Journal of Chromatographic Science 2012;50:721–726
doi:10.1093/chromsci/bms062 Advance Access publication May 28, 2012

Article

Development and Validation of a Stability-Indicating HPLC Assay Method for Simultaneous Determination of Spironolactone and Furosemide in Tablet Formulation

Vijay R. Ram [1], Pragnesh N. Dave [1] and Hitendra S. Joshi [2*]

[1]Department of Chemistry, Krantiguru Shyamji Krishna Verma Kachchh University, Bhuj-370 001, Gujarat, India, and
[2]Department of Chemistry, Saurashtra University, Rajkot-360 005, Gujarat, India

*Author to whom correspondence should be addressed. Email: ram_vijay1982@yahoo.co.in

Received 14 October 2011; revised 16 November 2011

The objective of the current study was to develop and validate a simple, precise and accurate isocratic stability-indicating reversed-phase high-performance liquid chromatography (RP-HPLC) assay method for the determination of spironolactone and furosemide in solid pharmaceutical dosage forms. Isocratic RP-HPLC separation was achieved on an SGE 150 × 4.6 mm SS Wakosil II 5C8RS 5-μm column using a mobile phase of acetonitrile–ammonium acetate buffer (50:50, v/v) at a flow rate of 1.0 mL/min. The detection was carried out at 254 nm using a photodiode array detector. The drug was subject to oxidation, hydrolysis, photolysis and heat to apply stress conditions. The method was validated for specificity, linearity, precision, accuracy, robustness and solution stability. The method was found to be linear in the drug concentration range of 40–160 μg/mL with correlation coefficients of 0.9977 and 0.9953 for spironolactone and furosemide, respectively. The precision (relative standard deviation; RSD) among a six-sample preparation was 0.87% and 1.1% for spironolactone and furosemide, respectively. Repeatability and intermediate precision (RSD) among a six-sample preparation were 0.46% and 0.20% for spironolactone and furosemide, respectively. The accuracy (recovery) was between 98.05 and 100.17% and 99.07 and 100.58% for spironolactone and furosemide, respectively. Degradation products produced as a result of stress studies did not interfere with the detection of spironolactone and furosemide; therefore, the assay can be considered to be stability-indicating.

Introduction

Stress testing is a part of developmental strategy under International Conference on Harmonization (ICH) requirements and is carried out under more severe conditions than accelerated conditions. These studies serve to give information on a drug's inherent stability and assist in the validation of analytical methods to be used in stability studies (1–3). It is suggested that stress testing should include the effects of temperature, light, oxidizing agents and susceptibility across a wide range of pH values. It is also recommended that analysis of stability samples should be accomplished through the use of a validated stability-testing method.

Spironolactone is chemically 7α-acetylthio-3-oxo-17α-pregn-4-ene-21,17-carbolactone (Figure 1). Its molecular formula is $C_{24}H_{32}O_4S$ and its molecular weight is 416.58 gm/mol. 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; similarly, the effect diminishes slowly. Spironolactone has anti-androgen activity by binding to the androgen receptor and preventing it from interacting with dihydrotestosterone (4).

Furosemide is chemically 4-chloro-2-(furan-2-ylmethylamino)-5-sulfamoylbenzoic acid (Figure 2). Its molecular formula is $C_{12}H_{11}ClN_2O_5S$ and its molecular weight is 330.74 gm/mol. Furosemide, an anthranilic acid derivative, is a potent diuretic that inhibits the active reabsorption of chloride in the diluting segment of Henle's loop, thus preventing the reabsorption of sodium, which passively follows chloride (5). This loop diuretic is commonly used for the treatment of renal diseases, congestive heart failure and hypertension (6).

Some methods, either individual or in a combination of spironolactone and furosemide, have been reported, including simultaneous determination of spironolactone with hydroflumethiazide and spironolactone with furosemide in combination formulations by ultraviolet (UV) absorption and UV derivative spectrophotometry, respectively (7–8); spectrophotometric determination using complex formation with Cu(II) (9); liquid chromatography for biological samples (10–12) and thin-layer chromatographic determination (13).

According to a literature survey, no validated stability-indicating high-performance liquid chromatography (HPLC) assay method is available for the simultaneous determination of spironolactone and furosemide in pharmaceutical formulations. This paper deals with the forced degradation of spironolactone and furosemide under acidic hydrolysis and alkali hydrolysis, and oxidation, thermal and photolytic stress conditions. This paper also describes the validation of the developed method for the assay of spironolactone and furosemide from its dosage form (tablets).

Experimental

Materials

Spironolactone and furosemide standards were provided by Alembic Pharmaceuticals Ltd. (Baroda, India). Spironolactone and furosemide tablets containing 50 mg spironolactone and 20 mg furosemide and the inactive ingredient used in drug matrix were obtained from a market. HPLC-grade acetonitrile and water were obtained from Spectrochem (Mumbai India). Analytical-grade ammonium acetate, hydrochloric acid, glacial acetic acid, sodium hydroxide pellets and 30% v/v hydrogen peroxide solution were obtained from Ranbaxy Fine Chemicals (New Delhi India).

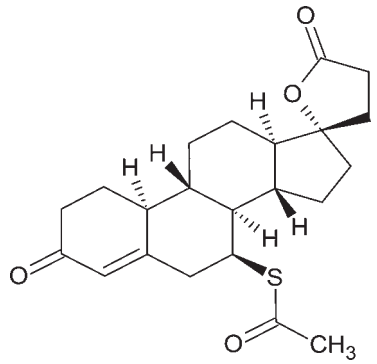

Figure 1. Chemical structure of spironolactone (7α-acetylthio-3-oxo-17α-pregn-4-ene-21,17-carbolactone).

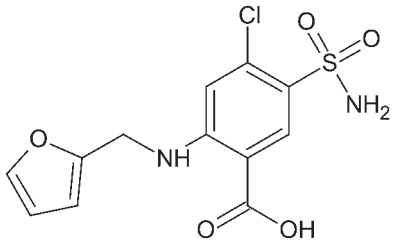

Figure 2. Chemical structure of furosemide [4-Chloro-2-(furan-2-ylmethylamino)-5-sulfamoylbenzoic acid].

Instrumentation

The chromatographic system used to perform development and validation of this assay method comprised an LC-10ATvp binary pump, an SPD-M10Avp photodiode array detector and a rheodyne manual injector model 7725i with a 20-μL loop (Shimadzu; Kyoto, Japan) connected to a multi-instrument data acquisition and data processing system (Class-VP 6.13 SP2, Shimadzu).

Chromatographic conditions

Chromatographic analysis was performed on an SGE SS Wakosil II 5C8RS column (150 × 4.6 mm i.d., 5-μm particle size) column. The mobile phase consisted of acetonitrile–0.01M ammonium acetate buffer, pH 3.9 (50: 50, v/v). To prepare the buffer solution, 0.7708 g ammonium acetate were weighed and dissolved in 1,000 mL HPLC-grade water and then adjusted to pH 3.9 with glacial acetic acid. The mobile phase was filtered through a 0.45-μm nylon membrane (Millipore; Bangalore, India) and degassed in an ultrasonic bath (Spincotech; Mumbai, India). The flow rate of the mobile phase was adjusted to 1.0 mL/min and the injection volume was 20 μL. Detection was performed at 254 nm.

Standard preparation

A spironolactone standard stock solution containing 500 μg/mL was prepared in a 100-mL volumetric flask by dissolving 50.00 mg of spironolactone and then diluting to volume with diluent. Next, 10 mL of this stock solution were placed in a 50-mL volumetric flask and made up to mark with diluent (the final standard solution was 100 μg/mL). A furosemide standard stock solution containing 200 μg/mL was prepared in a 100-mL volumetric flask by dissolving 20.00 mg of furosemide and then diluting to volume with diluent. Next, 10 mL of this stock solution were placed in a 50-mL volumetric flask and made up to mark with diluent (the final standard solution was 40 μg/mL).

Test preparation

Twenty tablets were weighed and the average weight of each tablet was determined. From these, five tablets were weighed and transferred into a 500-mL volumetric flask. Approximately 50 mL of diluent was added and the mixture was sonicated for a minimum of 30 min with intermittent shaking. Then content was brought back to room temperature and diluted to volume with diluent. The sample was filtered through a 0.45-μm nylon syringe filter. Next, 10 mL of this stock solution were placed in a 50-mL volumetric flask and made up to mark with diluent. The final concentration was 100 μg/mL of spironolactone and 40 μg/mL of furosemide.

Forced degradation study

The degradation samples were prepared by transferring powdered tablets, equivalent to 50 mg spironolactone and 20 mg of furosemide, into a 250-mL round-bottom flask. Then, prepared samples were employed for acidic, alkaline and oxidant media, and also for thermal and photolytic conditions. After the degradation treatments were completed, the stress content solutions were allowed to equilibrate to room temperature and diluted with mobile phase to attain 100 μg/mL concentrations of spironolactone and 40 μg/mL concentrations of furosemide. Specific conditions are described in the following.

Acidic degradation condition

Acidic degradation study was performed by adding the drug content to 0.01 N HCl at room temperature for 3.0 h until the mixture was neutralized.

Alkali degradation condition

Alkaline degradation study was performed by adding the drug content to 0.05 N NaOH at room temperature for 3.0 h until the mixture was neutralized.

Oxidative degradation condition

Oxidation degradation study was performed by adding the drug content to 30% v/v H_2O_2 at room temperature for 3 h.

Thermal degradation condition

Thermal degradation was performed by exposing the solid drug at 80°C for 72 h.

Photolytic degradation condition

Photolytic degradation study was performed by exposing the drug content to UV light for 72 h.

Method validation

Specificity study

The evaluation of the specificity of the method was determined against a placebo. The interference of the excipients of the claimed placebo present in pharmaceutical dosage form was derived from a placebo solution. Furthermore, the specificity of the method toward the drug was established by checking the interference of the degradation products in the drug quantification for assay during the forced degradation study.

Linearity

Linearity test solutions for the assay method were prepared at seven concentration levels from 40 to 160% of assay analyte concentration (40, 60, 80, 100, 120, 140 and 160 μg/mL). The peak areas versus concentration data were evaluated by linear regression analysis.

Precision

The precision of the assay method was evaluated in terms of repeatability by performing six independent assays of spironolactone and furosemide test sample preparation and calculating the percent relative standard deviation (%RSD) of the assay (intra-day). Intermediate precision of the method was checked by another person performing the same procedure on a different day (inter-day) under the same experimental conditions.

Accuracy

An accuracy study was performed by adding known amounts of spironolactone and furosemide to the placebo preparation. The actual and measured concentrations were compared. Recovery of the method was evaluated at three different concentration levels (corresponding to 50, 100 and 150% of test preparation concentration). For each concentration level, three sets were prepared and injected in duplicate.

Robustness

The robustness study was performed to evaluate the influence of small but deliberate variations in the chromatographic conditions. The factors chosen for this study were the flow rate ($\pm$0.1 mL/min), mobile phase composition [acetonitrile–buffer (48:52 and 52:48, v/v)], buffer pH ($\pm$0.2 pH) and using different lots of LC columns.

Solution stability

The stability of the solution was evaluated for test preparation. The solution was stored at ambient temperature and 2–5°C and tested at intervals of 12, 24, 36 and 48 h. The responses for the aged solution were evaluated using a freshly prepared standard solution.

Result and Discussion

To develop a rugged and suitable HPLC method for the quantitative determination of spironolactone and furosemide, the analytical conditions were selected after testing different parameters such as diluents, buffer, buffer concentration, organic solvent for mobile phase, mobile phase composition and other chromatographic conditions. Our preliminary trials, using different compositions of mobile phases consisting of water with methanol or acetonitrile, did not give good peak shapes. By using 0.01M ammonium acetate buffer, adjusted to pH 3.9 with glacial acetic acid and keeping the mobile phase composition as acetonitrile–ammonium acetate buffer (50:50, v/v), the best peak shape was obtained. For the selection of organic constituent of the mobile phase, acetonitrile was chosen to reduce retention time and to attain good peak shapes. A chromatogram of the standard preparation is shown in Figure 3. A system suitability test of the chromatographic system was performed before each validation run. Five replicate injections of standard preparation were injected and asymmetry, theoretical plate and %RSD of peak area were determined. For all system suitability injections, asymmetry was less than 2.0, theoretical plate was greater than 5,000 for spironolactone and 2,500 for furosemide and %RSD of peak area was less than 2.0. The specificity of the method was determined by checking the interference of the placebo with the analyte and the proposed method was eluted by checking the peak purity of spironolactone and furosemide during the forced degradation study. The peak purity of spironolactone and furosemide was found to satisfactory under different stress conditions. There was no interference of any peaks of degradation product with drug peaks. Major degradation was found in acidic

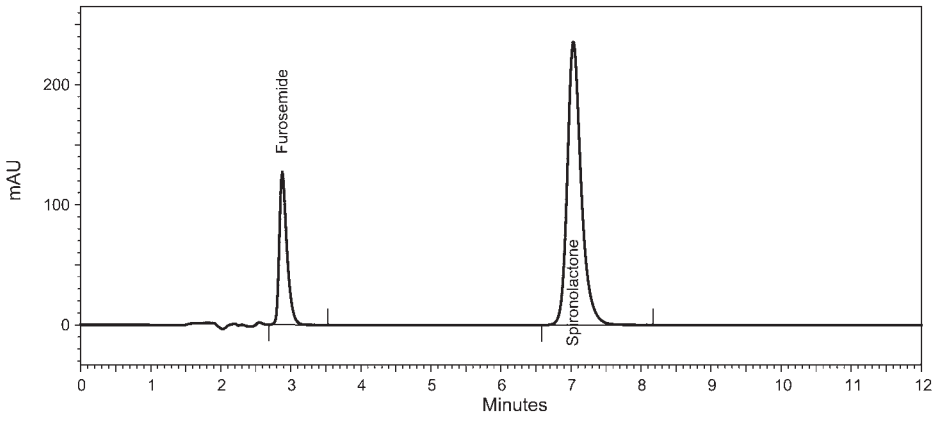

Figure 3. Chromatogram of standard preparation.

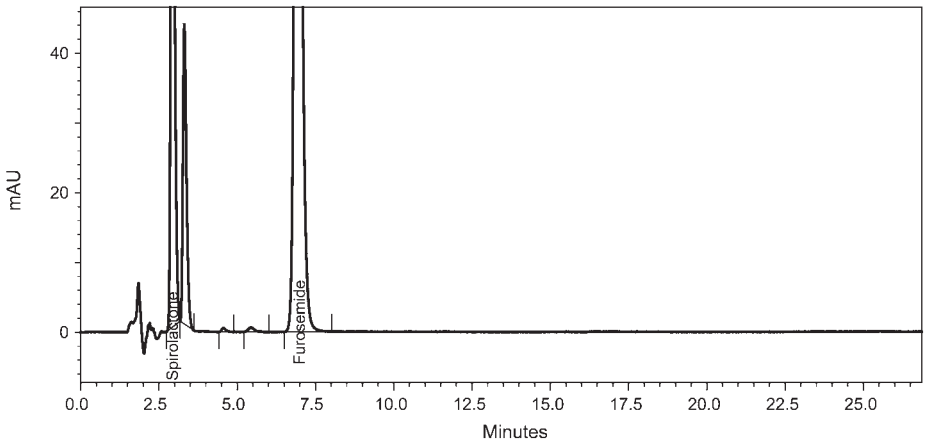

Figure 4. Chromatogram of acidic degradation study.

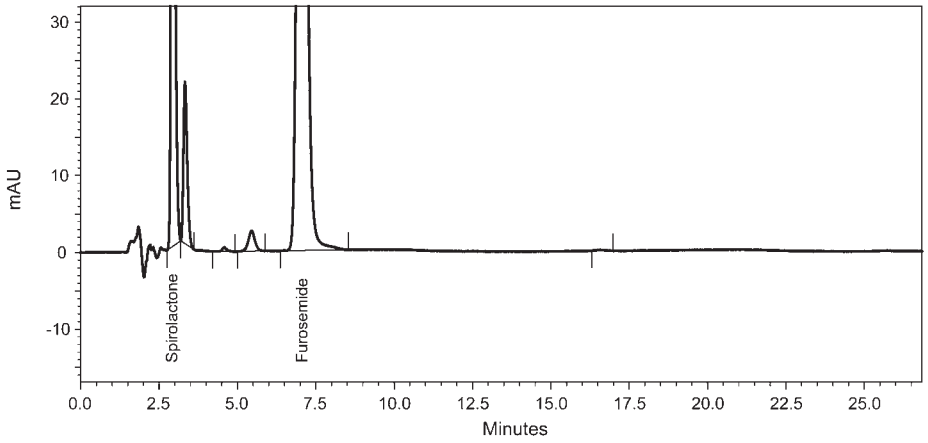

Figure 5. Chromatogram of alkali forced degradation study.

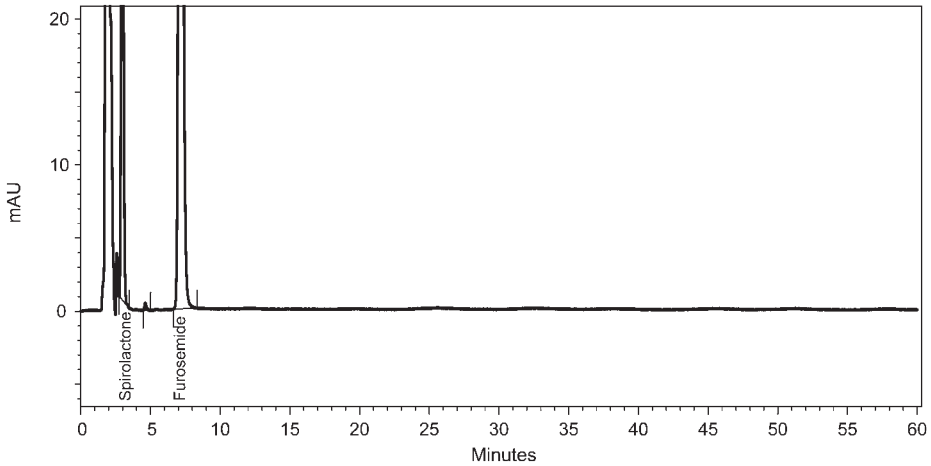

Figure 6. Chromatogram of oxidative forced degradation study.

conditions; the product was degraded up to 8.72%. The major impurity peak was found at 3.307 min (Figure 4). In alkali degradation, it was found that approximately 5.67% of the drug degraded at 3.328 min (Figure 5) and in oxidative conditions, approximately 0.1–0.2% of the drug degraded (Figure 6). Spironolactone and furosemide were found to be slightly degraded in photolytic conditions, but stable under the thermal degradation. Seven-point calibration curves were

Table I
Evaluation Data of Precision Study

Set	Spironolactone (% assay)		Furosemide (% assay)	
	Intra-day ($n = 6$)	Inter-day ($n = 6$)	Intra-day ($n = 6$)	Inter-day ($n = 6$)
1	100.7	100.7	100.4	98.9
2	101.1	100.9	100.5	98.9
3	99.0	100.3	98.4	98.7
4	100.7	99.7	98.0	98.5
5	100.2	100.8	99.3	98.5
6	101.6	100.3	100.9	98.4
Mean	100.5	100.4	99.6	98.6
Standard deviation	0.87	0.46	1.18	0.20
%RSD	0.87	0.46	1.19	0.20

Table II
Evaluation Data of Accuracy Study

	Level (%)	Theoretical concentration* (μg/mL)	Observed concentration* (μg/mL)	% Recovery	%RSD
Spironolactone	50	50.34	50.42	100.17	0.49
	100	99.40	97.58	98.17	1.68
	150	148.73	145.83	98.05	1.38
Furosemide	50	20.85	20.73	100.58	0.32
	100	40.03	40.00	100.08	1.23
	150	59.64	60.20	99.07	1.37

*Each value corresponds to the mean of three determinations.

obtained in a concentration range from 40–160 μg/mL for spironolactone and furosemide. The response of the drug was found to be linear in the investigated concentration range, with correlation coefficients of 0.9977 and 0.9953, respectively, for spironolactone and furosemide. The results of the repeatability and intermediate precision studies for spironolactone and furosemide are shown in Table I. The developed method was found to be precise, because the %RSD values for the repeatability and intermediate precision studies were <0.87% and <0.46%, for spironolactone and <1.19% and <0.20% for furosemide. The HPLC area responses for accuracy determination are depicted in Table II. The results show that the best recoveries (98.05–100.17%) of the spiked drug were obtained at each added concentration for spironolactone, and that best recoveries (99.07–10.58%) of the spiked drug were obtained at each added concentration for furosemide, indicating that the method is accurate. Table III shows the results obtained in the solution stability study at different time intervals for test preparation. The test preparation solution was found to be stable up to 48 h at 2–5°C and ambient temperature, because during this time, the result did not decrease below the minimum percentage. The results of the robustness study of the developed assay method are shown in Tables IV and V. The results show that during all variance conditions, the assay value of the test preparation solution was not affected and in accordance with that of actual values. System suitability parameters were also found to be satisfactory; hence, the analytical method can be concluded to be robust.

Table III
Evaluation Data of Solution Stability Study

Intervals	% Assay for test solution stored at 2–8°C		% Assay for test solution stored at ambient temperature	
	Spironolactone	Furosemide	Spironolactone	Furosemide
Initial	98.6	98.8	98.2	98.3
12 h	99.9	99.7	98.9	101.0
24 h	100.7	99.9	99.4	101.1
36 h	99.4	100.5	100.5	101.4
48 h	99.5	102.4	101.6	102.1

Table IV
Evaluation Data of Robustness Study of Spironolactone

Robust conditions	% Assay	System suitability parameters	
		Theoretical plates	Asymmetry
Flow 0.9 mL/min	101.8	6,332	1.55
Flow 1.1 mL/min	102.0	6,025	1.37
Buffer pH 3.7	97.9	5,987	1.35
Buffer pH 4.1	98.5	6,101	1.34
Buffer–ACN (48:52, v/v)	99.9	6,157	1.37
Buffer–ACN (52:48, v/v)	98.8	5,967	1.33
Column change	100.0	6,012	1.39

Table V
Evaluation Data of Robustness Study of Furosemide

Robust conditions	% Assay	System suitability parameters	
		Theoretical plates	Asymmetry
Flow 0.9 mL/min	101.9	3,391	1.55
Flow 1.1 mL/min	102.1	2,907	1.49
Buffer pH 3.7	99.9	3,001	1.46
Buffer pH 4.1	99.4	2,775	1.63
Buffer–ACN (48:52, v/v)	100.5	3,172	1.66
Buffer–ACN (52:48, v/v)	99.7	3,081	1.48
Column change	100.9	2,997	1.53

Conclusion

A new analytical method was developed to be routinely applied to simultaneous determination of spironolactone and furosemide in pharmaceutical dosage form. In this study, the stability of spironolactone and furosemide in present dosage forms was established through employment of ICH recommended stress conditions. The developed procedure was evaluated for specificity, linearity, accuracy, precision and robustness to ascertain the stability of the analytical method. The method was proved to be specific, linear, precise, accurate, robust and stability-indicating. Hence, the method is recommended for routine quality control analysis and stability sample analysis.

Acknowledgments

The authors are thankful for facilities & grants given under UGC- Special Assistance Programme (DRS) Department Research Support (Sanction letter No. 540/6/DRS/2004 (SAP-I) Dt. 26/03/2004), and Department of Science & Technology New Delhi Fund For Improvement of Science & Technology (FIST) (Sanction letter No. SR/FST/CSI-072/2003 Dt. 24/12/2003), National Facility for Drug Discovery and Department of

Chemistry, Saurashtra University, Rajkot – 360 005 (INDIA) for providing analytical facilities.

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