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Determination of carbamazepine in rat plasma in the presence of licorice juice by using HPLC and its pharmacokinetic applications

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ABSTRACT

*Carbamazepine (CBZ) is the first choice anticonvulsant and mood-stabilizing drug used primarily in the treatment of epilepsy and bipolar disorder. A simple, rapid and accurate method for determination of carbamazepine in rat plasma in presence of licorice has been developed by using High performance liquid chromatography. A mixture of 55 % of Water Contains (1 ml Triethylamine per 1 liter) and 45 % of Acetonitrile of pH of 6 (adjusted with phosphoric acid) was used as a mobile phase, BDS hypersil C18 column (150mm×4.6mm,i.d 5μ) and a flow rate of 1 ml/min were used, the auto-sampler injection volume was 15 μL, and metronidazole benzoate was used as internal standard. The retention time for carbamazepine and metronidazole benzoate were 2.8 and 3.7 minutes, respectively. The method was validated in terms of linearity (R^2= 0.9997) for the concentration (80-4800) ng/ml, precision, accuracy, stability and recovery. A and B groups of rats (n = 8 for each group) were used in the preclinical study. The first group (A), carbamazepine was administrated with water. While in group B, licorice was given instead of drinking water and pre-administrated to the rates before giving carbamazepine dose (10mg/kg). Plasma levels of carbamazepine were compared with plasma levels of carbamazepine with licorice. The maximum plasma concentration (Cmax), area under curve (AUC) and time to reach maximum concentration for carbamazepine with water were (4129.66 ng/ml, 31860.95 ng*hr/ml, and 1.00 hr) respectively. They were significantly reduced in presence of licorice juice (2885.53 ng/ml and 15776.70 ng*hr/ml, 0.50 hr), p value <0.05. In conclusion, we advise to avoid the licorice intake with CBZ or Caution should be considered when licorice is given with carbamazepine, since licorice lower plasma level of carbamazepine when pre-administrated.*

Key words: Carbamazepine, HPLC, Pharmacokinetic, drug interaction.

INTRODUCTION

Carbamazepine (figure 1) is carboxamide derivative antiepileptic drugs, used in the treatment of partial-onset seizures [1].as well as other neurological and psychiatric disorders [2]. In addition, it is the drug of choice for many combination therapies and used in treatment of geriatric patients with multiple disease states [3].

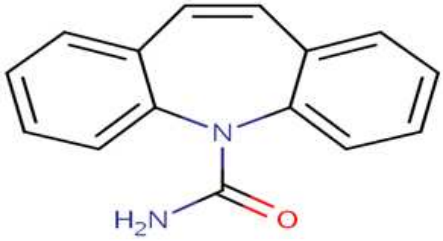

Figure 1: Carbamazepine chemical structure

Carbamazepine is extensively metabolized in the liver, primarily by CYP3A4, to carbamazepine-10,11-epoxide which is pharmacologically active (figure 2). Additional isoenzymes that contribute to the metabolism of carbamazepine include CYP2C8, CYP2B6, CYP2E1, CYP1A2, and CYP2A6. Carbamazepine-10,11- epoxide is in turn metabolized, via epoxide hydrolase, to an inactive trans-carbamazepine diol. Carbamazepine is an enzyme inducer, and additionally, carbamazepine undergoes auto induction so that its clearance can increase 3-fold within several weeks of starting therapy and this often requires an upward dosage adjustment.

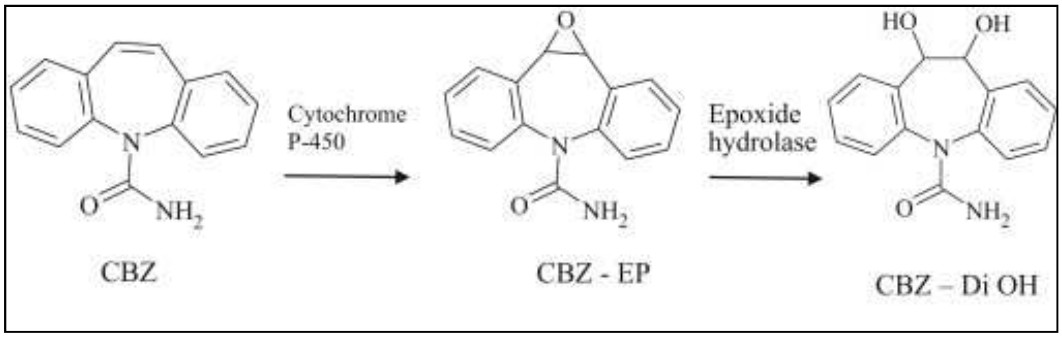

Figure 2: Metabolism of carbamazepine

Several HPLC and LC methods with UV detection for the determination of CBZ and its metabolite in drug products and human plasma have been developed and validated to address this issue[4, 5, 6, 7, 8].

Because of its widespread and long term use, carbamazepine is frequently prescribed in combination with other drugs, leading to the possibility of drug interactions [9, 10], and there is also possibility of co-administration with some juices[11, 12].

In the current study, a simple and accurate HPLC method has been developed and validated for determination of carbamazepine in rat plasma and study the effect of licorice juice on the pharmacokinetic profile of carbamazepine.

EXPERIMENTAL SECTION

2.1. Chemical and reagents

Carbamazepine and Metronidazole benzoate were purchased from Joswe medical. Acetonitrile, methanol and water for HPLC gradient grade were obtained from (Fisher scientific). In addition, OrthoPhosphoric Acid 85% from (GPR RECTAPUR) and triethylamine from (TEDIA)

2.2. Instrumentations

The study employed a high pressure liquid chromatography (FINNIGAN SURVEYOR) equipped with UV-VIS Plus Detector, ChromQuest software 4.2.34 Solvent delivery systems pump (LC Pump Plus), and an automatic sampling system (autosampler Plus). Separation was achieved using a 150 mm × 4.6 mm (i.d.) C_{18}, BDS, reversed phase column with an average particle size of 5 µm. The column effluent was monitored at 285 nm and the chromatographic data analysis was performed with computer System (Windows XP, SP3).

Table 1: Summary of chromatographic conditions

Mobile phase composition	55 % of Water Contains (1 ml Triethylamine per 1 liter) 45 % of Acetonitrile pH= 6.00, adjust with H_3PO_4
Column type	Hypersil Thermo Electron Corporation, BDS C18 (150mm x 4.6 mm, 5µm)
HPLC Conditions	
Wave length	285 nm
Pump flow rate	1.0 ml/min
Auto-sampler injection volume	15 µl
Auto-sampler Temperature	10 ˚C
Column oven temperature	25 ˚C
Expected Retention Times (minutes)	
Carbamazepine	2.8
Metronidazole benzoate (IS)	3.7

2.3 Chromatographic conditions

A mobile phase consisting of 55 % of Water Contains (1 ml Triethylamine per 1 liter) and 45 % of Acetonitrile of pH of 6 (adjusted with phosphoric acid) was circulated through a reversed-phase Thermo scientific column (BDS HYPERSIL C18) with particle size of 5 µm and dimensions of 150mm×4.6mm, further details and retention time of the analyte and internal standard (IS) are illustrated in table 1.

2.4 Preparation of stock and working solutions

2.4.1 Preparation of stock and working solutions of metronidazole benzoate (IS)

A stock solution of the internal standard (IS) at a concentration of 1 mg/ml was prepared by dissolving 10mg of metronidazole benzoate in 10ml acetonitrile. Working solutions of IS was prepared by taking 0.8ml of the stock solutions and diluted to 100ml of acetonitrile to get concentration of 8µg/ml.

2.4.2 Preparation of stock and working solutions of carbamazepine

Stock solution of CBZ was prepared by dissolving 10.0mg CBZ in 50ml methanol, resulting in a solution containing 200 µg/ml. Then, stock solution was stored at -20℃. This solution was diluted in methanol to give working solutions (3.2, 6.4, 12.8, 32.0, 64.0, 128.0, 192.0, 9.6, 96.0 and 160.0) µg/ml.

2.4.3 Preparation of calibration curve and quality control (QC) samples in plasma

In order to get the seven spiked levels (calibration curve) in plasma, 25 µl volume were taken from each working solution and spiked in 1000 µl of plasma. The obtained calibration curve concentrations were: (80, 160, 320, 800, 1600, 3200 and 4800) ng/ml in plasma. While the quality control (QC) concentrations were: (240, 2400 and 4000) ng/ml.

2.5. Method Validation

2.5.1 Precision and accuracy

The intra-day precision and accuracy of the method was determined by analysis of 6 replicates of the lower limit of quantification (LLOQ) and QC levels in the same day. The inter-day variability was determined by analysis of three runs of the lower limit of quantification (LLOQ) and QC levels in three different days. The relative standard deviation values (RSD) or CV% were calculated from the ratios of the standard deviation (SD) to the mean and expressed as percentage.

The accuracy of the method was determined by comparing practical amounts recovered from the control samples with actual values present in the samples (theoretical values).

The acceptable limits of intra-day and inter-day accuracy and precision were below 15% except at the LLOQ, for which accuracy and precision should be below 20% [13].

2.5.2 Linearity

The calibration curve of CBZ is a plot of the peak area ratio (PAR) of the drug to the internal standard as a function of the drug concentration (*C*). This gives the following equation: PAR = Slope × *C* + Intercept. The slope and the intercept are determined from the determined PAR and the nominal concentration of the drug. The unknown CBZ concentrations are determined from this equation.

Linearity of the plotted curve is evaluated through the value of the correlation coefficient (R^2) which should be more than 0.98

2.5.3 Stability

In this study, we performed auto sampler and freeze–thaw stability tests. The tests of stability were carried out using low and high concentrations of QC samples. The short-term (bench top) stabilities was assessed by keeping the plasma samples at room temperature for 24 h. For the freeze and thaw stability test, the samples were stored at -30℃ for 24 h and kept at room temperature until the samples were thawed completely, then refrozen for 24 h. This cycle was repeated three times (three cycles) and then analyzed. The autosampler stability was evaluated after keeping the samples in the auto sampler rack at 10℃ for 24 h after sample preparation. The analyte were considered stable if the assay values were within the acceptable limit of accuracy ±15%.

2.6. Preclinical study

2.6.1 Preparation of carbamazepine solution and licorice juice

Weight equivalent to 250.00mg of carbamazepine were dissolved in 25 ml of distilled water to get concentration 10 mg/ml suspension of carbamazepine. The preparation of licorice juice was done according to the traditional way; licorice root obtained from a local herb shop was soaked in clean water for 1 hour. The wet licorice paste was then wrapped in a clean white cloth without being squeezed and tied on a drinking water tap. Finally, cold water was

allowed to drizzle over the tied licorice cushion in a period of 2 hours. Licorice juice was supplied without any additives and was refrigerated and consumed within 48 hours.

2.6.2 Animal handling and study protocol

The study protocol was approved by ethical committee of the High Research Council, faculty of pharmacy and medical science, University of Petra, Amman, Jordan (No.17/2014). Adult male and female Sprague Dawley laboratory rats were supplied by the animal house of Applied Science University with average weight of (140-220g). They were placed in air-conditioned environment (20-25 C) and exposed to a photoperiod cycle (12 hours light/12 hours' dark) daily. All rats fasted 24hr before experiment day.

All rats were marked on tail for identification, weighed and randomized into groups. Two groups of 10 rats; control and licorice. Carbamazepine 50 mg\kg was given by oral gavage to control group.

Combination of licorice with the drug were administrated to the second group as follows: pre-administered with licorice in drinking water for 12 hours before the experiment and half an hour before carbamazepine was given, a booster dose of juice (5 ml) was administrated to each rat.

2.6.3 Sample collection and processing

Blood sample were taken from the rats optical vein at the following time points: zero, 0.25 hour, 0.5 hour, 1 hour, 2.0 hours, 3.5 hours, 4.5 hours, 6.5 hours and 24.0 hours. Blood samples were drawn into an Ethylenediaminetetraacetic acid (EDTA) containing micro-tubes. Blood samples were immediately centrifuged at 5,000 RPM for 10 minutes; plasma was obtained and placed into labeled Eppendorf tubes and stored at -30^{0}C till analysis.

In order to perform the sample extraction, the following experimental procedure was followed; this procedures were applied for rat samples, calibrator and quality control samples.

- 100 µl of each test sample (blank, zero, standards, QC low (QCL), QC mid (QCM), QC high (QCH) or Rat samples) were taken into the appropriate tubes.
- 150 µl of Internal Standard (8.0 µg/ml of metronidazole benzoate was prepared in acetonitrile) were added to it.
- Sample were vortex vigorously for 1.0 minute,
- then samples were centrifuged at 14000 rpm for 15 minutes.
- The clear supernatant was transfer to a flat bottom insert and 15µl was injected into the HPLC column.

RESULTS AND DISCUSSION

3.1 Validation

A full method validation was performed for described HPLC method to demonstrate the reliability of a particular method for the determination of carbamazepine concentration in a rat plasma. (Figure 3‹4‹5‹6)

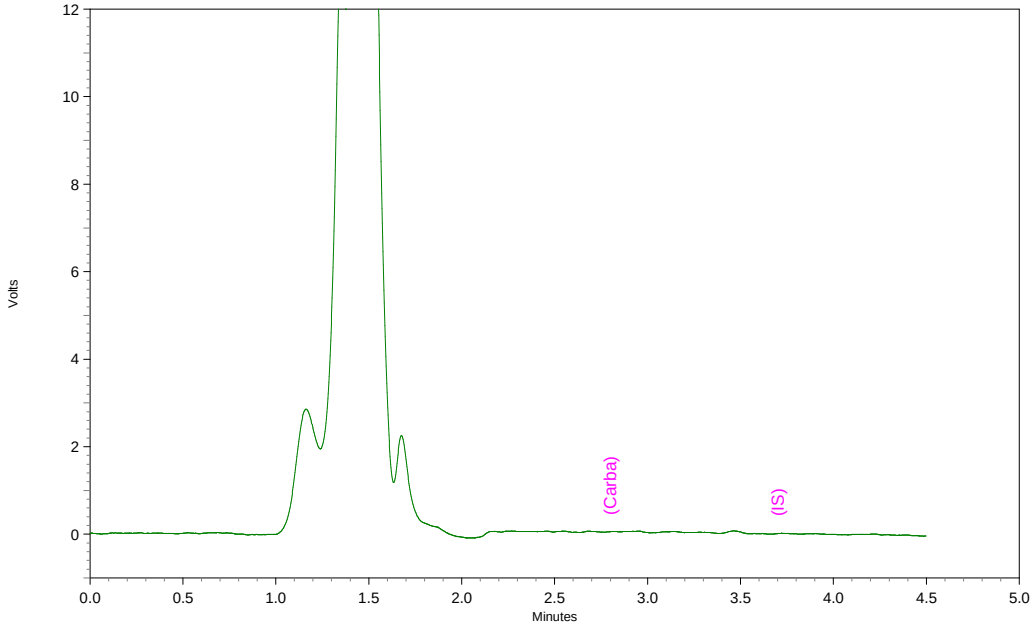

Figure 3 Blank sample

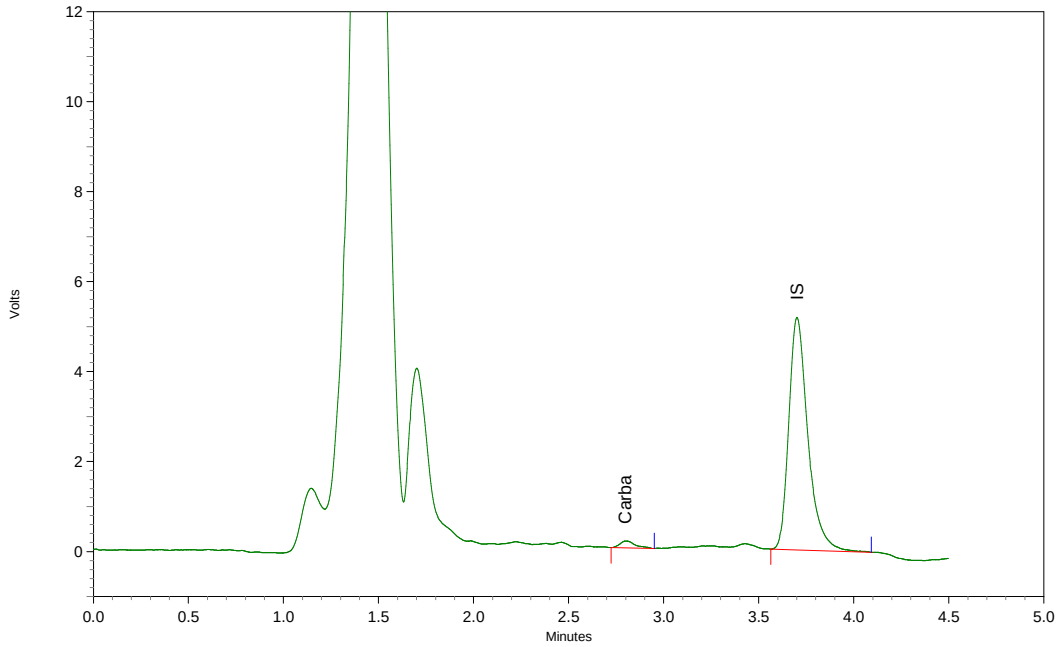

Figure 4: LLOQ sample

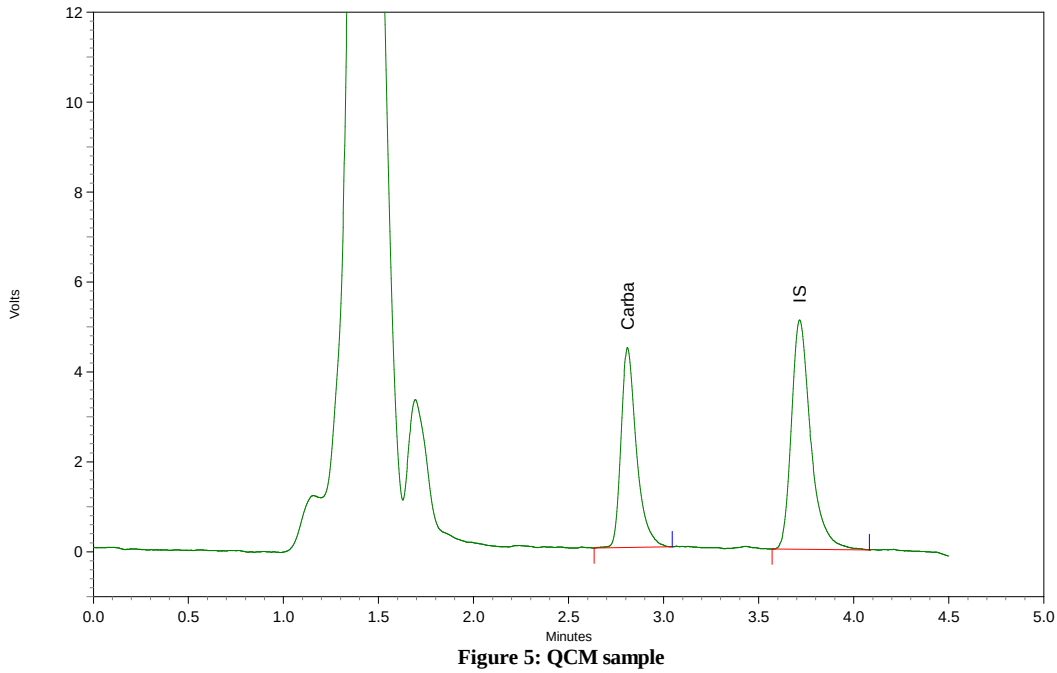

Figure 5: QCM sample

3.1.1 Accuracy and Precision

Inter-day accuracy over the concentration range were ranged between 99.94 and 106.36 % (table 2).

Comparing with the accepted criteria which is 85-115 % for all concentrations except for LLOQ which is 80-120 %, the accuracy obtained is within the required criteria in terms of accuracy.

Inter-day precision was evaluated over the three days. CV% was less than 4.16 % (LLOQ). CV% for QCL, QCM and QCH were 3.09, 3.41 and 4.06% (table 2).

The precision (CV%) did not exceed 20% for LLOQ and 15% for the other concentrations which prove the closeness of the measurements.

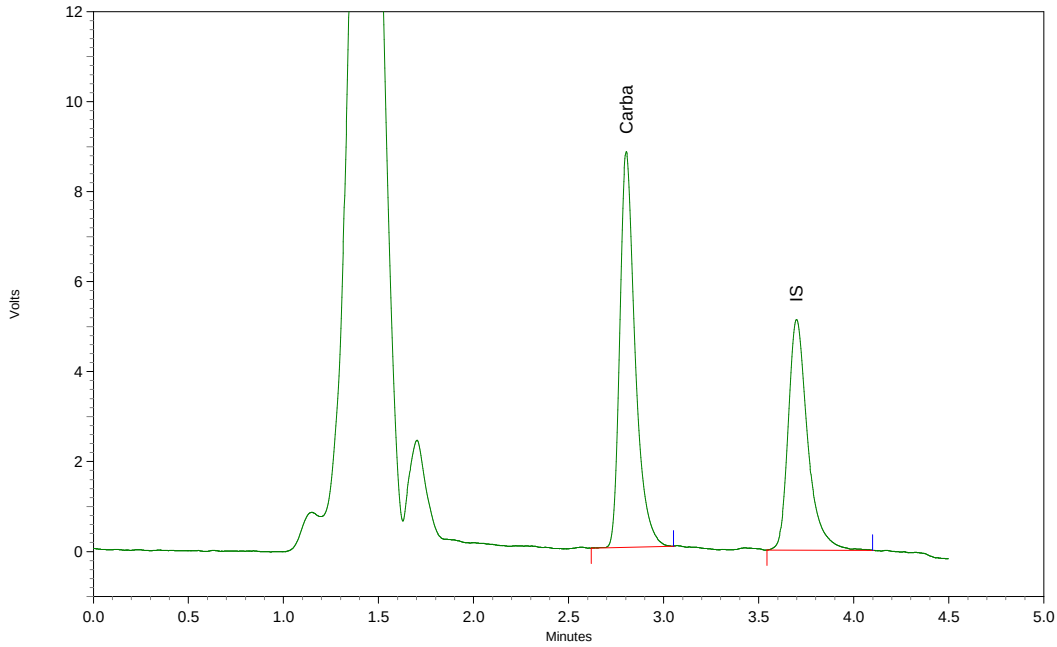

Figure 6: QCH sample

Table 2: Inter-day precision and accuracy for the quality control samples of carbamazepine in the three days of validation

	LLOQ (80 ng/ml)			QCL (240 ng/ml)			QCM (2400 ng/ml)			QCH (4000 ng/ml)		
	Day One	Day Two	Day Three	Day One	Day Two	Day Three	Day One	Day Two	Day Three	Day One	Day Two	Day Three
	84.79	86.94	84.84	259.25	241.3	241.71	2523.6	2297.6	2311.11	3995.22	3891.2	3970.85
	87.77	89.93	90.06	254.72	250.94	244.6	2478.54	2476.01	2303.22	4103.22	4054.36	4140.95
	80.95	87.24	83.77	246.71	237.26	254.45	2400.86	2403.61	2326.18	3981.86	3888.99	3826.16
	88.33	86.12	79.94	253.99	239.59	229.22	2546.79	2386.13	2388.12	4254.52	4074.27	3976.42
	86.67	85.76	77.22	245.51	248.58	246.76	2385.86	2524.6	2394.54	4405.28	4392.94	4022.36
	85.09	79.93	86.21	237.18	251.93	243.13	2401.81	2332.09	2295.39	4065.27	3955.04	3871.43
Mean	**85.09**			**245.93**			**2398.67**			**4048.35**		
STD	**3.54**			**7.6**			**81.75**			**164.46**		
CV%	**4.16**			**3.09**			**3.41**			**4.06**		
Accuracy %	**106.36**			**102.47**			**99.94**			**101.21**		

3.1.2 Linearity

Linearity is determined by calculating the regression line using a mathematical treatment of the results (i.e. least mean squares) vs. analyte concentration. The determination coefficient (R^2) measures the amount of variation in the response (dependent) variable explained by changes in the explanatory (independent variable). A value of 1 for R^2 indicates a perfect linear relation between target concentration and predicted concentration. The closer the value of R to 1 the stronger is the linear relation.

The linear regression equation was used for calculating the drug concentration at the each validation day, using one unique target concentration for getting the "D area/ IS area" at each of the 3 days of validation and in stability testing.

The R^2 were greater than 0.99 during the validation course. Data of the standard curve with regards to correlation, slope, R^2 and intercept are shown in tables 3, 4, and Figure 7.

Therefore, validation results of the three days are passed within the required criteria in terms of linearity.

Table 3: Linearity and linear working range of six calibration curves of carbamazepine data based on the measured concentration

Concentration for each calibration point (ng/ml)	Measured concentrations for each calibration point							
	1	2	3	4	5	6	Mean	Accuracy%
80	75.31	82.91	85.35	75.24	82.64	87.63	**81.51**	**101.89**
160	157.34	164.3	167.55	164.23	154.43	154.86	**160.45**	**100.28**
320	336.79	298.5	304.25	335.71	318.51	309.77	**317.26**	**99.14**
800	824.05	817.12	733.16	771.45	786.35	779.73	**785.31**	**98.16**
1600	1615.18	1583.76	1626.97	1639.28	1675.45	1570.51	**1618.52**	**101.16**
3200	3136.07	3102.42	3132.48	3205.3	3087.63	3204.43	**3144.72**	**98.27**
4800	4815.26	4910.98	4910.25	4768.8	4854.98	4853.06	**4852.22**	**101.09**

Table 4: Raw data for mean six calibration curves with regards to correlation, slope, R^2 and intercept for linearity

Correlation	Slope	R^2	Intercept
0.99985	0.000289	0.9997	-0.00439

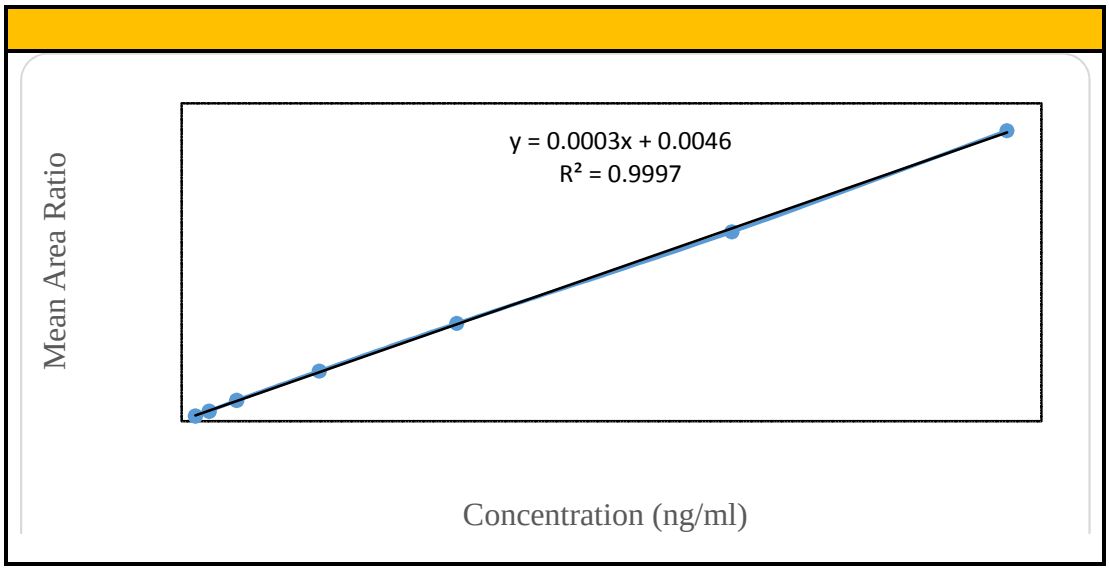

Figure 7: The plot of linearity for mean six calibration curves

3.1.3 Stability

3.1.3.1 Auto-sampler stability

Regarding the auto-sampler stability three samples with concentrations 240 ng/ml (QCL) and 4000 ng/ml (QCH) were processed as mentioned in the sample preparation section then directly injected (0.00 hr) and then were kept in the auto sampler and re-injected after 24.0 hours.

Table 5 and 6 show data for auto-sampler indicated by two QC concentrations (QCL and QCH) for carbamazepine after preparation procedure (auto-sampler stability), temperature=10°C.

From the table's data, we found the auto sampler stability test is passed according to the accepted criteria where the accuracy% doesn't exceed ±15%.

Table 5: Carbamazepine QCL samples stability at 10°C (auto sampler stability). QCL (240 ng/ml)

Time	AUC Drug	AUC IS	Ratio	Measured Concentration (ng/ml)	Accuracy %
00.00	2625	36429	0.072	**244.15**	101.73
Hour	2644	36196	0.073	**247.63**	103.18
	2656	36629	0.073	**245.75**	102.4
24.00 Hours	2525	35214	0.072	**242.91**	101.21
	2666	36598	0.073	**246.93**	102.89
	2671	36898	0.072	**245.32**	102.22

Table 6: Carbamazepine QCH samples stability at 10°C (auto sampler stability). QCH (4000 ng/ml)

Time	AUC Drug	AUC IS	Ratio	Measured Concentration (ng/ml)	Accuracy %
00.00	40764	36453	1.118	**3926.29**	98.16
Hour	42262	36325	1.163	**4085.3**	102.13
	42112	34792	1.21	**4250.55**	106.26
24.00 Hours	41906	35645	1.176	**4128.27**	103.21
	41682	35740	1.166	**4095.21**	102.38
	42422	36738	1.155	**4054.6**	101.36

3.1.3.2 Freeze and thaw stability

Regarding the freeze and thaw stability three samples with concentrations 240 ng/ml (QCL) and 4000 ng/ml (QCH) were stored and frozen in the freezer at the intended temperature and then thawed at room or processing temperature. After complete thawing, samples are refrozen again applying the same conditions. At each cycle, samples were frozen for 24 hours before they are thawed.

Table 7 and 8 shows data for freeze and thaw stability indicated by two QC concentrations (QCL and QCH) for carbamazepine.

From the table's data, we found that **freeze and thaw** test after 3 cycles is passed according to the accepted criteria where the accuracy% doesn't exceed ±15%.

Table 7: Carbamazepine QCL samples freeze and thaw stability. QCL (240 ng/ml)

Time	AUC Drug	AUC IS	Ratio	Measured Concentration (ng/ml)	Accuracy %
00.00 Hour	2625	36429	0.072	**244.15**	101.73
	2644	36196	0.073	**247.63**	103.18
	2656	36629	0.073	**245.75**	102.4
Cycle #3	2348	35136	0.067	**245.28**	102.2
	2164	32691	0.066	**243.07**	101.28
	2410	35557	0.068	**248.61**	103.59

Table 8: Carbamazepine QCH samples freeze and thaw stability. QCH (4000 ng/ml)

Time	AUC Drug	AUC IS	Ratio	Measured Concentration (ng/ml)	Accuracy %
00.00 Hour	40764	36453	1.118	**3926.29**	98.16
	42262	36325	1.163	**4085.3**	102.13
	42112	34792	1.21	**4250.55**	106.26
Cycle #3	42657	35187	1.212	**4256.36**	106.41
	42647	36307	1.175	**4124.44**	103.11
	42174	38269	1.102	**3870.28**	96.76

3.2 Effect of pre-administration of licorice on carbamazepine Pharmacokinetics

Carbamazepine (50mk/kg) was given by oral gavage to 8 rats. Blood sample were withdrawn at several time intervals to get carbamazepine plasma profile (figure 8). Carbamazepine reached its maximum plasma concentration 4129.66 ng/ml after an hour of administration (figure 9). Then it gradually decreased to reach its minimum concentration of 441.22 ng/ml after 24 hours.

Licorice juice was given instead of drinking water for 16 hr and another 5ml pre-administration before carbamazepine oral dose. Drug reached its maximum concentration after half an hour with concentration of 2885.53 ng/ml (figure 10). Then gradually decreased to reach a minimum concentration of 186.28 ng/ml.

Summary of pharmacokinetic parameters are illustrated in table 9. For carbamazepine, AUC 0-24 was 31860.95 ng*hr/ml. While AUC 0-∞ was37638.55 ng*hr/ml. Kel and half-life were found 0.08 hr^{-1} and 9.08 hr. While for carbamazepine with licorice, AUC 0-24 was 15776.70 ng*hr/ml. While kel and half-life were 0.08 hr^{-1} and 8.94 hr, respectively.

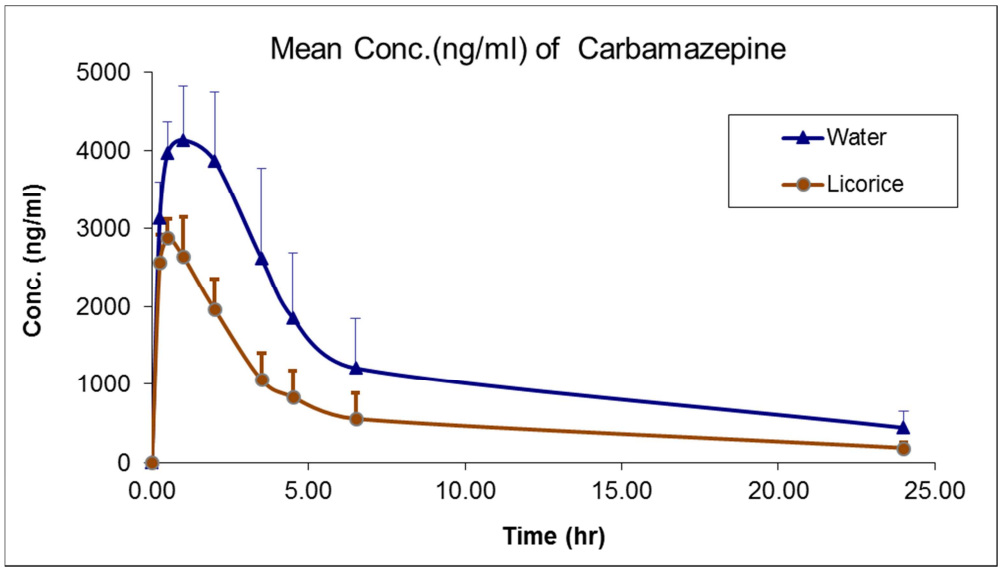

Figure 8: Carbamazepine – water and Carbamazepine – licorice, concentration- time profile (n=8)

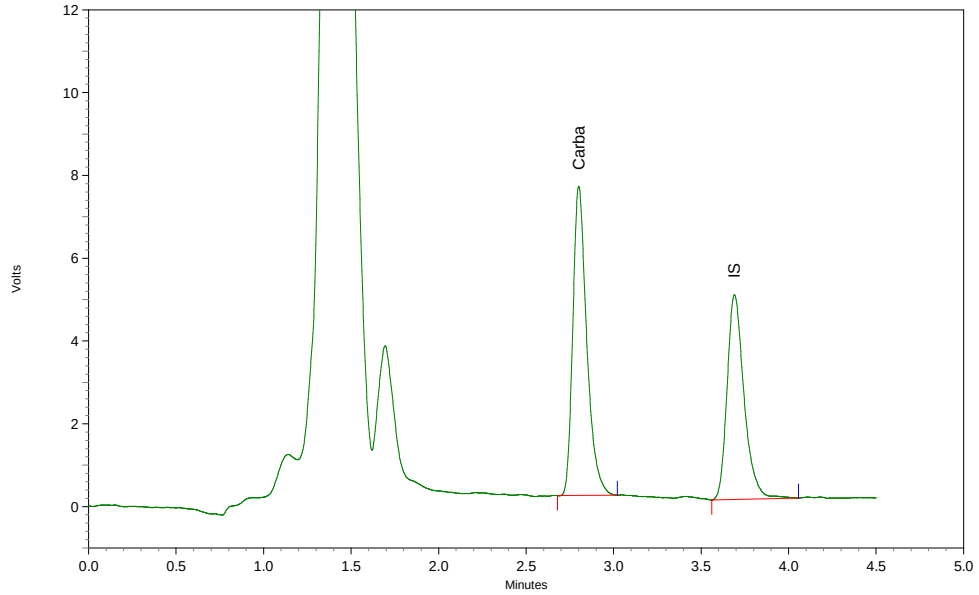

Figure 9: Control rat sample at 1.00 hr (carbamazepine alone)

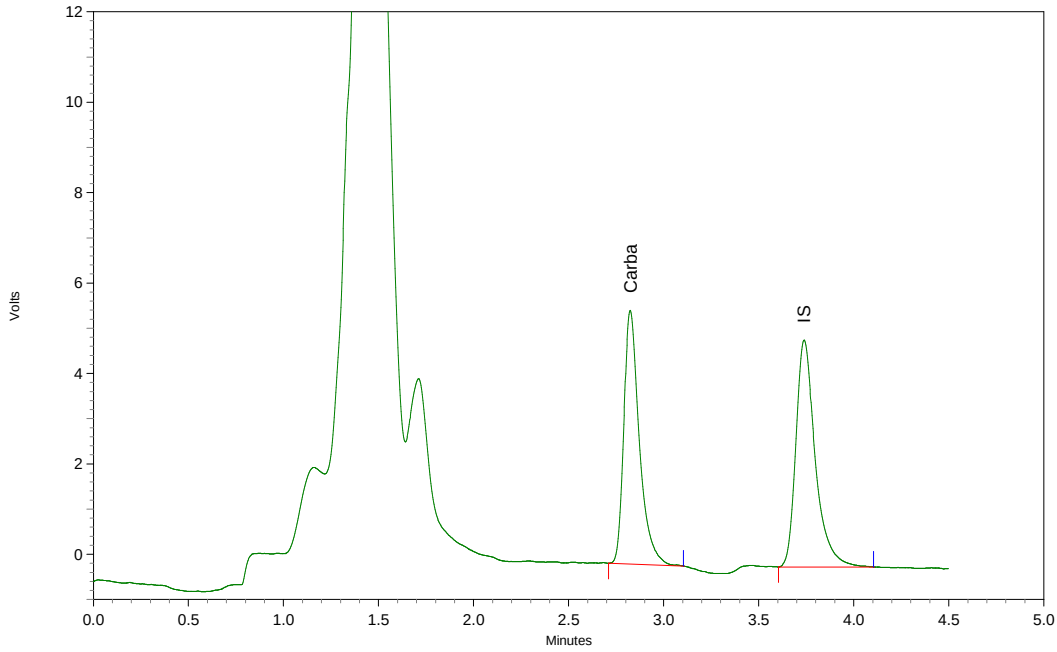

Figure 10: Rat sample at 0.50 hr (carbamazepine with licorice)

Table 9: Comparison in major pharmacokinetic parameters between carbamazepine alone and carbamazepine with licorice

Group 1 (carbamazepine alone)	**parameter**	**average**
	AUC 0-24	**31860.95**
	Tmax (hr)	**1.00**
	Cmax (ng/ml)	**4130.00**
	K	**0.076**
	Half Life	**9.08**
Group 2 (carbamazepine with licorice)	**parameter**	**average**
	AUC 0-24	**15776.70**
	Tmax	**0.50**
	Cmax	**2886.00**
	K	**0.078**
	Half Life	**8.94**

parameter	P-value
AUC 0-24	0.0034
Tmax	0.0589
Cmax	0.0000
K	0.9632
Half Life	0.9440

A food–drug interaction is the consequence of a physical, chemical, or physiologic relationship between a drug and a product consumed as food or a nutrient present in a botanically-derived food or dietary supplement [14].

Foods consumed as beverages account for a very high proportion of dietary antioxidant intake. Growing evidence supporting cardio protective benefits promotes moderate consumption as part of a healthy lifestyle [15]. However, certain beverages contain substances that can influence drug disposition via modulation of drug metabolizing enzymes and transporters in the intestine [14].

However, on the other hand, licorice had been reported to inhibit the functions of P-gp and CYP-dependent monooxygenase by *in vitro* studies[16]. Moreover, licorice significantly decreased the oral bioavailability of Cyclosporine. The major causative agent was glycyrrhetic acid, the major metabolite of glycyrrhizin, which activated P-gp and CYP3A and resulted in decreased absorption of cyclosporine in rats [17]. Accordingly, we reported some works in herb–drug and drug–drug interactions [18, 19, 20, 21, 22].

Carbamazepine is known as a substrate (metabolized) of cytochrome P450 3A4 (CYP3A4), indicating that any modulator of CYP3A4 may alter the pharmacokinetics and pharmacodynamics of carbamazepine.

As indicated and illustrated in the results, licorice reduces significantly both Cmax and AUC of carbamazepine in rat plasma. Since licorice was found as modulator of CYP3A4 and carbamazepine is a substrate of it, this interaction is most probably due to the induction of CYP 3A4, this need further elaboration in the future.

CONCLUSION

Herbal products are becoming popular as alternative medicines worldwide. Herb–drug and herb–herb interactions are a current topic of debate, while combination therapies have been validated and show potential clinical benefits.

The difference between Cmax (single administration vs. combination with licorices) was significant, and the difference in AUC was significant as well. This decrease in the plasma level of carbamazepine by licorice juice could be due to the induction of CYP3A4 activity since carbamazepine is metabolized primarily by CYP 3A4.

Future studies are needed to examine this possibility by working in-vitro on CYP3A4 enzyme. In addition, further investigations in humans are necessary.

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