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# Determination of carbamazepine and carbamazepine 10,11-epoxide in human plasma by tandem liquid chromatography–mass spectrometry with electrospray ionisation

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**Abstract**

A sensitive method for the determination of carbamazepine and carbamazepine 10,11-epoxide in plasma is described, using high-performance liquid chromatographic separation with tandem mass spectrometry. Samples were purified using liquid–liquid extraction and separated on a Phenomenex® Luna $C_{18}$ 5 μm, 150×2 mm column with a mobile phase consisting of acetonitrile, methanol and formic acid (0.1%) (10:70:20, v/v). Detection was performed by a Micromass Quattro Ultima™ mass spectrometer in the MRM mode (LC–MS–MS) using electro spray ionisation (ESI+), monitoring the transition of the protonated molecular ion for carbamazepine at *m/z* 237.05 and carbamazepine 10,11-epoxide at *m/z* 253.09 to the predominant ions of *m/z* 194.09 and 180.04, respectively. The mean recovery was 95% for carbamazepine and 101% for carbamazepine 10,11-epoxide, with a lower limit of quantification of 0.722 ng/ml for carbamazepine and 5.15 ng/ml for carbamazepine 10,11-epoxide, when using 0.5 ml plasma. This high-throughput method was used to quantify 230 samples per day, and is sufficiently sensitive to be employed in pharmacokinetic studies. 

*Keywords:* Carbamazepine; Carbamazepine 10,11-epoxide

## 1. Introduction

Carbamazepine is an established drug for the control of grand mal and psychomotor epilepsy and it is also effective in the treatment of trigeminal neuralgia. Furthermore, it is presently used in bipolar depression. It is predominantly eliminated in the liver, where it is metabolised to carbamazepine 10,11-epoxide and other derivatives. Carbamazepine 10,11-epoxide seems to have antiepileptic properties as well as carbamazepine itself.

Chelberg et al. [1] and Martens and Banditt [2] both published liquid–liquid extraction methods followed by HPLC and UV-detection to determine carbamazepine. Liu et al. [3] described a precipitation method with acetonitrile for the quantitation of carbamazepine in serum with a photodiode-array detector at 200 nm, while Romannyshyn et al. [4] described a liquid–liquid extraction followed by isocratic HPLC analysis with UV detection at 210 nm in a range of 0.5–20 μg/ml for carbamazepine and 0.25–15 μg/ml for carbamazepine 10,11-epox-

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ide. Yukimitsu and Chiyoji [5] published a solid-phase extraction method from serum followed by HPLC and UV-detection at 240 nm. Reported run-times for the determination of carbamazepine and carbamazepine 10,11 epoxide were between 10 and 27 min [2–4].

Our described LC–MS–MS method made it possible to inject the samples 4 min apart, increasing the through-put up to six times. The lowest LOQ (with high precision) from the references was 1 μg/ml [1], whereas our method has an LOQ of 0.722 ng/ml.

This paper thus represents the first quantitative LC–MS–MS method for the determination of carbamazepine and carbamazepine 10,11-epoxide in human plasma. A sensitive assay method, in the lower ng/ml levels in plasma, was optimised on a Micromass Quattro Ultima™ mass spectrometer with mass spectrum–mass spectrum (MS–MS) capabilities in tandem with liquid chromatography (LC).

## 2. Experimental

### 2.1. Materials and chemicals

A Phenomenex® Luna $C_{18}$ 5 μm, 150×2 mm column (Phenomenex, Torrance, CA, USA) was used for separation at a flow-rate of 0.25 ml/min and 20 μl was injected onto the column. The mobile phase was delivered and the samples injected by a Waters-Alliance 2790 system. Detection was performed by a Micromass Quattro Ultima™ mass spectrometer interfaced to an electrospray ionisation (ESI) source.

Acetic acid (Pro-Analysi) was obtained from Merck (Darmstadt, Germany), acetonitrile (B&J High Purity) was obtained from Baxter (Muskegon, USA) and formic acid (98/100%) was obtained from BDH Laboratory Supplies (Dorset, UK). All chemicals were used as received. Water was purified by RO 20SA reverse osmosis and Milli-Q® polishing system (Millipore, Bedford, MA, USA).

Carbamazepine (5*H*-dibenz[*b*,*f*]azepine-5-carboxamide, $C_{15}H_{12}N_2O$) (Fig. 1a) was supplied by VIS Farmaceutici (Hamburg, Germany) and carbamazepine 10,11-epoxide (10,11-epoxy-5*H*-dibenz[*b*,*f*]-azepine-5-carboxamide, $C_{15}H_{12}N_2O_2$) (Fig. 1b) was supplied by Sigma–Aldrich (St. Louis, MO, USA).

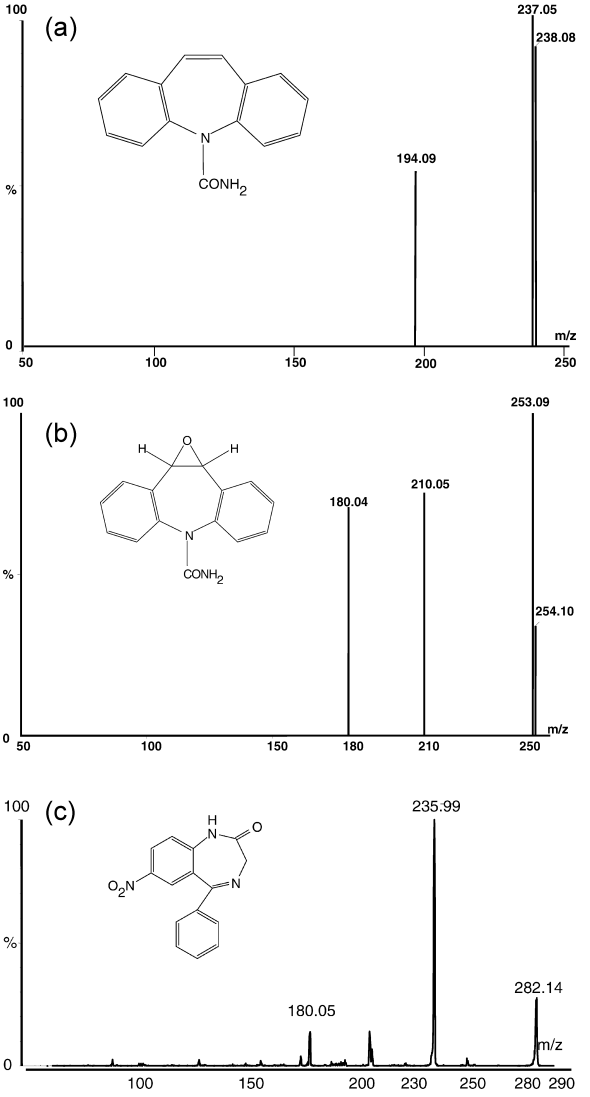


Fig. 1. (a) A full scan spectrum (MS–MS) of a pure solution of carbamazepine in acetonitrile:0.1% formic acid (1:1, v/v). The parent [M+1] ion with *m*/*z* 237.05 and the predominant product ion *m*/*z* 194.09 are shown. (b) A full scan spectrum (MS–MS) of a pure solution of carbamazepine 10,11-epoxide in acetonitrile:0.1% formic acid (1:1, v/v). The parent [M+1] ions with *m*/*z* 253.09 and predominant product ion *m*/*z* 180.04 are shown. (c) A full scan spectrum (MS–MS) of a pure solution of nitrazepam in acetonitrile:0.1% formic acid (1:1, v/v). The parent [M+1] ions with *m*/*z* 282.14 and predominant product ion *m*/*z* 235.99 are shown.

Nitrazepam was obtained from FARMOVS–PAREXEL Bioanalytical Services internal reference library.

### 2.2. Calibration standard preparation

Carbamazepine and carbamazepine 10,11-epoxide stock solutions were prepared in methanol and used immediately to spike blank plasma. The solutions were not retained for further use. Calibration standards and quality control standards were prepared in normal human plasma by spiking plasma to a known concentration and then serially diluting it with normal blank plasma to attain the desired concentration range. The prepared calibration standards and quality control standards were aliquoted into microfuge tubes and stored at −20 °C until analysis.

### 2.3. Extraction procedure

To 500 μl of plasma was added NaOH (0.1 *M*, 500 μl) and nitrazepam (internal standard) (100 μl, 800 ng/ml in water) in a 5-ml ampoule. Ethyl acetate (3 ml) was added and the samples were vortexed for 45 s and centrifuged at 3000 *g* for 3 min at 10 °C. The aqueous phase was frozen on a freezerbath at −30 °C for 3 min, the organic phase poured into another 5-ml ampoule and dried under a gentle stream of nitrogen at 40 °C. The analytes were redesolved in 200 μl of mobile phase and vortexed briefly to homogenise. The Waters Alliance series 2790 autosampler injected 20 μl onto the HPLC column. The samples were cooled to 4 °C on the autosampler.

### 2.4. Instrumental conditions

Chromatography was performed at ambient temperature, at a flow-rate of 0.25 ml/min with acetonitrile:methanol:formic acid (0.1%) (10:70:20, v/v) as mobile phase. All chromatographic solvents were sparged with helium before use.

### 2.5. Mass spectrometry

Electrospray ionisation was performed in the positive mode with the nebulizing gas (nitrogen), cone gas and desolvation gas set at 80, 135 and 640 l/h, respectively. The ionisation source was connected by a fused-silica (375 μm) capillary to the syringe pump for tuning and the instrument responses for carbamazepine and carbamazepine 10,11-epoxide were optimised using flow injection. Optimal responses were obtained with a cone voltage setting of 40 V, a heated capillary voltage of 2.8 kV and temperature at 100 °C.

The transition of the protonated molecular ion for carbamazepine at $m/z$ 237.05 and carbamazepine 10,11-epoxide at $m/z$ 253.09 to the predominant ions were $m/z$ 194.09 and 180.04, respectively. The transition of the protonated molecular ion for nitrazepam at $m/z$ 282.14 to the predominant ion was $m/z$ 235.99. The isolation width was 2 a.m.u. and the relative collision energy set at 30% for all three analytes. The mass spectrometer was interfaced to a computer workstation running MassLynx version 3.3 software.

### 2.6. Validation

The method was validated assaying plasma quality control samples ($n=5$) at 0.83, 1.66, 3.28, 13.10, 52.50, 105.00, 210.00, 1674, 3347 and 6693 ng/ml for carbamazepine and 5.84, 11.70, 23.10, 92.30, 369.00 and 738.00 ng/ml for carbamazepine 10,11-epoxide, to determine the accuracy and precision of the method. Quality control values were calculated from a standard regression curve, constructed from the ratio of analyte to internal standard peak areas, containing eight different concentrations, spanning the concentration range 0.83–6693.00 ng/ml for carbamazepine (Wagner $(\ln(y) = a(\ln(x)^2) + b(\ln(x)) + c$, $a$: −0.001110, $b$: 0.946032, $c$: −2.443848, $r^2$: 0.999534, mean RSD (%) 7.43), and 5.84–738.00 ng/ml for carbamazepine 10,11-epoxide (Wagner $(\ln(y) = a(\ln(x)^2) + b(\ln(x)) + c$, $a$: −0.016443, $b$: 1.130090, $c$: −4.300546, $r^2$: 0.999734, mean RSD (%) 7.18).

### 2.7. Matrix effects

Matrix effects were determined by analysing blank biological fluids from six different sources to determine possible interference.

### 2.8. Recovery

Absolute recovery of the analyte was determined in triplicate at high, medium and low concentrations in normal plasma by extracting drug free plasma samples spiked with carbamazepine and carbamazepine 10,11-epoxide. Recovery was calculated by comparison of the analyte peak-areas of the extracted samples with those of the unextracted analyte standards, representing 100% recovery.

## 3. Results and discussion

At first, plasma containing carbamazepine and carbamazepine 10,11-epoxide were extracted on $C_{18}$ solid-phase extraction columns. The columns were washed with $NH_3OH$ (0.01 *M*) and the analytes eluted with acetonitrile. The elute was dried in a Savant speedvac evaporator and the analytes reconstituted in mobile phase. The extracts were found to be dirty with poor chromatography and an increase in column pressure was observed after just five injections. Liquid–liquid extractions was performed with NaOH (0.1 *M*) as pH modifier, using ethyl acetate (5 ml) and diethyl ether (5 ml), respectively, as organic solvents in a 10-ml ampoule. The samples were vortexed and centrifuged at 3000 *g*. The aqueous phases were frozen on a freezerbath at −30 °C, the organic phases poured into 5-ml ampoules and dried under a gentle stream of nitrogen at 40 °C. The analytes were redesolved in mobile phase, vortexed briefly to homogenise and injected onto the HPLC column. The extraction with ethyl acetate gave the best results and it was decided to optimise this extraction.

The carbamazepine 10,11-epoxide stock solution had to be prepared in a polypropylene scintillation vial, since the aluminium in the lid of the glass scintillation vial reacted as a catalyst, causing a breakdown of the carbamazepine 10,11-epoxide dissolved in the methanol. The mean absolute recoveries of carbamazepine, determined in triplicate at 105, 411 and 3274 ng/ml, were 89.71, 94.46 and 101.27%, respectively. The mean absolute recoveries of carbamazepine 10,11-epoxide, determined in triplicate at 26.7, 107 and 418 ng/ml, were 96.19, 104.48 and 102.44%, respectively.

Results from the intra-day validation assay indicate a valid calibration range of 0.722–10 644 ng/ml for carbamazepine and 5.15–1204 ng/ml for carbamazepine 10,11-epoxide. Table 1 depicts the quality control data obtained during the validation of the method for carbamazepine and carbamazepine 10,11-epoxide, while Table 2 depicts the intra-day back calculated quality controls for carbamazepine and carbamazepine 10,11-epoxide. On-instrument stability was inferred from intra-day quality control data obtained during the pre-study validation. No significant degradation could be detected in the cooled samples (4 °C) left on the autosampler for at least 35 h for carbamazepine, 121 h for carbamazepine 10,11-epoxide and 13.5 h for nitrazepam (internal standard). The batches were divided into groups of

Table 1
Summary of intra-day quality control results for carbamazepine ($n=5$) and carbamazepine 10,11-epoxide ($n=5$)

(a) Carbamazepine

| | QC J | QC J (Dil.) | QC I | QC H | QC G | QC F | QC E | QC D | QC C | QC B | QC A |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Nominal (ng/ml) | 6693 | 6693 | 3347 | 1674 | 210 | 105 | 52.5 | 13.1 | 3.28 | 1.66 | 0.830 |
| Mean | 7145 | 6767 | 3454 | 1675 | 200 | 103 | 49 | 12 | 3 | 1.63 | 0.928 |
| %Nom | 106.8 | 202.2 | 103.2 | 100.1 | 95.4 | 98.4 | 94.2 | 97.2 | 92.2 | 98.4 | 111.8 |
| RSD (%) | 2.6 | 1.6 | 6.1 | 4.3 | 2.0 | 4.2 | 5.2 | 7.0 | 6.7 | 8.2 | 4.6 |

(b) Carbamazepine 10,11-epoxide

| | QC F | QC E | QC D | QC C | QC B | QC A |
|---|---|---|---|---|---|---|
| Nominal (ng/ml) | 738 | 369 | 92.3 | 23.1 | 11.7 | 5.84 |
| Mean | 762.54 | 364.29 | 92.11 | 22.04 | 11.54 | 5.67 |
| %Nom | 103.3 | 98.7 | 99.8 | 95.4 | 98.6 | 97.1 |
| RSD (%) | 7.4 | 7.9 | 6.1 | 5.9 | 4.7 | 7.7 |

Table 2
Summary of back calculated quality control concentrations of carbamazepine and carbamazepine 10,11-epoxide (inter-day variation) showing the repeatability of the method

(a) Carbamazepine

| | QC I | QC H | QC G | QC F | QC E | QC D | QC C | QC B |
|---|---|---|---|---|---|---|---|---|
| Nominal (ng/ml) | 3347 | 837 | 210 | 52.5 | 26.3 | 13.1 | 3.28 | 1.66 |
| Mean | 3229.97 | 873.59 | 205.71 | 55.10 | 26.33 | 13.34 | 3.01 | 1.68 |
| RSD% | 4.7 | 5.2 | 9.5 | 9.3 | 8.2 | 13.0 | 13.4 | 16.0 |
| *n* | 10 | 8 | 15 | 9 | 8 | 15 | 6 | 13 |
| %Nom | 96.5 | 104.4 | 98.0 | 105.0 | 99.7 | 101.8 | 91.8 | 101.5 |

(b) Carbamazepine 10,11-epoxide

| | QC G | QC F | QC E | QC D | QC C | QC B | QC A |
|---|---|---|---|---|---|---|---|
| Nominal (ng/ml) | 1476 | 369 | 185 | 92.3 | 23.1 | 11.7 | 5.84 |
| Mean | 1286.37 | 370.79 | 187.20 | 94.37 | 22.36 | 11.51 | 5.89 |
| RSD% | 8.8 | 7.6 | 7.4 | 7.3 | 11.4 | 7.7 | 5.1 |
| *n* | 11 | 9 | 8 | 14 | 7 | 16 | 10 |
| %Nom | 87.2 | 100.5 | 101.2 | 102.2 | 96.8 | 98.4 | 100.8 |

samples (two groups of ~115 samples each) which were extracted consecutively and loaded onto the autosampler ~4 h apart to ensure that samples did not remain on-instrument awaiting injection for longer than the tested on-instrument stability times. Each group of 100 assays contained an equal proportion of the calibration and quality control standards processed in the batch.

Freeze–thaw stability was not relevant in this study since samples were not subjected to multiple freeze–thaw cycles and were assayed immediately after thawing. Sample residues were discarded after analysis. Repeat assays were performed on the duplicate aliquots, which remained frozen until assayed. Carbamazepine and carbamazepine-10,11-epoxide are reported to be stable when frozen in serum for at least 4 months [6].

Due to the high specificity of MS–MS detection, no interfering or late eluting peaks were found when chromatographing the blank plasma extracts from six different sources.

Fig. 1a shows the single parent to product ion mass spectrum (MS–MS) of carbamazepine and Fig. 1b the single parent to product ion mass spectrum (MS–MS) of carbamazepine 10,11-epoxide acquired with the abundant product ions at $m/z$ 194.09 and 180.04, respectively. A strong daughter ion with $m/z$ 210.05 was observed, but the daughter formed at $m/z$ 180.04 was more stable.

Fig. 1c shows the single parent to product ion mass spectrum (MS–MS) of nitrazepam acquired with the abundant product ion at $m/z$ 235.99.

Quantitation was achieved at unit resolution in full scan MS–MS mode scanning the product ion spectrum from $m/z$ 50 to 250 and monitoring the transition of the protonated molecular ion at $m/z$ 237.05 for carbamazepine, to the largest product ion $m/z$ 194.09 and the protonated molecular ion at $m/z$ 253.09 for carbamazepine 10,11-epoxide to the largest product ion $m/z$ 180.04.

Typical retention times for carbamazepine were 2.65–2.84 min (mean RSD 0.62%), for carbamazepine 10,11-epoxide were 2.73–2.87 min (mean RSD 0.60%) and for the internal standard were 2.33–2.47 min (RSD 0.66%). A chromatography time of 4 min made it possible to analyse 230 samples per day. In order to test for the presence of matrix effects [7], six different plasma pools were extracted and then spiked with a known concentration of the analyte. These samples were injected and peak areas compared. The calculated RSD was used as measurement of reproducibility with a RSD of greater than 10% suggesting the presence of matrix effects [7]. The data showed no significant matrix effects either for carbamazepine (RSD 1.6%), carbamazepine 10,11-epoxide (RSD 3.1%) or nitrazepam at the tested concentrations.

Fig. 2a shows representative chromatograms of

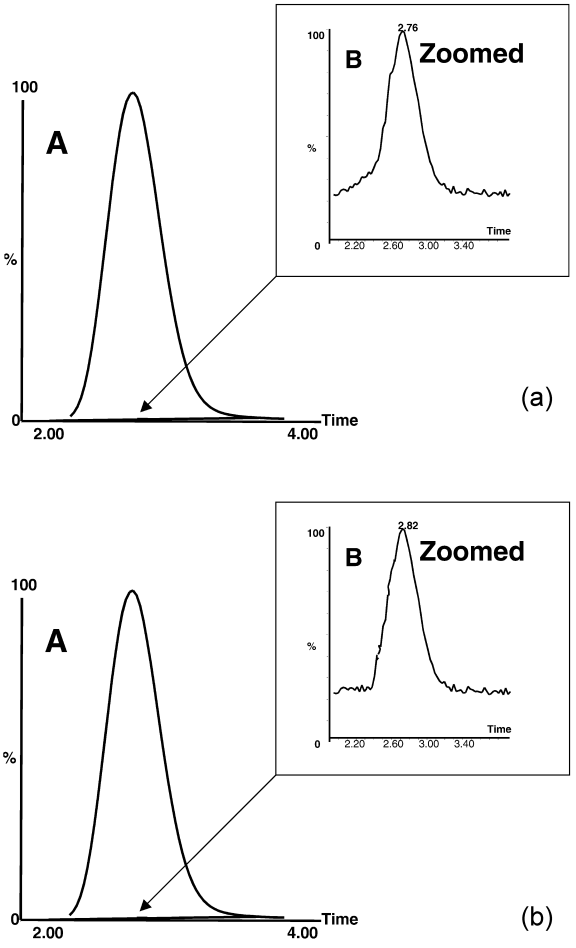


Fig. 2. (a) Chromatograms of calibration standards, containing 10 644 ng/ml (A) and 0.722 ng/ml (B), respectively, of carbamazepine in plasma. (b) Chromatograms of calibration standards, containing 602 ng/ml (A) and 5.84 ng/ml (B), respectively, of carbamazepine 10,11-epoxide in plasma.

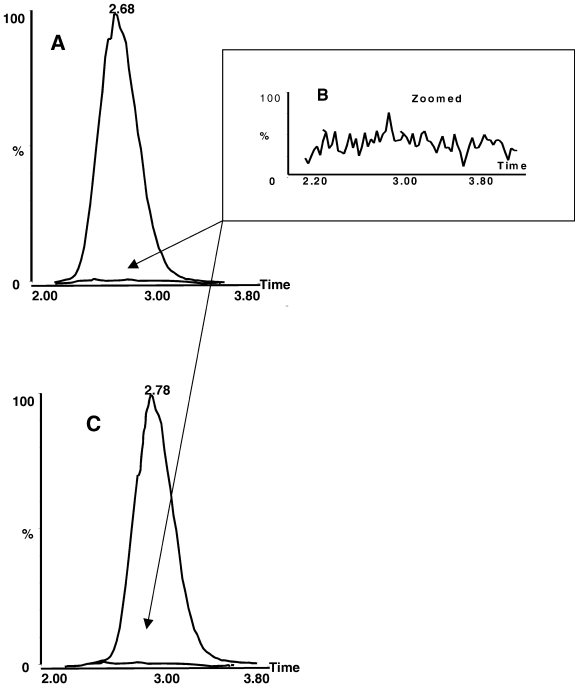


Fig. 3. Chromatograms of a subject sample containing 224.69 ng/ml (A) of carbamazepine in plasma, 74.59 ng/ml (C) of carbamazepine 10,11-epoxide in plasma, and blank plasma extract (B).

carbamazepine obtained at 10 644 ng/ml and 0.722 ng/ml (LLOQ), and Fig. 2b shows representative chromatograms obtained at 602 and 5.84 ng/ml, while Fig. 3 depicts chromatograms from a subject sample (both carbamazepine and carbamazepine 10,11-epoxide) and a blank plasma extract, zoomed in to show the absence of any interference.

The method was employed to analyse plasma samples containing carbamazepine and carbamazepine 10,11-epoxide obtained after a single 400-mg oral dose of carbamazepine in 22 healthy volunteers. The maximum plasma concentrations obtained varied between 1200 and 1000 ng/ml for carbamazepine and between 85 and 75 ng/ml for carbamazepine 10,11-epoxide (quantified using peak area ratios). Fig. 4a,b represents the mean pharmacokinetic profiles for carbamazepine and carbamazepine 10,11-epoxide for 22 human volunteers after receiving a 400-mg oral dose of carbamazepine each.

## 4. Conclusion

A highly sensitive and selective method for the quantification of carbamazepine and its metabolite, carbamazepine 10,11-epoxide, in human plasma has been developed and validated. Plasma concentrations of carbamazepine and carbamazepine 10,11-epoxide could be quantified from 0.722 to 10 644 ng/ml making it possible to analyse samples up to 144 h after a single oral dose of 400 mg of carbamazepine

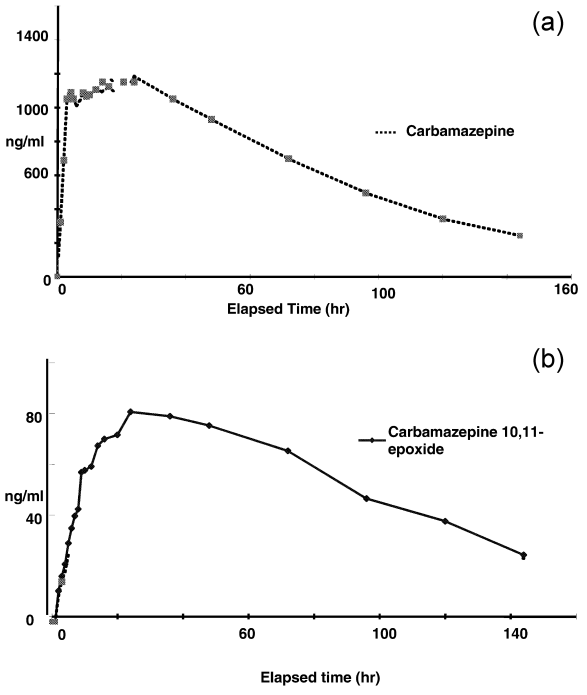


Fig. 4. (a) Representative mean carbamazepine plasma concentration/time profile ($n=22$) as obtained after a single 400-mg oral dose of carbamazepine to human volunteers. (b) Representative mean carbamazepine 10,11-epoxide plasma concentration/time profile ($n=22$) as obtained after a single 400-mg oral dose of carbamazepine to human volunteers.

to human volunteers. This LC–MS–MS method is far more sensitive and precise than any other method described. Radioimmunoassays [4] are much more sensitive, but due to cross-reactivity, it is advisable to determine only one analyte at a time.

This is the first chromatographic method for the quantitation of carbamazepine and carbamazepine 10,11-epoxide in plasma described using liquid chromatography, with tandem mass spectrometry.

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