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Validated HPTLC Method for Simultaneous Estimation of Ramipril and Metolazone in Bulk Drug and Formulation

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ABSTRACT

This paper describes a new, simple, precise, and accurate HPTLC method for simultaneous estimation of Ramipril and Metolazone as the bulk drug and in tablet dosage forms. Chromatographic separation of the drugs was performed on aluminum plates precoated with silica gel 60 F_{254} as the stationary phase and the solvent system consisted of toluene : ethyl acetate : methanol : glacial acetic acid (4 : 4 : 1 : 0.2 v/v/v/v). Densitometric evaluation of the separated zones was performed at 223 nm. The two drugs were satisfactorily resolved with R_F values 0.33 ± 0.02 and 0.59 ±0.02 for Ramipril and Metolazone respectively. The accuracy and reliability of the method was assessed by evaluation of linearity (600-2100 ng/spot for Ramipril and 100-350 ng/spot for Metolazone, precision (intra-day % RSD was 1.28 – 1.58 and inter-day % RSD was 1.14 – 1.83 for Ramipril and intra-day % RSD was 0.67 – 1.03 and inter-day % RSD was 0.49 – 1.18 for Metolazone), accuracy 99.44 ± 0.15 for Ramipril and 99.85 ± 0.39 for Metolazone), and specificity in accordance with ICH guidelines.

Keywords: Thin layer chromatography, Densitometry, Validation and Quantification, Ramipril and Metolazone.

INTRODUCTION

Ramipril is chemically (2*S*,3*aS*,6*aS*)-1-[(2*S*)-2-{[(2*S*)-1-ethoxy-1-oxo-4-phenylbutan-2-yl]amino}propanoyl]-octahydrocyclopenta[*b*]pyrrole-2-carboxylic acid [1, 2] **(Figure 1).** Ramipril is an angiotensin-converting enzyme (ACE) inhibitor, used to treat hypertension and congestive heart failure. ACE inhibitors lower the production of angiotensin II, therefore relaxing arterial muscles while at the same time enlarging the arteries, allowing the heart to pump blood more easily, and increasing blood flow due to more blood being pumped

into and through larger passage ways. Ramipril is a prodrug and is converted to the active metabolite ramiprilat by liver esterase enzymes. Ramiprilat is mostly excreted by the kidneys.

Metolazone is chemically 7-chloro-2-methyl-3-(2-methylphenyl)-4-oxo-1,2-dihydroquinazoline-6-sulfonamide [3] **(Figure 2).** Metolazone is an oral diuretic drug, commonly classified with the thiazide diuretics. It is primarily used to treat congestive heart failure and high blood pressure. Metolazone indirectly decreases the amount of water reabsorbed into the bloodstream by the kidney, so that blood volume decreases and urine volume increases. This lowers blood pressure and prevents excess fluid accumulation in heart failure.

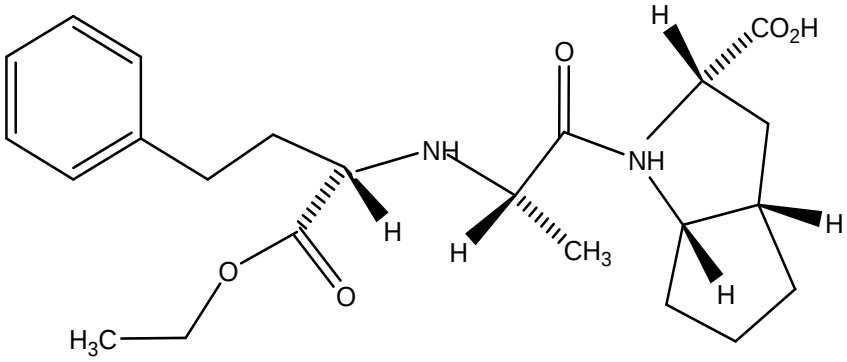

Figure 1: Structure of Ramipril

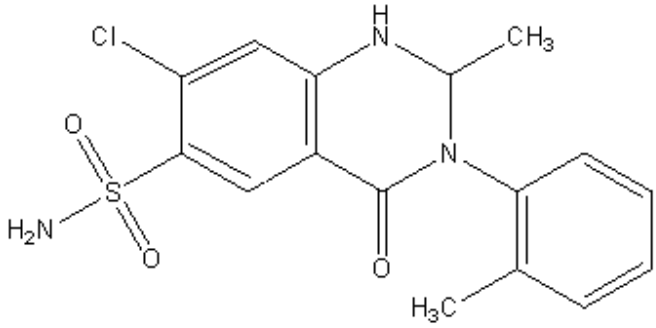

Figure 2: Structure of Metolazone

Today TLC is rapidly becoming a routine analytical technique due to its advantages of low operating costs, high sample throughput and the need for minimum sample preparation. The major advantage of TLC is that several samples can be run simultaneously using a small quantity of mobile phase unlike HPLC thus reducing the analysis time and cost per analysis.

Literature review reveals that methods have been reported for analysis of Ramipril by HPLC [4, 5] and HPTLC [6, 7, 8, 9, 10, 11, 12] in combination with other drugs. LC-MS-MS development and validation for simultaneous quantitation of Metolazone with other drug in human plasma have been also reported [13, 14, 15, 16, 17].

To date there has been no published reports on simultaneous quantitation of Ramipril and Metolazone by HPTLC in bulk drug and in tablet dosage form. This present study reports for the

first time the simultaneous quantitation of Ramipril and Metolazone by HPTLC in bulk drug and in tablet dosage form. The proposed method is validated as per ICH Guidelines [18].

MATERIALS AND METHODS

Working standards of pharmaceutical grade Ramipril (Batch No. 2126735) and Metolazone (Batch N0. 20103601P) were obtained as generous gifts from Ranbaxy Laboratories Ltd., Dewas (Madhya Pradesh, India) and Centaur Pharmaceuticals Pvt. Ltd., Ambarnath (Maharashtra, India). They were used without further purification and certified to contain 99.70 % (w/w) and 99.80 % (w/w) on dry weight basis for Ramipril and Metolazone respectively. Fixed dose combination tablets (Brand Name: METOZ R-2.5) containing 2.5 mg of Ramipril and 2.5 mg of Metolazone were procured from Centaur Pharmaceuticals Pvt. Ltd, India. All chemicals and reagents of analytical grade were purchased from Merck Chemicals, Mumbai, India.

Instrumentation

The samples were spotted in the form of bands of width 6 mm with a Camag 100 microlitre sample (Hamilton, Bonaduz, Switzerland) syringe on silica gel precoated aluminum plate 60 $_{F-254}$ plates [20 cm × 10 cm with 250 µm thickness; E. Merck, Darmstadt, Germany] using a Camag Linomat V (Switzerland) sample applicator. The plates were prewashed with methanol and activated at 110 ^{o}C for 5 min prior to chromatography. A constant application rate of 0.1 µL/s was used and the space between two bands was 5 mm. The slit dimension was kept at 5 mm × 0.45 mm and the scanning speed was 10 mm/s. The monochromator bandwidth was set at 20 nm, each track was scanned three times and baseline correction was used. The mobile phase consisted of toluene : ethyl acetate : methanol : glacial acetic acid (4 : 4 : 1 : 0.2 v/v/v/v) and 9.2 mL of mobile phase was used per chromatographic run. Linear ascending development was carried out in a 20 cm × 10 cm twin trough glass chamber (Camag, Muttenz, Switzerland) saturated with the mobile phase. The optimized chamber saturation time for the mobile phase was 40 min at room temperature (25 ^{o}C ± 2) at relative humidity of 60 % ± 5. The saturation time was kept 30 min for each chromatographic run. Each chromatogram was developed over a distance of 8 cm. Following the development, the TLC plates were dried in a stream of air with the help of an air dryer in a wooden chamber with adequate ventilation. The flow in laboratory was maintained unidirectional (laminar flow, towards the exhaust). Densitometric scanning was performed using a Camag TLC scanner III in the reflectance absorbance mode at 223 nm and operated by CATS software (V 3.15, Camag). The source of radiation used was deuterium lamp emitting a continuous UV spectrum between 190 and 400 nm. Concentrations of the compound chromatographed were determined from the intensity of the light. Evaluation was performed by linear regression of peak areas determined by UV absorption as a function of sample amounts.

Preparation of Standard Stock Solutions

Standard stock solutions with a concentration of 1000 µg/mL were prepared in methanol for Ramipril and Metolazone respectively. From the standard stock solutions, diluted mixed standard solutions were prepared containing 500 µg/mL for Ramipril and 500 µg/mL for Metolazone respectively. The stock solution was stored at 2-8 °C protected from light.

Optimization of the HPTLC method

The TLC procedure was optimized with a view to develop a simultaneous assay method for Ramipril and Metolazone respectively. The mixed standard stock solution (500 µg/mL of Ramipril and 500 µg/mL of Metolazone) was taken and 2 µL sample was spotted on to TLC plates and run in different solvent systems. Optimization of HPTLC method was very difficult in this case as Ramipril was not moving at all in toluene : ethyl acetate : methanol (4 : 4 : 1 v/v/v). After many trials it was found that glacial acetic acid is necessary for movement of Ramipril. Finally the mobile phase consisting of toluene : ethyl acetate : methanol : glacial acetic acid (4 : 4 : 1 : 0.2 v/v/v/v) was found optimum **(Figure 3).**

In order to reduce the neckless effect TLC chamber was saturated for 30 min using saturation pads. The mobile phase was run up to a distance of 8 cm; which takes approximately 20 min for complete development of the TLC plate.

Validation of the method

Validation of the optimized TLC method was carried out with respect to the following parameters.

Linearity and range

From the mixed standard stock solution, 300 µg/mL of Ramipril and 50 µg/mL of Metolazone was taken, 2 to 7 µL solution were spotted on TLC plate to obtain final concentration 600-2100 ng/spot for Ramipril and 100-350 ng/spot for Metolazone. Linearity of the method was studied by applying six concentrations of the drug, each concentration was applied three times to the TLC plates. The plate was then developed using the previously described mobile phase and the peak areas were plotted against the corresponding concentrations to obtain the calibration curves.

Precision

The precision of the method was verified by repeatability and intermediate precision studies. Repeatability studies were performed by analysis of three different concentrations (600 ng/spot, 1200 ng/spot and 1800 ng/spot for Ramipril and 100 ng/spot, 200 ng/spot and 300 ng/spot for Metolazone respectively) six times on the same day. The intermediate precision of the method was checked by repeating studies on three different days.

Limit of detection and limit of quantitaiton

Limits of detection (LOD) and quantification (LOQ) represent the concentration of the analyte that would yield signal-to-noise ratios of 3 for LOD and 10 for LOQ, respectively. LOD and LOQ were determined by measuring the magnitude of analytical background by spotting a blank and calculating the signal-to-noise ratio for Ramipril and Metolazone by spotting a series of solutions until the S/N ratio 3 for LOD and 10 for LOQ. To determine the LOD and LOQ, serial dilutions of mixed standard solution of Ramipril and Metolazone were prepared from the standard stock solution in the range of 20–1800 ng/spot. The samples were applied to TLC plate and the chromatograms were run and measured signal from the samples was compared with those of blank.

Robustness of the method

Following the introduction of small changes in the mobile phase composition (± 0.1 mL for each component), the effects on the results was examined. Mobile phases having different compositions, e.g. toluene : ethyl acetate : methanol : glacial acetic acid (4.1 : 4 : 1 : 0.2 v/v/v), (4 : 4.1 : 1 : 0.2 v/v/v), (4 : 4 : 1.1 : 0.2 v/v/v), (4 : 4 : 1. : 0.1 v/v/v) were tried and chromatograms were run. The amount of mobile phase was varied over the range of ± 5 %. The plates were prewashed with methanol and activated at 110 °C for 2, 5, and 7 min respectively prior to chromatography. The time from spotting to chromatography and from chromatography to scanning was varied by 10 min. The robustness of the method was determined at three different concentration levels for 600 ng/spot, 1200 ng/spot and 1800 ng/spot for Ramipril and 100 ng/spot, 200 ng/spot and 300 ng/spot for Metolazone.

Specificity

The specificity of the method was determined by analyzing standard drug and test samples. The spot for Ramipril and Metolazone in the samples was confirmed by comparing the R_F and spectrum of the spot with that of standard. The peak purity of Ramipril and Metolazone was determined by comparing the spectrum at three different regions of the spot i.e. peak start (S), peak apex (M) and peak end (E).

Recovery studies

Accuracy of the method was carried out by applying the method to drug sample (Ramipril and Metolazone combination tablet) to which known amount of Ramipril and Metolazone standard powder corresponding to 80, 100 and 120 % of label claim had been added (standard addition method), mixed and the powder was extracted and analyzed by running chromatogram in optimized mobile phase.

Analysis of a marketed formulation

To determine the content of Ramipril and Metolazone in conventional tablet (Brand Name: METOZ R-2.5, Batch No. 106, Manufactured by Centaur Pharmaceutical Pvt. Ltd., Label claim: Ramipril 2.5 mg and Metolazone 2.5 mg per tablet), twenty tablets were weighed, their mean weight determined and finely powdered. The weight of the tablet triturate equivalent to 2.5 mg of Ramipril and 2.5 mg of Metolazone was transferred into a 50 mL volumetric flask containing 35 mL methanol, sonicated for 30 min with occasional shaking and diluted to 50 mL with methanol. The resulting solution was centrifuged at 3000 rpm for 5 min and the drug content of the supernatant was determined (50 µg/mL for Ramipril and 50 µg/mL for Metolazone). Then 20 µL of the solution was applied which gave final concentration of 1000 ng/spot for Ramipril and 1000 ng/spot for Metolazone. The analysis was repeated in triplicate. The possibility of excipient interference with the analysis was examined.

RESULTS AND DISCUSSION

The results of validation studies on simultaneous estimation of the method developed for Ramipril and Metolazone in the current study using as the mobile phase toluene : ethyl acetate : methanol : glacial acetic acid (4 : 4 : 1 : 0.2 v/v/v/v) for TLC are given below.

Linearity

The drug response was linear (r^2 0.9974 for Ramipril and 0.9980 for Metolazone) over the concentration range between 600-2100 ng/spot for Ramipril and 100-350 ng/spot for Metolazone.

Precision

The results of the repeatability and intermediate precision experiments are shown in **Table 1**. The developed method was found to be precise as the RSD values for repeatability and intermediate precision studies were < 2%, as recommended by ICH guidelines.

LOD and LOQ

Signal-to-noise ratios of 3 : 1 and 10 : 1 were obtained for LOD and LOQ respectively. The LOD and LOQ were found to be 400 ng/spot and 600 ng/spot for Ramipril, 100 ng/spot and 120 ng/spot for Metolazone, respectively.

Robustness of the method

The standard deviation of peak areas was calculated for each parameter and the % RSD was found to be less than 2. The low values of the % RSD, as shown in **Table 2** indicated the robustness of the method.

Table 1: Precision Studies

Concentration (ng/spot)	Repeatability (n=6)			Intermediate precision (n=6)		
	Measured conc. ±SD	(%) RSD	Recovery (%)	Measured conc. ±SD	(%)RSD	Recovery (%)
Ramipril						
600	599.43 ± 3.52	1.28	99.90	591.69 ± 3.25	1.14	98.50
1200	1185.12 ± 3.61	0.94	98.75	1190.11 ± 4.77	1.24	99.16
1800	1780.37 ± 8.89	1.58	98.88	1785.51 ± 10.64	1.83	99.19
Metolazone						
100	98.74 ± 10.75	1.03	98.74	98.79 ± 11.07	1.08	98.79
200 -	198.96 ± 16.03	0.67	99.48	196.38 ± 11.97	0.49	98.19
300	295.19 ± 37.31	0.91	98.39	297.62 ± 1.50	1.18	99.20

Table 2: **Robustness testing**

Parameter	**SD of Peak Area for Ramipril**	**% RSD**	**SD of Peak Area for Metolazone**	**% RSD**
Mobile phase composition (±0.1 ml)	4.07	0.34	3.74	0.02
Amount of mobile phase (±5%)	21.09	0.83	8.72	0.15
Time from spotting to chromatography (+ 10 min.)	5.31	0.16	3.31	0.06
Time from chromatography to scanning (+ 10 min.)	6.12	0.25	2.63	0.03

Specificity

The peak purity of Ramipril and Metolazone was assessed by comparing their respective spectra at the peak start, apex and peak end positions of the spot i.e., r (S, M) = 0.9973 and r (M, E) = 0.9981. A good correlation (r = 0.9994) was also obtained between the standard and sample spectra of Ramipril and Metolazone respectively.

Table 3: Recovery studies

Label claim (mg/tablet)	Amount added (mg)	Total amount (mg)	Amount Recovered (mg) ± % RSD	% Recovery
Ramipril				
2.5	2.0 (80%)	4.5	4.49 ± 1.13	99.92
2.5	2.5 (100%)	5.0	4.94 ± 1.43	98.80
2.5	3.0 (120%)	5.5	5.47 ± 1.12	99.61
Metolazone				
2.5	2.0 (80%)	4.5	4.43 ± 1.21	98.63
2.5	2.5 (100%)	5.0	4.95 ± 0.78	99.11
2.5	3.0 (120%)	5.5	5.43 ± 1.56	98.82

Table 4: Analysis of commercial formulation

Ramipril (2.5 mg)	Ramipril found (mg per tablet)	
	Mean ± SD (n= 6)	Recovery (%)
1st Lot	2.48 ± 1.09	99.52
2nd Lot	2.49 ± 1.11	99.64
Metolazone (2.5 mg)	Metolazone found (mg per tablet)	
	Mean ± SD (n= 6)	Recovery (%)
1st Lot	2.49 ± 1.09	99.60
2nd Lot	2.48 ± 1.11	99.20

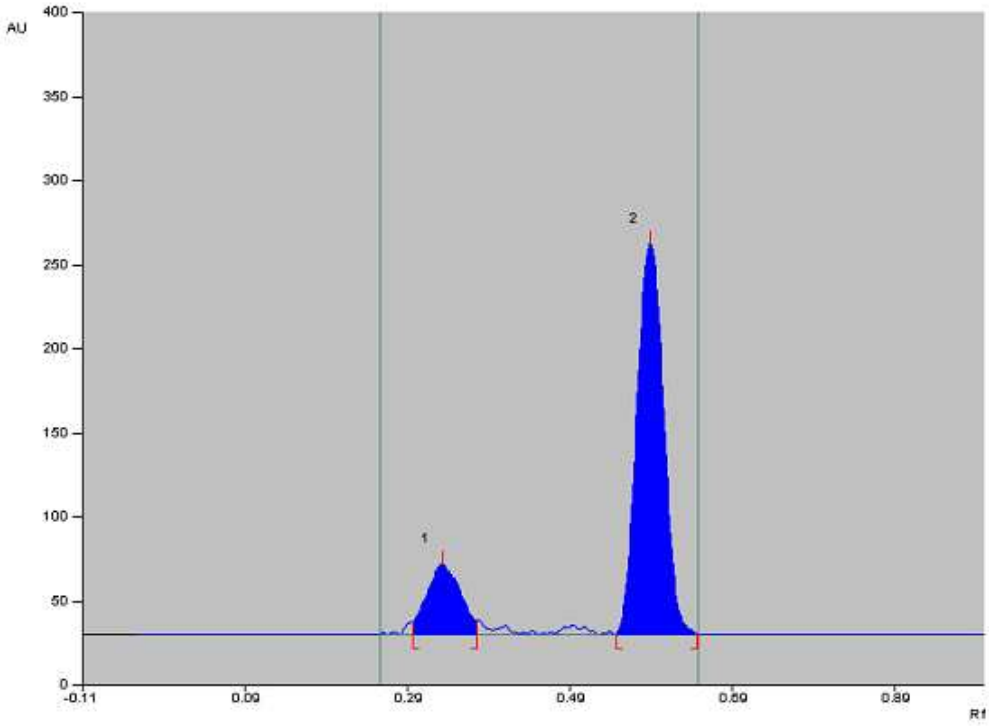

Figure 3: Densitogram of standard drugs

Mobile phase: toluene : ethyl acetate : methanol : glacial acetic acid (4 : 4 : 1 : 0.2 v/v/v/v)
Ramipril: R_F 0.33 ±0.02, Metolazone: R_F 0.59 ±0.02
Concentration: 1000 µg/mL for Ramipril and 1000 µg/mL for Metolazone
Application volume: 1 µL; Wavelength: 223 nm

Recovery studies
As shown from the data in **Table 3** good recoveries of the Ramipril and Metolazone in the range from 98.63 % w/w to 99.92 % w/w were obtained at various added concentrations.

Analysis of a formulation
Experimental results of the amount of Ramipril and Metolazone in tablets, expressed as a percentage of label claim were in good agreement with the label claims thereby suggesting that there is no interference from any of the excipients that are normally present in tablets. Two different lots of Ramipril and Metolazone combination tablets were analyzed using the proposed procedures **(Table 4).**

CONCLUSION

The developed TLC technique is precise, specific and accurate. Statistical analysis proves that the method is suitable for the analysis of Ramipril and Metolazone as bulk drug and in pharmaceutical formulation without any interference from the excipients. It may be extended to study the degradation kinetics of Ramipril and Metolazone also for its estimation in plasma and other biological fluids. The proposed TLC method is less expensive, simpler, rapid, and more flexible than HPLC.

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