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Ultra Sensitive Liquid Chromatographic Method for the Simultaneous Determination of Carbamazepine with Nsaids in API, Pharmaceutical Formulation and Human Serum by Programming the Detector: Application to *In Vitro* Drug Interaction

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Abstract

Here we describe an ultra-sensitive reverse phase liquid chromatographic method for the simultaneous determination of carbamazepine with NSAIDs in API, pharmaceutical formulation and human serum with UV detection. The separation of these multiple drug components was made by a Bondapak C_{18} column (10 µm, 25 cm×0.46 cm) using a moderate acidic mixture of mobile phase i.e., methanol:water 80:20 with pH adjusted to 3.0 using 85% o-phosphoric acid. The flow rate of the mobile phase was affixed at 1.0 ml min^{-1} for the best minimum interval of elusion of all the analytes. The detector response was monitored at isobestic point of 220 nm at ambient temperature. Calibration curves were obtained in the range of 0.4-12 µg mL^{-1} for carbamazepine, 0.5-16 µg mL^{-1} for meloxicam and 0.25-8.0 µg mL^{-1} for ibuprofen and mefenamic acid. Limits of detection were found to be 4.0, 3.0, 1.0, and 13.0 ng mL^{-1} respectively. The method was compared by programming the detector at individual wavelength of components which showed more sensitivity with linear range 0.10-3.0, 0.15-5.0, 0.10-3.0 and 0.125-4.0 µg mL^{-1} and detection limits 2.0, 2.0, 1.0, and 3.0 ng mL^{-1} respectively. ICH guidelines were followed for validation study. The method showed good precision and accuracy within acceptable range and can be successfully applied for the determination of these drugs in pharmaceutical formulations, human serum and clinical laboratories without interference of excipients or endogenous components of serum. *In vitro* interaction studies have also been carried out at physiological temperature in buffers of various pH (4, 7.4 and 9.0) simulating human stomach environments and % availability has been calculated. Moreover, the solid charge transfer complexes of carbamazepine with interacting NSAIDs were synthesized and characterized by IR spectroscopy and verified by computational molecular modeling.

Keywords: Carbamazepine; NSAIDs; HPLC; Time program; Drug interaction

Introduction

Epilepsy is a chronic brain disorder that causes people to have frequent seizures [1,2], the cause of which cannot be identified; however factors that are associated include brain trauma, strokes, brain cancer, and misuse of drug and alcohol. Carbamazepine (CBZ), chemically, *5H*-dibenzo [*b,f*] azepine-5-carboxamide, is an anticonvulsant agent, extensively used as antiepileptic and mood stabilizing drug. It is also approved to treat bipolar affective disorder like resistant schizophrenia, ethanol withdrawal, restless leg syndrome, psychotic behavior associated with dementia and post-traumatic stress disorders [3,4]. It is usually taken in combination with other antiepileptic drugs such as phenytoin or valproic acid. Non-steroidal Anti-inflammatory Drugs (NSAIDs) are widely recommended for the treatment of acute or chronic pains and inflammatory conditions such as rheumatoid arthritis, osteoarthritis and ankylosing spondilytis [5]. They are useful in primary dysmenorrhea, fever, sepsis-induced acute pneumonia [6], in retarding metastases of mammary carcinoma [7] and in preventing oxidative lesions of lungs caused by phosgene [8]. They also possess mild antiplatelet effect. Meloxicam (MEL), 4-hydroxy-2-methyl-N-(5-methyl-2-thiazolyl)-2H-1,2-benzothiazine-3-carboxamide-1,1-dioxide, ibuprofen (IBU), (*RS*)-2-(4-(2-methylpropyl)phenyl)propanoic acid and mefenamic acid (MEF), 2-(2,3-dimethylphenyl)aminobenzoic acid are commonly prescribed NSAIDs with other medication for the complain of pain and inflammation. Chemical structures of CBZ, MEL, IBU and MEF are given in figure 1.

Variety of techniques have been reported in literature for the determination of MEL including spectrophotometric and fluorimetric methods [9], flow-injection technique [10], electrochemical determination [11], HPLC-UV [12,13], LC-MS [14,15]. The methods have also been reported for the quantitation of IBU in commercial formulation and body fluids such as capillary isotachophoresis [16], spectrofluorometric determination [17], LC [18,19], spectrophotometric assay [20]. Determination of MEF has been reported by spectrophotometric [21,22], voltammetric [23], liquid chromatographic techniques [24,25].

Our research colleagues have reported a number of liquid chromatographic methods with UV detection for different classes of simultaneously prescribed drugs including statins [26], NSAIDs [27], diuretics [28], ACE inhibitors [29], quinolones [30], H_1-receptor antagonists [31], H_2-receptor antagonists [32], anti-diabetic [33] drugs and so on. Almost, all of these methods have been developed and validated isocratically at isobestic point of studied drugs. Also the *in vitro* interaction studies have been carried out with variety of drug classes at physiological temperature in buffers of various pH (4, 7.4 and

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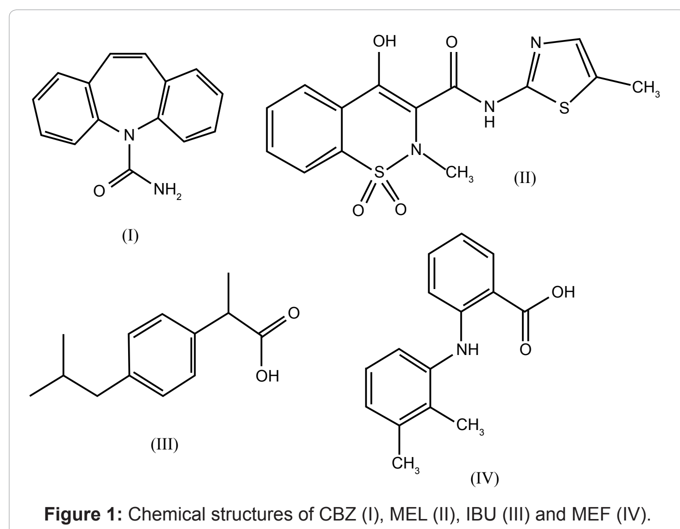

Figure 1: Chemical structures of CBZ (I), MEL (II), IBU (III) and MEF (IV).

9.0) simulating human stomach environment and % availability has been calculated.

In the present study, we described a rapid and efficient liquid chromatographic method with UV detection for the simultaneous determination of CBZ with NSAIDs at isobestic point of 220 nm and then compared the method by programming the detector adjusting the wavelength with time to match the individual analyte's chromophore which enhanced the sensitivity of method. The developed analytical method was validated following the ICH guidelines [34] and further applied to study the possible *in-vitro* interaction of CBZ with NSAIDs at physiological temperature (37°C) in different pH environments simulating full stomach juice (pH 4) and intestinal juice (pH 6.8-9). In addition, the solid charge transfer complexes of CBZ with interacting NSAIDs were synthesized and characterized by IR spectroscopy and verified by computational molecular modeling. The developed and validated HPLC method can successfully be applied for the determination of studied drugs with good percent recovery values in pharmaceutical formulation and human serum without interference of tablet excipients or endogenous components of serum.

Experimental

Material and reagents

Reference standard of CBZ was obtained from Novartis Pharma Pvt Ltd, MEL from AGP Pakistan (Pvt.) Ltd, IBU from Abbott Laboratories Pakistan Ltd and MEF from Pfizer Laboratories Pakistan Ltd. Pharmaceutical formulations Tegral® 200 mg, Melfax® 15mg, Brufen® 200 mg, and Ponstan 200 mg were purchased from local pharmacy. All the products were labeled and had expiry of not less than 365 days at the time of study. HPLC grade methanol (Merck, Germany), 85% *o*-phosphoric acid (Merck, Germany) and double distilled de-ionized water was used throughout the analysis.

Instrumentation

The dissolution equipment was manufactured according to B.P 2007 standard with slight modification. The dissolution assembly was immersed in a water bath previously maintained at 37°C. Shimadzu 1800 UV-visible spectrophotometer was used for the determination of isosbestic point and individual λ_{max} of analyte. Shimadzu liquid chromatographic system equipped with LC-20 AT VP solvent delivery pump, rheodyne manual injector fitted with 20 µL loop, SPD-20 AV Shimadzu UV visible detector, connected with Shimadzu CBM-102 communication Bus module. Chromatographic data acquisition was performed on CLASS-GC 10 software (version 2). The FT-IR spectra were obtained from KBr discs using Shimadzu Prestige-21200VEC version 1.2 software. Moreover, microliter syringe and micropore filtration assembly were used in this study.

HPLC condition

The mobile phase consisted of 80:20 methanol-water with pH adjusted to 3.0 with 85% *o*-phosphoric acid, filtered with 0.45 µm pore size filter millipore vacuum filter system and degassed with an ultrasonic bath (LC 30H). Chromatographic separation was achieved on a Bondapak, C_{18} (10 µm, 25×0.46 cm) column. All the analyses were carried out at ambient temperature under isocratic conditions employing the flow rate 1.0 mL min^{-1}. UV detector was operated at 220 nm for analysis at isobestic point. For time program analysis, the detector was programmed at 212, 205, 220 and 215 nm for CBZ, MEL, IBU and MEF respectively.

Standard solutions preparation

Stock solutions of 100 µg mL^{-1} of CBZ, MEL, IBU and MEF were separately prepared in 100 mL volumetric flask by dissolving accurately weighed 10 mg of reference standard in 80:20 methanol-water diluent. All the solutions were sonicated on LC-20 ultrasonic bath.

Buffer of pH 4.0 was prepared by taking 0.1 M potassium chloride whose pH was adjusted with 0.1 N hydrochloric acid. pH 6.8 buffer was prepared by taking 0.6 g potassium dihydrogen orthophosphate, 6.4 g disodium hydrogen orthophosphate and 5.85 g sodium chloride in 1000 mL deionized water and pH was adjusted with 17% ammonium hydroxide. pH 9 buffer was prepared by dissolving 17.4 g of potassium dihydrogen orthophosphate in 800 mL of deionized water and pH was adjusted with 17% ammonium hydroxide.

Calibration standards preparation

Working standard solutions were prepared in 25 mL volumetric flask by diluting the stock solutions with same solvent to bring the final concentration series of 0.4-12, 0.5-16, 0.25-8.0 and 0.25-8.0 µg mL^{-1} for analysis at isobestic point and 0.10-3.0, 0.15-5.0, 0.10-3.0 and 0.125-4.0 µg mL^{-1} for time program analysis for CBZ, MEL, IBU and MEF respectively. These working standard solutions were prepared once and analyzed daily for interday and intraday precision of the method. 20 µL of degassed and filtered (0.45 µm pore size) sample was injected into the system and peak response was recorded. Calibration curves were plotted between concentration and mean values of peak area.

Pharmaceutical formulations

Content of each drug in dosage formulations were determined by triturating ten tablets of each sample separately in mortar to obtain homogenous mixture. An amount equivalent to 10 mg of each drug was separately dissolved in mobile phase and allowed to stand for 30 min. These solutions were then sonicated for complete solubilization and filtered. Aliquots of each solution was accordingly diluted with mobile phase to get the required final concentration. All the samples were filtered with 0.45 µm membrane filter before delivering into the system.

Drug serum solution

The blood sample from a healthy person was collected at Fatmid Foundation Karachi and plasma was separated from it by centrifuging the blood sample at 1600 g for 10 min at 4°C. 1.0 mL of plasma along

with 9.0 mL acetonitrile was vortexed for few minutes followed by centrifugation for 10 minutes at 10,000 rpm. The clear serum solution obtained in supernatant was spiked with CBZ, MEL, IBU and MEF to obtain the desired concentrations of drugs in human serum for analysis at isobestic point and at individual λ_{max}.

Chromatographic conditions and experimental parameters optimization

Selection of stationary phase (column): Reversed-phase chromatographic columns of different manufacturer with different particulate were tried for the analysis of CBZ and NSAIDs, such as Bondapak, C_{18} (10 µm, 25×0.46 cm) column, Prospher Star C_{18} (5 µm, 25×0.46 cm) and Discovery C18 (5 µm, 25×0.46 cm).

Mobile phase composition and pH: Mixture of different organic solvents including methanol, acetonitrile, tetrahydrofuran and water in a variety of composition with varying pH were checked for better separation and resolution. The mobile phase composition that resulted in a better resolution with less retention times of the studied analytes was selected.

Flow rate: The flow rates of mobile phase were studied in the range of 0.8-2.0 ml/min and adjusted the appropriate flow rate.

Detector wavelength: Individual wavelength of each component was recorded on UV-Vis spectrophotometer. The isobestic point was then determined from the UV spectra and was chosen as the wavelength for simultaneous analysis of studied compounds. The detector was then programmed at individual wavelength of each component for time program analysis.

Method validation

The analytical validation was performed according to the recommendations of ICH 2006 guidelines [34] for system suitability test, specificity and selectivity, linearity, accuracy, precision, detection and quantitation limits and robustness.

System suitability of the method was evaluated on each day of validation and column efficiency was determined by calculating capacity factors (k'), theoretical plates (N), tailing factor (T), resolution (Rs), and separation factor (α). For specificity studies, blank solutions of solvent, placebo and serum sample and also the reference standard solution, solution of pharmaceutical formulation and spiked serum sample were chromatographed. Linearity was evaluated using calibration curves constructed for API and serum in triplicate at six concentration level by plotting the area under the chromatographic peak for each drug against its concentration at isobestic point and individual λ_{max} and regression characteristics including intercept, slope, correlation coefficient, standard error and standard error estimate were calculated. Accuracy was evaluated as the percentage of recovery of analytes to the pharmaceutical formulation and human serum by comparing the concentrations obtained from dosage formulation and drug-supplemented serum to the actual added concentrations. Inter-day and intra-day precision was determined by calculating the percent relative standard deviation for five determinations at each concentration of six samples for reference standard and three samples of human serum. The limit of detection and quantitation were taken to be the lowest concentration giving a signal three times and ten times as high as the signal-to-noise ratio respectively. In addition, the optimized chromatographic parameters like mobile phase composition, pH, flow rate and column were deliberately changed and their subsequent effect was observed on analytical results for robustness studies.

Availability studies

Prior to the interaction studies, the *in vitro* availability of CBZ and NSAIDs was determined in buffers of pH 4, 7.4 and 9.0. Samples were withdrawn periodically at an interval of 15 minutes for 3 h, diluted with methanol and chromatographed. The volume of dissolution fluid was maintained by adding an equal amount of dissolution fluid withdrawn, which had previously been maintained at the same temperature in the same bath.

In-vitro interaction studies by RP-HPLC

The *in-vitro* interaction studies of CBZ with NSAIDs was carried out by preparing separately 100 mL of 100 µg mL^{-1} stock solution of each drug in simulated gastric juice buffers of pH 4, 7.4 and 9 and sonicated for 10 min. Equivolume drug solutions of CBZ was individually mixed with each NSAID in each buffer separately to get the final concentration of 50 µg mL^{-1}. These flasks were kept at water bath previously maintained at 37°C for 3 h for interaction with constant stirring. 2 mL of these solutions were withdrawn at 0 min and periodically after every 30 min. Aliquots withdrawn were diluted to 10 µg mL^{-1} with methanol, filtered through 0.45 µm membrane filter and analyzed. Peak areas were recorded and compared with standard solution from which degree of interaction was evaluated.

Carbamazepine-NSAIDs complexes

Equimolar solutions of carbamazepine and NSAIDs were separately prepared in methanol. Equivolume solution of carbamazepine was individually mixed with MEL, IBU and MEF and the mixtures were refluxed on water bath for 3 hrs, filtered and left for crystallization at room temperature. These complexes were characterized by UV-visible and FT-IR spectroscopy.

Results and Discussion

Method development and optimization

The developed liquid chromatographic method for simultaneous determination of CBZ with NSAIDs was first optimized to choose the appropriate chromatographic conditions for efficient, accurate and economical analytical method. For stationary phase selection, Bondapak, C_{18} (10 µm, 25×0.46 cm) column, Prospher Star C_{18} (5 µm, 25×0.46 cm) and Discovery C_{18} (5 µm, 25×0.46 cm) were tested with a variety of mobile phases and the best separation with less retention time was achieved with Bondapak, C_{18} (10 µm, 25×0.46 cm) column. Mobile phase methanol-water and acetonitrile-water in different ratios with variable (pH 2.8-3.6) were tried to fix on the best ratio for separation of components. The mobile phase 80:20 methanol-water with pH adjusted to 3.0 exhibited good separation and high resolution with short analysis time. The flow rate of mobile phase greatly affected the analysis of the studied analytes. Although run time decreased significantly at higher flow rates, however resolution of peaks and sensitivity of the analytes were decreased. The flow rate was therefore adjusted to 1.0 mL min^{-1}. Separation was achieved at isobestic point of 220 nm. The UV spectra showing λ_{max} and chromatogram showing retention times of each component are given in figures 2 and 3 respectively.

Method validation

The validation parameters studied according to ICH guidelines were system suitability test, specificity and selectivity, linearity, accuracy, precision, detection and quantitation limits and robustness.

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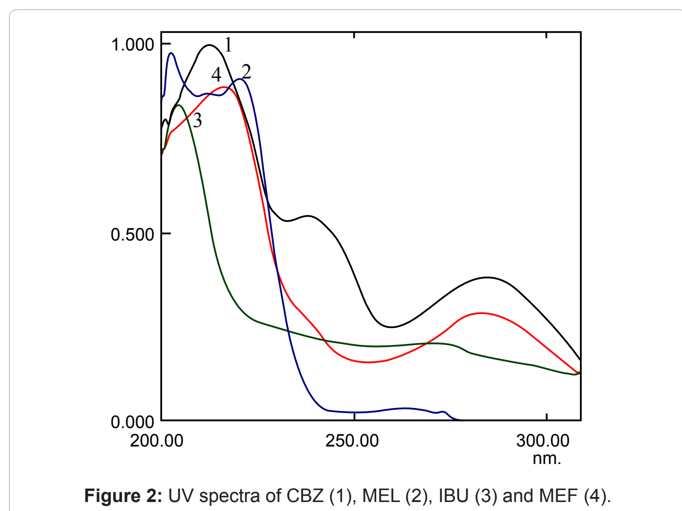

Figure 2: UV spectra of CBZ (1), MEL (2), IBU (3) and MEF (4).

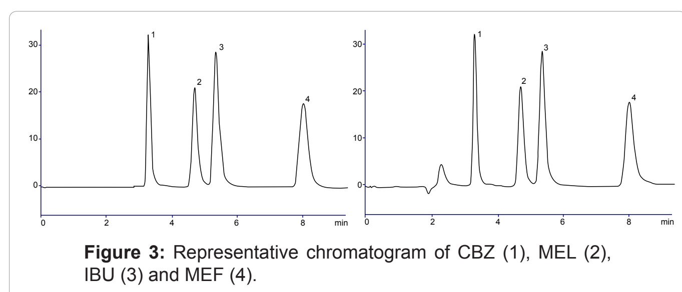

Figure 3: Representative chromatogram of CBZ (1), MEL (2), IBU (3) and MEF (4).

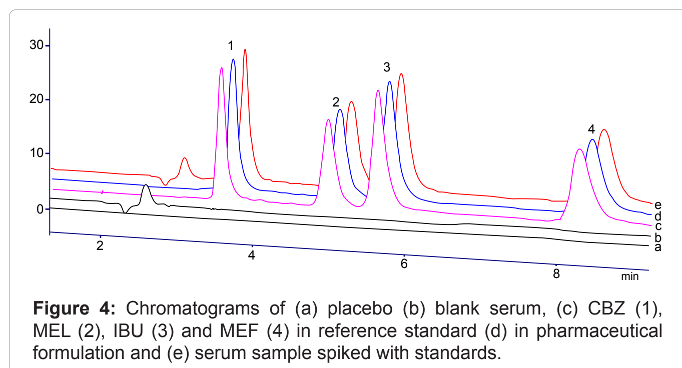

Figure 4: Chromatograms of (a) placebo (b) blank serum, (c) CBZ (1), MEL (2), IBU (3) and MEF (4) in reference standard (d) in pharmaceutical formulation and (e) serum sample spiked with standards.

Specificity

The mobile phase 80:20 methanol-water with pH adjusted to 3.0 was found to be suitable for separation and elution of drugs showing good peak shape and symmetry. The chromatograms of blank solvent, placebo and serum sample and also the reference standard solution, solution of pharmaceutical formulation and spiked serum sample were recorded. Chromatograms showed no other peak besides the four active components with retention times 3.26, 4.64, 5.28 and 7.88 min for CBZ, MEL, IBU and MEF respectively. Figure 4 showed the good separation of components in presence of excipients of pharmaceutical formulation and human serum.

System suitability test

The system suitability test was performed by 10 subsequent injections of mixture of reference standard solutions. The column efficiency was determined by calculating system suitability parameters. It was observed that the number of theoretical plates was found to be greater than 2000 and tailing factor was less than 2 for all the studied drugs. Also the resolution was less than 2 confirming the suitability of the method. The system suitability parameters calculated at isobestic point as well as at individual λ_{max} are presented in table 1.

Linearity

Calibration curves constructed between the concentration range of each drug i.e., 0.4-12, 0.5-16, 0.25-8.0 and 0.25-8.0 µg mL^{-1} for CBZ, MEL, IBU and MEF and respective chromatographic response were found to be linear for all the assayed drugs. The correlation coefficients (R^2) of calibration curve for all the studied analytes were consistently found not less than 0.998. The regression characteristics for drugs including slope, intercept, correlation coefficient, standard error and standard error estimate are given in table 2.

Precision

The precision of the method was verified for intra-day and inter-day study by means of percent relative standard deviation. Six different concentration of CBZ, MEL, IBU and MEF in the range of 0.4-12, 0.5-16, 0.25-8.0 and 0.25-8.0 µg mL^{-1} and three different concentrations of each of these drugs in serum i.e., 0.8, 1.5 and 3.0 for CBZ, 1, 2 and 4 for MEL and 0.5, 1 and 2 for IBU and MEF were analyzed and repeated the study for inter-day. The %RSD for intra-day and inter-day analysis of drugs in API and in human serum were found to be in the range of 0.02-2.10 (Table 3).

Accuracy

The accuracy of the method was evaluated in terms of percent recovery at six and three concentrations levels in a specified range in drug formulation and human serum respectively. Each of the analytical result is the representative of five replicate analyses. The results exhibit good accuracy of the method and recoveries were found to be 99.28-

Drug	t_R	*k'*	N	T	Res	α
Isobestic point						
CBZ	3.22	1.97	4325	1.62	1.22	1.39
MEL	4.24	1.59	5356	1.56	1.75	1.64
IBU	5.30	2.24	4791	1.71	1.92	1.41
MEF	6.66	3.07	5291	1.62	1.05	1.37
Time program						
CBZ	3.23	1.39	3883	-	1.92	1.32
MEL	4.25	2.14	5089	1.44	1.58	1.54
IBU	5.31	2.93	4617	1.60	1.85	1.37
MEF	6.68	3.94	5669	1.48	1.10	1.35

t_R=retention times, *k'*=capacity factor, N=theoretical plates, T=tailing factor, Res=resolution, α=separation factor

Table 1: System suitability Parameters.

Drug	Linearity µgmL^{-1}	Intercept	Slope	R^2	SE[a]	SEE[b]	LOD µgmL^{-1}	LOQ µgmL^{-1}
Isobestic point								
CBZ	0.4-12	33695	68281	0.9986	0.5939	0.9808	4.0	13
MEL	0.5-16	73993	92135	0.9987	0.2327	0.3803	3.0	10
IBU	0.25-8.0	-6294	191713	0.9999	0.0400	0.0226	1.0	3.0
MEF	0.25-8.0	39386	124225	0.9984	0.3661	0.6025	13	40
Serum								
CBZ	-	-15195	97939	0.9998	0.0123	0.0106	4.0	13
MEL	-	89152	86724	0.9988	0.3479	0.5512	3.0	9.0
IBU	-	-9271	193921	0.9989	0.0085	0.0071	2.0	5.0
MEF	-	6939	175424	1.0000	0.4588	0.5480	16	48

a=standard error estimate, b=standard error

Table 2: Regression characteristics and sensitivity of the method at isobestic point.

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CBZ			MEL			IBU			MEF		
Conc	% RSD[a]	% RSD[b]	Conc	% RSD[a]	% RSD[b]	Conc	% RSD[a]	% RSD[b]	Conc	% RSD[a]	% RSD[b]
Isobestic point											
0.4	0.18	0.16	0.5	0.36	0.30	0.25	0.18	0.36	0.25	0.83	0.96
0.8	0.05	0.18	1	0.19	0.05	0.5	0.30	0.37	0.5	0.44	0.13
1.5	0.42	0.63	2	0.05	0.16	1	0.23	0.11	1	0.12	0.11
3	0.23	0.14	4	0.27	0.02	2	0.13	0.07	2	0.10	0.10
6	0.02	0.05	8	0.02	0.11	4	0.14	0.98	4	0.04	0.12
12	0.21	0.11	16	0.02	0.12	8	0.05	0.42	8	0.05	0.76
Serum											
0.8	0.21	1.58	1	0.15	1.44	0.5	0.12	0.37	0.5	1.05	1.83
1.5	0.29	0.59	2	2.10	1.00	1	0.08	1.65	1	0.10	0.96
3	0.03	0.60	4	0.22	0.84	2	0.43	0.41	2	0.05	1.06

%RSDa = Intra-day, %RSDb = Inter-day,

Table 3: Precision of the proposed method at isobestic point.

Conc	%Rec	Conc	%Rec	Conc	%Rec	Conc	%Rec
CBZ		MEL		IBU		MEF	
Pharmaceutical formulations							
0.4	100.50	0.5	99.50	0.25	100.46	0.25	99.40
0.8	99.63	1	100.20	0.5	99.64	0.5	99.63
1.5	100.86	2	99.67	1	100.32	1	100.86
3	102.75	4	100.54	2	100.85	2	100.25
6	99.55	8	99.42	4	99.97	4	99.46
12	99.28	16	99.67	8	100.23	8	100.45
Serum							
0.8	100.37	1	100.20	0.5	99.62	0.5	99.94
1.5	101.57	2	100.49	1	99.67	1	100.49
3	100.59	4	99.06	2	100.60	2	100.32

Table 4: Recovery in serum and pharmaceutical formulations.

102.75% and 99.06-101.57%, which are near to the true value. The accuracy data is presented in table 4.

Detection and quantitation limits

The ratio of chromatographic signal when remained three times and ten times with their respective noise signals was concluded for LOD and LOQ respectively. LOD and LOQ for CBZ, MEL, IBU and MEF, determined on the basis of signal to noise ratio were 4.0, 3.0, 1.0, 13 and 13, 10, 3.0, 40 ng mL^{-1} in API and 4.0, 3.0, 2.0, 16 and 13, 9.0, 5.0, 48 ng mL^{-1} in human serum respectively. The LOQ of each analyte was found almost three times of their LOD values which also exhibits accuracy of the method (Table 2).

Robustness

Robustness of the developed method was studied by varying few of the chromatographic parameters by will and observing the effect on analytical results. It was accomplished that minor modification in mobile phase composition and flow rate show negligible effect on results leading to shifting of retention time up to 0.1%, change in pH did not affect the results, and however, variation in wavelength influenced the area under curve of peak. Overall, the method was found to be robust. Data is tabulated in table 5.

Programming the detector

UV Spectra, taken on Shimadzu 1800 UV-visible spectrophotometer showed individual λ_{max} 212, 205, 220 and 215 nm for CBZ, MEL, IBU and MEF respectively, that is why the detector was programmed at 212 nm for 0-3.5 min, 205 nm for 3.6-4.9 min, 220 nm for 5.0-6.5 min and 215 nm for 6.6-9.0 min for complete elution.

The method showed high sensitivity with good separation and resolution of each component (Figure 5). The linearity of the proposed method was found to be 0.10-3.0, 0.15-5.0, 0.10-3.0 and 0.125-4.0 µg mL^{-1} for CBZ, MEL, IBU and MEF respectively with correlation coefficient greater than 0.998. The regression data calculated from calibration curve constructed between peak area and concentration of each analyte including slope, intercept, correlation coefficient, standard error and standard error estimate are given in table 4.

The inter-day and intra-day precision was in the range of 0.02-2.10 and 0.02-1.83% confirming the precision of method. The sensitivity of the method was enhanced by programming the detector at individual wavelength and it showed LOD values 2.0, 2.0, 1.0 and 3.0 ng mL^{-1} for CBZ, MEL, IBU and MEF respectively (Table 6).

The technique is suitable for application of method for simultaneous determination of all the studied analytes in bulk drug, pharmaceutical formulation and human serum by programming the detector.

Interaction studies by HPLC

The availability of CBZ and all the studied NSAIDs in different pH environments, simulating human body compartments at 37°C in individual dosage formulations and in the presence of each other were calculated (Table 7). The interaction of CBZ with NSAIDs at 220 nm in all the buffer mediums showed considerable decrease in availability of CBZ i.e., 53.13, 51.46 and 53.23% available in presence of MEL, 47.66, 50.95 and 43.91 % available in presence of IBU and 47.76, 46.83 and

Parameters		CBZ		MEL		IBU		MEF	
		N	T	N	T	N	T	N	T
pH	2.9	3904	1.44	4866	1.59	4368	1.58	4738	1.22
	3.0	3871	1.51	4845	1.41	4342	1.56	4815	1.53
	3.1	3931	1.49	4907	1.38	4417	1.54	4854	1.54
Flow rate (ml min^{-1})	0.9	3701	-	4753	1.42	4213	1.53	5075	1.42
	1.0	3757	-	4761	1.40	4233	1.52	5022	1.41
	1.1	3713	-	4714	1.45	4204	1.54	5064	1.40
Mobile phase ($MeOH:H_2O$)	78:22	3602	-	4580	-	4025	1.50	5366	1.33
	80:20	3543	-	4574	-	4100	1.50	5365	1.32
	82:18	3537	-	4635	-	3878	1.49	5429	1.33
Column	Purospher STAR	3474	-	4267	-	4017	-	4606	-
	Discovery	3336	-	4068	-	3937	-	5546	-

N=Theoretical plates, T=Tailing factor

Table 5: Robustness.

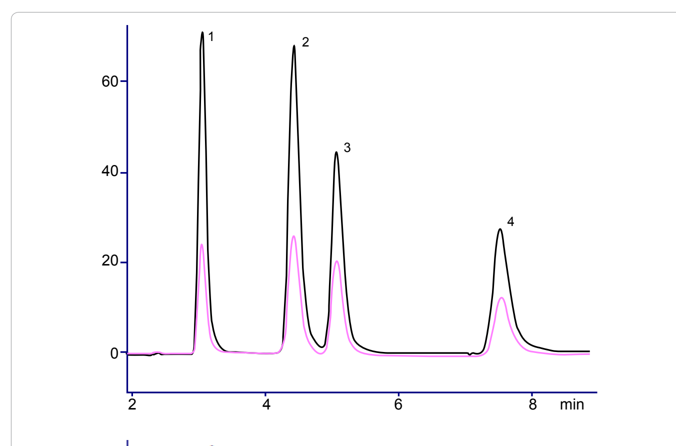

Figure 5: Representative chromatogram of CBZ (1), MEL (2), IBU (3) and MEF (4) at isobestic point (pink) and at individual λ_{max} (black).

Citation: Sultana N, Saeed Arayne M, Ali SN (2012) Ultra Sensitive Liquid Chromatographic Method for the Simultaneous Determination of Carbamazepine with Nsaids in API, Pharmaceutical Formulation and Human Serum by Programming the Detector: Application to *In Vitro* Drug Interaction. J Chromat Separation Techniq 3:156. doi:10.4172/2157-7064.1000156

CBZ		MEL		IBU		MEF	
Conc	**%RSD**	**Conc**	**%RSD**	**Conc**	**%RSD**	**Conc**	**%RSD**
0.1	0.65	0.15	0.08	0.1	0.62	0.125	0.82
0.2	0.25	0.3	0.57	0.2	0.14	0.25	0.28
0.4	0.26	0.6	0.29	0.4	0.24	0.5	0.15
0.8	0.31	1.25	0.59	0.8	0.68	1	0.24
1.6	0.16	2.5	0.33	1.5	0.27	2	0.42
3.2	0.10	5	0.87	3	0.15	4	0.19
Regression characterstics							
Parameters		CBZ		MEL		IBU	MEF
μgmL^{-1a}		0.10-3.0		0.15-5.0		0.10-3.0	0.125-4.0
Intercept		588		233139		53421	12459
Slope		509413		733554		551861	557057
R^2		0.9989		0.9986		0.9982	0.9995
SE[b]		0.0277		0.0730		0.1465	0.2292
SEE[c]		0.0488		0.1174		0.2408	0.3733
LOD ($ngmL^{-1}$)		2.0		2.0		1.0	
LOQ $ngmL^{-1}$)		6.0		6.0		3.0	

[a]Linearity, [b]standard error, [c]standard error of estimate

Table 6: Regression characteristics and precision of the proposed method at individual λ_{max}.

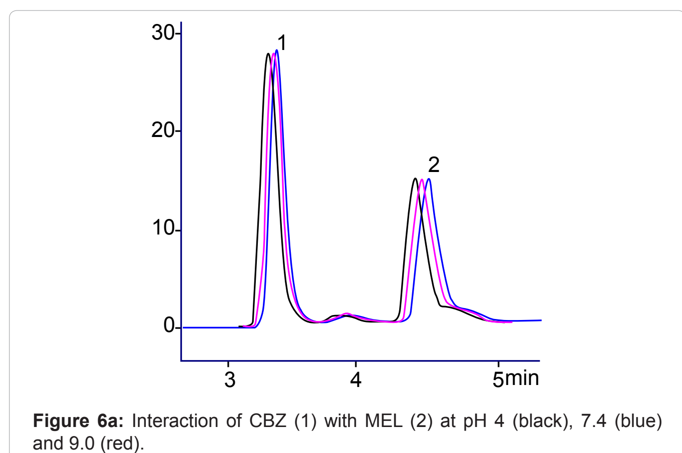

Figure 6a: Interaction of CBZ (1) with MEL (2) at pH 4 (black), 7.4 (blue) and 9.0 (red).

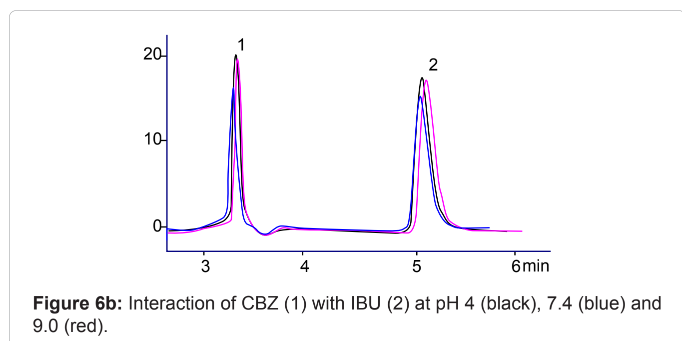

Figure 6b: Interaction of CBZ (1) with IBU (2) at pH 4 (black), 7.4 (blue) and 9.0 (red).

54.66 % available in presence of MEF at pH 4.0, 7.4 and 9.0. It was observed that the availability of interacted NSAIDs was also decreased in all the buffer mediums. MEL was 57.01, 56.50 and 50.44%, IBU was 59.29, 56.03 and 61.55% and MEF was 50.68, 52.92 and 52.48% available in buffer of pH 4.0, 7.4 and 9.0 respectively. The results indicate that the CBZ forms charge transfer complexes with interacting NSAIDs via nitrogen atom of amide group (Figures 6a-6c).

Synthesis and characterization of carbamazepiene complexes

The results of interaction studies were further supported by synthesizing the CBZ complexes with NSAIDs and characterizing by IR spectroscopy. The IR Spectra of CBZ showed distinct peaks at 3464 and 3340 cm^{-1} for stretching and at 1562 cm^{-1} for bending of N-H and the carbonyl peak of amide appeared at 1676 cm^{-1}. In MEL, N-H stretching and bending peaks appeared at 3291 and 1620 cm^{-1} and carbonyl peak was observed at 1580 cm^{-1} [35]. The IR spectra of IBU

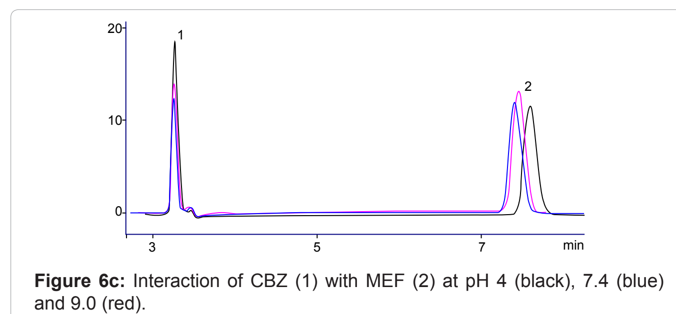

Figure 6c: Interaction of CBZ (1) with MEF (2) at pH 4 (black), 7.4 (blue) and 9.0 (red).

Time (min)	CBZ	MEL	CBZ	IBU	CBZ	MEF
	% Availability					
pH 4.0						
0	101.51	101.62	100.18	101.79	102.24	100.49
30	87.24	101.21	95.48	98.54	103.29	99.63
60	84.01	98.40	89.01	94.82	90.61	87.27
90	83.34	94.33	77.81	70.38	72.39	62.07
120	68.03	83.77	60.23	62.14	64.13	59.80
150	56.75	71.74	53.66	60.67	55.37	54.69
180	53.13	57.01	47.66	59.29	47.76	50.68
pH 7.4						
0	100.31	100.11	100.05	102.26	99.80	100.16
30	87.99	100.08	96.40	99.67	94.83	100.18
60	84.75	94.00	83.72	92.32	81.32	85.27
90	80.99	85.56	71.38	77.09	72.92	64.13
120	68.16	79.87	63.37	60.48	62.95	60.23
150	57.26	69.34	54.44	57.86	55.05	56.43
180	51.46	56.50	50.95	56.03	46.83	52.92
pH 9.0						
0	99.57	100.81	101.11	100.16	100.78	100.62
30	98.16	93.05	93.87	97.82	100.13	98.78
60	83.97	88.16	86.12	95.78	97.78	85.31
90	74.73	81.58	69.72	76.09	86.99	64.07
120	61.85	68.59	58.97	64.22	71.36	59.19
150	56.94	56.92	51.55	62.87	62.04	57.14
180	53.23	50.44	43.91	61.55	54.66	52.48

Table 7: Interaction studies.

CBZ	CBZ-MEL	CBZ-IBU	CBZ-MEF	Assignments
-	3549	-	-	*v* (O-H)
3464, 3340	3464, 3344	3464, 3340	3464, 3311	*v* (N-H) stretching
3159, 3020	3157, 3022	2954, 2922	3016, 2918	*v* (C-H)
1676	1680	1720, 1678	1678, 1649	*v* (C=O)
-	1658	-	-	*v* (C=N)
1562	1554	1562	1575	*v* (N-H) bending
1600, 1489	1598, 1490	1602, 1490	1508	*v* (C=C)
1458	1458	1458	1446	*v* (C-H) deformation
-	1124	1230	1255	*v* (C-O)
1149	1157	1120	1159	*v* (C-H) in plane
-	1303, 1267	-	-	*v* (S=O)
-	873	867	893	δ_{rock} CH_2

Table 8: Infrared frequencies and their assignments.

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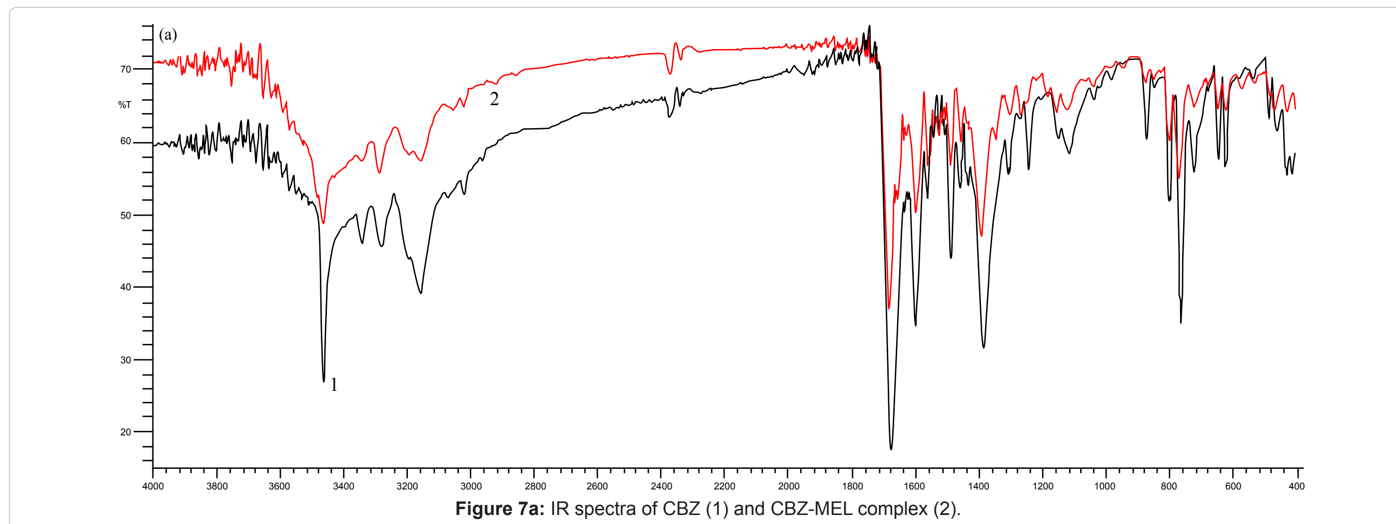

Figure 7a: IR spectra of CBZ (1) and CBZ-MEL complex (2).

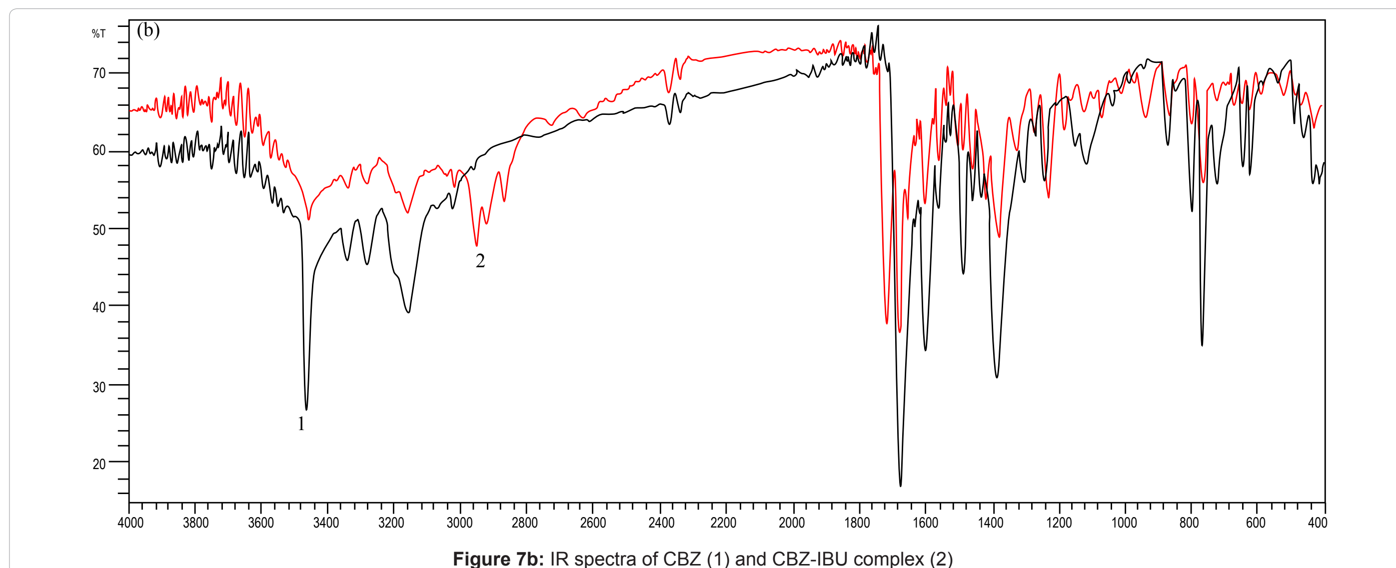

Figure 7b: IR spectra of CBZ (1) and CBZ-IBU complex (2).

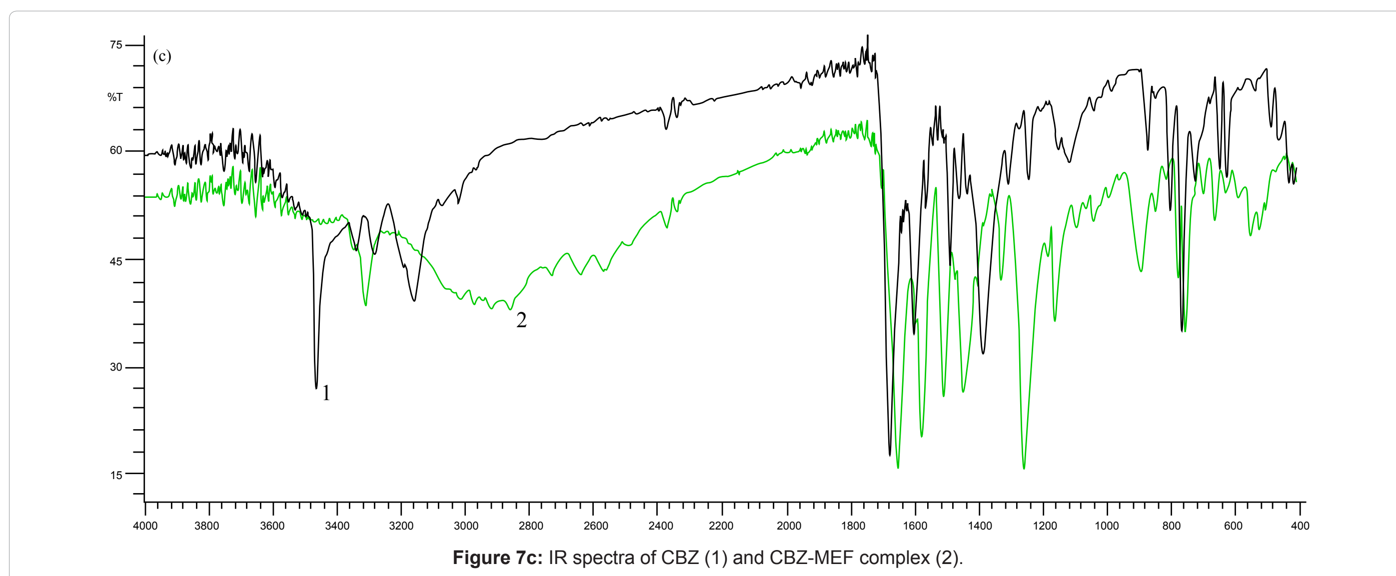

Figure 7c: IR spectra of CBZ (1) and CBZ-MEF complex (2).

showed characteristic broad band of hydroxyl group of carboxylic acid in the region of 2400-3200 cm^{-1}, whereas carbonyl peak appeared at 1720 cm^{-1}. MEF showed N-H stretching and bending at 3300 and 1572 cm^{-1} respectively & the carbonyl peak appeared at 1647 cm^{-1} [36].

The FT-IR spectra of CBZ-MEL complex showed N-H stretching at 3464 and 3344 cm^{-1} and bending at 1554 cm^{-1}. The carbonyl peak shifted to 1680 cm^{-1} assuring the formation of CT complex. In the CBZ-IBU complex, the distinct peak of hydroxyl group disappeared confirming the CBZ-IBU complex whereas two carbonyl peaks were observed at 1720 and 1678 cm^{-1} which are slightly shifted from parent drug ensuring the formation of complex. The N-H stretching and bending appeared at 3464 and 3340 cm-1 and 1562 cm^{-1} respectively. In CBZ-MEF complex, the disappearance of OH peak attributes to the interaction at carboxylate group of MEF. The infrared frequencies and their band assignments for alone CBZ and its complexes are given in table 8 and respective IR spectra are presented in figure 7.

Energy minimization studies

To establish the binding site and possible structures of CBZ and interacting NSAIDs, molecular modeling for the complexes were performed by using CS Chem 3D© Ultra version 8.03, implemented with Molecular Orbital Computations Software (MOPAC) and molecular dynamics computations software (MM2). Calculations of interactions and energies, resulting from bond stretching, angle bending, torsional energies and nonbonded were performed through molecular mechanics.

It was found that the CBZ forms complexes via nitrogen of amide group with all the studied NSAIDs, where as the carbonyl oxygen of acyl side chain in MEL and carboxylate group of IBU and MEF are involved in charge transfer complex formation. The structure of charge transfer complexes of drug-drug interaction are given in figure 8 and the minimization energies of these complexes are presented in table 9.

Application of proposed method

The proposed and validated LC method facilitates the simultaneous determination and quantification of CBZ, MEL, IBU and MEF in reference standard, pharmaceutical formulation and human serum without interference of undesired excipients of tablet or serum substances. The results were in good agreement with the declared contents and showed good percent recovery within the acceptable limits indicating the accuracy and precision of the method. Besides, the developed HPLC method has been effectively employed for the interaction of CBZ with NSAIDs (MEL, IBU and MEF).

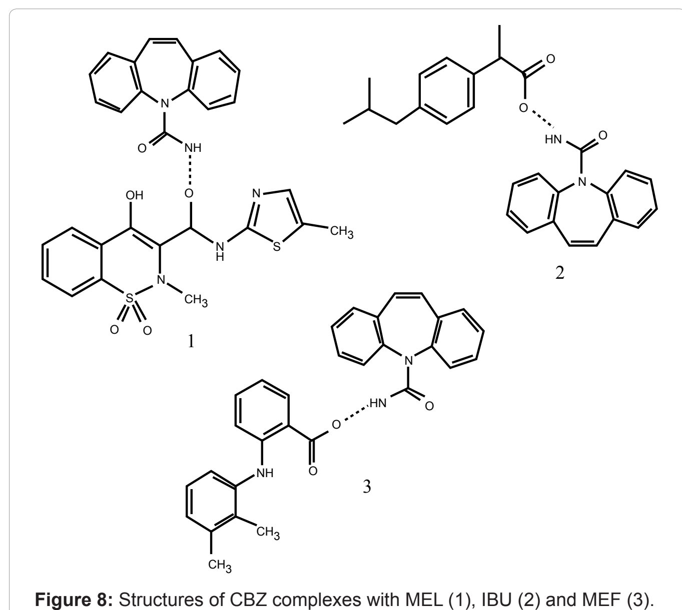

Figure 8: Structures of CBZ complexes with MEL (1), IBU (2) and MEF (3).

Parameters	CBZ-MEL	CBZ-IBU	CBZ-MEF
Iteration	95	69	362
Stretch	14	3.55	4.95
Bend	129	9.86	14.97
Stretch bend	-6.43	0.08	-4.59
Torsion	30	3.59	-28.65
Non-1,4 VDW	-9.39	-10.92	-6.87
Dipole/Dipole	33	21.18	15.57
Steric energy	-13.47	-4.33	-5.89
Total energy	178.02	23.02	-10.51

Table 9: Energy minimization calculations.

Conclusion

An accurate and reliable, isocratic reversed phase liquid chromatographic method has been developed for the simultaneous quantification of CBZ with NSAIDs with UV detection at isobestic point and at individual wavelength of each component with lowest detection limits. Linearity, precision, accuracy and suitability test were performed for method validation and were found parallel with the recommended standards. The advantage of this method is easy preparation of samples and mobile phase and separation of all the analytes with acceptable chromatographic resolution in a short period of time. All calibration curves were found to be linear with correlation coefficient greater than 0.998. The RSD values were less than 2%. High recovery values were obtained for pharmaceutical formulations and human serum. The proposed analytical method is therefore, suitable for routine analysis of CBZ and studied NSAIDs in quality control laboratories, pharmaceutical formulations and human serum. Chromatograms were free from excipients or serum interferences. Moreover, the developed LC method has been successfully applied for the interaction studies of CBZ with NSAIDs. On the basis of *in vitro* interaction results obtained from HPLC, it is concluded that CBZ interacts with studied NSAIDs through the formation of charge transfer complexes, and therefore simultaneous administration of these drugs may affect the bioavailability and loss of their therapeutic effect.

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