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Development and Validation of a HPTLC Method for Simultaneous Determination of Furosemide and Spironolactone in Its Tablet Formulation

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ABSTRACT

The objective of the current study was to develop a simple, precise and accurate High Perfomance Thin Layer Chromatographic [HPTLC] assay method and validated for determination of furosemide and spironolactone in solid pharmaceutical dosage forms. The mobile phase comprising of ethyl acetate: haxane in the volume ratio of [80: 20, v/v] was employed for the elution. Standard solution was prepared in methanol. The standard concentration was 40 µg ml^{-1} of furosemide and 100 µg ml^{-1} of spironolactone. Chromatographic analysis was performed on a HPTLC plates precoated with 0.25 mm layer of chromatographic silica gel mixture [Silica GF254] on aluminum sheets. After development of the chromatographic plate, the detection was carried out using an Ultraviolet scanning densitometer set at a wavelength of 254 nm. The method was validated for specificity, linearity, precision, accuracy, robustness and solution stability. The method was linear in the drug the concentration range from 0.016-0.064 mg ml^{-1} for furosemide and 0.040-0.160 mg ml^{-1} for spironolactone with correlation coefficient 0.9958 and for Spironolactone with correlation coefficient 0.9975. The (relative standard deviation – RSD) values for intraday precision study and interday precision study was < 2.0 % for furosemide and spironolactone. The mean recovery for furosemide was 98.51 – 98.81 % and 98.20 –98.98 % for spironolactone.
Keywords*:* Spironolactone, Furosemide, Assay method, HPTLC method, Development and Validation

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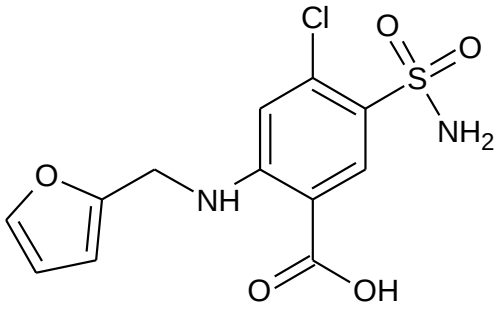
INTRODUCTION

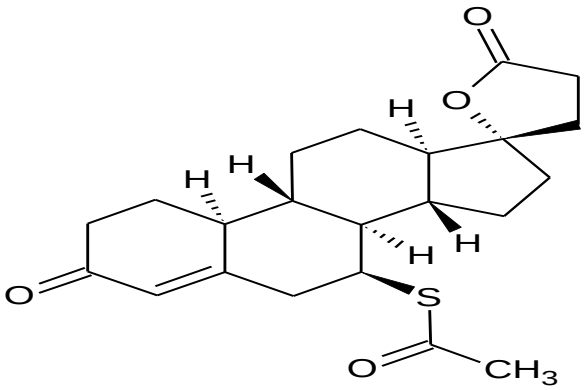
HPTLC [1-3] is the most simple separation technique today available to the analyst. It can be considered a time machine that can speed your work and allows you to do many things at a time usually not possible with other analytical techniques.

Figure 1: 4-Chloro-2-(furan-2-ylmethylamino)-5-sulfamoylbenzoic acid



Furosemide is chemically 4-chloro-2-(furan-2-ylmethylamino)-5-sulfa moylbenzoic acid (**Fig. 1**). Its CAS number is 54-31-9. Its molecular formula is $C_{12}H_{11}ClN_2O_5S$ having molecular weight 330.74gm/mole. Furosemide, an anthranilic acid derivative, is a potent diuretic that inhibits the active reabsorption of chloride in the diluting segment of the loop of Henle, thus preventing the reabsorption of sodium, which passively follows chloride [4]. This loop diuretic is commonly used for the treatment of renal diseases, congestive heart failure and hypertension [5]. Additionally, furosemide is a noncompetitive subtype-specific blocker of GABA-A receptors [6-8]. Furosemide has been reported to reversibly antagonize GABA-evoked currents of α6 β2 γ2 receptors at micromole concentrations, but not alpha1 β2 γ2 receptors [6-7]. During development, the alpha6 beta2 gamma2 receptor increases in expression in cerebellar granule neurons, corresponding to increased sensitivity to furosemide [7].

Figure: 2 7α-Acetylthio-3-oxo-17α-pregn-4-ene-21,17-carbolactone



Spironolactone is chemically 7α-acetylthio-3-oxo-17α-pregn-4-ene-21,17-carbolactone (**Fig. 2**). Its molecular formula is $C_{24}H_{32}O_4S$ having molecular weight 416.58 gm/mole.

Spironolactone inhibits the effect of aldosterone by competing for intracellular aldosterone receptors in the distal tubule cells (it actually works on aldosterone receptors in the collecting duct). This increases the excretion of water and sodium, while decreasing the excretion of potassium. Spironolactone has a fairly 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 preventing it from interacting with dihydro testosterone [8]. Spironolactone has anti-androgen activity by binding to the androgen receptor and preventing it from interacting with dihydrotestosterone[9].

Various publications are available regarding determination method of furosemide and spironolactone but most of the methods are applicable to alone spironolactone or furosemide in pharmaceutical dosage form or in biological fluids [10]. Potentiometric [11], colorimetric estimation[12], thin-layer chromatography [13], complexation [14], first-derivative and spectrophotometric [15], flow injection chemiluminescence method [16], proton nuclear magnetic resonance spectroscopic [17] and HPLC [18] methods are reported. Only few methods are reported for the simultaneous determination of spironolactone and furosemide [19]. Two methods are UV and UV derivative spectrophotometric determination of two-component mixtures which is able to determine spironolactone and furosemide in combined dosage form and fourth fluorimetric determination of spironolactone in the presence of frusemide and hydroflumethiazide in drug formulations [20]. As far as our knowledge is concern, No reported HPTLC method estimation of these drugs in tablet, taking into consideration the simplicity, cost effectiveness and reliability of HPTLC in the analysis of Drugs, an attempt was made to develop a new, simple and validated HPTLC method for simultaneous estimation of furosemide and spironolatone dosage form tablets. The HPTLC method was subjected to statistical validation and was applied for the determination of furosemide and spironolactone from its combined dosage form.

EXPERIMENTAL

Materials

Furosemide and spironolactone standard of was provided by Aarti Drugs Ltd., Boisar (India). Furosemide and spironolactone tablets containing 50 mg spironolactone and 20 mg furosemide and the inactive ingredient used in drug matrix were obtained from market. Ethyl acetate, hexane and methanol were purchased from Spechtrochem Ltd.

Instrumentation

Traditional Thin Layer Chromatography and its modern instrumental quatintative analysis version HPTLC are very popular for many resons such as visual chromatogram, simplicity, multiple sample handling, low running and maintenance costs, disposable layer etc. HPTLC is the fastest of all chromatographic methods. Very few HPTLC plates are required to handle a very large number of samples. Important scientific reasons for HPTLC's modest

popularity are that it is an open system due to which atmospheric conditions can affect the chromatographic process during development i.e. during separation. Humidity is found to be variable affecting reproduction of results. Most HPTLC's done today are performed on silica gel, a strongly hydrophilic substance. The nature of silica gel is such that it separates both by adsorption throughout its structural OH group as well as partition though its absorbed moisture content.

The HPTLC comprises of three sections. The lower section holds the 20 X 10 cm twin through chamber. The upper half is robotic where the plate is held for pre-conditioning the layer before development specified in the method as well as for drying the plate uniformly after development. The top section contains the electronics.

The chromatographic system used to perform development and validation of this assay method was Camag Linomat V Sample applicator, Camag Twin trough glass chamber and Camag TLC scanner III equipped with Cats 3 Version software.

Mobile phase preparation

The mobile phase comprising of ethyl acetate: haxane in the volume ratio of (80: 20, v/v) was employed for the elution.

Diluents preparation

Use methanol used as a diluent.

Standard preparation

Furosemide standard stock solution containing 200 µg ml^{-1} was prepared in a 100 ml volumetric flask by dissolving 20.00 mg of Furosemide and then diluted to volume with diluent. Further take 10 ml of this stock solution in 50 ml volumetric flask and make up to mark with diluent (this standard solution of 40µg ml^{-1}). Spironolactone standard stock solution containing 500 µg ml^{-1} was prepared in a 100 ml volumetric flask by dissolving 50.00 mg of spironolactone and then diluted to volume with diluent. Further take 10 ml of this stock solution in 50 ml volumetric flask and make up to mark with diluent (this standard solution of 100 µg ml^{-1}).

Test preparation

Twenty tablets were weighed and the average weight of tablet was determined. From these, five tablets were weighed and transfer into a 500 ml volumetric flask. About 50 ml of diluent was added and sonicate for a minimum 30 minute. with intermittent shaking. Then content was brought back to room temperature and diluted to volume with diluent. The sample was filtered through 0.45µm nylon syringe filter. Further take 10 ml of this stock solution in 50

ml of volumetric flask and make up to mark with diluent. The concentration obtained was 40 µg ml^{-1} of furosemide and 100 µg ml^{-1} of spironolactone.

Chromatographic conditions

Chromatographic analysis was performed on a HPTLC plates precoated with 0.25 mm layer of chromatographic silica gel mixture (Silica GF254) on aluminum sheets. After development of the Cromplate, The detection was carried out using an UV scanning densitometer set at a wavelength of 254 nm.

RESULTS AND DISCUSSION

Development and Optimization of the HPTLC Method

Selection of Chromatographic Condition

In the present work, an analytical method based on HPTLC was developed and validated for assay determination of furosemide and spironolactone in tablet formulation. The various steps involved in TLC/HPTLC are Sample preparation, TLC plate pretreatment, and Sample application, Drying, Evalution and Documentation. Thin layer chromatography is an off-line technique as compared to HPLC/GC but the properties that govern the selection of a method and its components are essentially same.

Following are the factors or steps involved in method development by TLC, which results in good separation, which is one of the most critical steps in qualitative and quantitative analysis 1)selection of stationary phase, 2)Selection of vapour phase, 3) Selection of suitable solvent, 4) Optimisation of mobile phase, 5)Selection of development mode ,6)Selection of other operating parameters.

Traditionaly TLC is inexpensive, simple to use and requires minimal instrumentation, laboratory space and maintenance. However, to achieve good precision, accuracy and reproducibility, a certain degree of instrumentation is required and dansitometric detection is necessary for quantification.

Standardization of experimental condition

The extent of separation of various components of a mixture by a given Thin layer chromatography method depends on the separation efficiency and selectivity of the separating system. The various factors influencing the separation are type of stationary phase, type of pre-coated plate(TLC/HPTLC), layer thickness, binder in the layer, mobile phase, solvent purity, size of the developing chamber ,saturation of the chamber (pre-equilibrium), relative humidity, temperature and separation distance

Mobile phase optimization

The mobile phase in TLC is generally selected by controlled trial and error method. In normal phase, TLC separation is carried out on a non-aqueous mobile phase(silica gel) using a non-aqueous mobile phase. Developing solvent usually, is a mixture of non-polar organic solvent with a polar modifier such as methanol, ethyl acetate, acetone, acetonitrile to control the solvent strength and selectivity.

Sometimes small amounts of third component such asacetic acid, ammonia, triethylamine and formic acid are added to mobile phase because they partially modify the surface of silica gel. Keeping the acidic and the basic centers in a molecule nonionised; leads to decrease in the tailing of polar sample components.
The selection of the mobile phase is of prime importance in the development of a chromatographic technique for proper elution, resolution, spot definition, symmetrical peak shapes and Rf reproducibility of the analytes.

In present research work, initial trials were done using mobile phase with used these solvent like chloroform, methanol, ethyl acetate and hexane. It was observed that resolution between the two actives was good but the Rf value was not meeting the acceptance criteria. Hence, the final method set was by using Ethyl acetate: Hexane (80:20) for better separation as well as reproducibility of Rf value.
Thus, optimized mobile phase used for separation was Ethyl acetate: Hexane in volume ratio of 80:20(v/v).

Both components i.e. furosemide and spironolactone are found to be soluble in methanol was choosen as diluents for standard as well as sample preparation. Recovery of actives in accuracy was good with the use of methanol as solvent.

Selection of the most suitable wavelength for densitometric scanning

In case of multi component pharmaceuticals preparations, containing more than one active ingredient with variable concentrations, if the TLC plate is scanned at maximum absorbance of each component the purpose of using TLC, as a high throughput device is lost. Hence, in such cases one has to explore the possibility of selecting a wave length at which the entire chromatogram can be scanned without losing any vital information about different components in the formulation. The single wavelength referred to as “most suitable wavelength” In the present research work the most suitable wavelength was found to be 254 nm as can be seen from the UV spectrum where in all both actives showed good absorption. (**Fig. 3**)

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Optimized chromatographic conditions for HPTLC

Optimized chromatographic conditions for HPTLC method as under,

Parameters	Chromatographic Conditions
Development chamber	CAMAGE Twin Trough Chamber
Stationary phase	Silica gel GF254 precoated on aluminum sheet
Mobile phase	Ethyl acetate: Hexane(80:20)
Chamber saturation	45 mins
Sample applicator	CAMAGE LINOMAT V
Band	6mm
Space	9 mm
Scanning speed	20mm/sec
Development distance	8 cm
Drying of plate	Room temperature
Densitometric scanner	CAMAGE TLC SCANNER
Lamp	Deuterium
Wavelength	254 nm
Volume	5µl

Fig. 4 and **5** represent the densitometry chromatograms of standard and test preparation respectively.

Figure 3: UV Scan Spectrum of standards

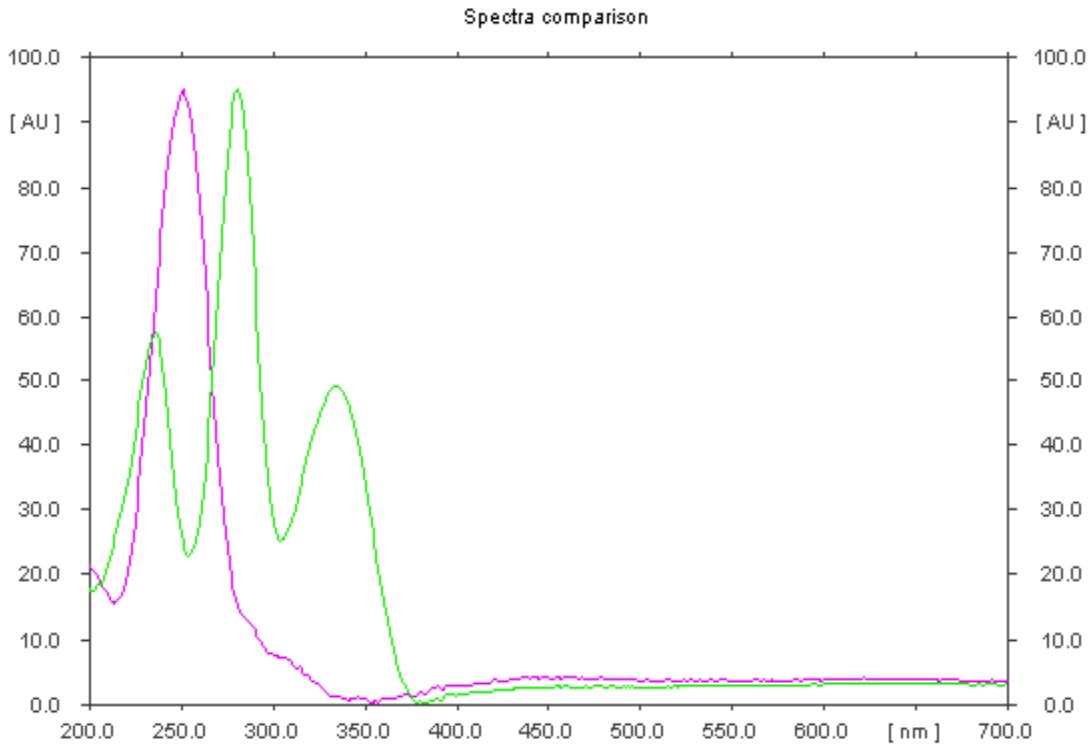

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Figure 4: Chromatogram of standard preparation

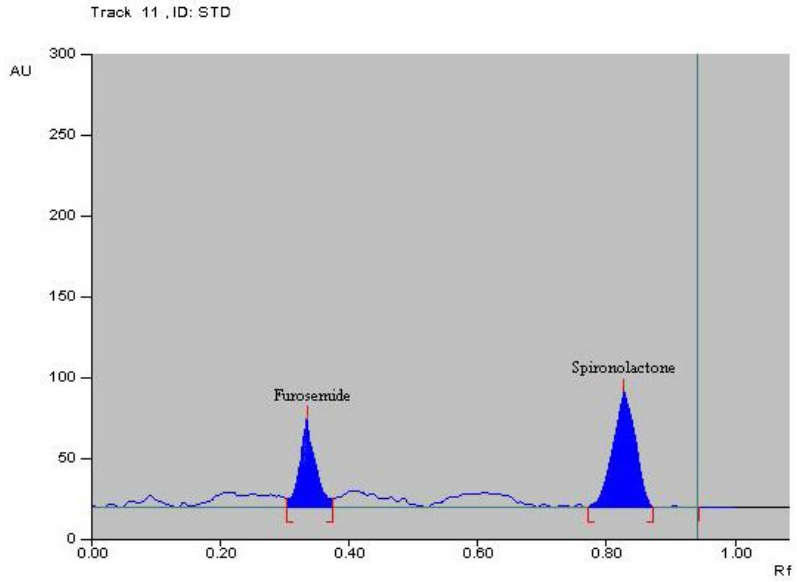
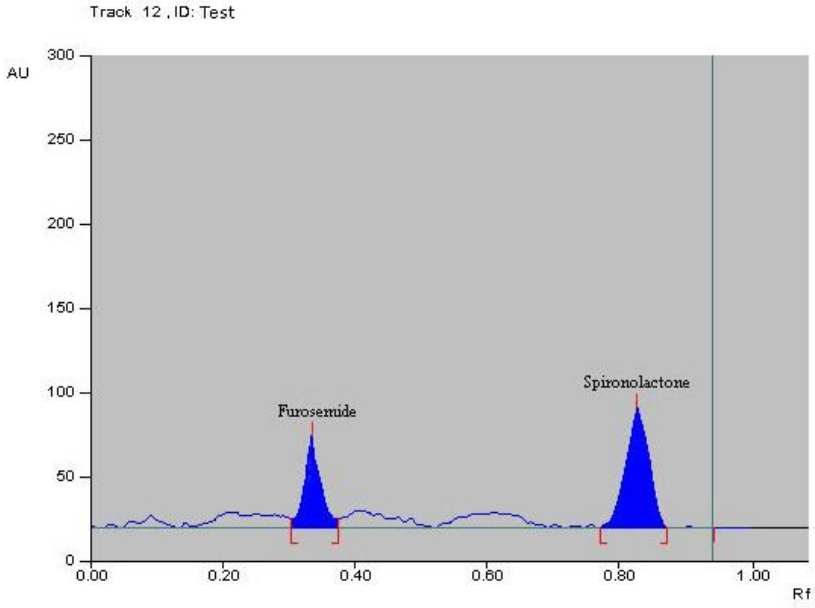


Figure 5: Chromatogram of test preparation



METHOD VALIDATION

Specificity

In an assay, demonstration of specificity requires that it can be shown that the procedure is unaffected by the presence of impurities or excipients. In practice, this can be done by spiking the drug substance or product with appropriate levels of impurities or excipients and demonstrating that the assay results is unaffected by the presence of these

extraneous materials. There should be no interference of the diluents / placebo at Rf value of drug substances.

Linearity

For linearity seven points calibration curve were obtained in a concentration range from 0.016-0.064 mg ml^{-1} for furosemide and 0.040-0.160 mg ml^{-1} for spironolactone. The response of the drug was found to be linear in the investigation concentration range and the linear regression equation for furosemide was y = 28661x + 38.477 with correlation coefficient 0.9958 (**Fig. 6)** and for spironolactone was y = 38237x + 47.754 with correlation coefficient 0.9975 (**Fig. 7**). Where x is the concentration in mg ml^{-1} and y is the peak area in absorbance unit.

Figure 6: Linearity curve for furosemide

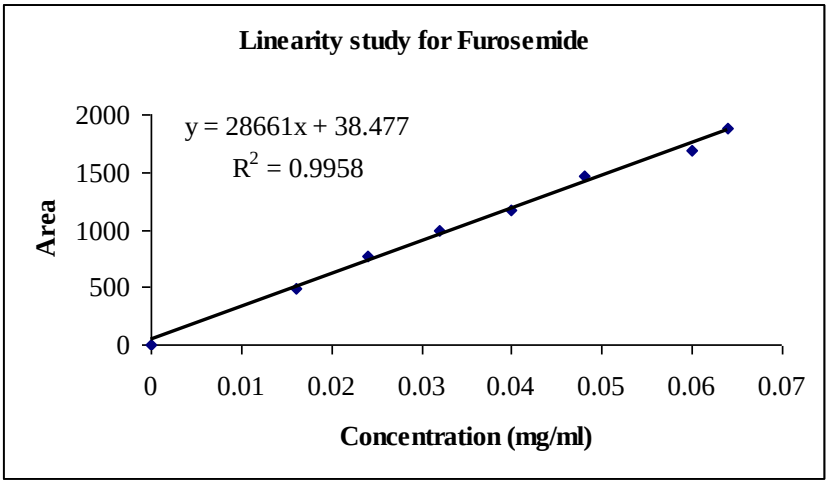
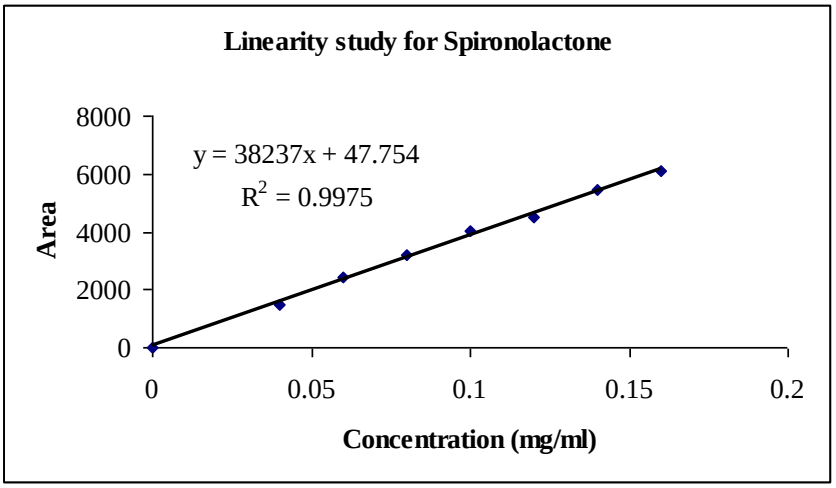


Figure 7: Linearity curve for spironolactone



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Precision

Data obtain from precision experiments are given in **Table 1** for intraday and interday precision study for both furosemide and spironolactone. The RSD values for intraday precision study and interday precision study was < 2.0 % for furosemide and spironolactone. Which confirm that the method was precise.

Table 1: Results of precision study

Set	Furosemide(%Assay)		Spironolactone(%Assay)	
	Intraday (n = 6)	Interday (n = 6)	Intraday (n = 6)	Interday (n = 6)
1	98.0	97.1	97.9	98.5
2	99.9	100.3	97.6	99.2
3	100.7	98.7	97.2	98.1
4	98.0	98.6	99.3	100.7
5	96.6	97.2	100.2	99.5
6	100.1	100.3	96.5	97.6
Mean	98.9	98.7	98.1	98.9
Standard deviation	1.58	1.42	1.35	1.10
% RSD	1.60	1.44	1.37	1.11

Table 2: Results of accuracy study

	Level (%)	Theoretical concentration[a] (µg ml^{-1})	Observed concentration[a] (µg ml^{-1})	% Recovery	% RSD
Furosemide	50	20.73	20.43	98.53	0.59
	100	40.00	39.40	98.51	1.01
	150	40.60	59.68	98.81	0.14
Spironolactone	50	50.47	49.94	98.95	0.32
	100	100.40	99.59	99.20	1.23
	150	150.33	148.80	98.98	1.37

[a] Each value corresponds to the mean of three determinations

Accuracy

Recovery of furosemide and spironolactone were determined at three different concentration levels. The mean recovery for furosemide was 98.51–98.81 % and 98.20–98.98 % for spironolactone (**Table 2**).

Solution stability study

Stability of sample solution was checked by using sample preparation from preparation from precision study stored at room temperature for 24 hours; withdrawn in the intervals of 2 hrs, 4hrs, 12hrs and 24hrs and applied on the chromatoplate. After development, the

chromatogram was evaluated for additional spots if any. There was no indication of compound instability in the sample solution.

Stability on the sorbent layer prior to development (spot stability): The time wherein the sample is left to stand on the sorbent prior to chromatographic development can influence the stability of separated spots and are required to be investigated for validation. Two dimensional chromatography using same solvent system was used to find out any decomposition occurs during spotting and development. In case, if decomposition occurs during development, peaks of decomposition products are obtained for the analyte both in the first and second direction of run. No decomposition was observed during spotting and development.

Robustness

The result of robustness study of the developed assay method was established in Table 4 and Table 5. The result shown that during all variance conditions, assay value of the test preparation solution was not affected and it was in accordance with that of actual. System suitability parameters were also found satisfactory; hence the analytical method would be concluded as robust.

Table 4: Evaluation data of robustness study of furosemide

Robust conditions	% Assay	System suitability parameters	
		Area	Rf value
Ethyl acetate.: Hexane(82:18)	99.4	1042	35
Ethyl acetate.: Hexane(78:22)	98.3	1243	31
Analyst Change	98.5	1144	33

Table 5: Evaluation data of robustness study for spironolactone

Robust conditions	**% Assay**	**System suitability parameters**	
		Area	**Rf value**
Ethyl acetate :Hexane(82:18)	98.1	3986	86
Ethyl acetate :Hexane(78:22)	98.3	4289	82
Analyst Change	98.5	4123	84

System suitability

A system suitability test of the chromatographic system was performed before each validation run to confirm the suitability and reproducibility of the system. The system suitability experiment as carried out using 40 ppm of furosemide and 100 ppm of spironolactone. This solution was spotted five times on the chromatographic plate under the optimized conditions. Parameters that were studied to evaluate the suitability of the system where RSD of peak area and % RSD of retention factor of drug peak The R_f value of individual spotted should be comparable with the corresponding Rf value of system suitability solution.(Table 6)

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Table 6: System suitability

No.	100% Level			
	Furosemide		Spironolactone	
	Peak area	Rf value	Peak area	Rf value
1	1220	34	4033	84
2	1243	33	4071	85
3	1236	33	4129	84
4	1205	32	4181	81
5	1280	31	4210	83
Mean	1237	32.6	4125	83.4
S.D.	28.27897	1.14	73.750932	1.52
%RSD	2.29	3.50	1.79	1.82

The value of % Relative standard deviation of 5 replicates of peak area of bands should be well less than 5.0 %. Whereas %Relative standard deviation of R_f value should be less than 10.0 %. From the above results, it can be seen that the %Relative standard deviation for all parameter is well below the required limit for all the parameters.

To demonstrated that that the proposed method is adequate for its intended use, the method was subjected to statistical validation to determine linearity, accuracy and precision.

CONCLUSION

A new analytical method has been developed to be routinely applied to determine furosemide and spironolactone in pharmaceutical dosage form. In this study, stability of furosemide and spironolactone in present dosage form was established through employment of ICH recommended stress condition. The developed procedure has been evaluated over the specificity, linearity, accuracy, precision and robustness in order to ascertain the stability of the analytical method. It has been proved that it was specific, linear, precise, accurate and robust. Hence, the method is recommended for routine quality control analysis and stability sample analysis.

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REFERENCES

[1] Sherma J, Fried B. Handbook of thin layer chromatography. New York: Marcel Dekker, Inc 3rd ed., 2003; 3-4.
[2] Sethi PD. HPTLC: Quantitative analysis of pharmaceutical formulations. 1st ed. New Delhi: CBS Publisher 1996; 44-57.
[3] Peter EW. Thin layer chromatography: A modern practical approach. UK: The royal society of chemistry 2005; 6-154.
[4] Berardesca E, Gabba P, Ucci G, Borroni G, Rabbiosi G. Int J Tissue React 1988; 10(2): 115.
[5] Jacobson HR, Kokko JP. Annu Rev Pharmacol Toxicol 1976; 16: 201.
[6] Brater DC. Drugs 1991; 41: 14.
[7] Korpi ER, Kuner T, Seeburg PH, Luddens H. Mol Pharmacology 1995; 47(2): 283–289.
[8] www.sanofi-aventis.com
[9] www.archneur.ama-assn.org.
[10] Dreux C, Halter D. Hop Serv Biochim, Annales de Biologie Clinique 1976; 34(2): 113-120.
[11] Santini AO, Pezza HR, Sequinel R, Rufino JS, Pezza L. J of the Brazilian Chem Soc 2009; 20(1): 64-73.
[12] Mishra P, Katrolia D, Agrawal RK. Current Science 1989; 58(9): 503-505.
[13] Hadzija BW, Mattocks AM. J Chromatography 1982; 229(2) : 425-32.
[14] Goelcue A. Journal of Anal Chem 2006; 61(8): 748-754.
[15] Abdel-Hay HM. Int J Pharma 1983; 99(2-3): 333-336.
[16] Shu-hua He, Chao-yin Li, Sichuan Shifan Daxue Xuebao. Ziran Kexueban 2007; 30(6): 787-789.
[17] Hanna GM, Lau-Cam CA. J AOAC Int 1993;76(3): 526-530.
[18] Denia Mendes de Sousa Valladao, Ionashiro M, Netto JZ. Quimica Nova 2008; 31(1): 44-46.
[19] Parimoo P, Bharathi A, Padma K. Ind J Pharm Sci 1995; 57(3): 126-129.
[20] Salem H, El-Maamli Magda, Mohamed El-Sadek, Afaf Aboul Kheir. Spectro Lett 1991; 24(3): 451-470.