Research Article

Development and Validation of Stability-Indicating RP-UPLC Method for Simultaneous Determination of Related Substances of S(–)Amlodipine and S(–)Metoprolol Succinate in Fixed Dose Combination Tablet Dosage Form

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A novel, rapid, accurate, sensitive, precise, and stability-indicating reverse-phase ultra-performance liquid chromatographic (RP-UPLC) method was developed and validated for determination of related substances of S(–)Amlodipine and S(–)Metoprolol Succinate in fixed dose combination tablet dosage form. The chromatographic separation was achieved with the use of Acquity UPLC HSS T3, 1.8 μm , 2.1 $\times$ 100 mm analytical column at 45°C employing a gradient elution. Mobile phase consisting of mobile phase-A (solution containing 5.0 gm of sodium dihydrogen phosphate monohydrate per liter of water and Acetonitrile in the ratio of 95 : 5) and mobile phase-B (Acetonitrile) was used at a flow rate of 0.5 mL min^{–1} with injection volume of 10 μL and the detection was done at 232 nm using UV detector. The retention times of S(–)Metoprolol Succinate and S(–)Amlodipine were found to be 2.8 minutes and 8.1 minutes, respectively. During method validation all the parameters were evaluated as per ICH guidelines, which remained well within acceptable limits. This method can be used for the estimation of related substances of S(–)Amlodipine and S(–)Metoprolol Succinate in fixed dose combination tablet dosage form.

1. Introduction

The combination of calcium channel blocker, that is, S(–)Amlodipine Besilate (Figure 1), and β_1 -adrenoceptor blocker, that is, S(–)Metoprolol Succinate (Figure 2), is more effective for the treatment of exercise-induced angina pectoris than β -adrenoceptor blocker immunotherapy. S(–)Amlodipine Besilate is a long-acting calcium channel blocker (dihydropyridine DHP class) used as an antihypertensive and in the treatment of anginal chest pain. S(–)Metoprolol Succinate is a selective β_1 receptor blocker used in treatment of several diseases of the cardiovascular system, especially hypertension. The formulation is available as bilayer tablets containing S(–)Amlodipine Besilate as immediate release and S(–)Metoprolol Succinate as extended release.

From the literature review, we found that there are some spectrophotometric methods available for simultaneous estimation of Amlodipine and Metoprolol in tablet dosage form [1, 2] and few high performance liquid chromatographic analytical methods are published for determination of Amlodipine Besilate and Metoprolol Succinate in combination of pharmaceutical formulations [3–7]; some analytical methods are also available for estimation of Amlodipine and Metoprolol in tablets having Amlodipine as immediate release and Metoprolol as extended release [8]. Also some HPTLC methods developed for estimation of Amlodipine Besilate and Metoprolol Succinate in combination [9]. Through literature survey also revealed some LC-MS/MS analytical methods for estimation of Amlodipine Besilate and Metoprolol Succinate in human plasma and its

FIGURE 1: Chemical structure of S(-)-Amlodipine Besilate.

FIGURE 2: Chemical structure of S(-)-Metoprolol Succinate.

TABLE 1: Gradient programme of mobile phase.

Time in minute	Flow rate mL/min	% of mobile phase-A	% of mobile phase-B
0.0	0.5	90	10
7.0	0.5	70	30
12.0	0.5	50	50
13.1	0.5	90	10
15.0	0.5	90	10

application in bioequivalence study [10]. Some analytical methods describe the estimation of Amlodipine with other drugs like telmisartan, clopidogrel bisulfate, and atorvastatin calcium in different formulations [11–13]. Few UPLC methods have been developed for quantification of impurities and degradation products of Metoprolol Succinate in tablet dosage form [14].

Hitherto, best to our present knowledge, there is no stability-indicating UPLC method available or published for the determination of related substances of S(-)-Amlodipine Besilate and S(-)-Metoprolol Succinate in fixed dose combination tablet dosage form. The scope of the present work is to expand the optimization of the chromatographic conditions to identify and quantify separately all the impurities and degradation products accurately and precisely at a minimum of runtime by using UPLC which in another way is more specific and also saves time and solvent. In addition, stress testing of the drug substance can be helpful for identifying the likely degradation products, which can in turn help to establish the degradation pathways and the intrinsic stability of the molecule and validate the stability-indicating power of the analytical procedures used. Moreover, the current method has been validated for known impurities, Amlodipine related compound-A and Metoprolol Succinamide impurity as well as S(-)-Amlodipine Besilate and S(-)-Metoprolol Succinate in terms of accuracy, precision, specificity, linearity, ruggedness, robustness, and system suitability as per the recommendations of ICH guidelines [15–17]. In addition, the proposed method was effectively applied for the routine analysis and quality control (QC) analysis.

2. Experimental

2.1. Chemicals and Reagents. Working standards of S(-)-Amlodipine Besilate, S(-)-Metoprolol Succinate, Amlodipine

related compound-A, Metoprolol Succinamide impurity, and tablet formulation (each tablet containing 5 mg of S(-)-Amlodipine Besilate and 25 mg of S(-)-Metoprolol Succinate) were provided by Emcure Pharmaceuticals Ltd., Pune, India. All the solvents used were of HPLC grade. HPLC water was generated in-house by using Merck Millipore, Milli-Q water purification system; hydrochloric acid was obtained from Rankem, Ltd., India. Sodium dihydrogen phosphate monohydrate, sodium hydroxide pellets, and Acetonitrile were procured from Merck, India, Ltd. 50% hydrogen peroxide was obtained from Fischer scientific.

2.2. Instrumentation and Chromatographic Conditions. Waters Acquity UPLC system (Waters Corporation, Milford, Massachusetts, USA) was used consisting of quaternary pump, photodiode array detector, an auto injector, and on-line degasser. The separation was achieved using Acquity UPLC HSS T3, 1.8 μ , 2.1 $\times$ 100 mm analytical column. Empower-2 software (Waters Corporation) was used for data acquisition. The analytical balance and pH meter used were manufactured by Mettler Toledo. The chromatographic separation was achieved using analytical column by a step gradient programme presented in Table 1. The mobile phase consisted of two parts: mobile phase-A (mixture of sodium dihydrogen phosphate monohydrate buffer and Acetonitrile with the ratio of 95 : 5 v/v) and mobile phase-B (Acetonitrile). Sodium dihydrogen phosphate monohydrate buffer was prepared by dissolving 5.0 gm of sodium dihydrogen phosphate monohydrate in 1000 mL of Milli-Q water, mixed well and sonicated. The mobile phases were filtered through 0.2 μ m filter and degassed in an ultrasonication bath before use. The flow rate and column temperature was maintained as

0.5 mL min⁻¹ and 45°C, respectively, throughout the analysis. The sample cooler temperature was maintained at 10°C. The injection volume was kept at 10 µL and wavelength was optimized at 232 nm which was found suitable for detection and quantification of all impurities of S(-)Amlodipine and S(-)Metoprolol Succinate. The stressed samples were analysed using Photodiode array detector in the wavelength range of 200–400 nm.

2.3. Preparation of Solutions. Diluent used for preparation of standard and sample solution was prepared by mixing buffer (5.0 gm of sodium dihydrogen phosphate monohydrate per 1000 mL of water) and Acetonitrile with the ratio of 20 : 80.

2.3.1. Preparation of S(-)Amlodipine Besilate Standard Stock Solution (Solution-A). 30 mg of S(-)Amlodipine Besilate working standard was weighed and transferred accurately into a 200 mL volumetric flask. About 70 mL of diluent was added and sonicated for dissolving and volume was made up to mark with diluent and mixed well.

2.3.2. Preparation of S(-)Metoprolol Succinate Standard Stock Solution (Solution-B). 50 mg of S(-)Metoprolol Succinate working standard was weighed and transferred accurately into a 100 mL volumetric flask; about 10 mL of mobile phase-A was added for dissolving and then 60 mL of diluent was added and sonicated for complete dissolving and volume was made up to mark with diluent and mixed well.

2.3.3. Preparation of Standard Solution. 1 mL of S(-)Amlodipine Besilate stock solution (Solution-A) and 1 mL S(-)Metoprolol Succinate stock solution (Solution-B) were pipetted into 100 mL volumetric flask. Volume was made up to mark with mobile phase-A and mixed well.

2.3.4. Preparation of Peak Identification Solution. About 5 mg of Amlodipine related compound-A reference standard was weighed and transferred accurately into 50 mL volumetric flask. About 30 mL of diluent was added and sonicated for dissolving and volume was made up to mark with diluent and mixed well. 1 mL of this solution was further diluted to 50 mL with mobile phase-A and mixed well.

2.3.5. Sample Preparation. Intact tablets (20 tablets) equivalent to about 500 mg of S(-)Metoprolol were weighed and transferred into 100 mL volumetric flask containing about 10 mL of diluent. About 60 mL of diluent was added and sonicated for about 30 minutes with intermittent shaking at bath temperature not exceeding 25°C. The flask was kept on bench top to attain room temperature and volume was made up to the mark with diluent, mixed well. It was allowed to settle for about 10 minutes. 10 mL of the supernatant was further diluted to 50 mL with mobile phase-A. The solution was filtered through 0.2 µ nylon filter paper by discarding initial 2 mL of filtrate (dilution scheme: sample → 100 mL/10 mL → 50 mL).

FIGURE 3: Typical UPLC chromatogram of blank preparation.

FIGURE 4: Typical UPLC chromatogram of peak identification solution.

2.3.6. Excipient Blend Preparation. About 6450.0 mg of excipient blend was weighed and transferred accurately into 100 mL volumetric flask containing about 10 mL of diluent. About 60 mL of diluent was added and sonicated for about 30 minutes with intermittent shaking at bath temperature not exceeding 25°C. The flask was kept on bench top to attain room temperature and volume was made up to the mark with diluent, mixed well. It was allowed to settle for about 10 minutes. 10 mL of the supernatant was further diluted to 50 mL with mobile phase A. The solution was filtered through 0.2 µ nylon filter paper by discarding initial 2 mL of filtrate.

2.3.7. Forced Degradation Study of S(-)Amlodipine Besilate and S(-)Metoprolol Succinate Tablet Formulation. Forced degradation study was performed to determine the ability of the proposed method for determination of S(-)Amlodipine, S(-)Metoprolol Succinate and its degradation products that are generated during forced degradation, for which S(-)Amlodipine and S(-)Metoprolol Succinate tablets were intentionally subjected to various stress conditions and chromatograms were recorded. The stability indicating nature of the analytical method was proved from the forced degradation studies and the typical UPLC Chromatograms were represented in Figures 3, 4, 5, 6, 7, and 8. Excipient blend preparations were treated in the same way as that of the sample for respective stress conditions. Conditions of forced degradation and results are summarized in Table 2.

TABLE 2: Forced degradation conditions and results.

Condition	Degrading agents/conditions	Exposure period	% degradation			
			Metoprolol Succinamide impurity	Amlodipine related compound-A	Any individual impurity	Total Impurities
Control sample	Untreated	NA	BLQ*	BLQ	0.071	0.177
Heat degradation (solid state)	—	60°C for 4 hours	BLQ	BLQ	0.078	0.186
Heat degradation (solution state)	—	60°C for 1 hour	BLQ	BLQ	0.077	0.185
Humidity degradation	75% R.H.	24 hours	BLQ	BLQ	0.073	0.120
Photolytic degradation	—	1.2 million lux hours and 200 watt hrs./m ²	BLQ	0.680	0.120	0.938
Acid degradation	0.5 N HCl, 2 mL	For 1 hour at 80°C	BLQ	0.301	0.390	0.749
Base degradation	0.5 N NaOH, 2 mL	For 1 hour at 80°C	BLQ	0.267	0.085	0.532
Peroxide degradation	5% H ₂ O ₂ , 5 mL	For 2 hours at 80°C	0.263	3.600	2.484	13.965

* BLQ: below quantitation limit.

FIGURE 5: Typical UPLC chromatogram of standard solution.

FIGURE 6: Typical UPLC chromatogram of excipient blend solution untreated.

FIGURE 7: Typical UPLC chromatogram of sample solution untreated.

FIGURE 8: Typical UPLC chromatogram of sample solution peroxide treated.

3. Results and Discussion

3.1. Optimization of the Chromatographic Conditions. The objective of this chromatographic method was to separate all the peaks of analytes, that is, S(-)Amlodipine and S(-)Metoprolol and its impurities, in a short runtime with good system suitability parameters. At the initial stage different columns like C8 and C18 with particle size of 1.7 μ were tried with mobile phase using different buffers like potassium dihydrogen phosphate, dipotassium hydrogen phosphate, and sodium dihydrogen phosphate with different

molar concentration in the pH range of 3.0 to 8. Flow rate was kept at 0.5 mL min⁻¹. Based on the above trials the chromatographic conditions, analytical column, mobile phase composition, pH of the buffer, and flow rate were finalized and validated as per ICH guidelines.

3.2. Method Validation. The analytical method validation was carried out as per ICH method validation guidelines (Q₂R1). The parameters include system suitability criteria, specificity, accuracy, precision (repeatability, intermediate precision),

TABLE 3: Results of system suitability test.

Peak name	Tailing factor	Theoretical plates	%RSD of six injections
S(-)Metoprolol	1.38	29070	0.09
S(-)Amlodipine	1.06	144639	0.73

TABLE 4: Results of specificity test (peak purity criteria).

Peak name	Peak purity angle	Peak purity threshold	Purity criteria
S(-)Metoprolol	1.167	4.324	Pass
S(-)Amlodipine	0.042	0.533	Pass
Metoprolol Succinamide impurity	14.674	90.000	Pass
Amlodipine related compound-A	13.153	90.000	Pass

linearity, limit of detection, limit of quantitation, robustness, filter compatibility, and solution stability.

3.2.1. System Suitability Test. The system suitability test was performed by injecting 6 replicates of standard solution into the chromatograph and the chromatograms were recorded. The relative standard deviation of the area for individual peaks, for six replicate injections of standard solution, should not be more than 5.0%. The tailing factor for the S(-)Amlodipine and S(-)Metoprolol peaks should be less than 2.0. The theoretical plates for the S(-)Amlodipine and S(-)Metoprolol peaks should be more than 5000. The results obtained for theoretical plates, USP tailing factor, were all well within acceptable limits. Results obtained are summarized in Table 3.

3.2.2. Specificity. A photodiode array detector was used for analysis of stressed solutions to determine the specificity of the method and to evaluate the homogeneity of the analyte peak. The peak purity obtained was found to be acceptable (purity angle < purity threshold) which shows that no additional peaks were coeluting with the analytes thus proving the ability of the method to access unequivocally the analyte of interest in the presence of any potential interference. The results of forced degradation studies proved that the proposed method has the ability to separate the analyte from its degradation products, indicating a high degree of specificity of the proposed method. The excipient blend preparations and diluent blank preparation did not show any peak at the retention time of S(-)Metoprolol, S(-)Amlodipine, and its known impurities. Results obtained for peak purity angle and purity threshold are summarized in Table 4.

3.2.3. Determination of Limit of Detection and Limit of Quantification (LOD and LOQ). The nominal concentration of the test solutions for S(-)Amlodipine and S(-)Metoprolol is 200 mgmL⁻¹ and 1000 mgmL⁻¹, respectively. The LOD and LOQ for S(-)Amlodipine Besilate and S(-)Metoprolol Succinate and its known impurities (Amlodipine related compound-A and Metoprolol Succinamide) were determined

TABLE 5: Limit of detection and limit of quantification.

Analyte	LOD	LOQ
S(-)Metoprolol	0.013% (0.130 ppm)	0.040% (0.400 ppm)
S(-)Amlodipine	0.013% (0.026 ppm)	0.040% (0.080 ppm)
Metoprolol	0.033% (0.330 ppm)	0.100% (1.000 ppm)
Succinamide impurity	0.066% (0.132 ppm)	0.200% (0.4000 ppm)

TABLE 6: Results of LOQ precision.

Analyte	%RSD of six injections	S/N value
S(-)Metoprolol	1.06	55
S(-)Amlodipine	1.78	24
Metoprolol Succinamide impurity	0.64	119
Amlodipine related compound-A	0.69	67

TABLE 7: Results of linearity (correlation coefficient).

Analyte	Correlation coefficient
S(-)Metoprolol	0.99963
S(-)Amlodipine	0.99950
Metoprolol Succinamide impurity	0.99967
Amlodipine related compound-A	0.99959

TABLE 8: Relative response factor.

Analyte	Slope	RRF
S(-)Metoprolol	18015	1.0
S(-)Amlodipine	48003	1.0
Metoprolol Succinamide impurity	20981	1.16
Amlodipine related compound-A	31669	0.66

by signal-to-noise ratio method, by injecting in triplicate a series of diluted solutions with known concentrations over a range starting from 10% to 120% of the specified limit concentration of known impurities and any unknown individual impurity. LOD and LOQ were obtained by plotting linearity graph of average area at each level against the concentration (ppm) and determined the slope.

LOD and LOQ concentration level determined are summarized in Table 5.

3.2.4. LOQ Precision. A solution containing S(-)Amlodipine, S(-)Metoprolol Succinate, Amlodipine related compound-A and Metoprolol succinamide impurity at LOQ level was prepared and injected in six replicate.

%RSD and S/N ratio obtained are summarized in Table 6.

3.2.5. Linearity and Range. Linearity of the method was tested by preparing and injecting in triplicate a series of standard preparations over a range starting from LOQ to 150%

TABLE 9: Recovery of Amlodipine related compound-A and Metoprolol Succinamide impurity.

Spiking level	Amlodipine related compound-A		Metoprolol Succinamide impurity	
	% mean recovery	%RSD	% mean recovery	%RSD
LOQ	94.78	1.13	105.30	0.33
Level-1 (50%)	92.17	1.04	101.71	1.79
Level-2 (100%)	94.45	0.73	100.05	1.22
Level-3 (150%)	97.31	0.45	101.45	0.97

TABLE 10: Results of method precision and intermediate precision.

Analyst	Metoprolol Succinamide impurity	Amlodipine related compound-A	% of any individual impurity	% of total impurities
I	0.539	0.926	0.050	1.562
	0.539	0.926	0.049	1.562
	0.541	0.926	0.049	1.562
	0.542	0.927	0.051	1.570
	0.539	0.927	0.048	1.561
	0.541	0.931	0.050	1.570
II	0.527	0.861	0.050	1.531
	0.522	0.861	0.052	1.529
	0.522	0.862	0.052	1.530
	0.526	0.862	0.051	1.581
	0.530	0.866	0.050	1.536
	0.529	0.863	0.052	1.576
Mean	0.533	0.895	0.050	1.556
SD	0.008	0.034	0.001	0.019
%RSD	1.50	3.80	2.00	1.22

of the standard preparations of S(–)Metoprolol Succinate (0.5%) and S(–)Amlodipine (0.5 %) and specification limit of known impurities. Average peak area at each level was plotted against drug concentration in ppm and linear regression analysis was performed on the resulting plot. Results obtained are summarized in Table 7.

3.2.6. RRF Calculation. Relative response factor for known impurities was calculated from the linearity study using the following formula:

$$\begin{aligned} \text{RRF of Metoprolol Succinamide} &= \frac{\text{Slope of Metoprolol Succinamide impurity}}{\text{Slope of S(–)Metoprolol Succinate}}, \\ \text{RRF of Amlodipine related compound-A} &= \frac{\text{Slope of Amlodipine related compound-A}}{\text{Slope of S(–)Amlodipine}}. \end{aligned} \quad (1)$$

Relative response factor of Metoprolol Succinamide and Amlodipine related compound-A is summarized in Table 8.

3.2.7. Accuracy. Accuracy of the method was determined by standard addition method by spiking known impurities in sample preparation. The % recovery of known impurities at

different levels was evaluated. The samples for accuracy were prepared as unspiked samples and at LOQ level, 50%, 100%, and 150% level of specification limit of known impurities for stability by spiking impurity stock solutions in triplicate. Relative response factors of the related impurities were used to calculate the percentage weight of related impurities in drug product. Results for each level of recovery are summarized in Table 9.

3.2.8. Method Precision and Ruggedness. During method precision six samples were prepared as per analytical method by spiking Amlodipine related compound-A and Metoprolol Succinamide impurity at limit level and compared with one unspiked sample. The %RSD for twelve cumulative samples was calculated (six from method precision and six from intermediate precision). Results of method precision and intermediate precision are summarized in Table 10.

3.2.9. Robustness. The robustness of an analytical method is the ability to remain unaffected by small changes in chromatographic parameters. For robustness study one unspiked sample and two samples spiked with known impurities at limit level were prepared. The parameters altered for robustness study are change in column (same make, different serial number/lot), change in flow rate (–0.1 to 0.05 mL/minute), change in wavelength (± 2 nm), and change in column

TABLE 11: Results of robustness.

Parameters	Values As per method	Tailing factor		USP plates		%RSD of STD		%RSD of AL Imp.-A	%RSD of MT Imp.	%RSD of Unknown Imp.	%RSD of total Imp.
		AL	MT	AL	MT	AL	MT				
Control											
Column (different serial number)	Precision	1.28	1.49	193410	28616	0.25	0.12	0.22	0.19	2.0	0.26
	Intermediate precision	1.23	1.20	97580	18686	0.41	0.09	0.23	0.57	1.96	1.55
	Flow	1.23	1.37	179599	31125	0.36	0.18	2.24	1.29	4.17	2.58
Wavelength (±2 nm)	0.4 mL/min	1.15	1.31	148500	24413	0.35	0.13	2.55	0.55	10.64	0.89
	0.55 mL/min										
Oven temperature (-5 and +2°C)	230 nm	1.06	1.06	65255	8954	1.40	1.18	2.55	2.25	4.08	0.57
	234 nm	1.10	1.10	70523	9120	1.23	0.74	0.87	3.80	4.00	1.96
	40°C	1.17	1.31	152064	25101	0.21	0.20	3.08	0.37	13.21	2.40
	47°C	1.20	1.44	279462	44894	0.80	0.19	2.29	1.31	3.92	0.45

TABLE 12: Results of filter validation.

Sample number	Metoprolol Succinamide impurity	Amlodipine related compound-A	Any individual impurity	Total Impurities
Unfiltered sample preparation	0.560	1.132	0.049	1.789
Filtered sample preparation-1	0.560	1.132	0.048	1.788
Filtered sample preparation-2	0.560	1.132	0.050	1.790
Filtered sample preparation-3	0.560	1.137	0.050	1.795
Filtered sample preparation-4	0.561	1.137	0.048	1.793
Filtered sample preparation-5	0.561	1.138	0.050	1.796
Absolute difference between unfiltered and filtered sample preparation				
Filtered sample preparation-1	0.000	0.000	0.001	0.001
Filtered sample preparation-2	0.000	0.000	0.001	0.001
Filtered sample preparation-3	0.000	0.005	0.001	0.006
Filtered sample preparation-4	0.001	0.005	0.001	0.004
Filtered sample preparation-5	0.001	0.006	0.001	0.007

oven temperature (-5 to $+2^{\circ}\text{C}$). % impurity results of two spiked sample preparations were calculated for each changed condition. Results obtained for each parameter changed for robustness are summarized in Table 11.

3.2.10. Stability of Analytical Solution. To determine the stability of solution, sample solution of S(–)Metoprolol and S(–)Amlodipine tablets spiked with known impurities and standard preparation were prepared as per method of analysis and stored at 2°C to 8°C for a period of five days. Sample and standard preparations were analyzed initially, after one day, after three days, and after five days. From the results solution was found to be stable up to 5 days at 2°C to 8°C .

3.2.11. Filter Validation. Sample and standard preparations were filtered using different filters and results compared against centrifuged sample and unfiltered. Results obtained are summarized in Table 12.

4. Conclusions

In pharmaceutical formulations, the impurities and degradation products can change the pharmacological and toxicological potency of the active pharmaceutical ingredient which has adverse effect on quality, safety, and efficacy of the drug. In this study, a rapid, precise, specific, and stability-indicating RP-UPLC method was developed. The effect of different stress conditions on the degradation of drug substance of S(–)Amlodipine Besilate and S(–)Metoprolol Succinate was studied and the data reflects satisfactory results for the validation parameters according to ICH guidelines. It could be applied for the routine analysis of S(–)Amlodipine Besilate and S(–)Metoprolol Succinate in quality control laboratories, research institutions, industries, approved testing laboratories, biopharmaceutics, bioequivalence studies, and clinical pharmacokinetic studies in near future.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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