma ratios ranged from 0.23 to 0.37 over similar plasma concentration ranges. It is therefore feasible that analysis of salivary samples can provide estimates of CBZ concentrations in plasma.

A more important advantage to analysis of drugs in saliva is that it often reflects the unbound, pharmacodynamically active fraction of drug in plasma. This is especially important for drugs that are highly or variably protein bound. We found that free plasma CBZ concentrations in our single subject were equivalent to salivary concentrations, and averaged 25% of total plasma concentrations. Using ultrafiltration techniques, Hooper et al. [21] reported a high degree of interindividual variability in the plasma protein binding of CBZ; free plasma CBZ concentrations ranged from 10 to 30% (mean 18%) in normal

subjects, and from 8 to 60% (mean 27%) in epileptic patients. Because of this variability in the free fraction of CBZ, interpretation of a therapeutic range of CBZ based on total plasma concentrations is difficult and may be facilitated by establishment of a therapeutic range for CBZ in saliva.

Assay sensitivity did not allow analysis of the epoxide metabolite in the saliva of the volunteer subject following a single dose of CBZ in this study. In contrast to a recent report [5], sensitivity of our HPLC method did allow quantitation of this metabolite in the saliva of epileptic patients who took CBZ chronically, as shown in Fig. 3. Although there was almost a four-fold difference between mean concentrations of parent drug and metabolite in plasma (8.5 and 2.4 μg/ml, respectively), the concentrations of CBZ and CBZ-EP in saliva were similar with average values of 1.7 and 1.2 μg/ml, respectively. Assuming that saliva is a reflection of free CBZ-EP in plasma, this apparent discrepancy can be explained by the fact that CBZ-EP is only 45—50% protein bound as compared to 75% for CBZ [18]. It is clear that further studies are necessary to evaluate the contribution of CBZ-EP to total anticonvulsant activity as well as the relationships between salivary, free and total plasma CBZ-EP concentrations in patients. Use of this sensitive and specific HPLC method for simultaneous analysis of CBZ and CBZ-EP in saliva may prove to be a more rational and convenient method for monitoring CBZ therapy in epileptic patients.

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