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DEVELOPMENT OF RP-HPLC METHODS FOR THE ESTIMATION OF NEBIVOLOL AND CARVEDILOL WITH SELECTED NSAIDS AND ITS APPLICATION TO DRUG DISPLACEMENT INTERACTION STUDIES

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Drug-drug interactions are gaining importance in practice nowadays. Many drugs bind to serum albumin and alter pharmacokinetics of the drugs such as distribution and elimination. Any small change in the extent of binding will largely affect the plasma concentration, especially those of highly bound drugs. The binding interactions of nebivolol (NEBI) and carvedilol (CAR) with BSA in presence of aceclofenac (ACE) and lornoxicam (LOR) were studied under simulated physiological conditions using validated reversed phase high performance liquid chromatographic (RP-HPLC) methods. The highest percentage protein binding of NEBI and CAR at saturation level were found to be 87.7±0.6% and 81.5±0.6%, respectively. In presence of ACE and LOR the maximum binding was 67.9±3.3% and 75.2±1.6% for NEBI and 80.4±0.03% and 42.0±0.3% for CAR. From the interaction study a significant decrease was observed in the protein binding of two drugs that may lead to increase in their plasma concentrations. Therefore careful monitoring should be exercised during the combination therapy of NEBI with ACE or LOR and CAR with LOR. Further in vivo studies are warranted for both NEBI and CAR in presence of these non-steroidal antiinflammatory drugs (NSAIDs) for which the developed RP-HPLC methods could serve purpose.

Keywords: Carvedilol, Nebivolol, Drug interaction, RP-HPLC

INTRODUCTION

Beta-blockers are an important class of drugs that are widely used to treat cardiovascular disorders such as cardiac arrhythmiasis, ischemic heart diseases and hypertension. Nebivolol (NEBI) is a highly selective third-generation beta-adrenergic blocker that has been used for the treatment of hypertension and heart failure (O'Neil 2006). It is preferentially β1-selective. NEBI is highly protein bound intravascularly, predominantly to albumin. The in vitro human plasma protein binding of NEBI is approximately 98% (Jaroslaw *et al.* 2008). Another β-blocker indicated for hypertension and heart failure is carvedilol (CAR), which blocks β1, β2 and α1- receptors (Theodore *et al.* 1991). More than 98% of CAR is bound to plasma proteins, preferably to albumin (Kahina *et al.* 2013).

Albumin is an abundant transport-protein found in plasma which binds a wide variety of drugs in two primary binding sites (I and II) that can have a significant impact on drug pharmacokinetics (Patrick and Yashveer 2001). Alteration in the albumin binding of drugs due to some physiological conditions may cause changes in the pharmacological and pharmacokinetic properties of drugs (Md Ashraful *et al.* 2007). Acidic drugs generally bind to plasma albumin, and basic drugs bind to either albumin or α1-acid glycoprotein. Displacement of a drug from its binding site by another drug leads to pharmacokinetic drug interactions. When a highly protein-bound drug is displaced from binding by a second

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drug, a sharp increase in the free drug concentration may occur, leading to toxicity. Drug displacement interactions depend upon binding affinities, specificity of binding, and relative concentrations of both displaced and displacer drugs. Non-steroidal antiinflammatory drugs (NSAIDs) represents one of the most commonly prescribed class of drug along with antihypertensive drugs. They are highly bound (>90%) to plasma albumin and displace many drugs from their albumin binding site.

In view of the above consideration, an in vitro investigation on displacement interactions of NEBI and CAR with two commonly prescribed NSAIDs, aceclofenac (ACE) and lornoxicam (LOR) was conducted. For NEBI, CAR, ACE and LOR various analytical methods like UV, high performance liquid chromatographic (HPLC) and high performance thin layer chromatographic (HPTLC) methods were reported individually and also with other combinations (Carolin *et al*. 2010; Santhosh *et al*. 2010; Shirkhedkar *et al.* 2010; Sohan *et al*. 2010; Kiran *et al*. 2009; Sahoo *et al*. 2009; Vivek *et al.* 2009; Bhinge *et al.* 2008; Patel *et al*. 2006). Hence, we developed HPLC methods (Snyder *et al.* 1988) for common antihypertensive drugs, NEBI and CAR in presence of ACE as well as LOR, and to observe the competitive effects of ACE and LOR on binding of NEBI and CAR when used simultaneously after method validation as per International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) guidelines (ICH 2005).

METHODS

Materials and Reagents

NEBI, CAR, ACE and LOR of pharmaceutical grade were used without further purification (99%–101%, w/w). Methanol, acetonitrile and water used were of HPLC grade and were purchased from Merck (Worli, Mumbai). Ammonium acetate was obtained from Hi Media Laboratories Pvt. Ltd. (Mumbai). The liquid chromatography mass spectrometer (LCMS) Shimadzu LCMS-2010EV (Singapore) consisted of the following components: a binary gradient pump, variable wavelength programmable photodiode array (PDA) detector with auto sampler system. The chromatographic analysis was performed using Compaq Intel Core-2 DUO HP W/907 software on a pre-packed RP-18 column (250 × 4.6 mm, 5 µm particle size). In addition, an electronic balance (Shimadzu, BL-220H, Kyoto), a pH meter (Elico L127, Hyderabad, India), a sonicator (Leclasonic ultrasonic cleaner, Sai Scientific Company, Coimbatore, India) and a hot air oven (Inlab Equipments Ltd., Coimbatore, India) were used in the study.

Selection of Chromatographic Mode for Separation

Proper selection of the methods depends upon the nature of the sample, its molecular weight and solubility. As NEBI, CAR, ACE and LOR are polar in nature, reversed phase chromatography was used because of its simplicity and suitability.

Preparation of Stock and Standard Solutions

Stock solutions were prepared by weighing 10 mg each of NEBI, CAR, ACE and LOR and then transferred to 4 separate 100 mL volumetric flasks, dissolved using methanol except for CAR, for which we used acetonitrile as the solvent. Further dilutions were made with 0.067 M phosphate buffer of pH 7.4 to obtain solutions each containing 100 µg/mL of NEBI, CAR, ACE or LOR. Aliquots of standard stock solutions of NEBI, CAR, LOR and ACE were transferred using graduated pipettes into 10 mL volumetric flasks and

4 different mixtures (NEBI-ACE, NEBI-LOR, CAR-ACE and CAR-LOR) were prepared and made up to the volume with the mobile phase to give the final concentration of 1–10 µg/mL for NEBI-ACE, NEBI-LOR CAR-ACE and CAR-LOR.

Fixed Chromatographic Condition for NEBI-ACE

By considering few parameters like solubility, polarity and maximum absorption the chromatographic conditions were selected as:

Column	: RP-18 column (250 × 4.6 mm, 5 µm particle size), cartridges
Mobile phase	: 20 mM ammonium acetate:methanol (30:70, v/v); pH 4 (adjusted using 1% orthophosphoric acid)
Detection wavelength	: 237 nm
Flow rate	: 1 mL/min
Operating temperature	: Room temperature

For NEBI-LOR, the fixed chromatographic conditions were similar to that of NEBI-ACE except the mobile phase ratio which was 35:65, v/v.

Fixed Chromatographic Condition for CAR-ACE

Column	: RP-18 column (250 × 4.6mm, 5 µm particle size), cartridges
Mobile phase	: Water:acetonitrile (40:60, v/v); pH 2.5 (adjusted using 1% orthophosphoric acid)
Detection wavelength	: 285 nm
Flow rate	: 0.8 mL/min
Operating temperature	: Room temperature

A flow rate of 1 mL/min was used for CAR-LOR. Other chromatographic conditions were similar to that of CAR-ACE.

Validation (ICH 2005)

Specificity

The chromatogram of the blank was observed for any additional peaks particularly, at the retention time of the analytes after injecting the blank and sample solutions under fixed chromatographic conditions.

Linearity and Range

Working standard solutions containing mixture of NEBI-ACE and NEBI-LOR as well as CAR-ACE and CAR-LOR in the ratio of 1:1 in the concentration range of 1–10 µg/mL were injected and chromatograms were recorded. Calibration curves were constructed by plotting peak area versus concentrations and regression equations were computed for all the drugs at the corresponding fixed chromatographic conditions.

Recovery

It was performed by standard addition method. Known amount of analytes were spiked at different levels into the pre-analysed samples. In this, 50%, 100% and 150% of the

expected analytes were added to the matrix. The unspiked and spiked samples were analysed by the proposed method.

Precision

Repeatability of measurements, intraday and interday precision studies were conducted for three different concentrations (4, 5 and 6 µg/mL) of NEBI and CAR with ACE and LOR and assayed under the same experimental conditions.

Limit of Detection and Limit of Quantification

To determine the limit of detection (LOD) and limit of quantification (LOQ) of NEBI and CAR in presence of ACE or LOR, the solutions were diluted successively and injected into the HPLC system at the fixed chromatographic conditions. The smallest level of analyte that gave a measurable response is called LOD. It was based on signal to noise ratio of 3:1. The smallest concentration of analyte which gave a response that could be accurately quantified is called LOQ. It was based on signal to noise ratio of 10:1.

Robustness

Robustness was studied by evaluating the effect of small, but deliberate variations in the chromatographic conditions. The conditions studied were flow rate (altered by ±0.1 mL/min), mobile phase composition (altered by ±1.0 mL/min) and buffer pH (altered by ±0.1). Robustness of the developed method was indicated by the overall relative standard deviation (RSD) of the data at each variable condition.

Solution Stability

The stability of the selected drugs in solution during the analysis was determined by repeated analysis of samples during the course of experimentation on the same day from 0 minute to 12 hours at room temperature and also after storage of the drug solution for 48 hours under laboratory bench conditions (32±1°C) as well as under refrigeration (4±0.5°C). The responses from the aged solutions were compared with those from freshly prepared standard solutions.

System Suitability

System suitability was carried out prior to the analysis of samples each day to ensure that the method could generate results of acceptability, accuracy and precision. The parameters include plate number, tailing factor, asymmetric factor, resolution and RSD of peak area of repetitive injection.

Application of the Developed Method for In Vitro Displacement Interaction Studies

Preparation of Reagents

Preparation of phosphate buffer of pH 7.4

A 0.067 M phosphate buffer solution of pH 7.4 was prepared by dissolving 3.532 g of potassium dihydrogen phosphate and 14.542 g of disodium hydrogen phosphate in deionised water and diluting to 1000 mL with the same.

Preparation of NEBI stock solution

NEBI stock solution was prepared by dissolving 10 mg of NEBI in 10 mL methanol and diluting to 100 mL with phosphate buffer of pH 7.4 to get the concentration 100 µg/mL. Serial dilutions were made in the range of 0.5–8 µg/mL using phosphate buffer (pH 7.4). Stock solution of ACE and LOR was also prepared by dissolving 10 mg of ACE and LOR in 10 mL of methanol and made up to 100 mL with phosphate buffer (pH 7.4) to get the concentration of 100 µg/mL. Further dilutions were made in the range of 5–40 µg/mL.

Preparation of CAR stock solution

Stock solution of CAR was prepared by dissolving 10 mg of CAR in 10 mL acetonitrile and diluting to 100 mL with phosphate buffer of pH 7.4 to get a concentration of 100 µg/mL. Serial dilutions were made in the range of 1–10 µg/mL using phosphate buffer (pH 7.4).

Preparation of stock solutions of ACE and LOR

Stock solution of ACE or LOR was also prepared by dissolving 10 mg of ACE or LOR in 10 mL of acetonitrile and made up to 100 mL with phosphate buffer of pH 7.4 to get concentration of 100 µg/mL. Further dilutions were made in the range of 5–40 µg/mL for ACE and 1–40 µg/mL for LOR.

Preparation of bovine serum albumin

A 1.5×10^{-4} M bovine serum albumin (BSA) solution was prepared by dissolving 0.512 g of BSA in 50 mL phosphate buffer of pH 7.4.

Equilibrium Dialysis

Optimisation of NEBI/CAR concentration and its equilibration period

In this experiment, the activated membrane bags (20 cm long, 3.63 mL/cm capacity) were filled with 5 mL solutions of BSA (1.5×10^{-4} M) and then immersed in a fixed volume (25 mL) of phosphate buffer containing varying concentration of NEBI (3.0×10^{-6} M – 2.4×10^{-5} M) and CAR (6.888×10^{-6} M – 1.3776×10^{-5} M) and the system was shaken gently in a horizontal wrist-action shaker at room temperature. One mL sample was withdrawn at different time intervals (from 0 time to 9 hours with 1 hour time interval) from the buffer solution outside the membrane bag and was replaced with 1 mL of buffer. Samples were then injected into the HPLC system until constant peak area was obtained at 237 or 285 nm. Twenty mM ammonium acetate and methanol (30:70%, v/v) at a flow rate of 1 mL/min was used as mobile phase for NEBI-ACE while the mobile phase ratio was 35:65 for NEBI-LOR. The pH of ammonium acetate was adjusted to 4 using orthophosphoric acid (1%, v/v). Water:acetonitrile (40:60, v/v) at a flow rate of 0.8 mL/min was used as the mobile phase for CAR-ACE, and the flow rate was 1 mL/min for CAR-LOR. Orthophosphoric acid (1%, v/v) was used to adjust the pH of water to 2.5. Each time, 20 µL of sample solution was injected and the peak area was measured. The concentrations of the bound and unbound drugs were found using the standard curve. The experiment was repeated six times for the selected concentration (1.85×10^{-5} M of NEBI and 1.0824×10^{-5} M of CAR).

Effect of ACE on NEBI and CAR Binding to BSA

Five mL of 1.5×10^{-4} M BSA buffer solution was taken in 9 different dialysis bags, and 25 mL of NEBI-ACE mixture was taken in 9 different conical flasks of 100 mL capacity. Concentration of NEBI was fixed as 1.8×10^{-5} M and ACE or LOR was added with increasing concentration ranges ($1.4 \times 10^{-5} – 1.1 \times 10^{-5}$ and $1.30 \times 10^{-5} – 1.07 \times 10^{-4}$ M) to 8 of the 9 conical flasks. ACE or LOR was not added to one of the conical flask which contained only NEBI (1.8×10^{-5} M) and this was used as equilibrium control. In addition, a blank was prepared using BSA and buffer solution. The bags were prepared without tension in order to ensure efficient mixing. Both sides of the tubes were clipped properly so that there was no leakage. Dialysis was carried out in 100 mL conical flask covered with aluminium foil. The conical flasks were placed on a horizontal wrist-action shaker with a capacity of 24 and rotated at 80 rpm for 6 hours at room temperature. Buffer samples were collected from each flask after complete dialysis. Free concentrations of NEBI were measured by newly developed and validated RP-HPLC methods. Each experiment was carried out using freshly prepared BSA and drug solutions. Stability of NEBI, CAR, ACE and LOR were studied at room temperature for 24 hours in 0.067 M phosphate buffer (pH 7.4). To study the effect of ACE or LOR on CAR binding to BSA, the above procedure was repeated using CAR at a concentration of 1.0824×10^{-5} M and ACE and LOR in range of $1.4 \times 10^{-5} – 1.1 \times 10^{-5}$ and $2.70 \times 10^{-6} – 1.07 \times 10^{-4}$ M, respectively.

Statistical Analysis

Statistical analysis was carried out using one-way Analysis of Variance (ANOVA) followed by Dunnett's test using GraphPad prism. Values are expressed as mean±SEM of three parallel measurements. The $p<0.05$ was considered statistically significant.

RESULTS

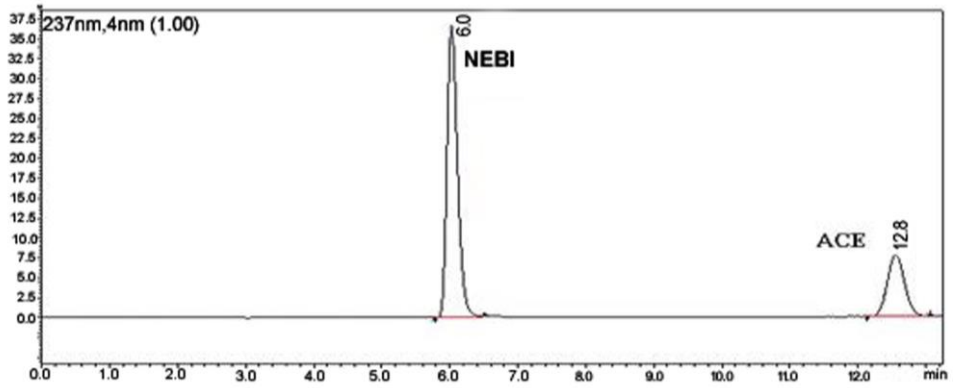
All the selected drugs for the interaction studies were practically insoluble in water. The present experiment and data reported in literature showed that methanol could dissolve NEBI, ACE and LOR. CAR was more soluble in acetonitrile than methanol, hence acetonitrile was chosen as the solvent of choice for CAR. Using the fixed chromatographic conditions, the retention time was found to be 6.0, 12.8 minutes for NEBI-ACE and 7.1, 8.8 minutes for LOR-NEBI [Figs. 1(a) and (b)]. Retention time of CAR-ACE was found to be 4.4 and 13.1 minutes while CAR-LOR showed retention time at 2.7 and 4.7 minutes, respectively [Figs. 1(c) and (d)].

UV spectra overlays of NEBI-ACE and NEBI-LOR showed the maximum absorbance at 237 nm for NEBI, so this wavelength was selected as the detection wavelength for the study. The detection wavelength selected for CAR-ACE and CAR-LOR was 285 nm. The peak purity index for all analytes were found to be close to one, proving the selectivity and specificity of the methods. A 10 point calibration curve was constructed with working standards and was found linear for each of the analyte over their calibration range of 1–10 µg/mL [Figs. 2(a)–(d)].

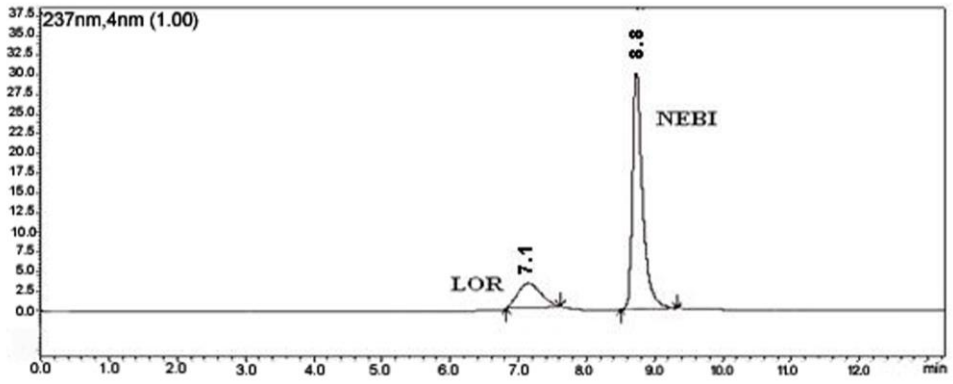
To prove the precision of the method, the intraday and interday precision studies were carried out and the % RSD values were found to be below two. The sensitivity of the methods was found out from the determination of LOD and LOQ of NEBI and CAR. For robustness evaluation, the method parameters were changed each time to estimate the effects. The % RSD values less than 2, indicating that the developed methods were robust. The results for validation are summarised in Tables 1–4. Recovery study was

assessed using six determinations over three concentration levels covering the linear range. Results shown in Table 5 confirmed the developed methods were highly accurate.

The system suitability parameters of NEBI, CAR, ACE and LOR were calculated using the standard chromatogram and the values are listed in Table 6. Selectivity was evaluated by injecting mobile phase alone and reference standard of each drug individually. No peaks were observed for mobile phase and no extra peaks were observed for all the selected drug solutions. The prepared solutions were injected at 1 hour interval under fixed chromatographic conditions in order to ascertain the stability of solutions. The % RSD of peak area obtained from each drug solution stability and mobile phase stability were found to be within 1%, showed no significant change in retention time, peak shape and column performance. It was found that the solutions were stable for more than 12 hours at room temperature and 48 hours under –20°C.

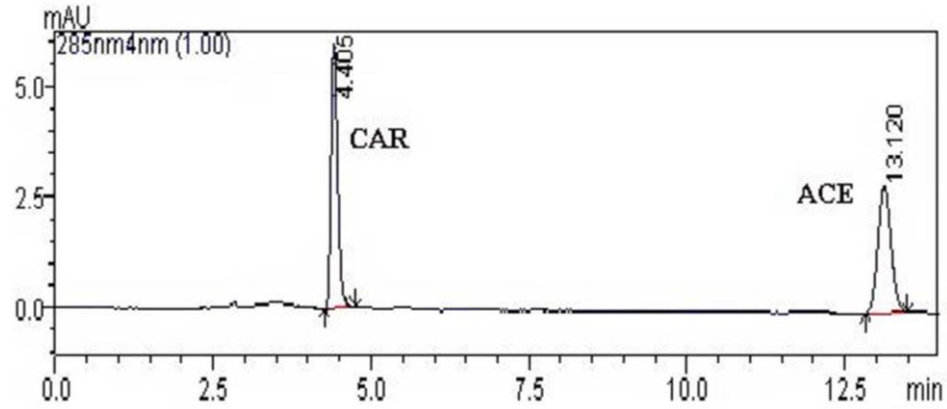


(a)

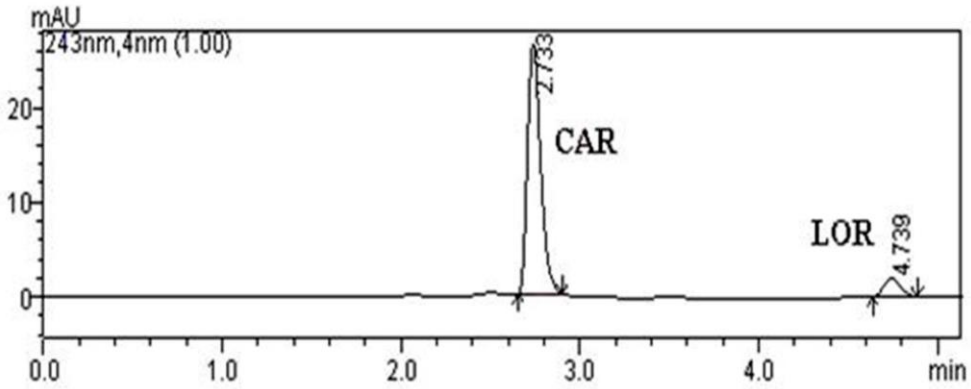


(b)

Fig. 1: RP-HPLC chromatograms for the simultaneous analysis of the selected drugs: (a) NEBI–ACE, (b) NEBI–LOR, (c) CAR–ACE and (d) CAR–LOR; peak purity index = 1 (*continued on next page*).

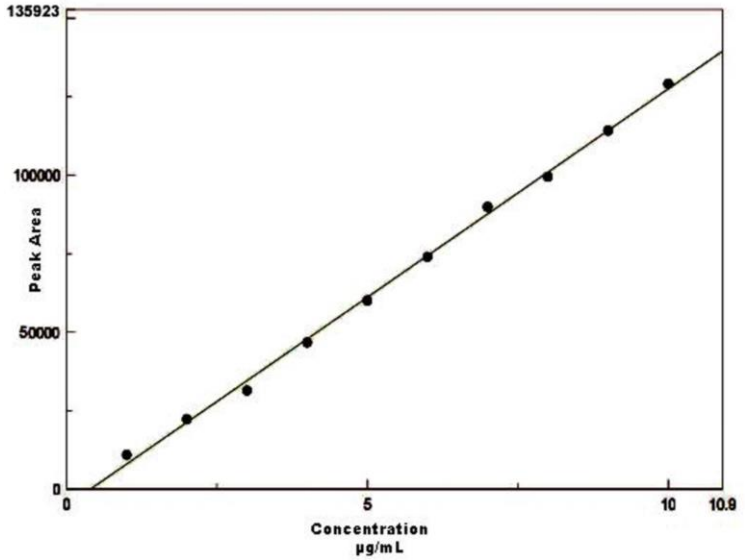


(c)

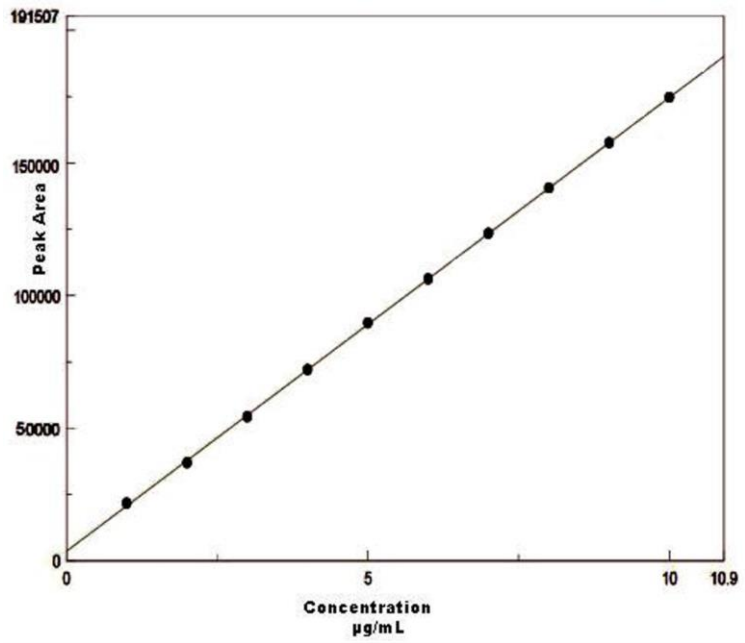


(d)

Fig. 1: (*continued*).

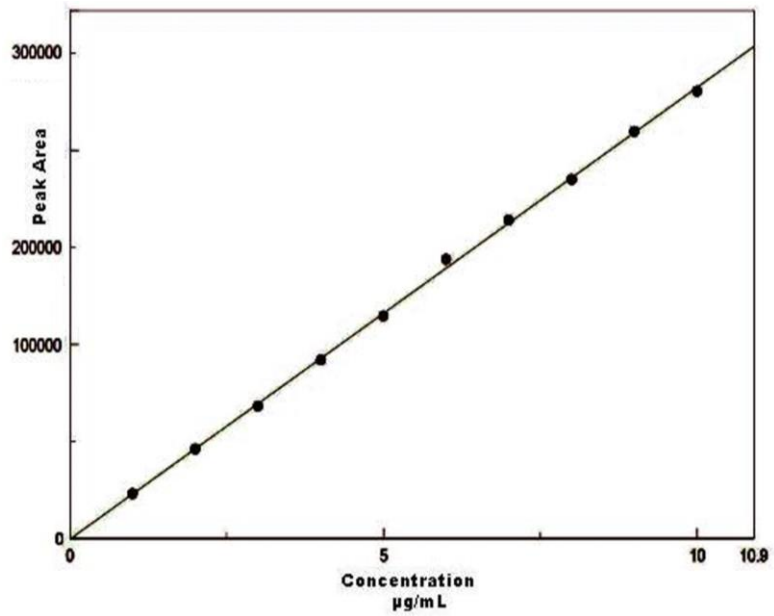


(a)

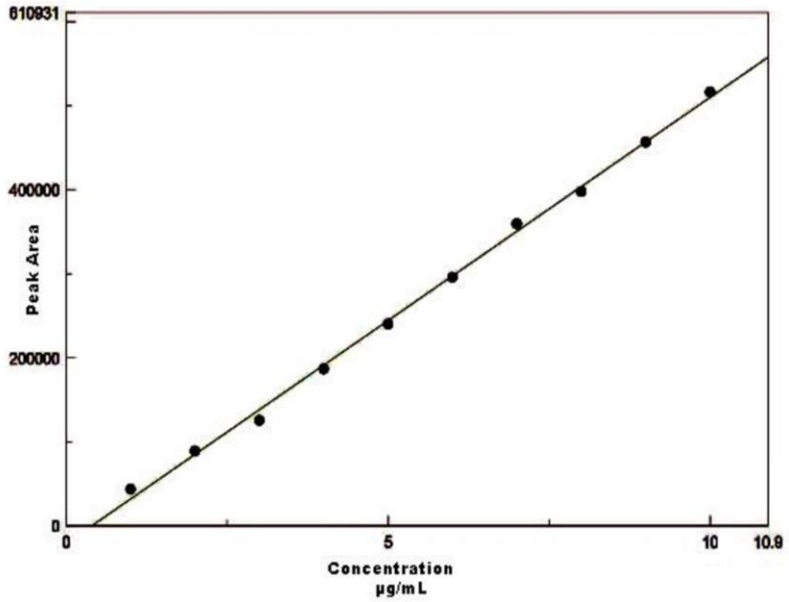


(b)

Fig. 2: Calibration curves of NEBI and CAR: (a) NEBI in presence of ACE, (b) NEBI in presence of LOR, (c) CAR in presence of ACE and (d) CAR in presence of LOR; correlation coefficient value >0.999 (*continued on next page*).



(c)



(d)

Fig. 2: (*continued*).

Table 1: Summary of validation parameters for NEBI and ACE.

Performance parameter	NEBI	ACE
Linearity and range (µg/mL)	1–10	1–10
Correlation coefficient	0.9998	0.9999
Precision (% RSD*)		
Interday	0.2979	0.7531
Intraday	0.1889	0.8400
LOD (ng/mL)	5	5
LOQ (µg/mL)	1.0	1.0
Robustness	Robust	Robust

Note: *Values of % RSD are expressed as mean±RSD of six determinations

Table 2: Summary of validation parameters for NEBI and LOR.

Performance parameter	NEBI	LOR
Linearity and range (µg/mL)	1–10	1–10
Correlation coefficient	0.9998	0.9998
Precision (% RSD*)		
Interday	0.2141	0.4847
Intraday	0.4051	0.5688
LOD (ng/mL)	5	10
LOQ (µg/mL)	1.0	1.0
Robustness	Robust	Robust

Note: *Values of % RSD are expressed as mean±RSD of six determinations

Table 3: Summary of validation parameters for CAR and ACE.

Performance parameter	CAR	ACE
Linearity and range (µg/mL)	1–10	1–10
Correlation coefficient	0.9999	1.0000
Precision (% RSD*)		
Interday	0.2072	0.6643
Intraday	0.3428	0.2924
LOD (ng/mL)	5	5
LOQ (µg/mL)	1.0	1.0
Robustness	Robust	Robust

Note: *Values of % RSD are expressed as mean±RSD of six determinations

Table 4: Summary of validation parameters for CAR and LOR.

Performance parameter	CAR	LOR
Linearity and range(µg/mL)	1–10	1–10
Correlation coefficient	0.9998	0.9994
Precision (% RSD*)		
Interday	0.1527	0.4991
Intraday	0.2435	0.4421
LOD (ng/mL)	5	5
LOQ (µg/mL)	1.0	1.0
Robustness	Robust	Robust

Note: *Values of % RSD are expressed as mean±RSD of six determinations

Table 5: Summary of recovery studies of all the selected drugs by standard addition method.

Concentration of standard added (µg/mL)		% recovery±RSD*	
NEBI	ACE	NEBI	ACE
2	2	100.18±0.3865	99.97±0.6845
4	4	100.09±0.2996	100.03±0.5333
6	6	100.15±0.4824	100.07±0.5986
NEBI	LOR	NEBI	LOR
2	2	99.99±0.3549	100.07±0.5846
4	4	100.13±0.1352	100.12±0.7423
6	6	100.04±0.4721	100.05±0.4977
CAR	ACE	CAR	ACE
2	2	100.04±0.3574	100.23±0.8649
4	4	100.16±0.4425	100.38±0.5631
6	6	100.09±0.2649	99.98±0.6577
CAR	LOR	CAR	LOR
2	2	100.18±0.4785	99.97±0.3623
4	4	100.26±0.5186	99.92±0.6370
6	6	100.09±0.3375	100.04±0.4038

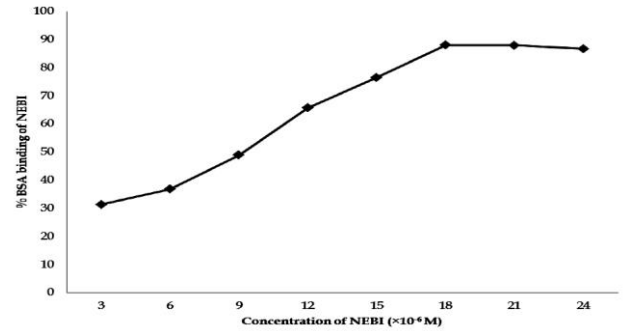
Note: *Each value is the mean of six determinations

Table 6: Summary of system suitability parameters for NEBI, CAR, ACE and LOR.

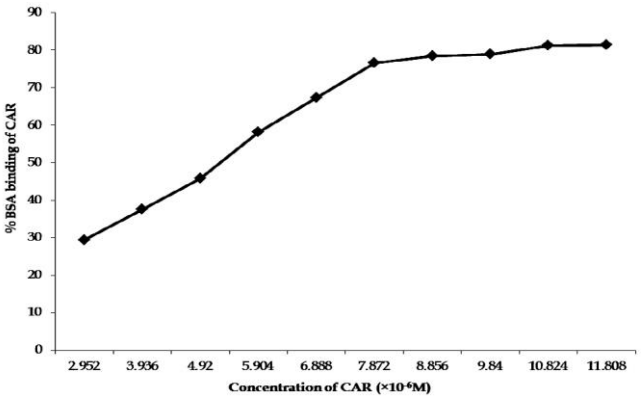
Drugs	Theoretical plate (N)	Asymmetric factor (A_s)	Tailing factor (T_f)
NEBI	6756	1.22	1.24
ACE	11299	1.11	1.10
NEBI	7583	1.30	1.33
LOR	9564	1.20	1.17
CAR	5386	1.30	1.4
ACE	11756	1.10	1.12
CAR	6037	1.30	1.30
LOR	7042	1.25	1.27

In Vitro Displacement Interaction Studies

The drug-drug interaction study of NEBI and CAR was conducted in presence of ACE or LOR. Standard curves were prepared using drugs at pH 7.4 by HPLC methods. In Figures 3(a) and (b), protein binding of NEBI and CAR showed that at low concentrations, the percentage of protein binding increased with an increase in the concentration of drug. But at higher concentrations, the protein binding percentage attained a steady plateau indicating the saturation zone for the binding of NEBI and CAR to BSA. Repeatability studies showed that the percentage binding of NEBI and CAR to BSA at saturation level (1.8×10^{-5} M/1.0824×10^{-5} M) was about 87.7±0.6% and 81.5±0.6%, respectively. The reproducibility of the experiment was excellent for both the drugs at saturation level. Degree of bag binding was also calculated, and included as a correction factor. The highest percentage protein binding of NEBI at saturation level was about 67.9±3.3% and 75.2±1.6% (Table 7) in presence of ACE and LOR whereas CAR showed 80.4±0.03% and 42.0±0.30% (Table 8), respectively. Unbound fractions of NEBI and CAR in presence of varying concentrations of ACE and LOR at room temperature (pH 7.4 and ionic strength 0.067 M) are shown in Figures 4 and 5. It can be inferred that both ACE and LOR have significant effects on the protein binding of NEBI. This is possibly due to good affinity of ACE or LOR for the same binding site in BSA. In vitro protein binding of CAR was found to be significantly altered by LOR. At saturation level, the percentage of free concentration of NEBI bound to BSA increased from 10.1±0.6% to 30.2±3.4% and 22.9±2.7% in presence of ACE and LOR respectively; while in the case of CAR, it was found to increase from 17.9±0.1% to 18.5±0.1% and 56.9±0.5%. The drug ACE displaced NEBI to a greater extent than LOR while LOR showed a major effect on in vitro protein binding of CAR. The selected drugs were found to be stable for more than 12 hours in 0.067 M phosphate buffer at pH 7.4.



(a)



(b)

Fig. 3: Percentage protein binding at various concentrations of (a) NEBI (3×10^{-6} M – 2.4×10^{-5} M) and (b) CAR (6.888×10^{-6} M – 1.3776×10^{-5} M). At saturation concentration NEBI showed 87.7% and CAR showed 81.1% binding with BSA. Concentration of BSA used was 1.5×10^{-4} M.

Table 7: Percentage protein binding (pb) of NEBI alone, NEBI in presence of ACE and LOR.

Concentration of ACE	% pb of NEBI	% pb of NEBI in ACE	Concentration of LOR	% pb of NEBI in LOR
1.4×10^{-5}	87.7±0.6	85.7±0.5[c]	1.30×10^{-5}	85.8±0.2[a]
2.8×10^{-5}		83.9±0.5[c]	2.60×10^{-5}	85.1±0.4[a]
4.2×10^{-5}		82.9±2.2[b]	4.00×10^{-5}	85.0±0.5[b]
5.6×10^{-5}		82.3±0.4[b]	5.40×10^{-5}	84.0±0.4[c]
7.0×10^{-5}		80.2±0.4[c]	6.70×10^{-5}	83.0±0.1[c]
8.4×10^{-5}		76.1±2.3[a]	8.10×10^{-5}	81.2±0.3[c]
9.8×10^{-5}		71.8±2.6[a]	9.40×10^{-5}	79.2±0.7[c]
1.1×10^{-5}		67.9±3.3[a]	1.07×10^{-4}	75.2±1.6[c]

Notes: *Values are expressed as mean±SEM of three parallel measurements. Statistical analysis was carried out using one-way ANOVA followed by Dunnett's test. [a]$p<0.01$, [b]$p<0.05$ and [c]$p>0.05$ when compared to % pb of NEBI alone at concentration 1.85×10^{-5} M.

Table 8: Percentage protein binding (pb) of CAR alone, CAR in presence of ACE and LOR.

Concentration of ACE	% pb of CAR	% pb of CAR in ACE	Concentration of LOR	% pb of CAR in LOR
–	81.5±0.6	–	2.70×10^{-6}	80.1±0.1[c]
–		–	5.40×10^{-6}	78.6±0.4[c]
–		–	8.10×10^{-6}	76.1±0.1[a]
–		–	1.10×10^{-5}	71.0±0.4[a]
1.4×10^{-5}		81.0±0.03[c]	1.30×10^{-5}	61.3±1.2[a]
2.8×10^{-5}		80.8±0.20[c]	2.60×10^{-5}	59.2±1.1[a]
4.2×10^{-5}		80.8±0.03[c]	4.00×10^{-5}	57.3±0.5[a]
5.6×10^{-5}		80.8±0.10[c]	5.40×10^{-5}	54.9±0.5[a]
7.0×10^{-5}		80.7±0.10[c]	6.70×10^{-5}	51.7±1.1[a]
8.4×10^{-5}		80.5±0.10[b]	8.10×10^{-5}	49.4±1.0[a]
9.8×10^{-5}		80.5±0.20[b]	9.40×10^{-5}	45.5±0.2[a]
1.1×10^{-5}		80.4±0.03[b]	1.07×10^{-4}	42.0±0.3[a]

Notes: *Values are expressed as mean±SEM of three parallel measurements. Statistical analysis was carried out using one-way ANOVA followed by Dunnett's test. [a]$p<0.01$, [b]$p<0.05$ and [c]$p>0.05$ when compared to % pb of CAR alone at concentration 1.0824×10^{-5} M.

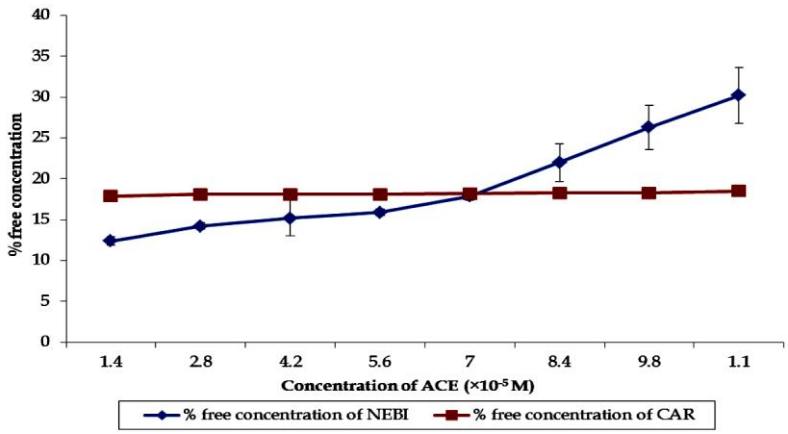


Fig. 4: Free fractions of NEBI and CAR as % of initial concentrations added (1.85×10^{-5} M and 1.0824×10^{-5} M) and when bound to BSA upon addition of ACE (1.4×10^{-5} – 1.1×10^{-5} M). Each value represents the average of three independent experiments ±SEM.

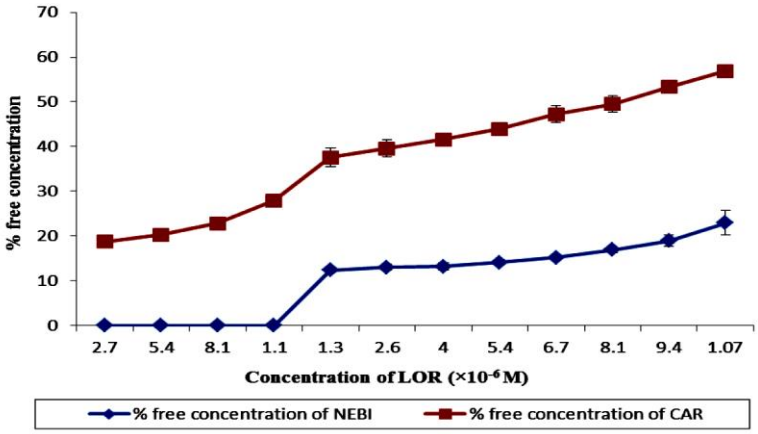


Fig. 5: Free fractions of NEBI and CAR as % of initial concentrations added (1.85×10^{-5} M and 1.0824×10^{-5} M) and when bound to BSA upon addition of LOR (2.70×10^{-6} – 1.07×10^{-4} M). Each value represents the average of three independent experiments ±SEM.

DISCUSSION

Reverse phase chromatography techniques with PDA detector have been routine for analysis of drug samples in bulk, formulation and biological fluids. Reverse phase column show excellent stability, column efficiency and versatility with number of mobile phases with various compositions for application of variety of compounds (Snyder *et al.* 1988). Palliative patients are frequently treated with chemotherapy and hormonotherapy, as a result of undesirable post drug effects for instance, hypoalbuminaemia that may occur in majority of patients, believed to be important in drugs with close affinity to albumin. NSAIDs are most frequently used drug in palliative medicine. They may enter into unfavourable pharmacodynamic interaction with drugs used in pharmacotherapy of cardio vascular system (CVS) diseases particularly NEBI and CAR. Hence it is necessary to perform drug-drug interaction studies of highly protein bound selected drugs (Jaroslaw *et al.* 2008).

For drug interaction study, phosphate buffer of 0.067 M solution is commonly used because it provides a stable physiological pH. At pH 7.4 the complex formation with anions apparently increases the net negative charge of albumin and makes more sites available for further binding, hence the pH was adjusted to 7.4 (O'Reilly 1969). Moreover at this pH the selected drugs were stable for more than 12 hours. In this study, the model protein is BSA. Binding chemistry of human serum albumin (HSA) and BSA is similar because of the high percentage of sequence identities between the two proteins (Alam *et al.* 2009). BSA, instead of HSA was used for the present study because of its low cost and easy availability. The concentration of BSA was selected as 1.5×10^{-4} M as per literature because the use of low concentration of albumin was desirable to avoid the Donnan effect and protein-protein interactions (O'Reilly 1967).

For the interaction studies equilibrium dialysis was preferred over ultrafiltration technique because equilibrium dialysis is the commonly employed technique (Jerome *et al*. 1985) which is inexpensive, simple, precise, reliable, accurate and easy to perform. Whereas ultrafiltration technique has a disadvantage of accumulation of protein at the membrane surface which in turn will adversely affect the liquid-protein bonds (Ahmed *et al*. 2007). Before performing the experiment, dialysis membrane was activated using

1M $NaHCO_3$ at 70°C for 4 hours and washing thoroughly with deionised water (Md. Shah *et al*. 2008). At equilibrium the concentration of unbound drug on both sides of the membrane must be equal, so it is necessary to conduct the experiment after equilibrium. In our study there were no physiological changes like denaturation of protein, pH changes during dialysis and alteration in membrane pore size observed (Patrick and Yashveer 2001), because the equilibrium was achieved within 6 hours for NEBI and 7 hours for CAR.

Generally in this type of experiment some amount of drugs are usually adsorbed on the dialysis bags. Hence a correction factor was applied. In our study, for calculating the bag binding, the dialysis was first carried out without addition of BSA and the sample was tested both from inside and outside the bag and used as correction factor (Robert 1969). From the results of analysis it was observed that the free concentrations of NEBI and CAR exceeded minimum toxic concentration in presence of NSAIDs which may lead to hypotension and heart failure. Hence it is necessary to maintain the free concentrations of NEBI and CAR.

CONCLUSION

Simple and sensitive RP-HPLC methods with PDA detector were developed and validated according to ICH guidelines for the simultaneous estimation of NEBI and CAR separately with ACE or LOR. The developed methods were applied for the investigation of drug displacement interactions studies with BSA as a protein model under equilibrium conditions. We found that ACE or LOR displaces NEBI and CAR from its binding sites on BSA. The pharmacological activity of a drug is related to its extent of protein binding. If a drug shows less affinity for albumin due to any alteration in protein binding, the pharmacological effect of the drug may be significantly altered. However, the results of the present studies in combination with NSAIDs might be helpful in realising to the overall binding behaviour of the selected drugs with BSA. Although the results presented here are significant, more elaborate studies including in vivo experiments are necessary to predict the actual changes in pharmacokinetic properties caused by the selected drugs.

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