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Validated And Stability Indicating Of Rp-Hplc Method For Simultaneous Estimation Of S(-)Metoprolol Succinate & Clopidogrel Bisulfate In Bulk And Tablet Dosage Form

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Abstract:
A simple, selective, accurate, and sensitive High Performance Liquid Chromatographic (HPLC) method was developed and validated for the simultaneous analysis of S(-)Metoprolol succinate(MS) & Clopidogrel bisulfate(CB). Chromatographic separation achieved isocratically on a Hypersil BDS C_8 (250mm X 4.6mm, 5µm) column utilizing a mobile phase of Methanol: Acetonitrile: Buffer (15:40:45) v/v , (pH 4.5 with OPA) at a flow rate of 1.5 mL / min and column oven temperature 40^o C with PDA detection at 220 nm. Statistical analysis proves that the method is reproducible and selective for the simultaneous estimation of MS and CB. Stability indicating studies shows both drugs are stable in oxidative stress condition and light. Metoprolol Succinate under goes degradation in both acidic and basic conditions but up to less extent than Clopidogrel bisulfate. The developed method was validated as per ICH guidelines in terms of accuracy, precision, linearity and specificity. Thus the study aimed at developing and validating new HPLC method, being simple, accurate, selective and sensitive and can be applied for the estimation of these drugs in combined dosage form

Key words: *S(-)Metoprolol succinate, Clopidogrel bisulfate, RP-HPLC, Validation*

1.Introduction

1.1.S(-)Metoprolol Succinate (MS)

(Fig. 1, a) is 2-Propanol,1-[4-(2-methoxyethyl) phenoxy]-3-[(1-methylethyl) amino]-,(±)-,butanedioate (2:1)(salt) with molecular formula of $(C_{15}H_{25}NO_3)_2$. $C_4H_6O_4$ and molecular weight of 652.8.[1]. It is official in IP, BP and USP [2-4].It is a cardio selective drug used in the treatment of hypertension and various cardiovascular disorders. The action of Metoprolol succinate is mediated through the beta1-selective adrenoceptor blockage, thus causing reduction in heart rate and cardiac output. It is a beta1-selective drug which belongs to the chemical class of beta blockers. The drug is quite sensitive, even a small dose of the drug giving a sufficient blockade of the beta-adrenergic receptors. which reduces chest pain and lowers high blood pressure[5-8].

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1.2.Clopidogrel Bisulfate (CB)

(Fig. 1, b) is Thieno [3,2-c]pyridine-5(4H)-acetic acid ,a-(2-chlorophenyl)-6,7-dihydro-,methyl ester,(S)-,sulfate (1:1). The empirical formula of Clopidogrel bisulphate is $C_{16}H_{16}ClNO_2S{\bullet}H_2SO_4$ and its molecular weight is 419.9.

The mechanism of action of clopidogrel is an inhibitor of adenosine diphosphate (ADP)-induced platelet aggregation acting by direct inhibition of ADP binding to its receptor and of the subsequent ADP-mediated activation of the glycoprotein GPIIb/IIIa complex.MS and CB is a well accepted combination in treatment of anti-hypertensive, strocks and anti-colting agent.[9-14]

Various methods of analysis were documented for MS and CB individually. Reported methods for analysis of MS alone and in combination with other drugs include AAS and spectrophotometry [15] HPLC [16], RP-HPLC [17], spectrophotometry and HPLC- UV [18], chiral LC [19], LC [20], and HPTLC [21]. Literature survey reveals the estimation of Clopidogrel bisulfate in pharmaceutical formulations by various chemometric, HPLC, HPTLC, TLC, and an LC-ESI-MS-MS method was developed [9].

None of the reported analytical HPLC methods enables simultaneous determination of MS and CB in tablet dosage forms.

Experimental:

2.Materials And Methods

2.1.Materials

Pure MS & CB was obtained as gift sample from Emcure Pharmacutical Ltd., Pune (India). HPLC grade Acetonitrile and Methanol were procured from Merck, India. Ammonium acetate, SLS, Triethalamine and orthophosphoric acid AR grade were from Merck, India. Highly pure water was prepared by using Millipore Milli Q plus purification system.The 0.45µm Nylon pump filter was obtained from Advanced Microdevices (Mumbai, India). Formulations of MS & CB used for the study were tablets (Metpure-AP, India) containing 50 & 75 mg of MS & CB respectively and were procured from local market.

2.2.Instrumentation And Chromatographic Condition

Analysis was performed on Waters HPLC 2695 separation module with built-in PDA detector and auto sampler. Chromatographic software Empower 2 was used for data collection and processing. The analytical column was Hypersil BDS C_8 (250 mm X 4.6mm, 5µm) with the mobile phase ammonium acetate buffer: acetonitrile: methanol (45:40:15 v/v), pH of buffer was maintained at 4.5 ± 0.05 with H_3PO_4. Mobile phase is filtered with 0.22 µm filter in Millipore vacuum filtration assembly and degassed prior to operating under isocratic condition at a flow rate of 1.5 mL/min. Sample injection volume was 20 µL and column oven temperature was 40^oC, elution was monitored at 220 nm with run time 20 min.

2.3.Preparation Of Mobile Phase

Accurately weighed about 7.7 g of ammonium acetate transferred to 980 mL of water, added 2 mL of triethyl amine and adjusted the pH to 4.5 with orthophosphoric acid, and made up to 1000 mL with water. Before use, the mobile phase was degassed by an ultrasonic bath and filtered through a 0.45 Nylon filter.

2.4.Preparation Of The Standard Solutions

Standard solution was prepared by transferring 50 mg of MS & 100 mg of CB working standard into a 100 mL volumetric flask, 50 mL mobile phase was added, and the mixture was sonicated to dissolve and make up the volume with mobile phase. 5 ml of these standard solution was transferred using A-grade bulb pipette into 50 mL volumetric flasks and made up to volume with mobile phase to get final concentrations of 50 & 100 µg/mL for MS & CB respectiveiy. The solutions were then filtered through a 0.45 Nylon filter. The filtered solutions were then injected into HPLC system.

2.5.Method Validation

As recommended in the ICH guidelines [23] all validation was performed during development of the procedure. The proposed method was validated for linearity, limit of detection (LOD), limit of quantitation (LOQ), precision, accuracy, specificity, and ruggedness.

2.6.Linearity

A stock solution of MS & CB (50 & 100 µg/ mL) was prepared by dissolving 50 mg &100mgof drug respectively in 100 mL mobile phase then transferring 5 mL of this solution to a 50-mL volumetric flask and diluting to volume. Solutions of different concentration (50-150 range) for construction of calibration plots were prepared from this stock solution. The mobile phase was filtered through a 0.45-µm membrane filter and delivered at 1.5 mL min−1 for column equilibration; the baseline was monitored continuously during this process. The detection wavelength was 222 nm. The prepared dilutions were injected in series, peak area was calculated for each dilution, and concentration was plotted against peak area in **.**

2.7.Analysis Of The Tablet Dosage Form

Twenty tablets (Metpure-AP) were weighed accurately and crushed to form fine powder. Powder weight equivalent to 50 & 75 mg of MS and CB each were dissolved in 100 mL volumetric flask with the Mobile phase. It was sonicated followed by filtration using Whatmann filter paper No. 41. Appropriate 5ml volumes of the aliquot were transferred into five different 50 mL volumetric flasks and the volume was made up to the mark with mobile phase to get concentration 50 & 75µg/mL. The solutions were subject to analysis and results obtained as in **.**

2.8.Limit Of Detection And Quantification

Limit of detection (LOD) and limit of quantification (LOD) were calculated based on the ICH guidelines.

2.9.Accuracy

It was found out by recovery study using standard addition method. Known amounts of standards of MS and CB was added to pre-analyzed samples at a level from 50% up to 150% and then subjected to the proposed HPLC method. Results of recovery studies are shown in **Table 1.**

2.10.Precision

Method and system precision of the assay samples containing MS and CB having concentration 50 &100 µg/mL for both were analyzed five times in the same day (intraday) and for three consecutive days by different analysts (interday). Precision was calculated as intra and interday coefficient of variation [% C.V. = (S. D. /mean) x 100] as shown in the **Table 2.**

2.11.Robustness
By introducing deliberately small changes in the mobile phase pH (± 0.2), flow rate (±0.1 mL/ min.), temp (±5^0 C), Organic composition robustness of the proposed method were studied. **Table 3.**

2.12.Solution Stability
Stability in solution was evaluated for the standard solution and the test preparation. The solutions were stored at 5º and at ambient temperature without protection of light and tested after 12, 24, 36 and 48 h. The responses for the aged solution were evaluated by comparison with freshly prepared solutions.

2.13.System Suitability
The suitability of the chromatographic system was tested before each stage of validation. Five replicate injections of standard preparation were injected and asymmetry, number of theoretical plates and relative standard deviation of peak area were determined.

2.14.Stability Studies Of MS & CB
In order to determine whether the analytical method and assay were stability-indicating, pure drug was stressed under various conditions to conduct forced degradation studies.

2.15.Acid- And Base- Induced Degradation
Acid-induced degradation was performed by adding 5 mL of stock solution of MS & CB to 5 mL 0.5N hydrochloric acid and was refluxed for 1 h at 60^{o}C. The resultant solution was diluted to obtain 50µg/mL and 20 µL were injected into the system and the chromatograms were recorded to assess the stability of sample. Base-induced degradation was performed by adding 5 mL of stock solution of MS & CB to 5 ml 1N sodium hydroxide and was refluxed for 1 h at 60^{o}C. The resultant solution was diluted to obtain 50µg/mL and 20 µL were injected into the system and the chromatograms were recorded to assess the stability of sample.

2.16.Oxidative Degradation
To study the effect of the oxidizing conditions, 5 ml of stock solution of MS & CB was added 5 mL of 10% hydrogen peroxide solution and solution was refluxed for 30 min at 60^{o}C. For HPLC study, the resultant solution was diluted to obtain 50µg/mL and 20 µL were injected into the system and the chromatograms were recorded to assess the stability of sample.

2.17.Photolysis
The photochemical stability of the drug was also studied by exposing the stock solution to UV-Light for 200 Watts/m2. The resultant solution was diluted to obtain 50 µg/mL of MS & CB and 20 µL of the solution was injected into the HPLC, and chromatogram was recorded.

3.Results And Discussions
The chromatographic conditions were optimized to develop a stability indicating assay method for MS & CB in tablet dosage forms. The basic chromatographic conditions were designed to be simple and easy to use and reproduce and were selected after testing the different conditions that affect HPLC analysis, for example column, aqueous and organic components of the mobile phase, proportion of mobile phase components, detection wavelength, diluents and concentration of analyte. Hypersil BDS C_8 (250mm X 4.6mm, 5µm) as used because of its advantages of high resolving capacity, better reproducibility, low-back pressure, and low tailing. The proportion of the mobile phase components was optimized to reduce retention time and enable good resolution of MS & CB from the degradation products. A detection wavelength of 222 nm was selected. Detection at 222 nm resulted in good response and good linearity.
The calibration curve was prepared by plotting the peak area of MS & CB against drug concentration (µg/mL) and was linear in the range of 50-150 µg/mL. The data were subjected to least-square linear regression analysis to calculate the calibration equation and correlation coefficient. The regression equation was found as y = 21534x + 4705. R^2 = 0.999 for MS & y = 20764x - 3930. R^2 = 0.999 for CB .The results show that there is an excellent correlation between the peak area and the concentration of MS & CB in the range tested. The limit of detection, with a signal to noise ratio of 3:1, was found to be 16.65 & 33.30µg/ml for MS & CB respectively. The limit of quantitation, with a signal to noise ratio of 10:1, was found to be 5 & 10µg/ml for MS & CB respectively. Results from the linear regression analysis with system suitability data were listed in Table 4. % RSD was less than 2 in method & system precision and in each parameter of robustness. So the proposed method is more precise and robust. To examine the accuracy of the method, recovery studies were carried out by standard addition method. The results were shown in Table 1. The average percent recoveries for MS and CB was obtained 99.92% & 100.42 % respectively, indicating that the method was accurate. The robustness of the method was assessed by assaying test solutions under different analytical conditions deliberately changed from the original conditions. For each different analytical condition the standard solution and test solution were prepared separately. The result obtained from assay of the test solution was not affected by varying the conditions and was in accordance with the true value. System suitability data were also found to be satisfactory during variation of the analytical conditions. The analytical method therefore remained unaffected by slight but deliberate changes in the analytical conditions. During study of the stability of stored solutions of standards and test preparations for assay determination the solutions were found to be stable for up to 48 h. Before each measurement of validation data a system suitability test was performed by measurement of general characteristics such as peak asymmetry, number of theoretical plates and RSD (%) of peak area observed for a standard solution. The values obtained were satisfactory and in accordance with in-house limits. The specificity of the method was also evaluated by checking the peak purity of the analyte peak during the forced degradation study. The peak purity of the MS & CB

peak under different stress conditions was 1.00, which is satisfactory and indicates there was no interference with the analyte peak from degradation products.. CB degraded negligibly in all conditions as its percentage degradation was found to be less than 10 % which may not be accountable as per ICH guidelines and might be the reason for absence of its degradant peak. MS degrade in acidic condition when kept combined in 0.5 N HCl for 60 min, one degradant peaks separate out from MS at RT 4.47 min, (Fig. 3, a) and MS And CB degrade in basic condition when kept combined in 1N NaOH for 60^0 C (60 min), two degradants peaks of MS at RT 3.8 & 6.7 min, (Figure 3b). When kept in H_2O_2 for 30 min, In MS one degradants peak separate out RT 5.6 min (Figure 3c) and CB depredated ≤ 10% in both acidic and H_2O_2 conditions and in1N NaOH for 60^0 C (60 min), no degradants peaks were seen.

The proposed method was applied to the analysis of marketed product and the results obtained were given in Table 3. The blank solution was prepared containing the components indicated in tablets except active principle. No interference was observed from the tablet excipients. The results were indicated that the method is suitable for routine analysis of MS & CB in pharmaceutical dosage forms

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4.Conclusion

The modalities adopted in experiment were successfully validated as per ICH guidelines analytical procedures laid down in routine analysis. The proposed method was validated by preliminary analysis of standard sample and by recovery studies. The percentage of average recoveries for MS and CB was obtained 99.92% & 100.42 % respectively.

This demonstrates that the developed HPLC method is new, simple, linear, accurate, sensitive and reproducible. Thus, the developed method can be easily used for the routine quality control of bulk and tablet form.

5.Acknowledgments

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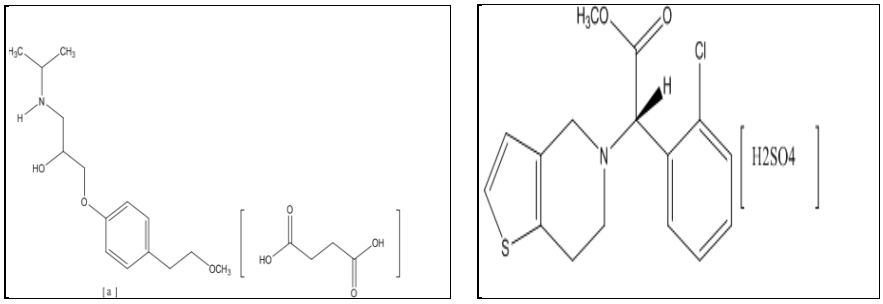


Figure 1(a) & 1(b): Chemical structures of MS (a) and CB (b)

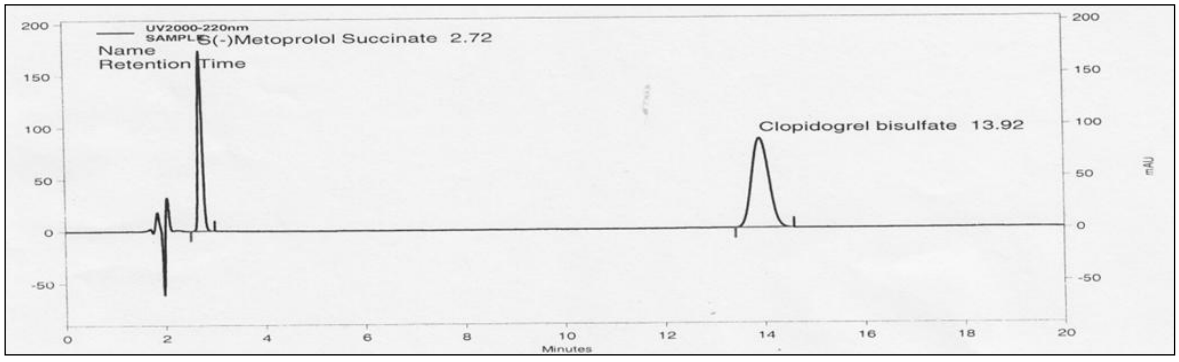
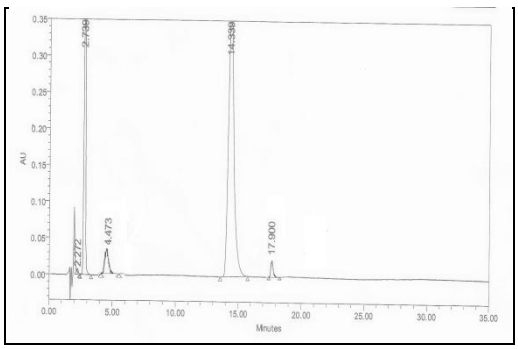
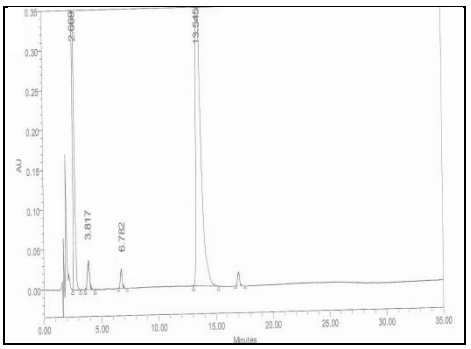


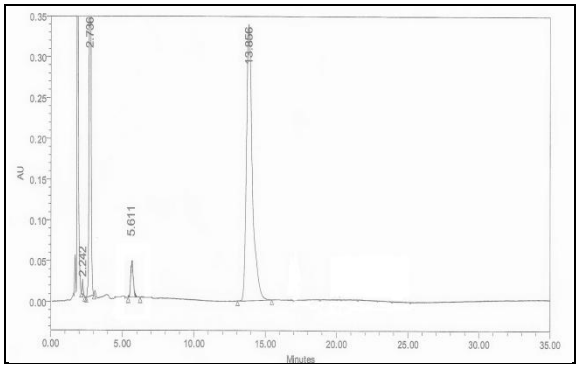
Figure 2: Typical Chromatogram Of MS & CB RT 2.7 &13.9 Respectively



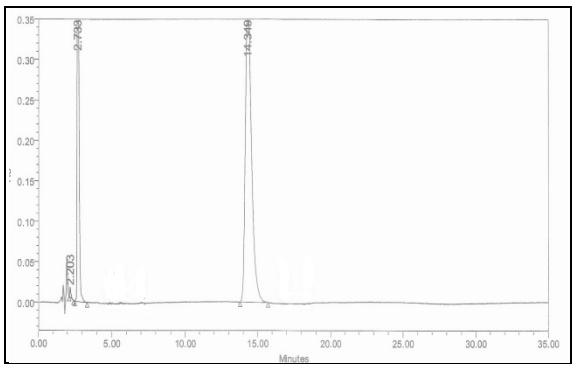
3(a)



3(b)



3(c)



3(d)

Figure 3:Typical HPTLC Densitograms Obtained From: Acid Hydrolysis-Treated Tablet Powder (A), Alkaline Hydrolysis Treated Tablet Powder(B), H_2O_2-Treated Tablet Powder (C), Photolysis- Treated Tablet Powder (D).

Sr. No.	Level of recovery	Amount of drug added		% Recovery		% RSD	
		MS	CB	MS	CB	MS	CB
1	50%	25	50	100.54	100.28	0.21	0.37
2	75%	32.5	75	100.26	99.96	0.29	0.20
3	100%	50	100	100.75	99.41	0.53	0.22
4	125%	62.5	125	99.45	100.05	0.87	0.98
5	150%	75	150	99.84	99.69	0.22	0.91

Table 1: Recovery Studies

Parameter	MS	CB
Concentration (µg/mL) [n=5]	25 to 75	50 to 150
Correlation coefficient (r^2)	0.999	0.999
% Recovery [n=9]	99.45 to 100.7	99.41to100.2
Precision [%RSD]		
Repeatability [n=5]	0.52	0.39
method [n=5]	1.46	0.33
system [n=5]	0.73	0.53
Robustness	Robust	Robust

Table 2: Summary Of Validation Data

Agent	Exposure time	Condition	Degradant peak		RT (min)		% Degradation	
			MS	CB	MS	CB	MS	CB
0.5 N HCl	60 min	60^0 C	1	1	4.47	17.9	13	6
1 N NaOH	60 min	60^0 C	2	1	3.8&6.7	-	25	3.2
3 % H_2O_2	30 min	60^0 C	1	-	5.6	-	16	-
U.V.Light	24 hours	Sunlight	-	-	-	-	-	-

Table 3: Forced Degradation Study

Parameter	Acceptance criteria	Result	
		MS	**CB**
Theoretical plate	More than 2000	6340	11912
USP Tailing factor	Less than 2	2.68	13.58
Capacity factor	Should be non-zero	0.64	4.31
USP Resolution	More than 2	11.13	

Table 4: System Suitability (N=5)

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7.Figure Captions

1. Fig. 1: Chemical structures of MS (a) and CB (b)
2. Fig. 2: Typical chromatogram of MS & CB, RT 2.7 &13.9 respectively
3. Fig. 3:Typical HPTLC densitograms obtained from: acid hydrolysis-treated tablet powder (a), alkaline hydrolysis treated tablet powder(b), H_2O_2-treated tablet powder (c), Photolysis- treated tablet powder (d).