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LC determination of carbamazepine in murine brain

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Abstract

A reversed phase HPLC method for the determination of carbamazepine (CBZ) in the brain of adult mice is described. CBZ was recovered from murine brain by solvent-extraction with ethyl acetate and resolved from imipramine (internal standard) and brain endogenous material using a Lichrospher RP select B column with a linear gradient of acetonitrile (40–80 v/v, 25 min) in ammonium acetate buffer (25 mM, pH 4.0) with UV detection at 285 nm. The method is selective, reproducible and precise with a limit detection of 45 ng/ml and is suitable for the determination of CBZ in murine brain after intra-peritoneal administration.

Keywords: Carbamazepine; RP-HPLC; Brain; Mouse

1. Introduction

Carbamazepine (CBZ) (5-H-Dibenz[b,f]azepine-5-carboxamide, CBZ) is an anticonvulsant used in clinical practice as first-line treatment for generalised tonic-clonic and partial seizures [1]. Thirty percent of patients are refractory to anticonvulsants even at maximal tolerable doses and optimum serum concentrations [2]. The reasons for this observed pharmaco-resistance have not been fully rationalised, but may involve pharmacodynamic and pharmacokinetic factors, the latter affecting drug transport across the blood-brain barrier [3].

With respect to CBZ the assessment of passage across the blood-brain barrier requires the determination of CBZ concentration in the brain, particularly when radiolabelled compound is unavailable. HPLC methods for the determination of CBZ in plasma, blood and in human and rat brain have been described earlier [4–9]. Mice with altered expression of membrane transport proteins such as P-glycoprotein encoded by the multidrug resistance (mdr) gene present a useful experimental model for studying the relationship between the expression of membrane transport proteins and alterations in drug pharmacokinetics [10,11]. This study describes the design and validation of an internal-standardised high-perfor-

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mance liquid chromatographic method for the determination of CBZ in murine brain tissue.

2. Experimental

2.1. Materials

CBZ and imipramine (3-{10,11-dihydro-5H-dibenz [b,f] azepine-5-yl}-*NN*-dimethyl-propylamine) hydrochloride were obtained from Sigma Chemicals (Poole, Dorset, UK). CBZ 10,11-epoxide (CBZE) and 10,11-dihydro-10,11-*trans*-dihydroxy-CBZ (DHCBZ) were gifts from Novartis (UK). Unless otherwise indicated, all solvents and chemicals were products of Sigma Chemicals Co. Ltd (Poole, Dorset, UK) and were of analytical grade quality or better.

2.2. Internal standard solution

Imipramine hydrochloride (IMP, 2.5 mg) was weighed accurately and transferred into a 100 ml volumetric flask. This was dissolved in and made up to the mark with ethyl acetate to produce a stock solution of concentration 25 μg/ml.

2.3. Sample preparation

Freshly removed murine brain tissue was homogenised in distilled water (1 ml) with a hand-held glass-teflon homogeniser. The IMP solution (5 ml) was subsequently added to each brain homogenate. The homogenate was rendered alkaline, vortexed (20 min) and the organic layer (upper layer) separated by centrifugation (1500 × *g*, 15 min) at ambient temperature. The organic layer was aspirated and reduced to dryness under a stream of nitrogen at 50 °C for 30 min. The residue was re-suspended in 40% v/v acetonitrile in ammonium acetate buffer (25 mM, pH 4.0) to a final volume of 1 ml and analysed by HPLC.

2.4. Calibration standards

Standard solutions of CBZ at concentrations of 4, 8, 16, 24 and 32 μg/ml were prepared in methanol. Homogenised murine brain tissues, as described under sample preparation, were spiked with the standard solutions of CBZ (50 μl). IMP (25 μg/ml, 5 ml) was subsequently added to each 1 ml of homogenate and mixed thoroughly. The samples were processed for HPLC analyses as described under sample preparation to yield CBZ standards of nominal concentration 0.2, 0.4, 0.8, 1.2 and 1.6 μg/ml.

2.5. High performance liquid chromatography (HPLC)

The chromatographic system consisted of a Dionex P580 pump (Dionex Corporation, Sunnyvale, CA) equipped with an autosampler and a Dionex UVDI70S detector. Peaks were monitored at 225 nm for simultaneous detection of CBZE, DHCB, CBZ and IMP, and subsequently at 285 nm for quantitative analysis. The chromatographic data was acquired and processed with Chromeleon® HPLC software (Dionex Corporation). Chromatographic separations were made at ambient temperature with a mobile phase flow rate of 0.5ml/min. Samples (25 μl) were injected onto a Lichrospher® 60 RP-select B column (E. Merck, Darmstadt, Germany, 125 × 4 mm I.D., 5 micron particle size, 60 Å pore size) and eluted with a linear gradient of acetonitrile in ammonium acetate buffer (25 mM, pH 4.0) from 40% v/v to 80% v/v over 15 min with a 10 min re-equilibration time. The ammonium acetate buffer was prepared by accurately weighing 1.52 g of ammonium acetate and making up to 450 ml with water. The pH was adjusted to 4.0 with glacial acetic acid and the final solution made up to 500 ml with water.

2.6. Precision and accuracy

The precision of analyses, expressed as inter-day and intra-day precision was calculated as,

$$\left[100 - \left(\frac{(\text{Standard deviation})}{\text{Mean}}\right)100\right]\%$$

Determinations for intra-day ($n = 5$) and inter-day precision ($n = 4$) were performed using extracts from the same brain samples.

The accuracy of the method was assessed at the low, intermediate and upper levels (0.2, 0.8 and 1.6 μg/ml, respectively) and determined as,

$$\left[\frac{\text{(Calculated amount of CBZ in brain)}}{\text{(nominal amount of CBZ added to brain)}}\right] 100\%$$

2.7. Application of method

Male fvb1 mice were given an intraperitoneal dose of CBZ (2 mg/kg or 20 mg/kg in 60% PEG400) and sacrificed 1 h after dosing. The brain tissue was removed, weighed and homogenised in 1.0 ml of water. IMP (5 ml) was subsequently added to each homogenate and mixed thoroughly. The samples were then processed for HPLC analyses as described under sample preparation. The amount of CBZ in brain was calculated as,

$$\frac{[V \times C]}{M} \text{ ng/g of brain tissue}$$

Where V represents the total volume of the reconstituted extract (ml), C represents the concentration of the reconstituted extract determined by HPLC (ng/ml) and M represents the weight of murine brain (g).

3. Results and discussion

The chromatographic peaks due to the major metabolites of CBZ, namely CBZE (t_R 4.2 min) and DHCBZ (t_R 3.3 min) were resolved from those of CBZ (t_R 6.5 min) and IMP (t_R 9.0 min) (Fig. 1 a) using the HPLC conditions. Endogenous material from homogenised brain did not impact on the quantification of the peaks due to CBZ and IMP (Fig. 1 b). The limit of detection of the CBZ was 45 ng/ml (3 × S/N ratio). Calibration solutions prepared within the range 0–1.6 μg/ml showed a linear relationship ($Y = [0.81 \pm 0.02]X - [0.02 \pm 0.01]$, $R^2 = 0.999 \pm 0.001$ (mean ± S.D., $n = 4$)) between UV-detector response (Y) and concentration of CBZ (X) recovered from spiked brain samples with small standard residuals randomly distributed about the regression line.

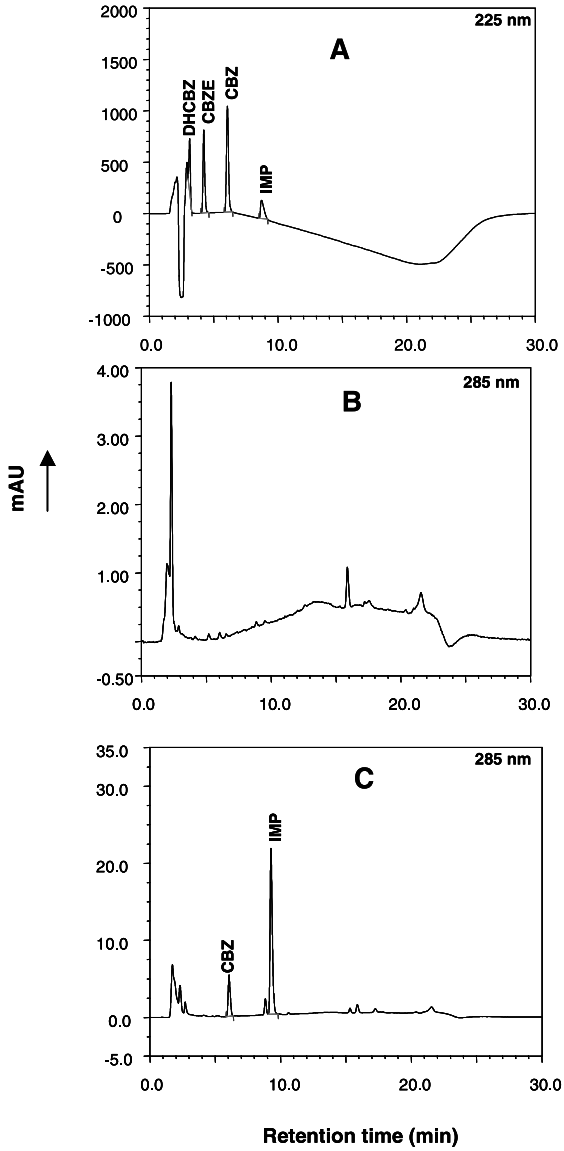

Fig. 1. HPLC chromatograms of (A) CBZ, IMP, CBZE and DHCBZ standards, (B) extract from control brain and (C) extract from brain at a concentration of 0.2 μg of CBZ per ml as described under sample preparation. Samples were separated using a 125 × 4 mm Lichrospher® 60 RP-select B column (mobile phase, linear gradient of acetonitrile in ammonium acetate buffer (pH 4.0, 25 mM) from 40 to 80% v/v over 25 min at 0.5 ml/min) with UV detection at 285 nm. The peaks for CBZE and DHCBZ were monitored at the 225 nm

Table 1
Intra-day precision, intra-day precision and accuracy of analysis of CBZ in murine brain at specified concentrations

Concentrations (μg/ml)	Intra-day precision	Inter-day precision	Accuracy
0.2	92.4, 94.1, 96.3, 96.5, 99.4	95.7 ± 2.6	92.0 ± 6.1
0.8	92.1, 93.8, 95.6, 98.8, 98.3	95.6 ± 2.7	103.4 ± 2.6
1.6	97.3, 98.5, 98.9, 99.1, 99.6	98.7 ± 0.9	99.3 ± 0.6

Values for intra-day precision are RSDs of quadruplicate determinations of five different samples at the stated concentration. Values for inter-day precision and accuracy are mean ± S.D. ($n = 4$).

The recovery of CBZ from brain was quantitative over the entire working range of 0.2–1.6 μg/ml as shown by the data for accuracy in Table 1. At the lowest working concentration (0.2 μg/ml), the determined values of accuracy, inter-day and intra-day precisions of the method were within an acceptable range of $\pm 10\%$ [12]. This criterion was satisfied at the intermediate and upper levels of 0.8 and 1.6 μg/ml, respectively.

Following the intraperitoneal administration of CBZ (2 and 20 mg/kg) to mice, brain concentrations after 1 h were determined as 193 ± 150 ng/g and 8.6 ± 2.7 μg/g, respectively. This method has been shown to be suitable for the determination of the concentrations of CBZ found in the brain of mice administered a near-therapeutic dose (2 mg/kg) and a non-lethal supra-therapeutic dose (20 mg/kg). In combination with the availability of experimental murine models with altered drug-transport characteristics such as the multidrug efflux *P*glycoprotein-deficient mdr1a/1b(−/−) mice [11], this method provides a useful tool for the assessment of impaired transport of CBZ into murine brain.

4. Conclusion

A liquid chromatographic method for the determination of CBZ in murine brain is described. The method is precise and facilitates quantitative recovery of CBZ from the brain matrix without interference from the major metabolites of CBZ and/or brain endogenous matter. The method, which is applicable to in vivo evaluation of the concentration of CBZ in mouse brain, may be suitable for the investigation of the contribution of altered transport of drugs across the blood-brain barrier in experimental mouse models on the observed pharmaco-resistance to CBZ.

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