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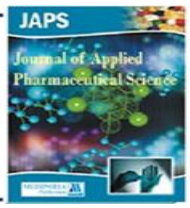

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Determination of Furosemide and Zonisamide as a Drug Substance and in Dosage Form by Ion Pair –Reversed Phase Liquid Chromatographic Technique

B. Udaykumar Rao and Anna Pratima Nikalje

ABSTRACT

An isocratic, selective, and accurate Ion pair - reversed phase liquid chromatographic method of analysis of Furosemide and Zonisamide both as a bulk drug and formulations was developed and validated. An ODS chromatographic column (250mmx4.6mm, 5µm) was used for the separation. The mobile phase consisted of a mixture of methanol and Tetrabutyl ammonium hydrogen sulphate (TBHS) 0.035 molar aqueous solution, pH adjusted to 6.0 using 1 N sodium hydroxide solution. The composition of TBHS with methanol used for Furosemide was 40:60(v/v) and that for Zonisamide was 50:50(v/v) delivered at a flow rate of 1.0ml/min and detection at wave length 240 nm. The developed method was validated in terms of selectivity, Linearity, limit of quantitation, precision, accuracy and solution stability. The proposed LC method achieved satisfactory resolution between Furosemide and 4-Chloro-5-sulphamoyl salicylic acid (CSSA), Zonisamide and CSSA an intermediate product possibly present in Furosemide and Zonisamide. The method can be employed as stability indicating method for both the drugs furosemide and zonisamide and its dosage form.

Keywords: Ion pair**,** Reversed phase, Zonisamide, Furosemide.

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INTRODUCTION

Zonisamide chemically known as [1, 2-benzisoxazole-3-methane sulfonamide] (Fig.1a) is used as an anticonvulsant in patients with epileptic disorders. Furosemide chemically known as [4-chloro-N-furfuryl-5-sulphamoylanthranilic acid] (Fig.1b) is used as a potent diuretic with a rapid action. It is used in the treatment of oedema associated with heart failure including pulmonary oedema and with the renal. Both furosemide and zonisamide are sulfonamides having different medicinal activity. The chromatographic condition used for both drugs are same except composition of mobile phase. Hence determination of both drugs are addressed under the same article. Few HPLC methods were reported in the literature for the quantitative determination of zonisamide in pharmaceutical dosage form using stability indicating HPLC method (Rao*et al.*,2006; Pathare *et al.*,2007), using coated monolithic column (Fritz *et al.*,2006), simultaneous determination with other antiepileptic drugs(Greiner-Sosanko *et al.*, 2007, Kaku *et al.*, 1991 and Vermeij *et al.*,2007), In human plasma or serum (Nakamura *et al.*, 2001; Shimoyama *et al.*,1999; Berry *et al.*,1991; Yoshida *et al.*, 2006 and Furuno *et al.*,1994) and by HPLC-Mass spectrometry (Subramanian *et al.*,2008).

Similarly for the quantitative determination of furosemide using HPLC (Roth *et al.*,1981), HPLC-DAD with home made column(Silva-Semaan F *et al.*,2005), HPLC with pulsed amperometric detection(Guermouche *et al.*, 1984), HPLC method in human plasma, serum, rat plasma and urine (Guzman *et al.*,2003; Barroso *et al.*,1996; Guermouche *et al.*,1985; Bauza *et al.*,1985; Lovett *et al.*, 1985; Galaon *et al.*,2007; Wenk *et al.*,2006; Okuda *et al.*, 1996; Mills *et al.*,1997 and Abou-Auda *et al.*, 1998). Attempts were made to develop a stability indicating HPLC method for the quantitative estimation of furosemide and zonisamide.

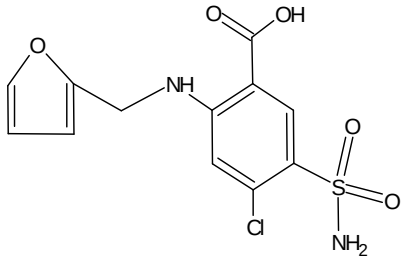

Fig. 1a: Chemical structure of Furosemide (FD).

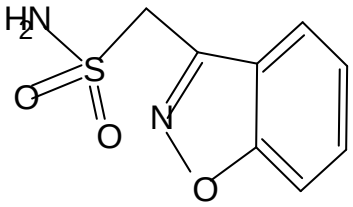

Fig. 1b: Chemical structure Zonisamide (ZN).

EXPERIMENTAL

Materials and Reagents

A sample of Furosemide and Zonisamide assigned purity 99.4 % and 99.6% of pharmaceutical grade were received from Torrent pharmaceuticals, Ahemedabad, India and Sun pharmaceuticals, Ahemedabad India. Furosemide tablet (Lasix-Aventis) of strength 40mg and Zonisamide capsule (Zonisef-sun pharma) of strength 50 mg procured from the market. Tetrabutyl ammonium hydrogen sulphate of analytical grade purchased from Merck, Mumbai, India. HPLC grade methanol was purchased from Qualigens –Mumbai, India. High purity water was prepared by Millipore milli Q plus purification system (France).

HPLC instrumentation and condition

A Shimadzu 2010 series LC system with photo diode array detector and in built auto injector (shimadzu corp., Kyoto, Japan) was utilsed for the method development and validation. LC solution software (shimadzu corp., Kyoto, Japan) was used for data acquisition and system suitability calculations. The chromatographic conditions were optimized using Phenomenex C-18 column, 250mm×4.6mm, 5µ (Shimadzu) column maintained at 25^0C using column oven, eluted with mobile phase at the flow rate of 1.0ml/min.The mobile phase were mixture of 0.035 molar TBHS in water adjusted the pH to 6.0 with 1N sodium hydroxide and methanol (40:60) v/v for Furosemide and (40:60) v/v for Zonisamide filtered through 0.45µm nylon filter and degassed by sonication. Measuments were made with injection volume 10µl and ultraviolet (UV) detection at 240 nm.

Standard and sample preparation

The standard stock solutions 1000 µg/ml each of furosemide and zonisamide were prepared separately by dissolving working standards in methanol and diluted to desired volume with methanol. Standard calibration solution of furosemide and zonisamide having the concentration in the range of 50µg/ml to 150µg/ml were prepared by diluting stock solution with methanol.

Analysis of dosage form

Ten tablets of furosemide and ten capsules of zonisamide separately were weighed, their mean weight, net content in case of capsule determined and crushed in mortar. An amount of powdered mass equivalent to 100 mg of furosemide and 100mg of zonisamide were transferred into a 100 ml volumetric flask separately containing 20 ml methanol, mechanically shaken for 10 min, ultrsoniccated for 5 min, and then diluted to volume with methanol and filtered. The first 10ml of the filtrate was rejected, and the subsequent was used to prepare sample solution by diluting 10 ml of filtered solution to 100ml with methanol.

RESULTS AND DISCUSSION

Method development

The primary target in developing this LC method is to achieve good resolution between 4-Chloro 5-sulfomoyl-salicylic acid (CCSA) an intermediate Fig.1c and those with Furosemide and Zonisamide. Mobile phase was selected in terms of its components and proportion. The work began with binary mixture of phosphate buffers and different organic modifier. After several trials tetra butyl ammonium hydrogen sulphate of 0.035 molar an ion pairing agent adjusted with pH 6.0 in place of phosphate buffer and organic modifier as methanol favoured the separation and elution of two drugs. With the increase of methanol, the retention time of both drugs can be shortened significantly. When the ratio of TBHS 0.035 molar gradually changed from 70:30 to 40:60 for furosemide and 50:50 for zonisamide. The retention time decreased from 15 to 5 min and 18 to 6 min. At the same time peak shapes of the two drugs improved. Finally mobile phase consisting of 40:60 v/v TBHS and methanol used for determination of furosemide, 50:50v/v TBHS and methanol used for determination of zonisamide were adopted which produces good resolution. The resolution between CCSA and furosemide, CCSA and zonisamide were more than 5.0. Furosemide and CCSA elutes at the RT 5.92 min and 4.1 min. Zonisamide and CCSA elutes at the RT 4.86 min and 7.0 min. The tablet and capsule matrix was also determined to see if any interference from them existed. No significant peaks from matrix were observed in the chromatogram, indicating no interference from the formulation matrix. A typical chromatogram for CCSA and furosemide, CCSA and zonisamide is shown in fig.2a and 2b.

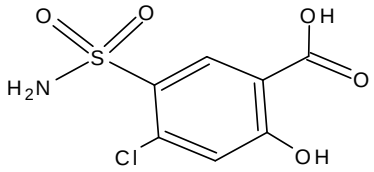

Fig. 1c: Chemical structure 4-Chloro-5-sulphamoyl salicylic acid(CSSA).

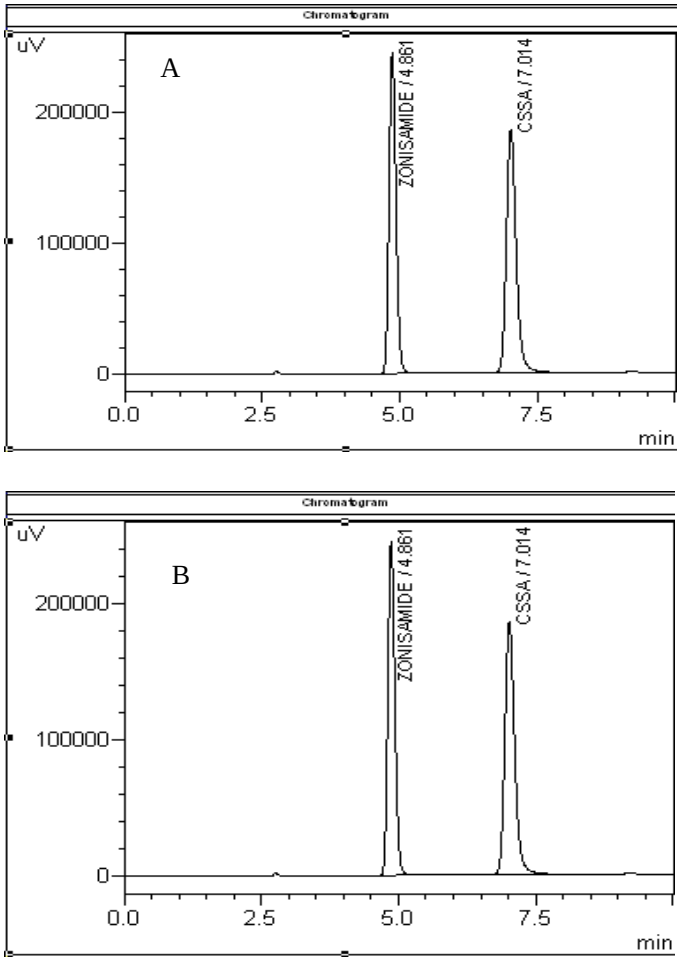

Fig. 2(a to b): Chromatograms of Furosemide, Zonisamide with CCSA .

Degradation studies

Forced degradation of furosemide tablet and zonisamide capsule samples under different stress condition (Heat, light, hydrogen-peroxide, acid and base) were prepared for further evaluation of the selectivity of the proposed LC method. For preparing acid and base induced degradation product 5ml of 0.1M HCl and 0.1M NaoH were separately added to 10 mg furosemide equivalent tablet powder and 10mg zonisamide equivalent capsule powder and exposed to 80°C for 6 h. The degraded samples were then neutralized and placed it in 100ml volumetric flask and prepared as described in the sample preparation. For preparing hydrogen peroxide induced degradation product 0.5 ml hydrogen peroxide (30%v/v) in 5ml water was added to 10 mg furosemide equivalent tablet powder and 10mg zonisamide equivalent capsule powder and exposed to 80°C for 6h. The degraded sample was placed it in 100ml volumetric flask and prepared as described in the sample preparation. The forced degradation in acidic, basic and oxidation media performed in the dark in order to avoid the possible effect of light. For preparing dry heat degradation product 10 mg furosemide equivalent tablet powder and 10mg zonisamide equivalent capsule powder stored at 80°C for 6h under dry heat condition in the dark and then cooled to room temperature. The degraded sample solution was prepared as described in the sample preparation. The photochemical stability of the drugs were also studied by exposing the tablet and capsule powder 1,200 K lux of visible light and 200 W h m^{-2} of UV light by using photo stability chamber. Proceeded the same as indicated for dry heat degradation. The resulting solutions were used as the degraded sample solution and determined under described chromatographic condition A typical chromatograms of oxidative degraded furosemide tablet and base hydrolysis zonisamide capsule samples are shown in Fig.3a and Fig.3b.

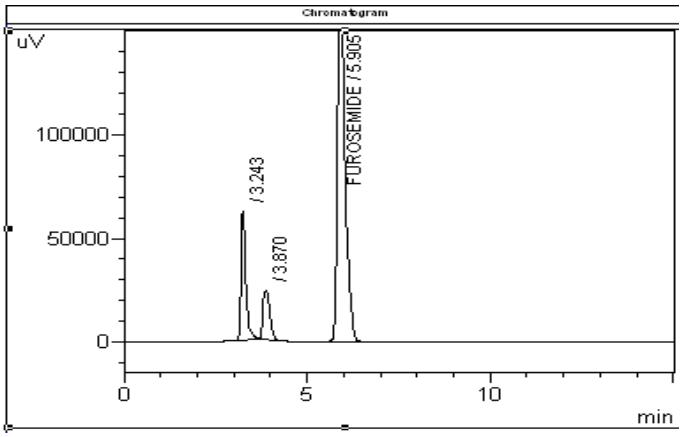

Fig. (3 a): Typical chromatograms of acid hydrolysis tablet sample of Furosemide.

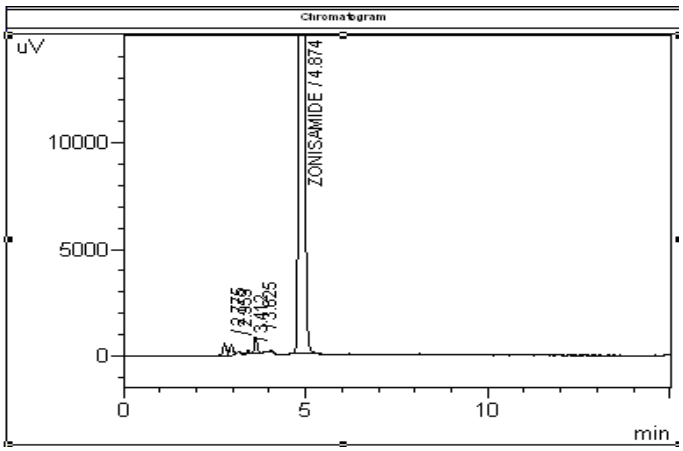

Fig. (3b): Typical chromatograms of base hydrolysis capsule sample of Zonisamide.

The degraded samples were compared to a capsule and tablet sample without degradation. The spectral homogeneity (Peak purity) 200-400nm was determined in the forced degraded samples. The threshold was set at ≥0.990. The peak purity, peak threshold and percent degradation (Table-1) for zonisamide and furosemide were demonstrated that the proposed LC method was able to separate each drug from degradants generated during forced degradation studies.

Table. 1: Forced degradation study Results

Stress condition	Drug Product					
	% degradation		Impurity		Peak purity index	
	FD	ZN	FD	ZN	FD	ZN
Sample without degradation	Nil	Nil	ND	ND	1.000	1.000
Refluxed with 2 N HCl solution for about 6 h at 80°C	25.2	0.1	ND	ND	1.000	1.000
Refluxed with 2 N NaOH solution for about 6 h at 80	0.1	1.7	ND	ND	1.000	1.000
Refluxed with hydrogen peroxide 3% solution	Nil	0.1	ND	ND	1.000	1.000
Exposed to Visible light for about 1200 K lux and UV light for 200 Whm^{-2}	Nil	nil	ND	ND	1.000	1.000
Dry heated for about 24h at 105 °C	Nil	nil	ND	ND	1.000	1.000

Table. 2: Furosemide and Zonisamide assay robustness result

Set Name	Initial precision (RSD%)		Tailing factor (≤ 1.5).		Resolution (>2%)		%purity	
	FD	ZN	FD	ZN	FD	ZN	FD	ZN
Standard condition	0.33	0.21	1.35	1.20	5.7	7.5	99.73	99.75
Flow rate 0.9ml/in	0.88	0.87	1.35	1.20	5.06	7.68	99.23	100.23
Flow rate 1.1ml/min	1.10	0.92	1.36	1.20	4.72	7.30	98.23	99.41
Mobile phase organic composition + 10% methanol	1.43	0.20	1.59	1.43	3.01	4.0	99.1.	98.29
Mobile phase organic composition - 10% methanol	0.13	0.22	1.63	1.44	7.45	5.0	100.47	98.9
Column temperature +5°C	1.64	1..0	1.36	1.21	5.03	8.23	98.01	100.26
Column temperature -5°C	1.08	0.21	1.39	1.19	4.50	6.8	99.33	100.0
pH 5.8	0.21	1.45	1.35	1.44	6.94	5.0	98.75	99.73
pH 6.2	0.26	1.32	1.34	1.17	6.92	6.1	99.07	99.42

FD: Furosemide ZN: Zonisamide.

Linearity

The linearity of the response of two drugs was verified at five concentration level ranging from 50µg/ml to 150µg/ml for both furosemide and zonisamide. The calibration curve was constructed by plotting mean area response A against concentration C of each drug. The regression equations obtained for the two drugs were A= 41293172C -43794 (r^2=1.0000, n=5) for furosemide and A=22380062C-11672 (r^2=0.9997, n=5) for zonisamide respectively. The result shows that an excellent correlation existed between peak area and concentration of each drug within the concentration range tested.

Limit of detection (LOD) and limit of quantitation (LOQ)

The LOD and LOQ for furosemide and zonisamide were determined at a signal to noise ratio of 3:1 and 10:1, respectively, by injecting a series of dilute solutions with known concentrations. Precision study was also carried out at the LOQ level by injecting six individual preparations of furosemide and zonisamide and calculated the % RSD of the area. The LOQ of furosemide and zonisamide were found to be 0.15 and 0.12 µg/ml respectively. The LOD of furosemide and zonisamide were found to be 0.12µg/ml and 0.10µg/ml.

Precision

The precision of the assay method was evaluated by carrying out six independent assays of furosemide and zonisamide test sample against a qualified reference standard and calculated the percentage % RSD of the assay. The mean % assay and % R.S.D. for assay values of furosemide were found to be 99.7% and 0.21% and for zonisamide were 99.8% and 0.33 % respectively, which is well within the acceptance criteria i.e. assay value should be between 97.0 and 103.0% and R.S.D. should be not more than 2.0%. The intermediate precision (inter-day precision) also evaluated by six independent assays of furosemide and zonisamide test sample against a qualified reference standard by different analyst, different HPLC system and different HPLC column in different days in the same laboratory. The mean % assay and % R.S.D. for assay values furosemide were found to be 99.2% and 0.24% and for zonisamide were 99.5% and 0.20 respectively which is well within the acceptance criteria.

Accuracy

Accuracy was determined by applying the developed method to synthetic mixtures of excipients to which known amounts of each drug corresponding to 80, 100 and 120% of label claim had been added. The accuracy was then calculated as the percentage of analyte recovered from the formulation matrix. Mean recoveries (Mean±S.D) for furosemide and zonisamide from the formulation are 100.73 ± 0.24% and 98.33 ± 0.12% respectively. The obtained result suggested the accuracy of the developed method for the determination of the two drugs in the formulation.

Robustness

The robustness of the method was determined by analyzing same sample at standard operating conditions and also by changing analytical conditions such as mobile phase composition, temperature, pH and flow rate. In all the deliberate varied chromatographic condition carried out i.e. organic phase composition, column temperature and flow rate in mobile phase. The system suitability parameter and % assay for the furosemide and zonisamide from the six replicate injections of test solution was found to be within the acceptable limits.The robustness of the method is established as the percentage deviation from the mean assay value obtained from precision study is less than ± 2%. Table-2 represents the robustness of the method.

Assay of Formulation

The validated LC method was applied to the determination of furosemide tablets and zonisamide capsule. Two batches were assayed and results are shown in Table-3 indicating

that the amount of each drug in the tablet and capsule samples met with requirements (90-110% of the capsule and tablet claim).

Table. 3: Result of furosemide and zonisamide in marketed formulation

Marketed formulation	Drug	% Amount found±SD	% RSD
Lasix tablet	Furosemide	99.33±0.21%	0.21
	40mg	99.53±0.18%	0.18
Zonsef capsule	Zonisamide	99.15±0.24%	0.24
	50mg	99.75±0.33%	0.33

Solution Stability and Mobile Phase Stability

The R.S.D. of assay of furosemide and zonisamide during solution stability and mobile phase stability experiments was within 1%. The solution stability and mobile phase stability experiments data confirms that sample solutions and mobile phase used during assay determination was stable up to 72 h.

CONCLUSION

An isocratic ion pair reversed phase liquid chromatographic method has been developed and validated for the quantitative determination of furosemide and zonisamide is precise, accurate, rapid and selective. The method was fully validated showing satisfactory data for all the method validation parameters tested. The developed method is a stability indicating and can be conveniently used by quality control department to determine the assay and other related sulphonamides in regular furosemide and zonisamide production samples and stability samples.

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