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Original Article

Analytical Validation and Stability Indicating Studies for Simultaneous Estimation of Benidepine and Metoprolol by Strong Cation Exchange (SCX) Chromatography

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ABSTRACT

Introduction: Polar anti-hypertensive drugs like benidepine and metoprolol often suffer from peak fronting and peak tailing effects in RP-HPLC. SCX chromatography which is alternative and complimentary to this RP-HPLC also has the capability to separate benidepine and metoprolol. Considering these aspects, SCX method was developed with performing stability indicating studies for the simultaneous quantification of benidepine (BEN) and metoprolol (MET) in bulk and tablet formulation. **Methods:** This investigation was performed on the Phenomenex Luna SCX column (100 × 2.1 mm, ID, particle size 5 µm) where both BEN and MET were eluted with ammonium formate (15mM) buffer: MeOH in ratio of 75:25 v/v for 12 min with isocratic elution at the flow rate of 1.2 ml/min; performed at 28°C and monitored at 226 nm wavelength. **Results:** The average retention time of BEN and MET were 1.290 and 3.520 min, respectively. The validation studies revealed good linearity over different concentration ranging between 7.5-250 µg/ml for BEN and 15.5-250 µg/ml for MET with R^2 values were 0.999 recorded for both drugs. Average drug recoveries of BEN and MET were ranging between 99.36±0.83% and 100.51±1.03%, respectively. The acid (0.1N HCl; 50°C), dry heat (50°C) and alkali (0.1N NaOH; 50°C) have not made any significant changes in both BEN and MET but peroxide (3% H_2O_2; 28°C) have degraded the BEN but MET was unaffected. **Conclusion:** The present SCX method represented the shortest run time for the investigation of benidipine and metoprolol where the results of linearity, accuracy, precision, robustness and specificity were under the obligation of ICH guidelines.

Key words: Benidipine, Metoprolol, SCX chromatography, RP-HPLC, ICH guidelines.

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INTRODUCTION

Basic strength ionic functionalities of several pharmaceutical drugs are intrinsic to understand their pharmacokinetic and pharmacodynamics properties in human application. In addition, the separation, characterisation and isolation of such complex pharmaceutical amines, illustrated with their net negative charges are also crucial to understand their tentative physiological roles as well as their action mechanism and pathways which however, still remain a big challenge.[1] It is specifically needed in separation of extremely polar amines and such analytical techniques are now desperately needed due to increasing popularity to understand their post-translational approaches like metabolomics and proteomic studies. Therefore, a great demand required for development and the need of efficient and alternative analytical tools that could not only separate or characterise the proteins/amines but also important to isolate them from several equally important drugs and excipients from pharmaceutical formulation.

Very few articles have been published to recommend the application of the most versatile C_{18} column for the concurrent estimation of benidipine (BEN) and metoprolol (MET);[2] presumably, the utilisation of C_{18} phase is very much challenging since probably the polar amines like metoprolol do not retain in ion pairing mode and hence elute with the void volume[3] that means, the strongly hydrophilic and ionic characteristics of metoprolol and benidipine might lowering their binding capacities to the ODS.[4] Therefore, to enhance the drug-C_{18} interaction, the *'ion suppression effects'* with additional basic buffers were recommended to improve the drugs selectivities but this ionic suppression, further develop the peak tailing/peak fronting effects and hence extend the elution order.[5] Specifically, earlier such types of erratic and irreproducible retention behaviour were observed in simultaneous quantification of two or more polar pharmaceutical amines.[6] Including RP-HPLC[7-10] few other alternative techniques including LC-MS/MS,[11] spectrophotometry,[12,13] radioimmunoassay[14-16] were recommended and demonstrated but their significance in simultaneous analysis of metoprolol and benidipine is questionable.

Therefore, alternative to above mentioned separation techniques, strong cation exchange liquid chromatography (SCX-LC) offers concurrent estimation of several amines to ensure and validate the comprehensive analytical studies. Although, SCX chromatography is relatively old technique, but still it has gained more popularity towards analysis of pharmaceutical amines. Importantly, this SCX based chromatography is complementary to the conventional ODS which however did not use the ion exchange mechanism. Moreover, pharmaceutical drugs and natural products analysis usually required preceding enrichment methods, owing to the involvement with high complexities and most often, low abundance of drug detection sensitivity.[17,18] Therefore, the importance of this study is to ensure the effectiveness of cation exchange (SCX) chromatography as an alternative tool towards simultaneous estimation of two antihypertensive drugs like BEN and MET from pharmaceutical formulation which were not reported earlier other than RP-HPLC.

Benidipinehydrochloride(Figure1A),(±)-(4R)-3-(R)-1-benzylpiperidin-3-yl5-methyl1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)pyridine-3,5-dicarboxylate hydrochloride is a racemic mixture of isomers RR-(−) and SS-(+). Benidipine is a dihydropyridine vasoselective calcium channel blocker[19] and it work differently than several other calcium channel blockers due to its stronger binding capacity with vascular, renal and

cardiac cellular membrane as well as its positive effect on nitric oxide production. As a solid, benidipine hydrochloride is stable to variations in heat and moisture and it is moderately stable to light exposure.[20]

Metoprolol succinate (Figure 1B), 2-propanol, 1-[4-(2- methoxyethyl) phenoxy]-3-[(1-methylethyl) amino]-,(±)-, butanedioate is a β1-selective adrenoceptor blocking drug.[8] In hypertension, due to its prolonged duration of action, presumably because of higher half-life, it is recommended twice daily administration. It is equally effective like other β-adrenoceptor blocking drugs used in angina pectoris and hypertension. Importantly, metoprolol is well tolerated and has less side-effect compared with other anti-hypertensive drugs.[21]

MATERIALS AND METHODS

Generous gifts of standards; BEN and MET were received from UltraChrom Innovatives Pvt. Ltd, India. The Benidin-M® tablets, containing 4mg of BED and 25mg of MET, manufactured by Lloyd healthcare Pvt. Ltd were purchased from local pharmacy store. All HPLC grade chemicals and solvents were purchased from Merck (Mumbai, India). The HPLC columns included Phenomenex-Luna® SCX (100 x 2.1 mm i.d., 5µ) purchased from UltraChrom Innovatives Pvt. Ltd. (Nagpur, India). HPLC analysis were performed on Shimadzu Class A-10 VP instrument, equipped with UV-Vis detector (SPD-10A VP), binary pumps (LC-10AT VP), system controller (SCL-10A VP) with manual rheodyne injector (20µl), controlled by LC-solution software. Analytical balance (ME-205, Mettler-Toledo), pH meter (FiveEasy-A211, Mettler-Toledo) and sonicator (9L250H, PCI) were used throughout the analysis.

Chromatographic conditions

Analysis were performed on Shimadzu HPLC system. Mobile phases A and B were water and methanol, respectively. Both contained 15 mM ammonium formate (AF). BEN and MET were eluted with AF (15mM): MeOH in ratio 15:75 v/v for 12 min with isocratic elution at 1.2 mL.min^{-1} flow rate. All separations were performed at 28°C and recorded at 225 nm wavelength.

Standard stock preparation

Accurately weighed 25 mg of each standard, BEN and MET were diluted with 25ml blank eluents separately in 50 ml volumetric flask and sonicated for 20 min. Furthermore, the stock solution was filtered through the 0.20 µ nylon filters and volume was adjusted to 50 ml with relevant solvents to make 500 ppm. Equal volumes of both standards were mixed to prepare 250 ppm (250µg.mL^{-1}). Furthermore, seven proportional serial dilutions (250, 125, 62.5, 31.25, 15.75, 7.87µg.mL) of freshly prepared stock solution (250µg.mL^{-1}) were prepared and analysed with chromatographic condition to investigate the linearity.

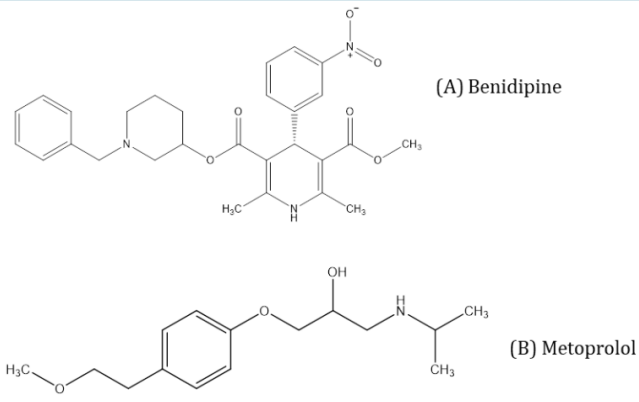

Figure 1: Molecular structures of (A) benidipine and (B) metoprolol.

Sample preparation

Twenty tablets of Benidin-M® were weighed separately and the average weight was calculated. They were crushed to fine powder and exactly 153 mg was transferred to the 50 ml volumetric flask to make the concentration of 1000 µg.mL-1 of both MET and BEN with 25ml of MeOH-H_2O (15:10 v/v). Serial dilutions were made to evaluate the drug recovery by knowing the peak area against the concentration. Prior to the analysis, samples were sonicated for 20 min and filtered through 0.20µ nylon filter. All separations were performed as mentioned in chromatographic condition.

Acidic, base, neutral hydrolysis and oxidation sample preparation

Forced degradation studies of BEN and MET were performed as per the ICH guideline.[22] 8 mL of freshly prepared homologous mixture of stock solution, containing BEN (500 µg.mL^{-1}) and MET (500 µg.mL^{-1}), prepared in H_2O-CH_3OH eluents was equally distributed into 4 different 25 mL volumetric flasks and further diluted with equal volume of HPLC grade H_2O, 0.1 N HCl, 0.1N NaOH and 3% H_2O_2 to get final concentration of 250 µg.mL^{-1} of both BEN and MET. Sample prepared in 3% H_2O_2 was kept at room temperature for 6 hr whereas the acid-base and neutral hydrolysed samples were kept at 60°C temperature for 6 hr. Furthermore, all samples were sonicated, filtered through 0.20µ nylon filters and then exactly twenty µL of each sample was analysed by HPLC using specified chromatographic method mentioned in chromatographic condition.

RESULTS AND DISCUSSION

The SCX adsorbents including two main bonding phases; sulfonic acid (SCX-1) and benzene sulfonic acid (SCX-3) were tested and evaluated in terms of their performances and as observed their retention behaviour varied in terms of separations of selected benidipine and metoprolol. Comparatively, benzene sulphonic acid (SCX-3) proved the most efficient adsorbent since most probably the existence of aromatic phenyl ring in SCX-3 is more hydrophobic and electrostatic towards enhancing the non-polar secondary interostatic affinity and increase binding strength with selected analytes. Apart from this, SCX-3 might also have some more characteristic features which make it enable for better separation of aliphatic amines like metoprolol and benidipine (Figure 2). Considering these aspects, the method development and validation studies were performed on Phenomenex Luna column packed with SCX-3 phase for simultaneous quantification of BEN and MET using 15mM AF-methanol (25:75% v/v) at the flow rate of 1.2 mL/min; and the resultant chromatogram were demonstrated in Figure 3.

The SCX-3; modified with benzene sulfonic acids are negatively charged in aqueous buffers to exhibit stronger binding with basic analytes. The SCX-3 residues then interact with counter-ion from buffers to replace and elute the amines. Importantly, these amines elute at low pH buffer between 2.7-3. Therefore, at this pH, those analytes have the net charges of ≤+1 eluted with void volume (t_0) whereas others, mostly amines/peptides characterized by ≥+2 net conjugate positive charges, retained in the column.[1] That's why, SCX chromatography is most preferred technique to isolate the peptides/phytoamines/pharmaceutical amines from acids and neutral hydrophobic compounds which without interfering with basic analyses, elutes with the void volume. Although, this depends on the type of eluent and buffer selected for the separation. Ammonium formate (pKa=3.7) is most preferred over ammonium acetate (pKa=4.7) since it is volatile and behaves as a salt rather than un-dissociated acid like acetate. Importantly, the application of TFA,

formic acid (FA) and other buffers which act above pH 7 would affect the SCX column performance. SCX based chromatography of selected amines is based on the fact that between BEN and MET, MET is enriched with N-terminal free amines, those contributes to its stronger retention and thus enables to elute slightly later than BEN (t_R = 1.24min) (Figure 2). Moreover, as displayed in all chromatogram (Figure 3), both drugs revealed good resolution and peak selectivity; most likely because of the differences in their amino functionalities where their separation was achieved within six minutes which is comparatively quite shorter than previously reported RP-HPLC method by Patel *et al.* 2019.[2]

System suitability studies

The proposed HPLC method for simultaneous quantification of BEN and MET was validated as per the ICH guidelines and including system suitability studies, other separation variables such as linearity, repeatability, accuracy, precision, robustness and specificity studies were tested and evaluated.

As demonstrated in Table 1, the system suitability parameters of the proposed HPLC method represents a high degree of reproducibility for simultaneous quantification of BEN and MET. For BEN, developed method expressed average retention time (t_R) of 1.21±0.04 min with mean k' of 1.84 whereas the t_R and k' for MET were 4.34 min and 9.17, respectively (Table 1). The tailing factor (*T*) values <2 have signified, no specific tailing in both analyses. Symmetric peaks represent an ideal Gaussian peak; where for both compounds, the symmetric and asymmetric factors were of almost equal magnitude. Moreover, the separation factor (α) and resolution (R_s) for MET were significantly higher than the minimum requirement as per the ICH guidelines.

Linearity and range

The linearity of HPLC method, is its ability to explicit the results that should be proportional to the concentration of studied analyses within a selected range. Therefore, over the selected range of 3.9–250 μg.ml^{-1} for BEN and 7.8–250 μg.ml^{-1} for MET, exceptionally high linearity was observed between the concentration against peak area with linear regression data represented for BEN and MET were; y = 47867x + 27624; and y = 29619x + 3788.5, respectively (Figure 4). Moreover, the regression coefficients (r^2) were observed 0.999 for both drugs which altogether signified a high degree of linearity (Table 1; Figure 4).

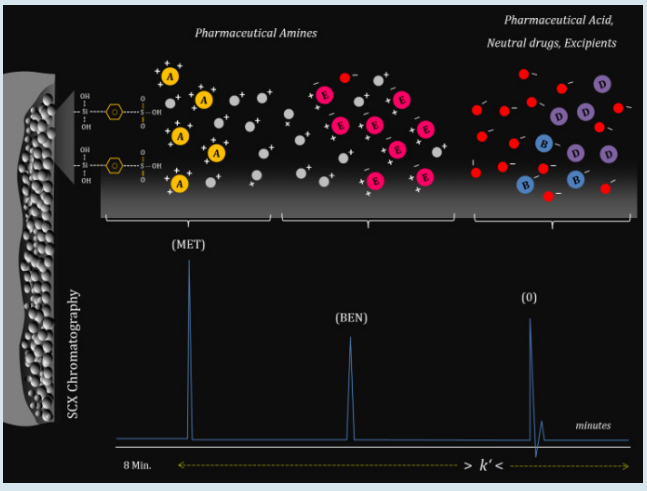

Figure 2: Schematic representation of SCX Chromatography.

Limit of detection and quantification

Limit of Detection (LOD) and Limit of Quantitation (LOQ) were estimated based on the standard deviation of the response and the slope of the regression equation. As observed, The LOD and LOQ of BEN were 3.97 and 12.04 μg.ml^{-1} whereas for MET they were 3.70 and 11.22 μg.ml^{-1}, respectively. It represented the higher detection ability of the proposed method for the lowest possible concentration of simultaneous investigation of selected drugs from the combination; displayed in Table 1.

Accuracy

Percentage recoveries of three different concentrations (injected twice) to investigate the BEN and MET were calculated to demonstrate the accuracy in Mean±SD and RSD% for the selected combination and the data obtained was reported in Table 2. Applying the calibration curve, the Y-intercept and the slope of the graph were used to determine the % recovery, attributed to the proposed method for the simultaneous quantification. The yielded Mean±SD and RSD% for both selected drugs were within the acceptance limit of 100±2% and <2%, respectively. Overall, the developed HPLC method displayed good accuracy from the obtained recovery data.

Precision

The precision of HPLC method expresses the closeness of the agreement between a series of measurements evaluated from multiple sampling of the same homogeneous sample under the given conditions. Both intra and inter-day precision variability's were extremely precise over the tested range of 62.5–250 μg/ml for both BEN and MET. Moreover, the peak areas of studied samples were also correlated with selected concentration where for both drugs the RSDs were less than 2%. As resulted, the RSDs of the intraday precision were in the range of 0.44% – 1.62% for BEN and 0.35% – 0.87% for MET whereas the RSDs of

Table 1: System suitability data of benidipine and metoprolol.

Selective variables	Benidipine (BED)	Metoprolol (MET)
Theoretical plates (N)	197	712
Capacity factor (K')	1.84	9.17
Resolution (R)	---	6.27
Selectivity/Separation factor (α)	---	4.97
Asymmetry/Tailing factor (T)	1.91	1.89
Retention time (t_R)	1.21 min.	4.34 min.
Wavelength of detection (nm)	225 nm	225 nm
Repeatability (%RSD)	1.91%	1.20%
Intra-day precision (%RSD)	0.44 – 1.62	0.35 -0.87
Inter-day precision (%RSD)	0.26 – 1.61	0.87 – 1.52
Linearity range	3.9 – 250 μg.ml^{-1}	7.8 – 250 μg.ml^{-1}
Regression equation	Y= 47867x + 27624	Y= 29619x + 3788.5
SE of intercept (S_e)	3177.71	13573.8
SD of intercept (S_a)	7105.57	33248.88
Correlation coefficient (r^2)	0.9999	0.9999
LODa (μg.mL^{-1})	3.97 μg.ml^{-1}	3.70 μg.ml^{-1}
LOQa (μg.mL^{-1})	12.04 μg.ml^{-1}	11.22 μg.ml^{-1}

intermediate precision were in the range of 0.26%–1.61% for BEN and 0.87%–1.52% for MET which is an acceptable precision with minimum variations of the proposed method (Table 1).

Robustness

Robustness of HPLC method represents its ability to remain unaffected by small but deliberate variations in separation parameters to provide its reliability during routine analysis. In this method, robustness was established by making deliberate changes in flow rate (1.2±0.2 mL.min^{-1}), organic modifier (75±2% mL) and temperature (28±2°C). Therefore, increased the flow rate by 1.2+0.2 mL.min^{-1}, reduced the t_R values to 1.04 and 3.71 min of BEN and MET, respectively whereas reduced the flow rate by 0.2 mL.min^{-1} has extended the t_R values to 1.42 and 5.17 min of similar drugs; although the variation was almost 17% (Figure 5A). However, as displayed, altering the concentration of CH_3OH as mobile phase by 85±2%, have not made any significant changes in the retention pattern of both BEN and MET since the differences in their retention time were appeared quite negligible (Figure 5B). Similarly, deliberate but small variation in column temperature by 28±2°C; have also not made any major changes in retention pattern of both selected drugs since as observed the variation in their t_R values was less than 1% (Figure 5C). Thus, increasing the flow rate, organic modifier and temperature, both BEN and MET were appeared earlier whereas decreasing these variables by same extents, their routine elution order were elongated. Although, the effect of flow rate (±0.2mL.min-1) was quite distinguishable compared with effects of solvent composition and temperature. It might presume that selecting the small column dimension (100 x 2.1mm id) might have exhibited this wide differences for variation in flow rate. Importantly from Table 3; excluding the theoretical plates (N); other variables like capacity factor (k'), resolution (R_s) and peak tailing factor (T_f) were almost remain unchanged and it clearly signified that the proposed method obliged all minimum requirements led by the ICH guidelines (Table 3).

Most often, the separation behaviour of pharmaceutical amines in SCX chromatography is based on their net ionic charges, electrostatic repulsion, charge distribution, steric selectivities and hydrophobicity.[23,24] Thus, improving the selectivities of simultaneous quantification of BEN and MET, 5-15mM AF with decreased MeOH was preferred to exhibit good results since as reported the extensive use of SCX column the hydrophilic terminus of SCX bases surrounded with aqueous mobile phase and thus exhibit the swelling of hydrophilic sulphonic acid residues which apparently, irreversibly increases the column back pressure and even additional swelling was observed with its persistent use. However, increased swelling extended the retention values, capacity factor, tailing factor and resolution by almost 10-30%. Therefore, to ovoid such evidences, flushing the column with 0.2M NaH_2PO_4 and 0.3M CH_3COONa by 15 column volume, followed by storing the column between 4-15°C for 2-5 days prior to the analysis was recommended.[25]

Table 2: Drug Recovery data of benidipine and metoprolol.

	BEN						MET					
(%)	Sample (µg/mL)	Drug (µg/mL)	Recovery (µg/mL)	Recovery %	Mean±SD	RSD %	Sample (µg/mL)	Drug (µg/mL)	Recovery (µg/mL)	Recovery %	Mean±SD	RSD %
80	4	3.2	7.11	98.75			25	20	44.89	99.75		
80	4	3.2	7.12	98.88	98.37±0.76	0.76	25	20	45.22	100.48	100.17±0.37	0.37
80	4	3.2	7.02	97.5			25	20	45.12	100.26		
100	4	4	8.21	102.62			25	25	51.27	102.54		
100	4	4	8.11	101.37	101.62±0.90	0.90	25	25	51.40	100.8	102.06±1.10	1.08
100	4	4	8.07	100.87			25	25	51.43	102.86		
120	4	4.8	8.56	97.27			25	30	54.21	98.56		
120	4	4.8	8.63	98.06	98.10±0.85	0.85	25	30	54.01	98.2	99.32±1.64	1.64
120	4	4.8	8.71	98.97			25	30	55.67	101.21		

Table 3: Robustness data of BEN and MET.

	BEN					MET					
Variables	t_R (min)	variation %	k'	T_f	N	t_R (min)	variation %	k'	T_f	R_s	N
Flow rate (+0.2 mL.min^{-1})	1.04	-14.04	1.76	1.86	191	3.71	-14.51	8.89	1.83	6.27	722
Flow rate (-0.2 mL.min^{-1})	1.42	+17.35	1.80	1.93	195	5.17	+19.12	9.17	1.96	6.27	690
CH_3OH (+2%)	1.20	-0.82	1.63	1.90	199	4.21	-2.99	8.78	1.88	6.50	725
CH_3OH (-2%)	1.23	+1.65	1.99	1.87	218	4.39	+1.15	9.40	1.82	6.50	802
Temperature (+2°C)	1.19	-1.65	1.76	1.86	191	4.31	-0.69	8.89	1.83	6.27	722
Temperature (-2°C)	1.20	-0.82	1.78	1.85	201	4.33	-0.23	8.91	1.82	6.32	727
Mean±SD	1.15±0.157	---	1.78±0.11	1.87±0.03	199±10	4.16±0.58	---	9.00±0.23	1.85±0.05	6.35±0.11	731±37

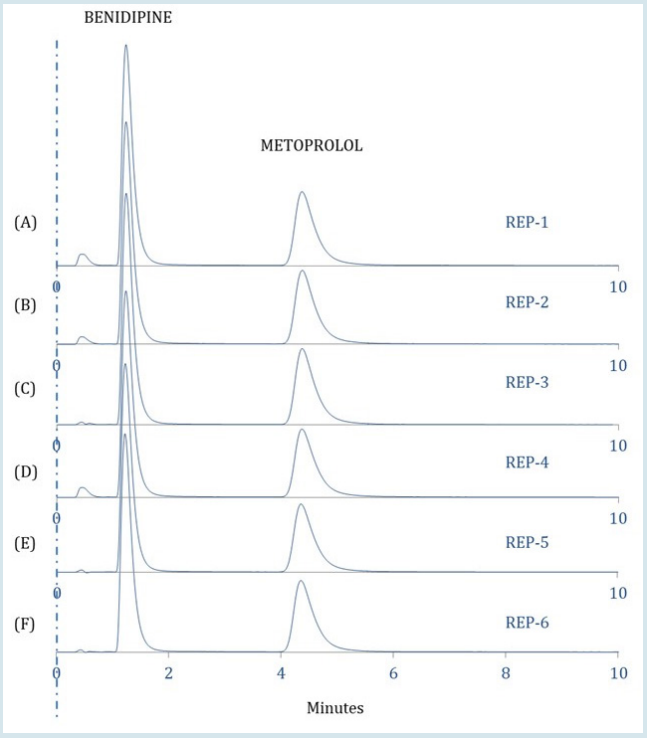

Figure 3: Repeatability data (A-F) six replicates of BEN and MET at 226 nm wavelength.

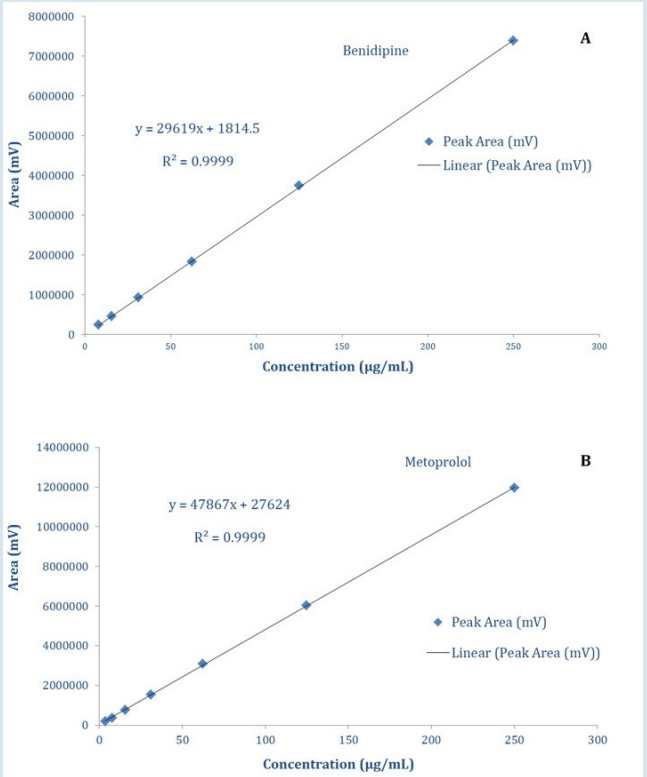

Figure 4: Linearity data of benidipine and metoprolol.

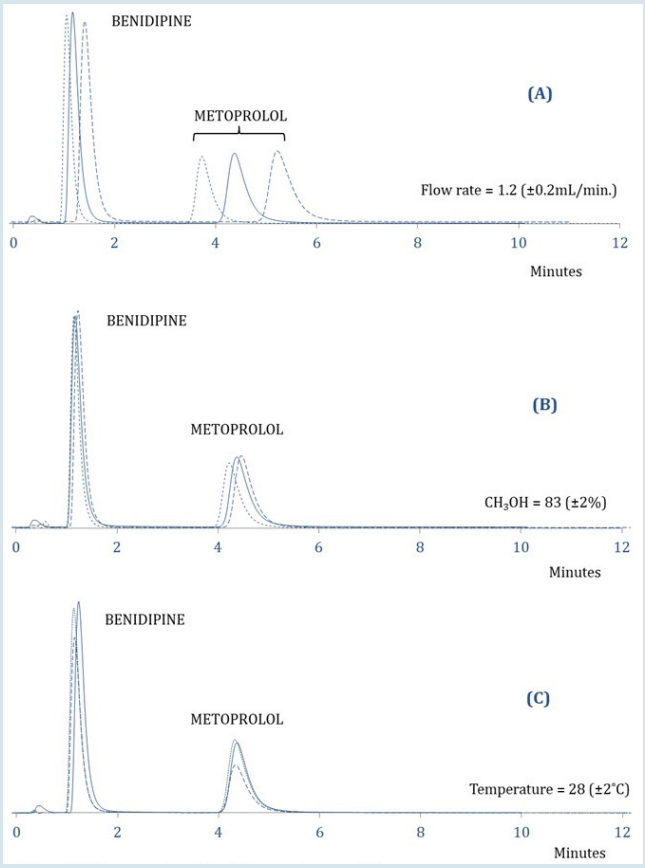

Figure 5: Robustness data of benidipine and metoprolol.

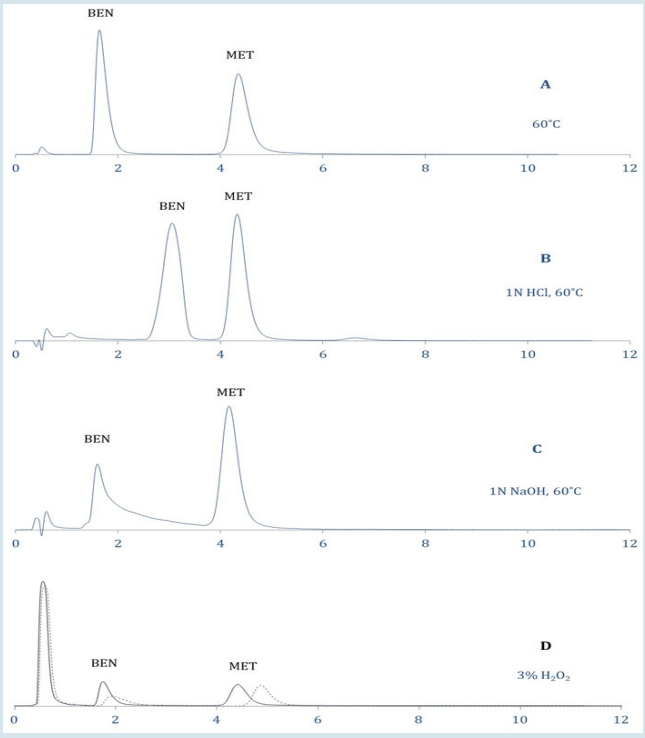

Figure 6: Force degradation data of benidipine and metoprolol.

Forced degradation studies

The forced degradation studies of BEN and MET using SCX chromatography revealed possible degradation under the influence of acid-base strength, peroxide and thermal environment (Figure 6).[26] As observed, both BEN and MET were resistant to the thermal stress at 60°C since no any degraded products were appeared in the HPLC chromatograph (Figure 6A). Nevertheless, the treatment under 1N HCl has made some changes in selectivity and stability of both drugs since as observed t_R of BEN was extended by 20% whereas the MET was degraded to develop one additional component at 6.85 min (Figure 6B). Similarly, the 1N NaOH, has not develop any new fragment for both compounds but it significantly affected the BEN selectivity since as displayed in Figure 6C, the peak shape of BEN was quite erratic and irreproducible. Furthermore, the treatment under 3% H_2O_2, produced degradation of the BEN to the most severe form since as observed there was almost 50-70% decrease in integrity of BEN and it is further accelerated by prolonged storing. In contrast, under the same peroxide treatment there was no degradation of metoprolol was appeared (Figure 6D). Altogether, to understand the susceptibility mechanisms of peroxide and 1N HCl; further studies involved the exact prediction of both BEN and MET by applying LC-MS/MS or LC-NMR techniques.

CONCLUSION

The present HPLC method represented the shortest run time for the simultaneous investigation of benidipine (BEN) and metoprolol (MET) where the results of linearity; accuracy, precision, robustness and specificity were found satisfactory and validated as per the ICH guidelines. The established method was stability indicating since there was no interference of degradants in force degradation studies was observed. Therefore, this established method is conducive for routine analysis and characterisation of either benidipine or metoprolol and the combination of both with other co-existing drugs from bulk and finished pharmaceutical formulations. All this further underlines the importance of SCX chromatography for its potential to be used in analysis of other relevant aliphatic/aromatic pharmaceutical amines based on their net positive charges and steric selectivities.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

RP-HPLC: reverse phase high performance liquid chromatography; **SCX:** strong cation exchange chromatography; **BEN:** benidipine; **MET:** metoprolol; **ICH:** international conference of harmonisation; **SE of intercept:** standard error of intercept; **SD of Intercept:** standard error of intercept; **N:** resolution; **t_R:** retention time; **T_f:** tailing factor; **RSD:** relative standard deviation.

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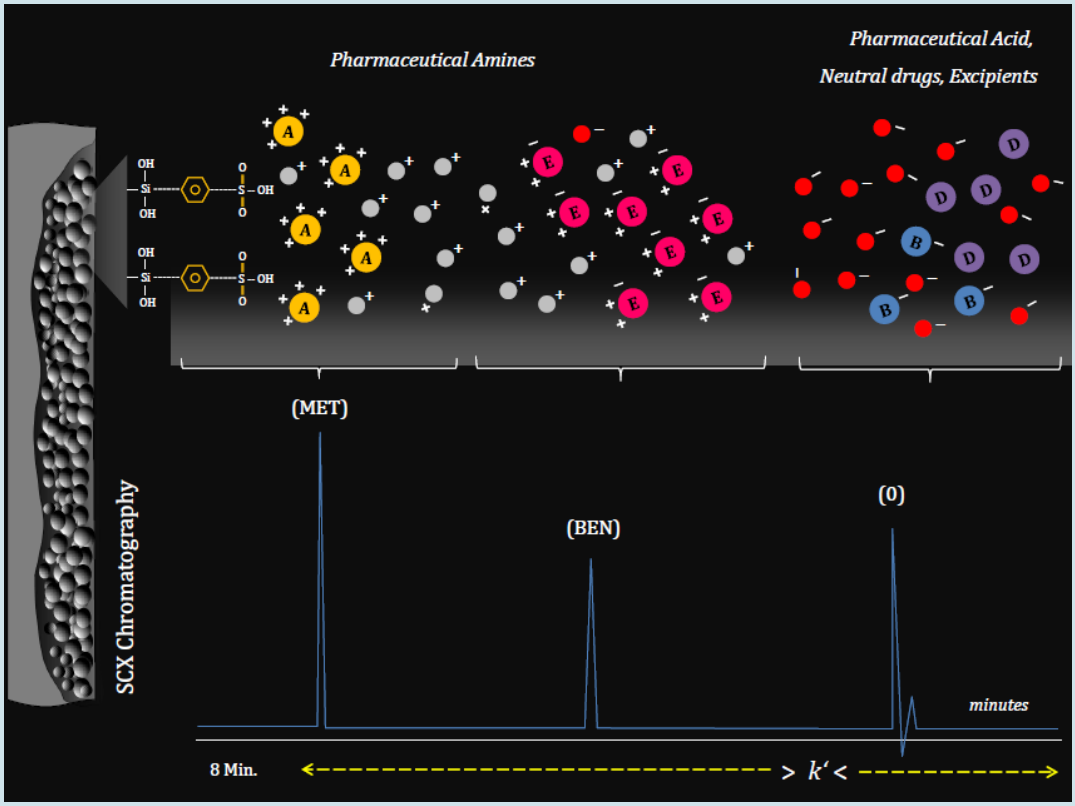
PICTORIAL ABSTRACT



SUMMARY

- Moderately polar antihypertensive drugs like metoprolol in combination with other equally potent drugs exhibit peak tailing/fronting effects along with the development of irreproducible selectivities on routinely used C_{18} column.
- Considering above aspects, since no any research article has been published on the simultaneous quantification of benidipine and metoprolol which are mostly prescribed in INDIA as anti-hypertensive drugs using either C_{18} or other relevant techniques.
- Therefore, this study demonstrates the simultaneous quantification along with stability indicating studies of benidipine and metoprolol, attempted using SCX chromatography.
- As resulted, this proposed method is innovative, reproducible, economical and efficient.

ABOUT AUTHORS

Dr. Pankaj Manohar Kharabe is a founder and director of UltraChrom Innovatives Pvt. Ltd. Main area of research activities involved; drug discovery and drug development, phytochemical analysis, metabolomics and proteomics studies, method development and validation studies of multidrug combination. Working in collaboration with research institutes, pharmaceutical and agricultural companies.