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DEVELOPMENT AND VALIDATION OF RP-HPLC AND ULTRAVIOLET SPECTROPHOTOMETRIC METHODS FOR SIMULTANEOUS DETERMINATION OF SPIRONOLACTONE AND TORSEMIDE IN PHARMACEUTICAL DOSAGE FORM

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ABSTRACT
HPLC and spectrophotometric methods were developed and validated for the quantitative determination of two diuretics, spironolactone and torsemide. The different analytical performance parameters such as linearity, precision, accuracy, specificity and robustness were determined according to ICH Q2B guidelines. Chromatography was carried out by isocratic technique on a reversed-phase C-18 Inertsil (250mm x 4.60mm), 5µ column with mobile phase composed of methanol:water (80:20). The UV spectrophotometric determinations (dual wavelength) were performed at 210 and 268nm for spironolactone and 243 and 330.5nm for torsemide. Both the methods were accurate and precise with recoveries ranging from 98.12% and 101% for both drugs and RSD < 2%.
KEYWORDS: Spironolactone; Torsemide; UV spectrophotometry; RP-HPLC

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INTRODUCTION

Diuretics increase the rate of urine flow and sodium excretion and are used to adjust the volume and/or composition of body fluids in a variety of clinical situations, including hypertension, heart failure, renal failure, nephrotic syndrome and cirrhosis. Drugs such as spironolactone (17-hydroxy- 7 -mercapto-3-oxo-17- pregn-4-ene-21-carboxylic acid lactone -7-acetate) competitively inhibit the binding of aldosterone to the mineralocorticoid receptor (MR). Unlike the MR-aldosterone complex, the MR-spironolactone complex is not able to induce the synthesis of multiple gene products called aldosterone induced proteins (AIPs). Since spironolactone blocks the biological effects of aldosterone, referred as aldosterone antagonist. Torsemide (1-isopropyl-3-(4-m-toluidino-3-pyridyl) sulfonylurea) is a loop diuretic, acts with in the lumen of the thick ascending portion of the loop of Henle, where it inhibits the Na+/K+/2Cl carrier system[1].

Literature survey revealed several analytical method for the determination of diuretics in formulation, which employ techniques such as high performance liquid chromatography (HPLC)[2, 3] and high performance thin layer chromatography (HPTLC)[4]. In all these reported method diuretics were analyzed individually and yet no method has been reported for simultaneous determination of the combination of spironolactone and torsemide. In biological fluids, the active principles as well as their metabolites have been quantitatively determined by HPLC with UV detection, electrochemical detection and LC/MS/MS[5-10].

The HPLC method is widely employed in quality control assessment of drugs because of their sensitivity, repeatability and specificity. On the other hand, the use of spectroscopic techniques can be considered a promising simple, faster, direct and relatively less expensive alternative for the determination of active drug content in pharmaceutical formulations with sufficient reliability. Hence, simple and specific RP-HPLC as well as UV procedure have been developed to determine spironolactone and torsemide simultaneously in pharmaceutical dosage forms .Both methods fulfilled the analytical requirement necessary to be applied to the content uniformity tests for finished pharmaceutical products when these are present in combinations as active principles and hence can be successfully applied for routine quality control.

MATERIALS AND METHODS

Chemicals

The bulk drugs of spironolactone and torsemide were obtained as gift samples from Cipla Pharmaceuticals Ltd. All solvents and reagents used were of HPLC or analytical grade. HPLC grade methanol, acetonitrile and ortho phosphoric acid were obtained from Merck India Ltd. Tablets of spironolactone (50 mg) and torsemide (20mg) (Dytor plus20 Cipla Pharmaceuticals Ltd) were procured from local market. Triple distilled water obtained from Younglin ultra 370 series instrument (reverse osmosis of demineralized water) was used in all experiments. All the solutions for analysis were freshly prepared and analyzed.

Instrumentation and analytical conditions

Chromatography was performed using a LC-10ATvp Shimadzu equipped with a SPD-M10 AVP-Shimadzu, UV/Vis diode array detector. Data acquisition and processing was performed using SPD-MXA automation system software. The method was conducted using an isocratic reverse phase technique. The analytical conditions (mobile phase composition, flow rate and analytical wavelengths) for the drugs have been summarized in **Table 1**. Mobile phase was prepared freshly, filtered through 0.45 µm membrane filter (Millipore, USA) and sonicated (Frontline Ultrasonic Cleaner FVS-10) for 20 min before use in order to deaerate the mobile phases. A C18 reverse phase Inertsil (250mm x 4.60mm), particle size 5µ column was used for analysis.

The UV method was performed on Shimadzu 1700 UV-Vis spectrophotometer at appropriately selected wavelengths for dual wavelength method of analysis. One-centimeter quartz cells were used for measuring absorbance. A Citizen electronic balance was used for the weighing deeds.

Preparation of standard and quality control solutions

HPLC method

Primary stock solutions (1000 µg/ml) and substock solutions (250 µg/ml) of both the drugs were prepared in methanol which were further diluted to obtain separate and mixed working standards in the concentration range of 25-125 µg/ml and 10-50 µg/ml for spironolactone and torsemide respectively. Both drugs were available as free bases, i.e. no salt form was used. Quality control (QC) samples were run with each batch of working standards in order to calculate the validation parameters. QC samples were prepared in methanol spiked with analytes at different concentrations following the same procedure as for calibration standards, using a different primary stock. The samples were analyzed with reagent blanks. All the dilutions were prepared in triplicates.

Spectrophotometric method

Primary stock solutions of spironolactone (1000 µg/ml) and torsemide (1000 µg/ml) were prepared in methanol, which have been further diluted by 0.1N HCl to obtain substocks of 250 µg/ml. Further mixed working range dilutions (10-50 µg/ml for spironolactone and 4-20 µg/ml for torsemide) were also prepared with 0.1N HCl. All measurements were made at room temperature. The quality control samples were prepared in the range of the calibration curve at different concentration levels in triplicates. The absorbances of these solutions were then put in the calibration curve to calculate the accuracy and precision of the method.

METHOD DEVELOPMENT

HPLC method

A simultaneous RP-HPLC method was developed for two diuretic drugs, which can be conveniently employed for routine quality control in pharmaceutical dosage forms. The chromatographic conditions were optimized in order to provide a good performance of the assay[11-13]. Based on the solubility, partition coefficient and polarity of the drugs, different mobile phases in varying combinations were tried and the best one was selected for the combination of spironolactone and torsemide as listed in **Table 1**. The flow rate was optimized to 1ml/min after considering the extent of longitudinal broadening and retention time. In either case of high or low flow rates, an ideal Gaussian curve of the peak is not obtained as the peak symmetry parameters are affected, i.e. asymmetry factor deviates from unity. The retention times for spironolactone and torsemide were 4.87 and 2.69 min respectively. Thus the total run time was short for the combination. The chromatogram has been shown in **Fig. 2**.

UV method

The principle for dual wavelength method is "the absorbance difference at two points on the spectra is directly proportional to the concentration of the component of interest". The dual wavelength method is based on above principle and can be utilized to a great extent without much complication to calculate the concentration (unknown) of particular component of interest in a mixture.

For dual wavelength method for analysis of two drugs wavelengths were selected where one component is having remarkable absorbance difference and other is having same absorbance (**Fig. 3**). The two selected wavelengths for spironolactone were 210nm & 268nm and for torsemide 243nm & 330.5nm. Concentration of samples was determined from standard calibration curves which were plotted between absorbance differences at selected wavelengths vs. concentration.

METHOD VALIDATION

Linearity

The methods were validated according to international conference on harmonization Q2B guidelines for validation of analytical procedures in order to determine the linearity, sensitivity, precision and accuracy for each analyte[14, 15]. Calibration curves were generated with appropriate volumes of working standard solutions for both UV and HPLC methods. In case of UV the range was optimized at 10-50µg/ml for

spironolactone and 4-20µg/ml for torsemide. The calibration ranges were 25-125µg/ml and 10-50µg/ml respectively for spironolactone and torsemide in the HPLC method. The linearity was evaluated by the regression analysis.

Precision and accuracy

Both precision and accuracy were determined with standard quality control samples (in addition to calibration standards) prepared in triplicates at different concentration levels covering the entire linearity range. Precision is the degree of repeatability of an analytical method under normal operational conditions. The precision of the assay was determined by repeatability (intra-day) and intermediate precision (inter-day and inter analyst) and reported as %R.S.D. The intermediate precision was documented as standard deviation and %R.S.D. Accuracy is the percent of analyte recovered by assay from a known added amount[15]. The repeatability of the method was determined by assaying minimum nine replicates of sample solutions by taking three test concentrations in the calibration range for both the methods (**Table-2&4**).

Specificity

The method specificity was assessed by comparing the HPLC chromatograms and UV scans obtained from the drug and the most commonly used excipients mixture with those obtained from blank (excipients solution in water without drug). The excipients chosen are the ones used commonly in tablet formulation, which included lactose, starch, microcrystalline cellulose, PVP and magnesium stearate. The drug to excipient ratio used was similar to that in the commercial formulations.

Commercial formulations to be evaluated

Dytor plus 20 tablets (50mg spironolactone and 20mg torsemide) manufactured by Cipla Pharmaceuticals were purchased and in vitro quality control tests like friability, disintegration, hardness and weight variation were performed according to the Indian Pharmacopoeial procedures[16].

Extraction of active ingredient

The tablets were accurately weighed and powdered. The amount of the drug in weighed quantity of powder was calculated based on the label claim and then the active ingredients were extracted in methanol. The solutions were sonicated for 20 min and then filtered through Whatman No. 1 filter paper. Multiple extractions were performed to assure complete extraction. Appropriate dilutions were made and the samples were subjected for both HPLC and UV analysis.

RESULTS AND DISCUSSION

HPLC method

For the selection of a detection wavelength the solvent interference factor and absorbance of both the components was considered and 260nm was found as most suitable. At absorbance maxima of both drugs i. e. 238nm for spironolactone and 288nm for torsemide, the absorbance of other component is not prominent. Therefore detection was made at 260nm where both drugs show significant absorbance. For optimization of the mobile phase, varying combinations of organic and aqueous phase (methanol & water respectively) were employed. Resolution between the two peaks decreased to 0.4 when organic phase was increased to 90% which was 2.2 in 80% organic phase. On the other hand, when organic phase was decreased to 70% the tailing factor increased to 1.68 instead of 0.96 in 80% organic phase with remarkable increase in retention times of both the drugs. After considering the varying combinations of various mobile phases methanol-water (80:20) was finalized as it was showing good peak shapes and a significant amount of resolution. The method was specific as none of the excipients interfered with the analytes of interest. Hence, the method was suitably employed for assaying the commercial formulation. Calibration curves were constructed with five standards and were found linear ($r2 \geq 0.999$) for each of the analyte over their calibration ranges (25-125µg/ml, $y = 19831x - 4473$ for spironolactone and 10-50µg/ml, $y = 28758.5x + 8960$ for torsemide). The slopes were calculated using the plot of drug

concentration versus area of the chromatogram. The developed HPLC method was accurate, precise and reproducible. All the validation parameters of the method were shown to be within the specified limits (**Table 2**). Accuracy and precision were determined by elaboration of three standard calibration curves, two from the same day (intra-day) and third one from a different day (inter-day). The intra- and inter-day precisions (%R.S.D.) at different concentration levels were found to be less than 2%. Both the drugs showed 98–101% recoveries from the commercial formulation when assayed with the developed HPLC method (**Table 3**). Moreover the %R.S.D. (less variation) shows good precision of the developed HPLC method.

Spectrophotometric method (Dual wavelength)

A spectrophotometric dual wavelength method was also developed for simultaneous analysis of the same combination of drugs. In this method, two wavelengths were selected for determination of one component out of which one corresponds to its λmax and other is the wavelength where the interfering component has the same absorbance as at the first wavelength. It is clear that absorbance difference between two selected wavelengths for each drug on the mixture curve is directly proportional to the concentration of the component of interest independent of the interfering component (other component) as it has same absorbance at the selected wavelengths. The selected wavelengths for spironolactone are 210nm & 268nm whereas for torsemide are 243nm & 330.5nm. The development of simple, rapid, sensitive and accurate analytical method for routine quantitative determination of samples will reduce unnecessary tedious sample preparations and cost of materials and labor. Spironolactone and torsemide are UV absorbing molecules with specific chromophores in their structures that absorb at a particular wavelength and this fact has been successfully employed for their simultaneous quantitative determination by dual wavelength spectrophotometric method.

The correlation coefficient of the standard curve was greater than 0.999. All the method validation parameters are well within the limits as specified in the ICH Q2B guidelines[14] as shown in Table 4. Table 3 lists the percent recovery (content uniformity) of both drugs in the commercial formulation by the developed method. The commercial dosage form showed 98.12–101% recovery by this method which were within the specified limits of content uniformity. Moreover, the UV method offers a cost effective and time saving alternative to HPLC method of analysis.

CONCLUSION

The proposed RP-HPLC and UV methods are simple, reliable and selective providing satisfactory accuracy and precision. The spectrophotometric method has an added advantage of being more economic and rapid alongwith comparable accuracy and precision as compared to HPLC method. Moreover the shorter duration of analysis for spironolactone and torsemide make these reported methods suitable for routine quantitative analysis in pharmaceutical dosage forms. The recoveries achieved are good by both the methods.

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Table 1: Various optimized chromatographic conditions for analysis

Variable	Conditions
Column	
Dimension	250mm x 4.60mm
Particle size	5µ
Bonded phase	Octadecylsilane (C18)
Mobile Phase	
Methanol **:** Water	80 **:** 20
Flow rate	1.0 ml/min.
Temperature	Ambient
Sample size	20 µl
Detection wavelength	260nm

Table 2: Validation Parameters of the HPLC Method of Spironolactone and Torsemide

Validation parameter	Spironolactone	Torsemide
Range (µg/ml)	25-125	10-50
Regression equation	y = 19831x – 4473	y = 28758.5x + 8960
% R.S.D. of slope	1.01	0.26
r2	0.9991	0.9997
Precision		
% R.S.D.	0.70 ± 0.33	0.67 ± 0.28
Percent recovery	100.04 ± 0.51	99.8 ± 0.98
Robustness (%value ± S.D. ± %R.S.D.)		
Flow rate (±0.2ml/min)	99.68 ± 1.08 ± 0.77	99.54 ± 1.22 ± 0.86
Mobile phase composition (±2%)	99.44 ± 0.82 ± 0.38	100.12 ± 1.07 ± 0.46

Three calibration graphs were generated within the same day and on three consequent days (n = 3). The standard concentrations were evenly distributed in the linear range. Precision and accuracy were determined with quality control samples at three concentration levels. Data showed the precision of the method at mean of three concentration levels within the calibration range. The slopes are represented as mean ± S.D. with the % R.S.D. given in parentheses.

Table 3: Percent Recoveries of Spironolactone and Torsemide in Commercial Formulation by UV and HPLC Methods of Analysis

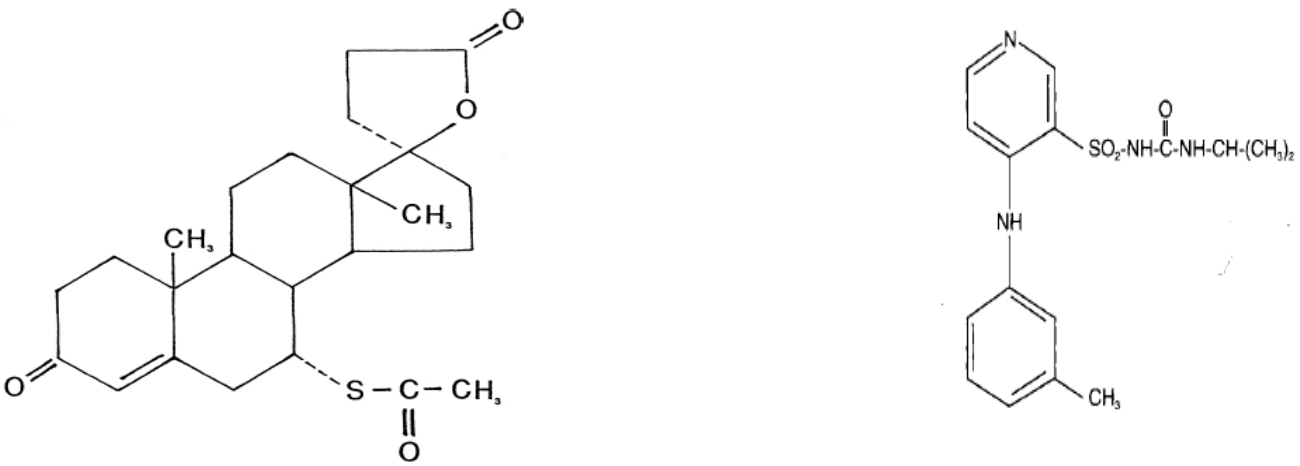
Product	Component	UV method		HPLC method	
		Mean ± S.D.	%R.S.D.	Mean ± S.D.	%R.S.D.
Dytor Plus 20	Spironolactone	99.82 ± 1.18	1.12	99.78 ± 0.88	0.81
Dytor Plus 20	Torsemide	98.12 ± 1.92	1.77	99.60 ± 1.56	1.48

The percent recoveries are represented as mean ± S.D. for which n = 3, R.S.D. = relative standard deviation.

Table 4: Validation Parameters for UV Method of Analysis of Spironolactone and Torsemide

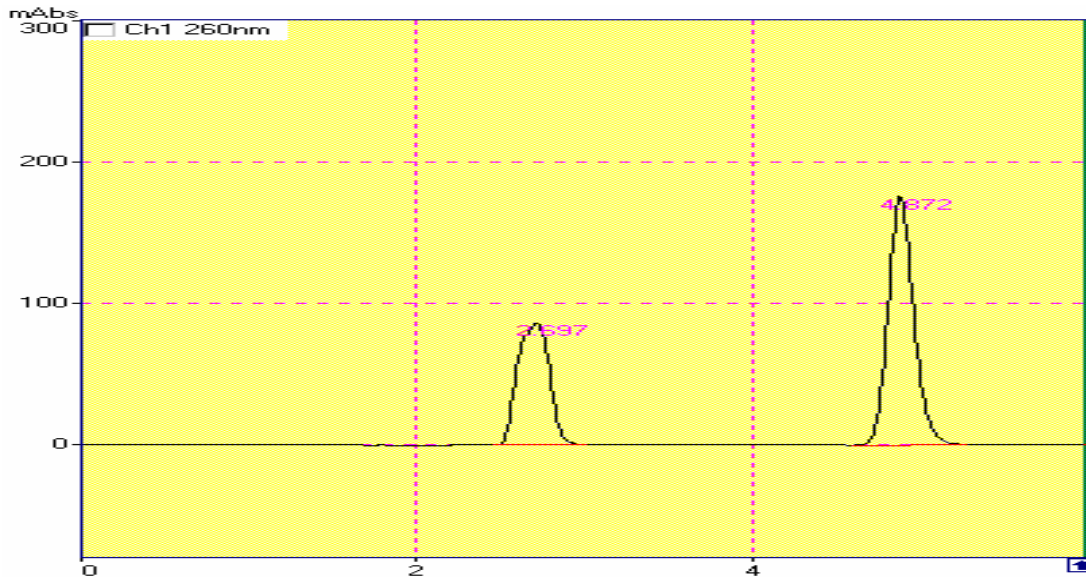
Validation parameter	Spironolactone	Torsemide
Range (µg/ml)	10-50	4-20
Regression equation	y = 0.1049x – 0.00125	y = 0.581x + 0.04194
% R.S.D. of slope	1.44	1.23
r2	0.9997	0.9988
Precision		
% R.S.D.	0.74 ± 0.40	0.97 ± 0.57
Percent recovery	99.56 ± 0.58	100.003 ± 0.46

Three calibration graphs were generated on three consequent days (n = 3) with minimum of six concentrations evenly distributed throughout the entire range. The accuracy represented by percent recovery and Precision was determined using quality control (QC) samples. Precision (%R.S.D.) is calculated as mean ± S.D. with n = 3 for each concentration.



(a) (b)

Fig 1: Chemical structures of (a) spironolactone and (b) torsemide



(a) (b)

Fig 2: Typical chromatogram showing the elution of (a) Torsemide (20µg/ml) and (b) Spironolactone (50µg/ml) at their respective retention times

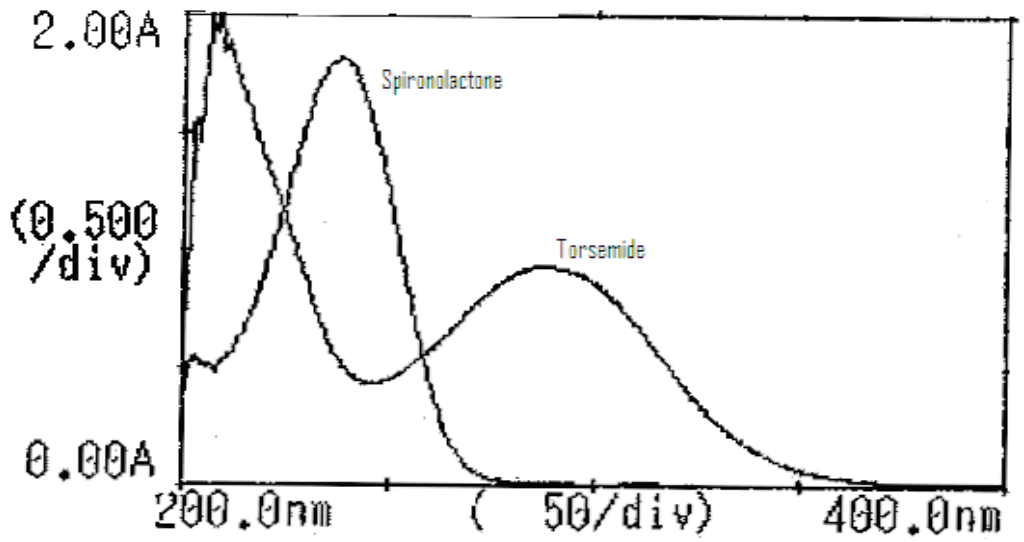


Fig 3: Overlay spectra of Spironolactone and Torsemide

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