

Determination of Carbamazepine and its Active Metabolite Epoxycarbamazepine from Plasma by Liquid Chromatography-Mass Spectrometry

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High performance liquid chromatography-mass spectrometry method is described for the determination of carbamazepine and epoxycarbamazepine in human plasma using fluconazole as internal standard. Good separation of the target compounds and short run time were obtained using an elution system of methanol : water (90 : 10% v/v). Carbamazepine and epoxycarbamazepine were isolated by solvent extraction. No significant interference caused by endogenous compounds was observed. This simple and rapid assay can be successfully used in pharmacokinetic studies of carbamazepine and epoxycarbamazepine.

Key Words: Carbamazepine, Epoxycarbamazepine, Fluconazole, High performance liquid chromatography-mass spectrometry, Human plasma.

INTRODUCTION

Carbamazepine is a white to off white powder soluble in alcohol¹ and used for partial seizures with complex symptomatology and generalized seizures^{2,3}. Carbamazepine is indicated in treatment of pain associated with true trigeminal neuralgia^{4,5}. The chemical name for carbamazepine is 5H-dibenz-[b,f]azepine-5-carboxamide (m.f. = C₁₅H₂N₂O₅ and m.w. = 236.27). Cytochrome P450 3A4 was identified as major isoform responsible for formation of carbamazepine-10,11-epoxide from carbamazepine⁶⁻⁸. Pharmacokinetic parameters of carbamazepine after a single oral 400 mg dose of parent drug in 24 healthy volunteers are given below:

Parameters	Carbamazepine	Carbamazepine 10,11-epoxide
C _{max} (µg/mL)	2–3	0.175
T _{max} (h)	4–24	35
t _{1/2} (h)	32	40

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There was no high performance liquid chromatography-mass spectrometry (HPLC-MS) method reported in literature and therefore an attempt has been made to develop a simple, accurate, precise and reproducible method for the determination of carbamazepine and epoxycarbamazepine together in human plasma. The method employs HPLC-MS and online solid-phase extraction for sample analysis.

EXPERIMENTAL

Instrument: HPLC-MS/MS system using turbo ion spray ionization (ESI).

Biological Matrix: Pooled human plasma, commercially procured and chromatographically analyzed to ensure non-interference.

Chromatographic Conditions:

Mobile phase	Water : Methanol (10 : 90)
Detector	Perkin-Elmer Sciex API 365 LC-MS system using turbo ion spray ionization (ESI)
Internal standard	Fluconazole
Flow rate	0.25 mL/min
Injection volume	5 μ L
Column	Xterra C18 (2.1 \times 50 mm) 3.5 μ
Scan	MRM
Polarity	Positive
Pause time	5 ms

Preparation of aqueous and plasma standards: Stock solution of carbamazepine, epoxycarbamazepine and internal standard of 1 mg/mL were prepared in methanol. Standard solutions containing a mixture of carbamazepine and epoxycarbamazepine of concentration 0.1 μ g/mL and 10 μ g/mL were also prepared using methanol. Calibration standards of mixture of carbamazepine and epoxycarbamazepine of concentrations (0.5 & 1.0 ng/mL), (1.0 & 5.0 ng/mL), (10.0 & 10.0 ng/mL), (100.0 & 50.0 ng/mL), (500.0 & 100.0 ng/mL), (1000.0 & 250.0 ng/mL), (2000.0 & 350.0 ng/mL), (4000.0 & 500.0 ng/mL), (8000.0 & 800.0 ng/mL) were prepared respectively and an LOQ sample at 0.50 & 1.0 ng/mL was prepared by spiking appropriate amount of the standard solutions in control plasma obtained from healthy human non-smoking volunteers. Quality samples were prepared in the blank control plasma at the concentrations of (0.50 & 1.0 ng/mL), (5.0 & 7.5 ng/mL), (700.0 & 150.0 ng/mL), (6000.0 & 600 ng/mL).

Sample Preparation: The following extraction procedure was used for preparation of biological matrix samples, *i.e.*, all calibration levels, QC samples and volunteer's plasma samples before injecting into HPLC-MS system. To 0.5 mL of plasma in a clean dry stoppered test tube 50 μ L of internal standard solution, 500 μ L of 1% trisodium orthophosphate (buffer), 5 mL of *n*-hexane : dichloromethane mixture (2 : 1) were added, vortexed for 2 min, centrifuged at 3500 rpm for 5 min. The upper organic layer was collected and evaporated to dryness in a water bath kept at 50–60°C using a constant stream of nitrogen. The

residue was reconstituted with 200 μ L mobile phase in 5 μ L aliquot and then injected into the LC-MS system.

Assay Validation

Specificity: 10 bags of fresh frozen plasma obtained from different sources were analyzed to ensure non-interference.

Linearity and Sensitivity: A calibration curve in the range of 0.5–8000.0 ng/mL and 1.0–800.0 ng/mL for carbamazepine and epoxycarbamazepine was constructed by plotting the area ratios of carbamazepine and epoxycarbamazepine to internal standard against carbamazepine and epoxycarbamazepine concentrations in plasma. LOQ was established based on an S/N ratio of 5.

Precision and accuracy: The precision of the assay was determined by replicate analyses of four different concentrations LOQ (0.5 & 1.0 ng/mL), LQC (5.0 & 7.5 ng/mL), MQC (700 & 150 ng/mL) and HQC (6000 & 600 ng/mL). Intra-day precision was determined by repeated analysis of each of QC samples on one day (n = 5) and the inter-day precision and accuracy was determined by repeated analysis on four consecutive days (n = 1 series/day). The concentration of each sample was determined using calibration standards prepared on the same day.

Stability: Analytes at low and high concentrations were tested for freeze-thaw (five cycles): bench top stability (up to 24 h), auto sampler stability (up to 24 h), long-term stability (12 weeks) and stock solution stability (up to 24 h).

Extraction Recovery: The absolute recoveries of carbamazepine and epoxycarbamazepine through extraction procedures were determined at low, medium and high concentrations by external standard method. A known amount of carbamazepine, epoxycarbamazepine and internal standard was added to human plasma prior to extraction. The concentration of carbamazepine and epoxycarbamazepine was calculated using the calibration curves prepared on the same day and was compared to nominal concentration to estimate extraction recovery.

Pharmacokinetics and study: Each of 40 healthy male volunteers received 1 \times 400 mg capsule of carbamazepine after overnight fasting. Blood samples were drawn at appropriate intervals centrifuged to obtained plasma samples.

Representative chromatograms: Representative chromatograms are shown in Figs. 1–3 in which the retention times were 0.41 min for carbamazepine, 0.38 min for epoxycarbamazepine and 0.36 min for internal standard.

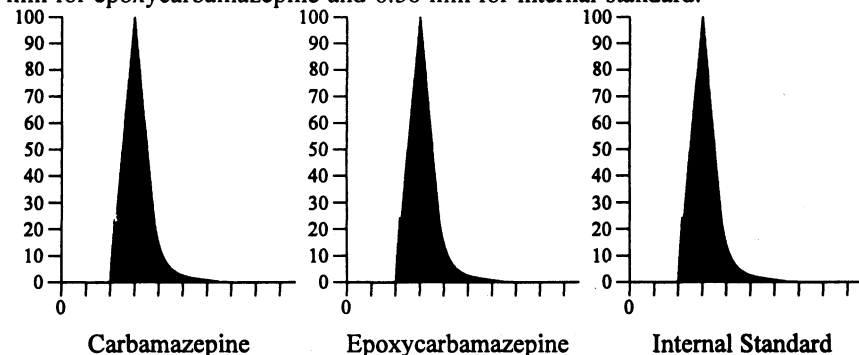


Fig. 1. Representative chromatogram for system suitability

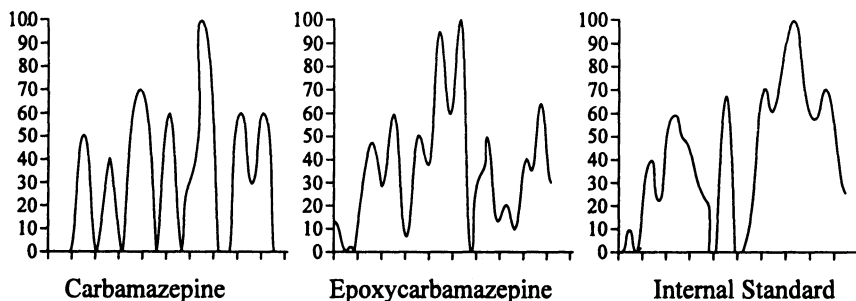


Fig. 2. Representative chromatogram for blank plasma

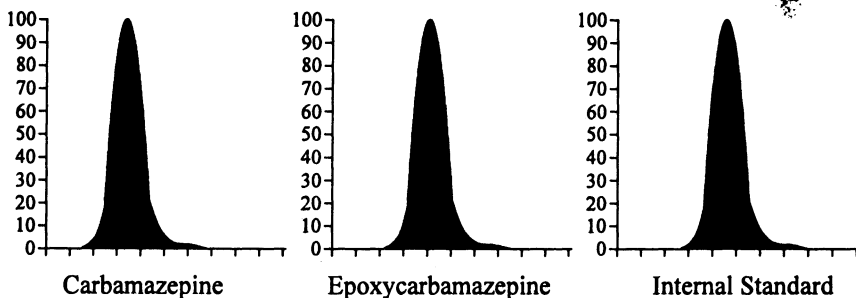


Fig. 3. Representative chromatogram for LOQ (Limit of Quantitation)

Conditions for ESI-MS: The ESI-mass spectrum at fragment voltages of 30 and 40 V showed that the protonated molecular ion $[M + H]^+$ of carbamazepine, epoxycarbamazepine and internal standard was at 237.3, 253.0 and 307.2 respectively. By increasing the fragmentor voltage, the fragmentation patterns of these protonated molecular ions were observed. The product ion mass spectrum of this protonated molecular ion with the most intensive product ions was observed at m/z 194.2 and 180.1 for carbamazepine and epoxycarbamazepine, respectively. By monitoring this product ion, a highly sensitive assay for carbamazepine and epoxycarbamazepine was developed.

The intensity of product ion of carbamazepine and epoxycarbamazepine at m/z was compared at fragmentor voltages of 10, 20 and 50 V in order to determine the optimal collision energy. The result showed that the highest sensitivity was obtained using a fragmentor voltage of 30 V and 32 V for carbamazepine and epoxycarbamazepine, respectively. Therefore, a fragmentor voltage of 30 V and 32 V was used to carry out LC-ESI-MS in the MRM mode. At this collision energy the most intensive product ion of I.S. protonated molecular ion was at m/z 238.4.

RESULTS AND DISCUSSION

The results are presented in Table-1.

Application: The method described above was successfully applied to the

pharmacokinetic study in which plasma concentrations of carbamazepine and epoxycarbamazepine in 40 healthy volunteers were determined up to 120 h after the administration of 400 mg capsule. The pharmacokinetic parameter values are calculated. The maximum plasma concentration of 2705.72–3158.56 ng/mL for carbamazepine and 103.51–114.57 ng/mL for epoxycarbamazepine after the administration.

TABLE-1

Test	Acceptance criteria	Results for carbamazepine	Results for epoxycarbamazepine	Conclusion
Specificity	Non-interference at the retention time of carbamazepine, epoxycarbamazepine and internal standard	No interfering peak at the retention time of carbamazepine	No interfering peak at the retention time of epoxycarbamazepine and internal standard was found	Method was found to be specific
Sensitivity	S/N more than 5 : 1	6.57 : 1	5.83 : 1	Method was found to be sensitive
Linearity	Regression to be more than 0.95	0.999	0.999	Method was found to be linear
Precision and accuracy	Intra day 1. % Accuracy for LOQ: 80–120% LQC: 85–115% MQC: 85–115% HQC: 85–115% 2. % RSD for LOQ: nmt 20% LQC: nmt 15% MQC: nmt 15% HQC: nmt 15% Inter day 1. % Accuracy for LOQ: 80–120% LQC: 85–115% MQC: 85–115% HQC: 85–115% 2. % RSD for LOQ: nmt 20% LQC: nmt 15% MQC: nmt 15% HQC: nmt 15%	Intra day 90.11%–100.73% 100.75–112.78% 106.46–113.54% 88.96–97.74% 5.97%–10.39% 5.30–10.19% 0.75–9.38% 1.14%–2.81% 96.72% 107.13% 109.71% 93.41% 10.19% 7.36% 7.01% 5.29%	Intra day 99.83–113.63% 93.23–100.02% 87.71–93.36% 97.64–114.21% 4.72%–10.77% 1.12%–7.71% 1.91%–6.73% 1.33%–2.47% 105.66% 96.78% 90.13% 107.91% 8.54% 7.24% 4.25% 8.64%	Method was found to be precise and accurate
Recovery	Precise, consistent and reproducible	55.51–60.03%	39.54–47.55%	Recovery was found to be precise, consistent and reproducible

EFFICACY RESULTS

A. For carbamazepine

Measure			C_{\max}	AUC_{0-t} (h.ng/mL)	$AUC_{0 \rightarrow \infty}$ (h.ng/mL)	T_{\max} (h)
Test product (TI)	N		36	36	36	36
	Mean		161.02	117481.69	138801.61	19.47
	SD		622.96	52634.78	63828.17	8.16
	CV (%)		38.7	44.8	46.0	41.89
	Geometric mean		1488.99	106651.78	125428.27	17.72
Test product (TII)	N		36	36	36	36
	Mean		3158.56	230374	266891.24	18.44
	SD		982.28	86827.06	103646.34	6.79
	CV (%)		31.1	37.7	38.8	38.83
	Geometric mean		2983.04	211341.48	245335.32	17.17
Reference product	N		36	36	36	36
	Mean		270.72	214804.09	249605.43	21.06
	SD		689.47	72729.31	1033747.93	7.96
	CV (%)		25.5	33.9	41.6	37.82
	Geometric mean		2609.30	201525.25	229650.42	19.51
ANOVA	Ln transformed	Formulation	0.0000	0.0000	0.0000	—
		Sequence	0.2297	0.1293	0.0815	—
		Period	0.9907	0.7185	0.4751	—
Least square mean	Ln transformed	Test (I)	1469.01	1041568.93	122248.28	—
		Test (II)	2943.47	20616893	238676.28	—
		Reference	2574.92	19841803	223037.19	—
Ratio least square mean T/R	Ln transformed test (I)		57.05	5303	54.80	—
Ratio least square mean T/R	Ln transformed test (II)		114.31	104.96	106.99	—
Confidence interval	Ln transformed (I)	Lower	52.32	49.18		—
		Upper	62.21	58.52	61.06	—
		Power (%)	99.48	98.06	95.95	—
Confidence interval	Ln transformed (II)	Lower	104.86	95.13	96.04	—
		Upper	124.62	115.82	119.19	—
		Power (%)	99.49	98.10	96.02	—

B. For epoxycarbamazepine

Measure		C_{max}	AUC_{0-t} (h.ng/mL)	$AUC_{0 \rightarrow inf}$ (h ng/mL)	T_{max} (h)	
Test product (TI)	N	36	36	36	36	
	Mean	53.42	1568.00	5428.85	34.56	
	SD	32.70	2742.14	3970.31	20.61	
	CV (%)	61.2	62.8	72.4	59.63	
	Geometric mean	43.49	3535.77	4314.93	28.92	
Test product (TII)	N	36	36	36	36	
	Mean	114.57	8932.69	10762.93	37.39	
	SD	67.15	4263.18	5016.94	15.41	
	CV (%)	58.6	47.5	46.6	41.21	
	Geometric mean	99.15	7959.86	9526.134	33.79	
Reference product	N	36	36	36	36	
	Mean	103.51	8851.39	10561.11	31.94	
	SD	50.08	4476.87	7686.32	12.49	
	CV (%)	48.4	50.8	72.8	39.10	
	Geometric mean	93.17	7877.78	8978.09	29.25	
ANOVA	Ln transformed	Formulation	0.00	0.00	0.00	—
		Sequence	0.8921	0.8941	0.7404	—
		Period	0.1815	0.8424	0.8019	—
Least square mean	Ln transformed	Test (I)	43.25	3544.48	4336.57	—
		Test (II)	99.43	7976.23	9562.24	—
		Reference	93.92	7857.00	8957.25	—
Ratio least square mean T/R	Ln transformed test (I)	46.26	45.11	48.41	—	
Ratio least square mean T/R	Ln transformed test (II)	103.87	101.52	106.75	—	
Confidence interval	Ln transformed (I)	Lower	40.40	38.79	41.60	—
		Upper	52.97	52.47	56.34	—
		Power (%)	85.76	78.55	78.32	—
Confidence interval	Ln transformed (I)	Lower	92.48	87.31	91.77	—
		Upper	121.20	118.03	124.18	—
		Power (%)	85.90	78.71	78.48	—

Conclusion

This assay achieved higher sensitivity and better specificity for the analysis of carbamazepine and epoxycarbamazepine in human plasma. The limit of quantitation of ng/mL for carbamazepine and epoxycarbamazepine was thus attainable by HPLC-MS. The internal standard proved to be good internal standard for this assay. No significant interference caused by endogenous compounds was observed. This simple and rapid assay can be successfully used in pharmacokinetic studies of carbamazepine and epoxycarbamazepine.

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