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Validated HPTLC Method Development for Simultaneous Estimation of Spironolactone and Hydrochlorothiazide in Formulation

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Abstract*— This study describes a new TLC method for simultaneous quantification of Spironolactone and Hydrochlorothiazide in bulk and tablet dosage forms that is simple, precise and accurate. The drugs were separated using aluminium precoated plate with silica gel 60 F-254 as the stationary phase using Toluene: Ethyl acetate: Methanol 6: 4: 1 (v/v/v) as the mobile phase. At 231 nm, the separated zones were densitometrically evaluated. The two drugs were satisfactorily resolved with RF values of 0.78 ± 0.02 and 0.33 ± 0.02 for Spironolactone and Hydrochlorothiazide. The accuracy and reliability of the method were assessed by evaluation of linearity (120-270 ng/spot for Spironolactone and 100-350 ng/spot for Hydrochlorothiazide), precision (intra-day RSD 0.98-1.80 % and inter-day RSD 0.76-1.92 % for Spironolactone, and intra-day RSD 0.84-1.85 % and inter-day RSD 0.31-1.68 % for Hydrochlorothiazide), accuracy (100.78 ± 0.49 % for Spironolactone and 98.54 ± 1.80 % for Hydrochlorothiazide, by ICH guidelines. These methods have been applied to formulation and no interference of excipients is seen which proves that the method is specific.

Keywords: Spironolactone; Hydrochlorothiazide; HPTLC; Validation.

I. INTRODUCTION

Spironolactone, 7α-Acetylthio-17α-hydroxy-3-oxopregn-4-ene-21-carboxylic acid γ-lactone inhibits the effect of aldosterone by competing for intracellular aldosterone receptors in the distal tubule cells (it works on Aldosterone receptors in the collecting duct). This increases the secretion of water and sodium while decreasing the excretion of potassium. Spironolactone has a relatively slow onset of action, taking several days to develop and similarly the effect diminishes slowly. Spironolactone has anti-androgen activity by binding to the androgen receptor and thus preventing it from interacting with dihydrotestosterone[1].

Hydrochlorothiazide, 6-chloro-1,1-dioxo-3,4-dihydro-2H -1,2,4-benzothiadiazine-7-sulfonamide, inhibits sodium chloride transport in the distal convoluted tubule. More sodium is then excreted in the kidney with accompanying fluid. Pharmacological effects begin in about 2 hours after an oral dose, peak in 4 hours, and lasts for about 6 to 12 hours. Hydrochlorothiazide is not metabolized, and a majority is excreted in the urine unchanged. It also causes a loss of potassium and bicarbonate[2].

Hydrochlorothiazide has been used in combination with several drugs. Spectrophotometric quantitative determination of Cilazapril and Hydrochlorothiazide in tablets by chemometric methodsis reported. In addition, quantitative analysis of binary mixtures of Hydrochlorothiazide-Amiloride has also been determined by spectrophotometry and HPLC[4], determination of Hydrochlorothiazide and Spironolactone in pharmaceutical formulation in presence of impurities and degradants, simultaneous estimation of Hydrochlorothiazide-Losartan potassium by HPLC, Enalapril-Hydrochlorothiazide by HPLC and Hydrochlorothiazide-Spironolactone by HPLC[8]. There is also a reported method by HPTLC for Olmesartan medoxomil and Hydrochlorothiazide [3]

There have been no published reports for the simultaneous estimation of Spironolactone and Hydrochlorothiazide by TLC in bulk drugs and pharmaceutical dosage forms. This work is used to simultaneously measure Spironolactone and Hydrochlorothiazide by TLC in bulk medication and prescription dose forms.

II. EXPERIMENTAL

2.1. Materials

Lupin Pharmaceuticals Ltd, Aurangabad, India, kindly supplied a pure drug sample of Spironolactone as a gift sample of Batch No.: PC-76DFG. Cipla Pharmaceutical Ltd, Panvel, India, kindly provided a pure drug sample of Hydrochlorothiazidez of Batch No.: HC-12DRT. It was used without further purification and verified to have a dry content of 98.55 % (w/w). Fixed-dose combination pills (Aldactazide) comprising 25 mg Spironolactone and 25 mg Hydrochlorothiazide[7] was obtained from Pharmacy Escrow.com in London because this tablet was not available in Indian market. Merck Chemicals, Mumbai, India, provided all analytical-grade chemicals and reagents.

2.2. Instrumentation

The samples were spotted on silica gel precoated aluminium plate 60 F–254 plates, [20 cm x 10 cm with 250 m thickness; E. Merck, [5] Darmstadt, Germany] with a Camag 100 microlitre sample syringe (Hamilton Bonaded,

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Vol 9, Issue 6, June 2022

Switzerland). They were utilising a Camag Linomat V sample applicator (Switzerland). Before chromatography, the plates were prewashed with methanol and activated for 5 minutes at 110°C. Bands were applied at a steady rate of 0.1 mL/s, with 5 mm spacing between the two bands. The slit size was fixed to 5 mm x 0.45 mm, and the scanning speed was set to 10 mm/s. The monochromator band width was set at 20 nm, each track was scanned three times and the baseline correction was used. Toluene: Ethyl acetate: Methanol (6: 4: 1, v/v/v) was employed as the mobile phase. In a 20 cm x 10 cm twin trough glass chamber[6], saturated with the mobile phase, linear ascending development was carried out (Camag, Muttenz, Switzerland). The chamber saturation time for the mobile phase was 30 min at room temperature (25ºC ± 2) at a relative humidity of 60% ± 5. The plate was allowed to run at a distance of 8 cm. After the development, HPTLC plates were dried with the current of air using air dryer in a wooden chamber with adequate ventilation. The flow rate in the laboratory was maintained unidirectionally (laminar flow, towards exhaust). Densitometric scanning was performed on a Camag HPTLC scanner III in the reflectance absorbance mode at 231 nm and operated by CATS software (V3.15, Camag). Radiation source used was deuterium lamp emitting continuous UV spectrum between 190 and 400 nm.

2.3 Preparation of Standard Stock Solutions

Standard stock solutions with a concentration of 1 mg/mL of Spironolactone and Hydrochlorothiazide were prepared separately using methanol. From the usual stock solution, mixed standard solution was prepared using methanol to prepare 0.1 mg/mL of Spironolactone and Hydrochlorothiazide. Application volume of 1.5 µL was applied on the TLC plate to obtain a final concentration of 150 ng/spot for both Spironolactone and Hydrochlorothiazide.

III. OPTIMIZATION OF HPTLC METHOD

The HPTLC procedure was optimised to develop assay method for Spironolactone and Hydrochlorothiazide; standard stock solution (0.1 mg/mL of Spironolactone and 0.1 mg/mL of Hydrochlorothiazide) were prepared and 1.5 µL volume was spotted on TLC plates, and ran in different solvent systems. Various solvent systems were used to find the best resolution. The mixture needs a polar mobile-phase combination for good separation of the two drugs. Initially, mobile phase composition used was in the ratio of 4: 3: 3 (v/v) for toluene: ethyl acetate: methanol, but the resolution was not good and not suitable for quantification purposes because samples travelled to the solvent front. The polarity of the mobile phase combination was then reduced by lowering the polarity of the mobile phase combination. Among the different mobile-phase combinations, toluene: ethyl acetate: methanol (6: 4: 1) gave well-resolved spots for Spironolactone and Hydrochlorothiazide and gave suitable Rf values of 0.78 and 0.33. To avoid neckless effect; the chamber was saturated for 30 mins using saturation pads. The mobile phase was run upto a distance of 8 cms and it took around 20 mins to develop the plate.

IV. VALIDATION OF THE METHOD

The optimised HPTLC method was validated covering the following parameters.

4.1 Linearity and range

From the mixed standard stock solution (1 mg/mL of Spironolactone and 1 mg/mL of Hydrochlorothiazide), the final concentration of 0.12-0.27 mg/mL was prepared for Spironolactone and 0.10-0.35 mg/mL was prepared for Hydrochlorothiazide. One µL spot was applied on the HPTLC plate to obtain final concentration of 170-270 ng/spot and 100-350[9]. ng/spot for Spironolactone and Hydrochlorothiazide. The sample was applied three times on the HPTLC plate. After developing the plate using the previously stated mobile phase, the peak areas were plotted against the relevant concentrations to produce the calibration curves.

4.2 Precision

Repeatability and intermediate precision experiments were used to confirm the methods precision. Three distinct concentration levels of 120, 150, 180 ng/spot for Spironolactone and 100, 150, 200 ng/spot for Hydrochlorothiazide were analyzed by HPTLC on the same day. The intermediate precision of the method were also checked by repeating studies on three different days.

4.3 Limit of detection and limit of quantification

The analyte concentration that would result in signal-to-noise ratios of 3 for LOD and 10 for LOQ. The LOD and LOQ were calculated by determining the signal-to-noise ratio for Spironolactone and Hydrochlorothiazide by identifying solutions until the balance reached 3 for the LOD and 10 for the LOQ. Serial dilutions of a mixed standard solution of Spironolactone and Hydrochlorothiazide[10] was produced from a common stock solution in the range/spot to determine the LOD and LOQ. The samples were applied to the HPTLC plate, and the chromatograms were run.

4.4 Robustness of the method

Minor changes in the mobile phase composition were done to evaluate variation in the results. Mobile phases having different compositions, e.g., toluene: ethyl acetate: methanol (6: 4: 1.1 v/v/v), (6: 4.1: 1 v/v/v), (6.1: 4: 1 v/v/v), (6: 4: 0.9 v/v/v) were tried and chromatograms were run. Prior to chromatographic development, the plates were prewashed with methanol and activated at 60 ºC for 2, 5, and 7 minutes. The time from spotting to chromatography and from chromatography to scanning was varied by ±10 mins. Robustness studies were determined at three different concentration levels of 120, 150, 180 ng/spot for

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Spironolactone and 100, 150, 200 ng/spot for Hydrochlorothiazide.

4.5 Specificity

Analysing the blank, standard and test samples determines the specificity of the developed method. The R_f values and spectrum for Spironolactone and [11] Hydrochlorothiazide were compared with that of the standard R_f values. Peak purity for both the drugs was found to be 0.999 which was obtained by comparing the spectrum at three different regions i.e. at peak start (S) and peak apex (M) peak-end end (E).

4.6 Accuracy

The approach was used on a medication sample (Spironolactone and Hydrochlorothiazide combination tablet) to which a known amount of Spironolactone and Hydrochlorothiazide standard powder corresponding to 80, 100, and 120 per cent of the label claim was added (Standard addition method), The powder was extracted and analysed using a chromatogram in an optimised mobile phase.

4.7 Analysis of a marketed formulation

The proposed method was applied to determine Spironolactone and Hydrochlorothiazide in commercial tablets of Aldactazide (Label claim: 25 mg Spironolactone and 25 mg Hydrochlorothiazide per tablet); twenty tablets were weighed, their mean weight was determined and the tablets were triturated to obtain a fine powder. The weight of tablet equivalent to 25 mg of Spironolactone and Hydrochlorothiazide was weighed and transferred to a 50 mL volumetric flask which contained 35 mL of methanol. The sample was sonicated for a duration of 30 minutes and diluted upto 50 mL with methanol concentration of 0.5 mg/mL for Spironolactone and Hydrochlorothiazide). To allow the excipients to settle at the bottom, the solution was centrifuged at 3000 rpm for 5 mins and further dilutions were performed to obtain a concentration of 0.15 mg/mL for Spironolactone and Hydrochlorothiazide. One µL sample was applied in triplicate on a TLC plate to obtain a concentration of 150 ng/spot for Spironolactone and Hydrochlorothiazide. The plate was further developed in the optimized mobile phase and analyzed.

V. RESULTS AND DISCUSSION

In the existing study, validation of simultaneously estimated method for Spironolactone and Hydrochlorothiazide was using toluene: ethyl acetate: methanol (6:4:1, v/v/v) as the mobile phase for HPTLC.

5.1 Linearity

The Spironolactone and Hydrochlorothiazide showed a good correlation coefficient (R^2 0.997 for Spironolactone and 0.998 for Hydrochlorothiazide) in the given concentration range (120-270 ng/spot for Spironolactone and 100-350 ng/spot for Hydrochlorothiazide) by HPTLC.

5.2 Precision

The repeatability and intermediate precision RSD (%) values by the HPTLC method for Spironolactone and Hydrochlorothiazide were found to be in the range of 0.98-1.80 and 0.84-1.58, respectively, and the RSD (%) values for Hydrochlorothiazide were found to be in the field of 0.76-1.39 and 0.52-1.68, respectively. As recommended by ICH guidelines, the developed method was precise as the RSD values for repeatability and intermediate precision studies were < 2%, respectively as shown in Table 2.

5.3 LOD and LOQ

For LOD and LOQ, signal-to-noise ratios of 3:1 and 10:1 were attained. The LOD and LOQ concentrations were determined to be 40 ng/spot and 50 ng/spot for Hydrochlorothiazide and 80 ng/spot and 100 ng/spot for Spironolactone.

5.4 Robustness of the method

The standard deviation peak of the areas was calculated for each parameter, and the % RSD was found to be less than 2% by HPTLC methods. Lower values of % RSD indicates that the method is robust which is shown in Table 3.

5.5 Specificity

By comparing their respective spectra at the peak start, apex and peak-end locations, r (S, M) 0.9991 and r (M, E) 0.9990, the peak purity of Spironolactone and Hydrochlorothiazide was determined. The standard and sample spectra of Spironolactone and Hydrochlorothiazide, shows a strong correlation (r=0.9993). There was no interference of excipients with that of Spironolactone and Hydrochlorothiazide peak in the assay.

5.6 Recovery studies

At three levels i.e. 80%, 100% and 120%, each for Spironolactone and Hydrochlorothiazide, standard drug was spiked and % Recovery was calculated which was found to be in the range of 98.11 % to 100.42 % for Spironolactone and 98.36 % to 101.13 % for Hydrochlorothiazide.

5.7 Analysis of a formulation

The drug content for Spironolactone and Hydrochlorothiazide in tablet formulation was found to be 98.5% and 102.7 %, respectively. There was no interference of excipient in recovering the drugs nor did the excipients interfere with the standard peaks.

VI. CONCLUSION

Introducing HPTLC into pharmaceutical analysis represents a significant step in terms of quality assurance. HPTLC has become one of the standard analytical technique because of many advantages like inexpensive operating costs, several samples can be run simultaneously, high sample throughput, minimal sample preparation and mobile phase requirements, reduced analysis time and cost per analysis. The developed HPTLC method is specific, precise and

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Vol 9, Issue 6, June 2022

accurate. Kinetics of Spironolactone and Hydrochlorothiazide can be studied and qualification in plasma and other biological fluids can be done.

REFERENCES

[1] Berardesca E, Gabba P, Ucci G, Borroni G, Rabbiosi G. Topical spironolactone inhibits dihydrotestosterone receptors in human sebaceous glands: an autoradiographic study in subjects with acne vulgaris. International journal of tissue reactions. 1988 Jan 1;10(2):115-9.

[2] Dinç E, Baleanu D. Spectrophotometric quantitative determination of cilazapril and hydrochlorothiazide in tablets by chemometric methods. Journal of pharmaceutical and biomedical analysis. 2002 Oct 15;30(3):715-23.

[3] Abdelaleem EA, Naguib IA, Zaazaa HE, Draz ME. Spectrophotometric methods for quantitative determination of binary mixture of hydrochlorothiazide and amiloride hydrochloride without prior separation. Asian journal of biomedical and pharmaceutical sciences. 2014 Jul 1;4(34):27.

[4] Hegazy MA, Metwaly FH, Abdelkawy M, Abdelwahab NS. Validated chromatographic methods for determination of hydrochlorothiazide and spironolactone in pharmaceutical formulation in presence of impurities and degradants. Journal of chromatographic science. 2011 Feb 1;49(2):129-35.

[5] Argekar, P. A., Sawant, G. J., "A gradient reversed-phase HPLC method for simultaneous determination of Hydrochlorothiazide and Losartan potassium from tablets" Analytical Letters, 2008 Feb 27;33, 869-880.

[6] Shetkar PB, Shinde VM. Simultaneuous determination of enalpril maleate and hydrochlorothiazide in tablets by reversed phase HPLC. Analytical letters. 1997 Apr 1;30(6):1143-52.

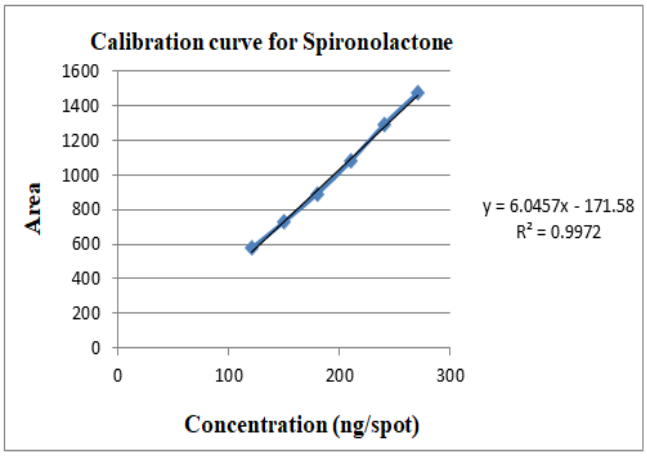
[7] Ebru Tekerek, Murat Süküroğlu, Okan ATAY. Quantitative determination of hydrochlorothiazide and spironolactone in tablet by spectrophotometric and HPLC method, Turk J. Pharm. Sci., 2008 April 02;5 (2) 53-66.

[8] Shah NJ, Suhagia BN, Shah RR, Patel NM. Development and validation of a simultaneous HPTLC method for the estimation of olmesartan medoxomil and hydrochlorothiazide in tablet dosage form. Indian Journal of Pharmaceutical Sciences. 2007;69(6):834.

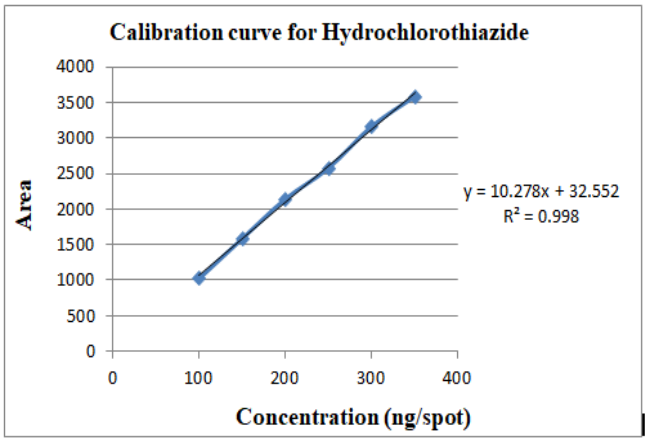
[9] ICH Guidance on Analytical Method Validation, in Proceedings of the International Convention on Quality for the Pharmaceutical Industry, Toronto, Canada, September 2002.

[10] ICH, Q2A Validation of Analytical Procedure: Methodology International Conference on Harmonization, Geneva, October 1994.

[11] ICH, Q2B Validation of Analytical Procedure: Methodology International Conference on Harmonization, Geneva, March 1996.



Standard linearity curve of Spironolactone (120-270 ng/spot)



Standard linearity curve of Hydrochlorothiazide (100-350 ng/spot)

Table 2. Precision studies

Drug	Amount (ng/spot)	Intraday precision RSD [%] n=6	Interday precision RSD [%] n=6
Spironolactone	120	1.80	1.39
	150	1.67	0.76
	180	0.98	1.92
Hydrochlorothiazide	100	1.58	0.31
	150	1.85	1.68
	200	0.84	0.52

Table 3. Robustness testing (n = 6)

Parameter	SD of peak area for Spironolactone	% RSD	SD of peak area for Hydrochlorothiazide	% RSD
Mobile phase composition (± 0.1 mL)	5.64	0.055	2.14	0.09
Amount of mobile phase (± 5%)	4.45	0.064	3.76	0.12
Time from spotting to chromatography (± 10 min.)	5.72	0.170	1.98	0.07
Time from chromatography to scanning (± 10 min.)	3.34	0.033	4.26	0.05

Table 1. Linearity studies

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International Journal of Science, Engineering and Management (IJSEM)

Vol 9, Issue 6, June 2022

Table 4. Applicability of the HPTLC method for the analysis of the pharmaceutical formulations

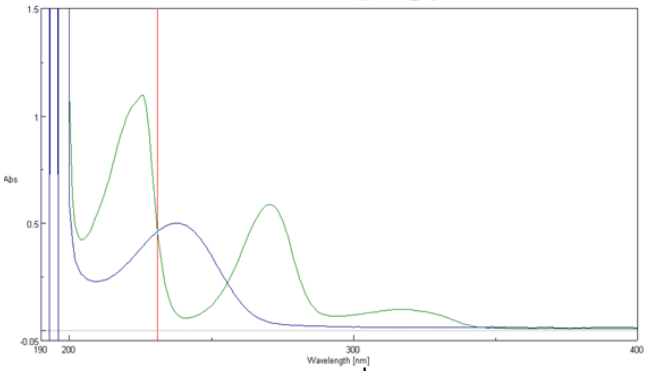
Drug	Label Claim (mg)	Drug content (%)	%RSD
Spironolactone	25	100.78	0.49
Hydrochlorothiazide	25	98.54	1.80

Table 5. Recovery studies

Drug	Label claim (mg per tablet)	Amount added (%)	Total amount (mg)	Amount recovered (mg)	Amount recovered (%)
Spironolactone	25	80	45	17.63	98.11
		100	50	19.64	100.42
		120	55	21.83	99.38
Hydrochlorothiazide	25	80	45	45.04	100.09
		100	50	49.18	98.36
		120	55	55.62	101.13

Table 6

Sr. No.	Property	Value
1.	Linear range i. Hydrochlorothiazide ii. Spironolactone	 120-270 ng/spot 100-350 ng/spot
	Regression equation i. Hydrochlorothiazide ii. Spironolactone	 y = 6.0457x - 171.58 y = 10.02x + 68.49
	Correlation coefficient i. Hydrochlorothiazide ii. Spironolactone	 R^2 = 0.9972 R^2 = 0.998
2.	Limit of detection for Hydrochlorothiazide Limit of quantitation for Hydrochlorothiazide Limit of detection for Spironolactone Limit of quantitation for Spironolactone	40 ng/spot 50 ng/spot 80 ng/spot 100 ng/spot
3.	% Recovery ± RSD i. Hydrochlorothiazide ii. Spironolactone	 98.11 %-100.42 % 98.36 %-101.13 %
4.	Precision ± RSD i. Hydrochlorothiazide ii. Spironolactone	 0.76-1.92 0.31-1.68
5.	Robustness	Robust
6.	Specificity	Specific



Overlay of Spironolactone + hydrocholorothiazide