Journal of Chromatography, 474 (1989) 223–230
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 518

APPLICATION OF THERMOSPRAY LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY TO THE SIMULTANEOUS QUANTIFICATION OF TRACER CONCENTRATIONS OF ISOTOPICALLY LABELLED CARBAMAZEPINE EPOXIDE AND STEADY-STATE LEVELS OF CARBAMAZEPINE AND CARBAMAZEPINE EPOXIDE

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SUMMARY

A thermospray high-performance liquid chromatography–mass spectrometry method for the separation and quantification of tracer concentrations of isotopically labelled carbamazepine epoxide ([^{15}N, ^{13}C]CBZE) in the presence of steady-state levels of the anticonvulsant carbamazepine (CBZ) and its epoxide metabolite (CBZE) has been developed. The technique does not require derivatization, demonstrates little or no thermal degradation of the analytes, provides increased specificity not available from conventional high-performance liquid chromatography, and has a detection limit of 500 pg for CBZE on-column. The method, incorporating d_4-CBZ and d_4-CBZE as internal standards, allows precise and accurate determination of the analytes with good reproducibility and stability.

INTRODUCTION

The epoxide hydrolase (EH) enzyme system is mainly involved in detoxification reactions of cytotoxic and genotoxic arene oxide xenobiotic metabolites[1]. Knowledge of the overall activity of this enzyme system *in vivo* may be of clinical importance enabling the prediction of individual susceptibility to adverse drug and environmental effects.

Carbamazepine-10,11-epoxide (CBZE, Fig. 1) is one of the principle metabolites of the anticonvulsant carbamazepine (CBZ, Fig. 1). The majority of the epoxide

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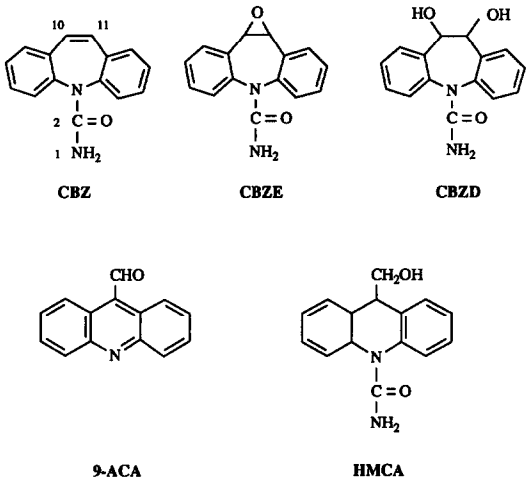

Fig. 1. Structures of Carbamazepine (CBZ); carbamazepine-10,11-epoxide (CBZE); carbamazepine-10,11-transdihydrodiol (CBZD); 9-hydroxymethyl-10-carbamoylacridan (HMCA); and 9-acridinecarboxaldehyde (9-ACA).

formed is ultimately converted to carbamazepine-10,11-transdihydrodiol (CBZD, Fig. 1) by EH. The ratio of CBZE to CBZ is reported to be higher in children than in adults, with the ratios being highest in very young children[2,3]. To examine this implied age dependent development of the EH enzyme system, CBZE pharmacokinetics must be evaluated in epileptic children on CBZ monotherapy. Such an evaluation requires an analytical method capable of distinguishing administered CBZE from the CBZE formed as a metabolite of CBZ.

Several gas chromatographic (GC) methods have been developed for the analysis of CBZ and CBZE[4,5], some of which have utilized mass spectrometric (MS) detection techniques[6,7]. However, the thermal degradation of the epoxide metabolite to 9-acridinecarboxaldehyde (9-ACA, Fig. 1) during GC analysis[8] has precluded this detection method from use with regard to isotopic labelling in the 1- and 2-positions of the molecule. High-performance liquid chromatography (HPLC) in combination with less specific detection methods, on the other hand, are routinely employed in the clinical assay of CBZ and CBZE and demonstrate no such degradation difficulties[9,10].

The thermospray liquid chromatography–mass spectrometry (TSP LC–MS) technique has proven to be a valuable and reliable tool in the routine detection and analysis of underivatized endogenous compounds that are intractable to GC–MS methods[11]. This paper describes a TSP LC–MS analytical methodology for the simultaneous determination of administered [1-^{15}N,2-^{13}C]carbamazepine epoxide ([^{15}N,^{13}C]CBZE), CBZE and CBZ in human blood utilizing stable isotope labelled internal standards and offers an assessment of the sensitivity, precision and accuracy of the technique in the quantification of these compounds.

MATERIALS AND METHODS

Reagents

Carbamazepine and 9-hydroxymethyl-10-carbamoylacridan were a generous gift from Ciba-Geigy (Summit, NJ, U.S.A.). Iminostilbene (>97% pure) and *m*-chloroperbenzoic acid (85% pure) were purchased from Fluka (Ronkonkoma, NY, U.S.A.) and used without further purification. Phosgene (^{13}C, 99%) 6.30% (w/w) in benzene, gaseous ammonia (^{15}N, 99%) and sulfuric acid ($^{2}H_2SO_4$, 99%) were supplied by Cambridge Isotope Laboratories (Woburn, MA, U.S.A.). Anhydrous ethanol (OD, 99%) was obtained from ICN Biomedicals (Cambridge, MA, U.S.A.). All solvents used in synthesis were reagent grade. Acetonitrile for HPLC was obtained from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Ammonium acetate and polyethylene glycol (PEG-200) were purchased from J. T. Baker (Phillipsburg, NJ, U.S.A.) and Aldrich (Milwaukee, WI, U.S.A.), respectively.

Synthetic procedures

[1-^{15}N,2-^{13}C]Carbamazepine epoxide ([^{15}N,^{13}C]CBZE). [^{15}N,^{13}C]CBZ was synthesized by the sequential reaction of iminostilbene with [^{13}C]phosgene and [^{15}N]ammonia according to Osterloh and Bertilsson[12]. The epoxidation of the purified [^{15}N,^{13}C]CBZ was achieved using *m*-chloroperbenzoic acid and the product, [^{15}N,^{13}C]CBZE, was recrystallized from ethanol. Purity and identity of the synthesized compound were verified by thin-layer chromatography (TLC) and melting point analysis and further confirmed by NMR and MS[8,13]. The ^1H NMR (300 MHz) in deuterochloroform using tetramethylsilane as internal standard, was found to be identical to that of the authentic unlabelled compound with the exception of the amino group protons at position 1. These appeared as a doublet at δ 4.59 ($^1J_{H,N}$ = 90 Hz) due to the ^{15}N–H coupling. The electron impact mass spectrum was in agreement with that published earlier[8], but exhibited the ions *m/z* 254 $[M]^{+\cdot}$ and 225 $[M-CHO]^+$ in contrast to *m/z* 252 $[M]^{+\cdot}$ and 223 $[M-CHO]^+$ as seen for authentic CBZE.

[$^2H_4$]Carbamazepine (d_4-CBZ) and [$^2H_4$]carbamazepine epoxide (d_4-CBZE). These two compounds were prepared as above using [$^2H_4$]iminostilbene (d_4-IMS), phosgene and ammonia as starting materials. The d_4-IMS was obtained by twice heating iminostilbene for 24 h at 78°C with anhydrous ethanol-d_1 in the presence of $^2H_2SO_4$ ($^2H_2SO_4$–$C_2H_5O^2H$, 1:100, v/v) according to a published procedure[12]. Electron impact mass spectra of the recrystallized d_4-CBZE and d_4-CBZ showed identical isotopic composition. The compounds contained 63% of the d_4 isomer and 4% d_6, 20% d_5, 12% d_3 and 1% d_1.

Chromatography

HPLC analysis was carried out under isocratic conditions using a LKB 2150 HPLC pump combined with a LKB 2152 controller unit (LKB, Bromma, Sweden). Samples were injected via a Valco C6W manual injector (Valco Instruments, Houston, TX, U.S.A.) equipped with a 100-μl sample loop. Separations were achieved on a Spherisorb C_8 analytical column (25 cm × 4.6 mm I.D., 5 μm Phase Separation, Norwalk, CN, U.S.A.) connected to a Brownlee RP-8 guard column (1.5 cm × 3.2 mm I.D., 7 μm, Brownlee Labs., Santa Clara, CA, U.S.A.). The mobile phase was acetonitrile–0.1 *M* ammonium acetate (40:60) at a flow-rate of 1.2 ml/min.

Mass spectrometry

TSP LC–MS analysis was performed in positive ion mode with the filament off, utilizing a Vestec Model 201 dedicated thermospray masss spectrometer (Vestec, Houston, TX, U.S.A.) with a directly heated probe vaporizer[14]. Thermospray probe control and tip temperatures were 120 and 180°C, respectively, and adjusted to maintain a jet temperature of 250°C. The source block was held at 300°C. A Hewlett-Packard 59979C ChemStation™ (Palo Alto, CA, U.S.A.) data system was used to control the mass analyzer and the data acquisition in selected-ion monitoring (SIM) mode. Selected ions of a 100 ppm solution of PEG 200 in 0.1 *M* ammonium acetate were used to provide mass calibration of the data system. Ions monitoring were *m/z* 253.1, 255.1 and 257.1, corresponding to the MH^+ ions of the CBZE analytes and their d_4-labelled internal standard and *m/z* 237.1 and 241.1, corresponding to the MH^+ of CBZ and its d_4-analogue. The dwell-times were set at 350 ms/ion for CBZE analogues and at 750 ms/ion for CBZ and its internal standard.

Quantitative analysis

Sample preparation. To each 250 μl blood sample, 50 μl of each of the two methanolic internal standard solutions containing d_4-CBZE (5 μg/ml) and d_4-CBZ (25 μg/ml), respectively, was added. Following the addition of 5 ml of dichloromethane, the samples were shaken for 20 min in a horizontal shaker and centrifuged at 500 *g* for 5 min at room temperature. The organic phase was then transferred to a disposable glass tube and again to a glass storage vial using a pasteur pipet. After evaporation to dryness the vials were capped and stored at −20°C to await TSP LC–MS analysis. For the generation of standard curves, 25 μl aliquots of each of five methanolic stock solutions containing increasing amounts of [^{15}N,^{13}C]CBZE (0.5, 1.0, 2.5, 5.0 and 10.0 μg/ml), CBZE (2.5, 5.0, 12.5, 25.0 and 50.0 μg/ml) and CBZ (5.0, 10.0, 25.0, 50.0 and 100 μg/ml) were added to 250 μl of blank human blood in separate vials and processed in the same manner as the samples.

With each sample set, two 250 μl control blood samples were assayed to monitor the precision and reproducibility of the method. Stocks of these samples containing 0.10, 0.50 and 5.0 μg/ml and 0.75, 2.0 and 10.0 μg/ml of [^{15}N,^{13}C]CBZE, CBZE and CBZ, respectively, were stored at −20°C and used throughout the study.

Analysis. Chromatographic analysis was performed using 20 μl aliquots of the samples reconstituted in 100 μl of mobile phase. Individual ion intensities were integrated using standard Hewlett-Packard MSD-GC™ software. Peak areas of [^{15}N,^{13}C]CBZE were corrected for the natural abundance derived from the unlabelled CBZE in the samples. The correction factor was determined empirically by LC–MS analysis of CBZE stock-solutions.

Response ratios for each analyte relative to its internal standard were calculated and used for the generation of the standard curves or the determination of the analyte concentration in patient and control samples.

RESULTS AND DISCUSSION

Optimization of chromatographic and instrumental parameters

Previously reported HPLC methods[9,10] were modified by the incorporation of ammonium acetate into the mobile phase to allow for TSP ionization of the analytes.

As may be seen in the reconstructed ion chromatogram illustrated in Fig. 2A, the acetonitrile–0.1 *M* ammonium acetate (40:60) mobile phase employed, resulted in excellent separation of CBZE (peak 3) and CBZ (peak 4) which eluted at 4.3 min and 6.4 min, respectively. In addition to the peaks of CBZE and CBZ, two other CBZ metabolites were detected. The diol, CBZD and [^{15}N,^{13}C]CBZD, was found to elute at 2.7 min (Fig. 2A, peak 1) and could have been monitored if the corresponding MH^+ of these compounds at *m/z* 271 and 273 had been acquired. The peak eluting at 3.9 min (Fig. 2A, peak 2) was identified as 9-hydroxymethyl-10-carbamoylacrian (HMCA, Fig. 1), whose MH^+ is isobaric with that of the labelled CBZE at *m/z* 255. Under the LC conditions employed, baseline separation of these two compounds was achieved (Fig. 2B).

The TSP LC–MS spectra of CBZE and CBZ showed protonated molecular ions

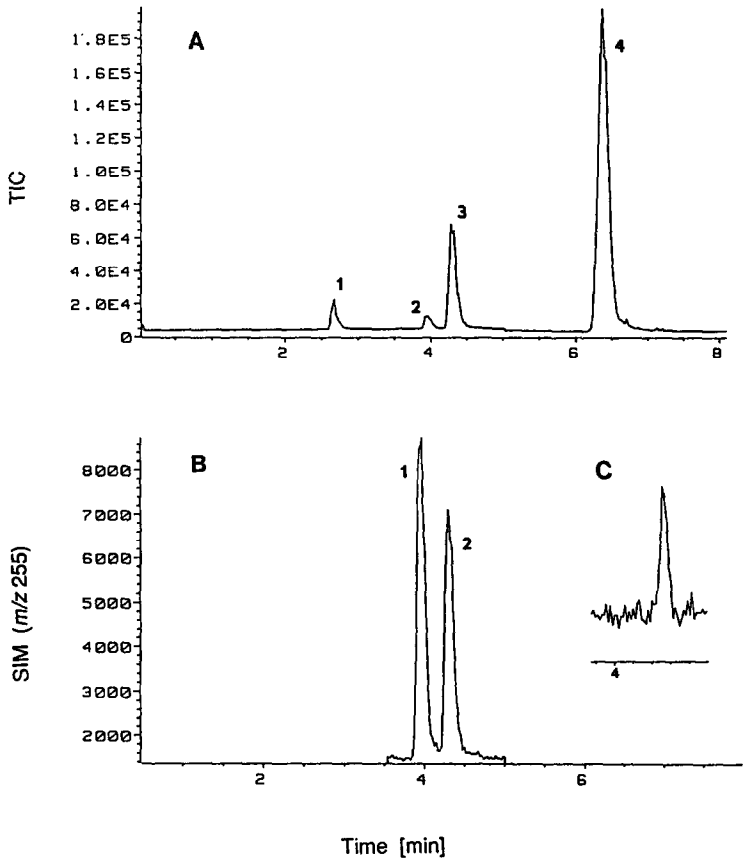

Fig. 2. TSP LC–MS ion current chromatograms from a patient blood sample. (A) Total-ion current chromatogram with the compounds being detected and the ions monitored for each peak as follows: 1 = *m/z* 271 and 273 (CBZD and [^{15}N,^{13}C]CBZD); 2 = *m/z* 255 (HMCA); 3 = *m/z* 253, 255 and 257 (CBZE, [^{15}N,^{13}C]CBZE and d_4-CBZE); 4 = *m/z* 237 and 241 (CBZ and d_4-CBZ). (B) Selected-ion current chromatogram *m/z* 255; 1 = HMCA; 2 = [^{15}N,^{13}C]CBZE at a blood concentration of 0.204 μg/ml. (C) Selected-ion current chromatogram *m/z* 255; detection of 1 ng [^{15}N,^{13}C]CBZE on-column. Signals in each chromatogram are normalized to the most intense peak.

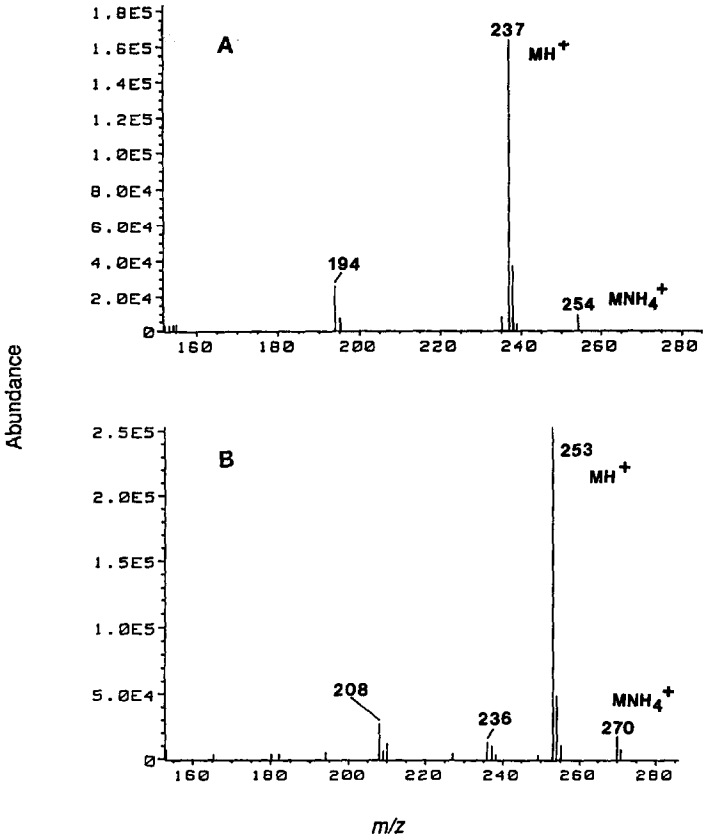

Fig. 3. TSP mass spectra of CBZ (A) and CBZE (B).

as their base peaks (Fig. 3). In addition, minor ammonium adducts were observed. Quantitative analysis was performed in the SIM mode for the protonated molecular ions. Thus, ions of *m/z* 253, 255 and 257 were monitored for the CBZE analogues in the time window between 3.5 and 5.0 min and ions of *m/z* 237 and 241 for CBZ between 5.0 and 8.0 min.

Critical TSP temperature parameters were optimized by injection of pure analytes on column. It was observed that source temperature variations over the range of 280 to 320°C did not significantly effect the TSP ionization. However, as observed by others[15], high jet temperatures resulted in pronounced instabilities of the jet leading to "spiking" in peak chromatographic profiles, while low jet temperatures yielded significant losses in sensitivity. To overcome this difficulty the peak width of the analytes were determined and dwell times chosen to enable at least 12 to 15 sampling across the peak of each ion monitored. The jet temperature was then adjusted to provide the optimal combination of sensitivity and stability. By this method the jet temperature of 250°C and dwell times of 350 and 750 ms for the CBZE and CBZ analogues, respectively, were established.

The ion chromatogram illustrated in Fig. 3C shows 1 ng of standard [^{15}N,^{13}C]CBZE on-column obtained by monitoring the MH^+ ion at a dwell time of 350 ms. The detection limit was 500 pg with a signal to noise ratio of 3:1.

Standard curves

For all the quantitative calculations peak areas of [^{15}N-^{13}C]CBZE were corrected for contributions resulting from the natural abundance of the unlabelled CBZE. Correction factors were determined empirically by the TSP LC–MS analysis of CBZE. For the generation of the standard curves, peak area ratios of analytes to internal standards were plotted against standard sample concentrations. Over the concentration ranges of 0.05–1.0 μg/ml, 0.25–5.0 μg/ml and 0.50–10.0 μg/ml for [^{15}N,^{13}C]CBZE, CBZE and CBZ, respectively, the standard curves were found to be linear with correlation coefficients greater than 0.999.

As discussed elsewhere[16,17], the detection of natural isotopic species from an analyte in the mass window of the internal standard results in standard curves which diverge from linear. In all cases presented, these isotopic contributions were found to be minor and an insignificant source of non-linearity. Of greater consequence was the observed 2.02% isotopic contribution of CBZE to the measured abundance of [^{15}N,^{13}C]CBZE at *m/z* 255. Since all of the standard solutions contained CBZE and [^{15}N,^{13}C]CBZE in the ratio of 5:1, the use of the observed peak areas in the generation of standard curves for the latter would have resulted in an approximate 10% systematic error. In the quantification of patient samples, where the ratios were as high as 40:1, the correction for isotopic contribution was found to be correspondingly more critical.

Precision and accuracy data of the assay are summarized in Table I. The intra-day variability was determined by analysis of triplicate extracts of the control samples throughout the day and was less than 1% for CBZ and CBZE, and 6% for [^{15}N,^{13}C]CBZE at a blood concentration of 100 ng/ml. The interday reproducibility showed coefficients of variation of less than 9% in all cases. Furthermore, it was found that the calculated concentrations were in good agreement with the predicted values.

TABLE I

INTRA- AND INTER-DAY VARIATIONS FOR [^{15}N,^{13}C]CBZE, CBZE AND CBZ IN HUMAN BLOOD CONTROL SAMPLES

Compound	*Nominal concentration (μg/ml)*	*Intra-day variation (n = 3)*		*Inter-day variation (n = 3)*	
		Mean (μg/ml)	*C.V. (%)*	*Mean (μg/ml)*	*C.V. (%)*
[^{15}N,^{13}C]CBZE	0.100	0.10	5.7	0.11	6.2
	0.750	0.76	3.9	0.72	7.3
CBZE	0.500	0.58	1.0	0.54	8.9
	2.000	2.05	1.0	1.98	7.7
CBZ	5.000	5.05	0.4	5.12	1.3
	10.000	10.94	0.1	10.66	5.4

CBZ, CBZE and [^{15}N-^{13}C]CBZE concentrations in patient blood samples

This method has been used to measure CBZ, CBZE and [^{15}N,^{13}C]CBZE in over 200 blood samples of children on CBZ monotherapy. The steady-state blood concentrations for CBZ and CBZE were found to be in the range of 8–12 and 1–2 μg/ml, respectively. Values for [^{15}N,^{13}C]CBZE varied in the range 50–800 ng/ml.

CONCLUSIONS

A TSP LC–MS method which allows the simultaneous quantification of [^{15}N,^{13}C]CBZE, CBZE and CBZ from blood samples after a single extraction procedure has been developed. The technique has proven to be rapid, sensitive and reliable, demonstrating little or no thermal degradation of the analytes, and unlike GC–MS methods does not require derivatization. The high specificity of LC–MS in combination with the exact control of sample recovery, using d_4-CBZE and d_4-CBZ as internal standards, provides precise and accurate determination of the analytes, with a detection limit for CBZE of 500 pg on-column. To date, this analytical procedure has been successfully utilized in the analysis of [^{15}N,^{13}C]CBZE, CBZE and CBZ in over 200 blood samples collected in single dose pharmacokinetic studies conducted in children on CBZ monotherapy.

ACKNOWLEDGEMENTS

This research was supported by research grant GM 37752 from the National Institutes of Health. We would like to thank Ms. Shannon West for her efforts in manuscript preparation and Ms. Martha Moore for her fine technical assistance.

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