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Development and validation of stability indicating UPLC method for the simultaneous determination of beta-blockers and diuretic drugs in pharmaceutical dosage forms

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Abstract: With the objective of reducing analysis time and maintaining good efficiency, there has been substantial focus on high-speed chromatographic separations. In this work, rapid, precise and specific stability indicating ultra performance liquid chromatography (UPLC) method has been developed and validated for the simultaneous determination of beta-blockers and diuretic drugs in pharmaceutical formulations. The chromatographic separations of all the drugs were achieved on a Waters Acquity BEH C18, 50×2.1 mm, 1.7 µm UPLC column within a short runtime of 3.3 min. The newly developed method was validated according to the ICH guidelines with respect to specificity, linearity, accuracy, precision and robustness. Forced degradation studies were also performed for all the drug samples to demonstrate the stability indicating power of the developed UPLC method. Two unknown degradants were detected in the alkaline degradation of Amiloride hydrochloride with sodium hydroxide having not been reported previously. The structure of the degradants was determined using a combination of UPLC/MS and mechanistic chemistry. This degradant result from destruction of the guanidine moiety of amiloride hydrochloride. The two impurities were characterized as 3,5-diamino-6-chloropyrazine-2-carboxylic acid (impurity A) and 3-chloropyrazine-2,6-diamine (impurity B), respectively. Comparison of system performance with conventional HPLC was made with respect to analysis time, efficiency and sensitivity.

Keywords: UPLC; Beta-blockers, Diuretic, Simultaneous determination; validation

1. Introduction

β-blockers are a well-known and characterized group of pharmaceutical compounds. The goal of antihypertensive drug therapy is to prevent complications of hypertension. β-blockers have been shown to reduce total and cardiovascular morbidity and mortality of hypertensive diabetic patients. They can be used in the treatment of hypertension, angina, pectoris, arrhythmia and congestive heart failure [1,2].

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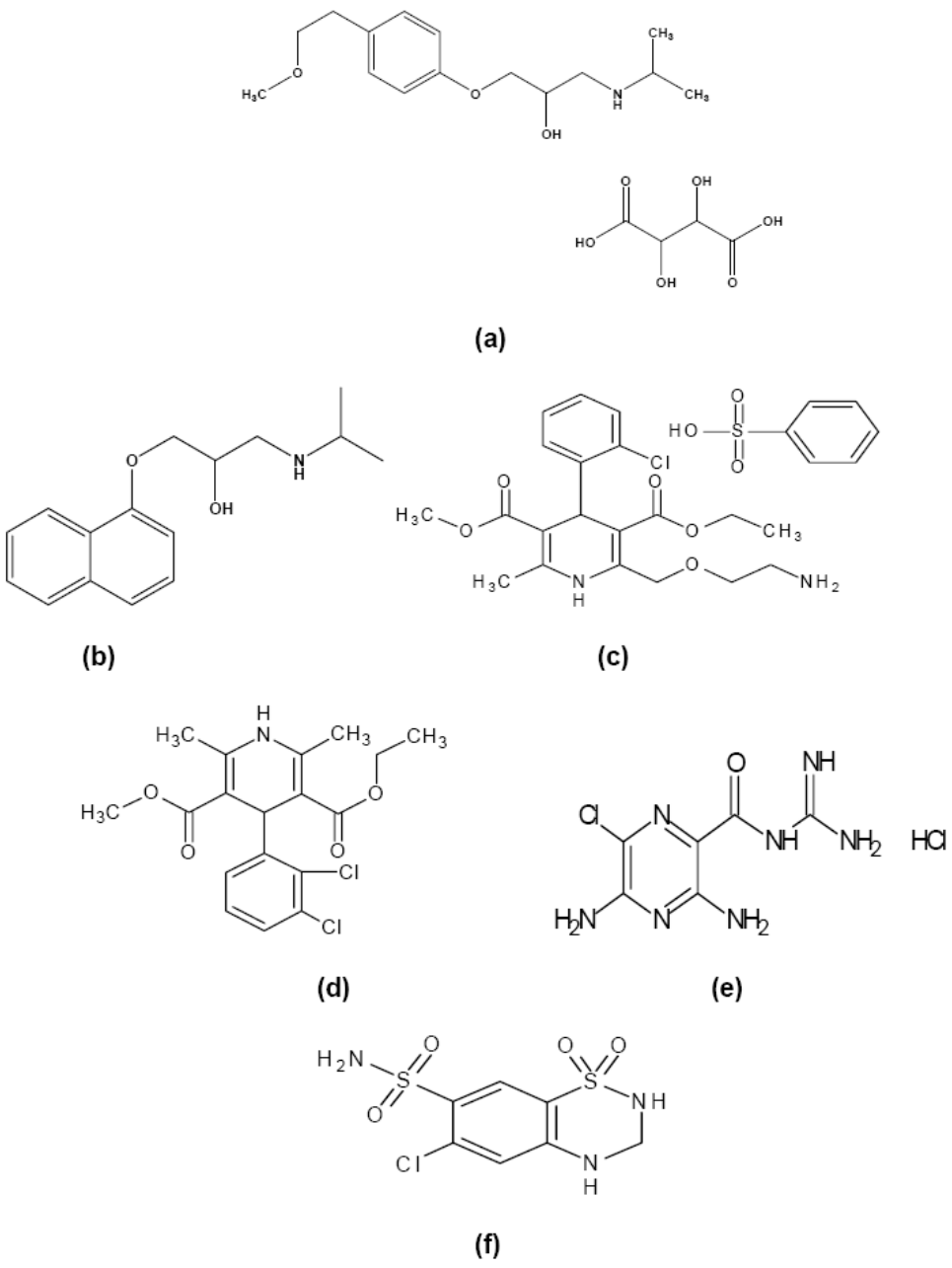


Fig. 1: Structures of (a) Metoprolol Tartrate (MT); (b) Propronolol - HCl (PRO); (c) Amlodipine Besylate (AB); (d) Felodipine (FEL) (e) Amiloride- HCl (AML) and (f) Hydrochlorothiazide (HCTZ).

Metoprolol tartarate (MT) (Fig 1a) is a selective and propranolol hydrochloride (PRO) (Fig 1b) is a non selective beta receptor blocker used to decrease the heart rate, force of contraction and cardiac output. Amlodipine besylate (AB) (Fig 1c) and felodipine (FEL) (Fig 1d) are calcium channel blockers used as anti-hypertensive drugs in the treatment of angina. It acts by relaxing the smooth muscle in the arterial wall, decreasing peripheral resistance and hence reducing blood pressure; in angina it increases blood flow to the heart muscle. Amiloride hydrochloride (AML) (Fig 1e) and hydrochlorothiazide (HCTZ) (Fig 1f) are diuretic drugs used in the treatment of hypertension and congestive heart failure [3,4].

Combination therapy with a diuretic (e.g. AML and HCTZ), beta-blocker (e.g. MT, PRO) and calcium channel blocker (AB and FEL) becomes often necessary and such dosage forms have been formulated [5]. These combination drugs are commonly used in the treatment of hypertension and congestive heart failure. Therefore, simultaneous determination of these drugs is meaningful.

A few methods are reported in the literature for combination hypertensive drugs by spectrophotometric [6-9], HPTLC [10] and HPLC [11-18]. Patel et al [17] described an isocratic HPLC method for the simultaneous estimation of six drugs for combined hypertension therapy.

UPLC is a new category of separation science which builds upon well-established principles of liquid chromatography, using sub-2µm porous particles. These particles operate at elevated mobile phase linear velocities to produce rapid separation with increased sensitivity and increased resolution [19]. Several reports are available in the literature on the HPLC-MS and UPLC-MS for the different combination of β-blockers and hypertensive drugs [20-32]. Stephen et al developed an UPLC/MS method for simultaneous identification of β-blockers [24]. However, none of these methods describes the UPLC assay for simultaneous determination of β-blockers and hypertensive drugs. This prompted us to develop a rapid, sensitive and specific UPLC assay method for the simultaneous determination of these drugs.

The objective of this work was to develop and validate a stability indicating UPLC method for the simultaneous determination of drugs, viz. AML, HCTZ, MT, PRO, AB and FEL in a single run for application in pharmaceutical formulation with UV detection. During stability studies, an unknown degradants of amiloride hydrochloride were observed during alkaline degradation when exposed to sodium hydroxide. This paper deals with the investigation of a novel degradation mechanism for amiloride hydrochloride in a pharmaceutical formulation.

2. Materials and Methods

2.1. Reagents

An active pharmaceutical ingredient (API) working standard of AML was obtained from BAL Pharma Limited, Bangalore, India; HCTZ, MT, PRO and AB were obtained from IPCA Laboratories Limited, Mumbai, India and FEL was purchased from Sigma-Aldrich. The test samples obtained from commercial drug store had the following combinations: AML – HCTZ (5 mg, 50 mg); MT – AB (25 mg, 5 mg); PRO (10 mg) and FEL (10 mg). HPLC grade acetonitrile and ammonium acetate were obtained from Merck, Mumbai, India. Formic acid (85%) was from Thomas Baker, Mumbai, India. High purity deionised water was obtained from Millipore, Milli-Q (Bedford, MA, USA) purification system.

2.2. Apparatus

2.2.1 High-performance liquid chromatography

A Serveyor HPLC system (Thermo Fisher, USA) equipped with quaternary gradient pump, auto sampler, column oven and photodiode array detector (PDA) was employed for analysis. Chromatographic data was acquired using ChromQuest 4.2 software.

2.2.2 Ultra performance liquid chromatography

A Waters Acquity UPLC system (Waters, USA) equipped with binary gradient pump, auto sampler, column oven and photodiode array detector (PDA) was employed for analysis. Chromatographic data was acquired using Empower 2 software.

2.2.2 Ultra high performance liquid chromatography – mass spectrometry (UHPLC-MS)

A Thermo-Finnigan Accela UHPLC system consisting of auto sampler, Accela pump, photo diode array detector was interfaced with a Thermo Finnigan LCQ Advantage Max ion trap mass spectrometer via an ESI probe.

2.3. Chromatographic conditions

2.3.1 Method 1

An X-Terra C18, 250×4.6 mm, 5µmcolumn was used for separation. Chromatographic separation was achieved using timed gradient. The mobile phase consisting of A: buffer (5 mM ammonium acetate pH 4.0 adjusted with formic acid) and B: a mixture of 90% acetonitrile and 10% buffer with a timed gradient programme was used. The gradient condition of the mobile phase was: 0 min 0% solvent B, 10.0 min 30% solvent B, 15 min 60% solvent B, 20 min 80% solvent B, 24 min 100% solvent B, 28 min 100% solvent B and 30 min 0% solvent B with further 2 min for system equilibration. The flow rate of the mobile phase was 1.0 mL/min with detection at 230 nm. The column temperature was kept at ambient and the injection volume was 10 µL.

This method is applied for the simultaneous determination of all the selected drugs by HPLC.

2.3.2 Method 2

An Acquity UPLC BEH C18 (50mm × 2.1 mm, 1.7 µm) (Waters, Ireland) column was used as a stationary phase. The mobile phase consisting of A: buffer (5 mM ammonium acetate pH 4.0 adjusted with formic acid) and B: a mixture of 90% acetonitrile and 10% buffer with a timed gradient programme was used. The gradient condition of the mobile phase was: 0 min 0% solvent B, 1.5 min 20% solvent B, 1.8 min 50% solvent B, 2.5 min 100% solvent B, 2.6 min 100% solvent B and 2.8 min 0% solvent B with further 0.5 min for system equilibration. The flow rate of the mobile phase was 613 µL/min with detection at 230 nm. The column temperature was kept ambient and the injection volume was 0.2 µL. Forced degradation studies were carried out with a 2996 photo diode array detector.

This method is applied for the simultaneous determination of all the selected drugs by UPLC.

2.3.3 LC/MS/MS analysis

All data were collected in the positive ion mode. Instrument parameters were heated capillary 320°C; sheath gas (N_2) 55; auxiliary gas (N_2) 15; total two micro scans; maximum injection time 200

ms. For MS experiments, the mass range scanned was *m/z* 50–900. MS/MS parameters were IW 1.0; RCE 40%. Chromatography was performed using method 2.

2.4. Solution preparation

2.4.1. Standard stock solution

Individual standard stock solution of AML, PRO, FEL, AB (each 5 mg/mL); HCTZ (8 mg/mL) and MT (12.5 mg/mL) was prepared separately by dissolving appropriate amounts of the compounds in methanol. These stock solutions were stored at around 5°C and found to be stable for a week.

2.4.2. System suitability solution

Composite system suitability standard solution was prepared by further diluting respective standard stock solutions with mobile phase in 5 mL volumetric flask to get final concentration of AML (100 µg/mL); HCTZ (1000 µg/mL); MT (500 µg/mL); PRO (200 µg/mL); AB (100 µg/mL) and FEL (200 µg/mL).

2.5. Sample solution

To determine the content of specific beta-blocker or diuretic drugs simultaneously in conventional tablets, 10 tablets each of the selected combined pharmaceutical dosage forms (section 2.1) were weighed individually, their mean weight determined and were ground to a fine powder using a glass mortar and pestle. An equivalent of 10 mg of AML, 100 mg of HCTZ, 50 mg of MT, 20 mg of PRO, 10 mg of AB and 20 mg of FEL was accurately weighed and transferred to a separate 100 mL volumetric flask, respectively. The volume was adjusted with methanol and water (1:1 *v/v*) and the resultant solution was sonicated for 15 minutes, filtered through a 0.2µm nylon filter (Millipore, Milford, USA) and injected directly onto the UPLC column using the optimized conditions.

2.6 Validation procedure

System suitability test was performed by making six repeated injections of standard solution to check parameters such as % relative standard deviation, theoretical plates, capacity factor, asymmetry factor and peak purity. The specificity of the method was determined by injecting the sample solution containing excipients without drug having concentration same as that of the sample.

Generation of force degradation sample

Stress degradation studies were performed for AML, HCTZ, MT, PRO, AB and FEL in tablet dosage form to provide an indication of the stability-indicating property and specificity of the proposed method. Intentional degradation was attempted to stress condition exposing it with an acid (0.5 N hydrochloric acid at 100° C for 1 hour), alkali (0.5 N sodium hydroxide at 100 ° C for 1 hour), hydrogen peroxide (30% at 100 ° C for 1 hour), heat (105° C) and UV light (254 nm and 366 nm wavelength) to evaluate the ability of the proposed method to separate active ingredients from its degradation products. The photo degradation study was performed by exposing the tablets to light in photo stability chamber for 7 days. Samples were also exposed to heat at 105° C for 24 hours and the contents were analyzed immediately. Sample solutions of AML, HCTZ, MT, PRO, AB and FEL tablets were prepared as per section 2.5. Peak purity test was carried out for the drugs using a PDA detector.

2.6.1 Linearity

Linearity solutions were carried out at six concentration levels from 25% to 150% of analyte concentration in triplicate. The curves were constructed by plotting drug concentration verses peak areas. Linear calibration curves were generated by linear regression analysis and obtained over the respective standard concentration ranges. The standard solution for linearity was prepared as per section 2.4.

2.6.2 Accuracy

The accuracy of the method was carried out by adding known amount of each drug corresponding to the concentration levels 50%, 80%, 100%, 120% and 150% of the label claim along with the excipients in triplicate. The samples were given the same treatment as described in Section 2.5.

2.6.3 Precision

Precision of the method was checked by carrying out six replicate assays of combination drugs against qualified working standard. Intermediate precision was performed analyzing the samples by two different analysts using different instruments on different days.

2.6.4 Solution stability

The stability of each drug in solution was determined for 48 hours at room temperature. The samples were checked at periodic intervals and the data were compared with freshly prepared samples.

2.6.5 Robustness

Robustness was performed by deliberately changing the chromatographic conditions. The flow rate of the mobile phase was changed from 613 µL/min to 575 µL/min and 650 µL/min. Standard solution was injected five times in replicate for each change. % RSD for all the drug components and resolution between drug components and their impurities were monitored for all robustness parameters.

Respective peak areas, dilution factors, sample and standard weights were taken into account to quantitate the amounts of drug substance in mg per tablet.

2.7 Computation

The UPLC method was obtained by converting the HPLC method using the Method Translator and Cost Saving Calculator version 2.0 (www.chem.agilent.com).

The flow rate of the UPLC method was obtained using the equation 1,

$$Q_{column\ 2} = Q_{column\ 1} \times \left(\frac{d_2}{d_1}\right)^2 \times \frac{d_{p1}}{d_{p2}} \qquad \text{Eq. 1}$$

where $Q_{column\ 1}$ and $Q_{column\ 2}$ are the flow rates, d_1 and d_2 are the diameters and d_{p1} and d_{p2} are the particle size of the HPLC and UPLC columns, respectively.

Capacity factor ‘k`’ gives an indication of how long each component is retained on the column. In the present study, the capacity factor of each peak in both HPLC and UPLC was obtained using the equation 2.

$$k` = \frac{t_R - t_m}{t_m} \qquad \text{Eq. 2}$$

Where 't_m' is the unretained peak's retention time and 't_R' is retention time of the peak of interest. Unretained peak's retention time (t_m) is determined by injecting uracil under same chromatographic conditions.

Column volume (CV) for both HPLC and UPLC columns were calculated by using the expression '$\pi r^2 L$'. Approximately 70% of the column volume then constitutes the void volume (is the volume that is not taken up by the stationary phase) of the column.

The dwell volume becomes increasingly important when employing a fast gradient with small columns typically used with UPLC system because it effects the time taken for the gradient to reach the head of the column. The dwell volumes for both HPLC and UPLC systems were estimated by employing respective columns as used in this study. The mobile phase consisting of A: methanol and B: 0.1% acetone in methanol was used. The flow rates for HPLC and UPLC were kept at 1.0 mL/min and 0.613 mL/min, respectively. Detector was set at 260 nm. Gradient programme was started from 100% A to 100% B in 20 minutes and then calculated the time taken to reach 50% absorbance ($T_{0.5}$). The difference in time interval between $T_{0.5}$ and 10 minutes (half the gradient time) was multiplied by the flow rate to determine the dwell volume.

Since the peak capacity [33] is a good tool to determine the quality of a gradient separation, performance of both the methods to separate all the selected drug compounds in the present study was compared with the peak capacity. The peak capacity (P) was calculated using the equation 3.

$$P = 1 + \frac{t_g}{(1/n)\sum_1^n w} \qquad \text{Eq. 3}$$

Where, 'n' is the number of peaks selected for the calculation, tg is the gradient run time and 'w' is peak width. Thus peak capacity is simply the gradient run time divided by the average peak width.

The mobile phase linear velocity (mm/sec) at a given flow rate was calculated by dividing the column length (mm) by the retention time of unretained peak (sec) i.e, void volume of peak time.

3. Results and Discussion

3.1. LC method development and transfer to UPLC

Initially, the isocratic and gradient HPLC conditions were optimized with an objective to separate all the six drug components selected in this study. The UV spectra of the analytes were independently determined. Each drug has exhibited different maximum UV absorbance (AML: 221 and 285; HCTZ: 221 and 285; MT: 225; PRO: 231; AB: 240 and FEL: 240). At a UV_{max} range of 210–225 nm, baseline drift towards the negative absorbance was observed in the chromatograms, whereas at wave length 230 nm we could detect all beta-blockers and diuretic drugs simultaneously with good separation, sensitivity and consistent baseline.

The chromatographic separation was achieved on X-Terra C18, 250×4.6 mm, 5µm column maintained at ambient temperature. The feasibility of various mixture(s) of solvents such as acetonitrile and methanol using different buffers such as ammonium acetate and formic acid with variable pH range of 3–6, along with altered flow-rates (in the range 1.0 – 1.5 mL/min), was tested for complete chromatographic resolution of the beta-blockers and diuretic drugs. The use of ammonium acetate buffer (pH 6) led to a poor resolution between AML and HCTZ while formic acid buffer (pH 3) resulted in lower capacity factor for AML. Finally, the use of ammonium acetate (pH 4) based buffer with a timed gradient was optimized as mentioned in Section 2.3.1, which provided adequate peak separation, with less tailing, and resulted in the best resolution amongst the buffers tested.

The basic chromatographic conditions like stationary phase, solvents and UV detection, employed in HPLC were taken into account while developing the new UPLC method. The detection

wavelength, column temperature, buffer and solvent used in HPLC were kept constant. The stationary phase C18 was chosen in order to have similar chemistry as that used in the HPLC. A BEH C18, 50×2.1 mm, 1.7µm column was employed for the separation. The injection volume was scaled down to 0.2 µl from 10µl as used in HPLC. As per van Deemter curves [34] for 2.1 mm i.d columns with 1.7 µm particle size, the maximum efficiency can be achieved between 3 and 7 mm/sec of linear velocity. Based on this theory, initial mobile phase linear velocity was kept at 4.2 mm/sec (0.5 mL/min). Unretained peak was found at 11 seconds. Under these conditions, though a satisfactory separation was achieved between all the selected drug components, tailing was observed for FEL in a total run time of 7–8 min. A backpressure of 6500 psi was observed. Taking in to account the capability of high operating pressure of UPLC the mobile phase linear velocity was increased to 5.6 mm/sec (0.613 mL/min) with a backpressure of 8,600 psi. This linear velocity, by using the ammonium acetate (pH 4) based buffer with a timed gradient as mentioned in Section 2.3.2, provided adequate peak separation, with less tailing, and resulted in the best resolution. The runtime was decreased to 3.3 min without affecting the separation of all the drug components. Hence, the gradient mode was preferred for UPLC analysis.

3.2. Comparison study of chromatographic performance

A comparative data on chromatographic performance of HPLC and UPLC has been obtained by injecting a solution of system suitability standard. Column volumes for HPLC and UPLC columns were found to be 4.15 mL and 0.19 mL, respectively. The dwell volumes for both HPLC and UPLC systems were found to be 1.6 mL and 0.11 mL, respectively.

The elution time of all the drug compounds in UPLC were observed to be reduced by 10-fold to that of HPLC employing 5-micron columns. However, based on van Deemter curves, it would have been 3 to 4 fold reduction in analysis as compared to HPLC method, had we employed 3-micron column as demonstrated by Jerkovich et.al. [35].

The resolution obtained for all the drug compounds selected in this study by UPLC showed comparatively better separation efficiency than HPLC. The higher peak capacity (P = 1494) in UPLC as against HPLC (P = 942) conforms to better gradient separation efficiency and resolving power of UPLC. The performance parameters of both the systems are shown in Table 1.

Table 1 System Suitability Parameters* and Comparison of system performance of HPLC and UPLC

Name of the drug component	Retention time		Capacity Factor		Resolution		USP Tailing		Peak capacity[33]	
	HPLC	UPLC	HPLC	UPLC	HPLC	UPLC	HPLC	UPLC	HPLC	UPLC
Amiloride	10.2	1.1	3.1	4.7	-	-	1.1	1.2	942	1494
Hydrochlorothiazide	12.8	1.3	4.1	5.4	13.9	5.0	1.0	1.1		
Metoprolol	13.4	1.9	4.3	8.9	2.7	26.9	1.4	1.2		
Propranolol	16.4	2.1	5.6	9.8	15.5	6.4	1.2	1.3		
Amlodipine	17.6	2.2	6.0	10.4	6.8	4.4	1.2	1.6		
Felodipine	24.3	2.6	8.7	11.9	41.2	9.9	0.9	1.5		

*USP 31, NF26 <621>

Under these optimized conditions, the analyte peaks were well resolved and free from tailing. The tailing factors were <2.0 for all the peaks. The nominal retention times of AML, HCTZ, MT, PRO, AB and FEL were found to be 10.17, 12.8, 13.37, 16.44, 17.58 and 24.35, respectively, at a flow rate of 1.0 mL/min using HPLC while with UPLC it was found to be 1.11, 1.3, 1.95, 2.12, 2.24 and 2.63 minutes at a flow rate of 1.0 mL/min and 0.613 mL/min, respectively. The typical chromatograms obtained from final HPLC and UPLC conditions are depicted in Fig. 2a and 2b.

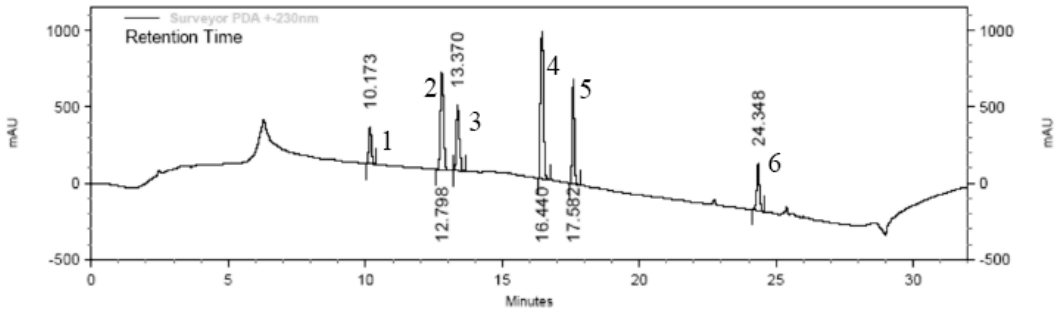


Fig. 2a. HPLC Chromatogram of the beta-blockers and diuretic drugs analyzed

[1] Amiloride, [2] hydrochlorothiazide, [3] metoprolol, [4] propranolol, [5] amlodipine and [6] felodipine.

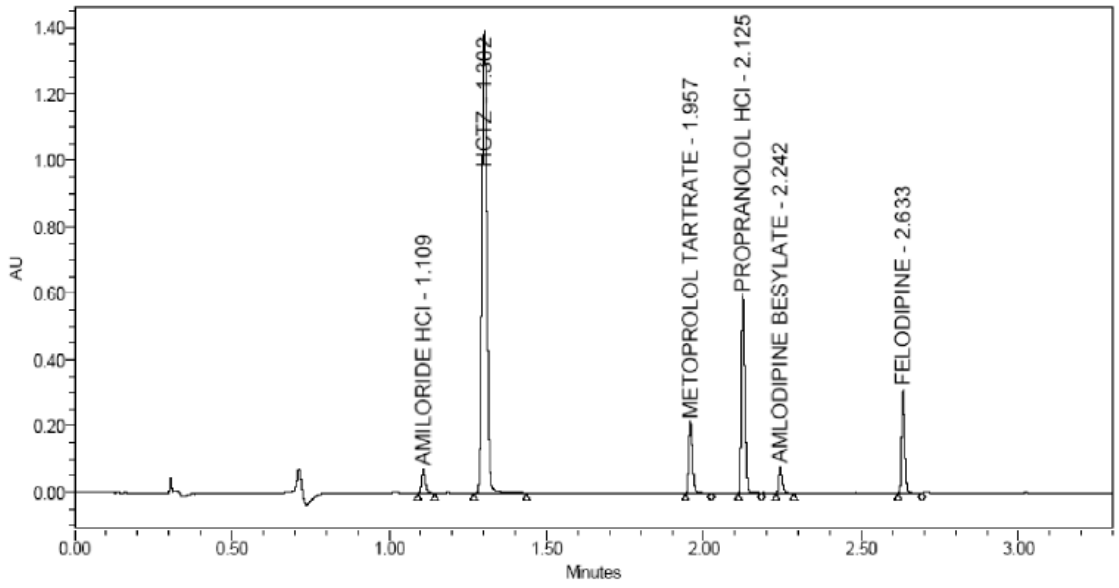


Fig. 2b. UPLC Chromatogram of the beta-blockers and diuretic drugs analyzed

3.3. UPLC Method validation

The newly developed method was validated according to the ICH guidelines with respect to specificity, linearity, accuracy, precision, and robustness [36]. System suitability parameters like

capacity factor (k'), resolution (R) tailing factor (T) and peak capacity (P) were calculated and presented in Table 1.

3.3.1. Specificity

Different force degradation samples were analyzed and it was found that the drug peaks in acid, alkali, peroxide, UV and photo degraded drug sample solutions have passed the purity test. Purity angle for the selected drug components in all stress conditions were found to be less than the threshold angle. This study confirms the specificity of the developed method.

The overall degradation in acidic and basic condition was found to be around 15 % for all the drug compounds except for AML and AB. In basic stress condition, AML and AB showed around 50% degradation. The degradation of AB is ascribed to the acid or alkaline hydrolyses of the acetyl groups of amlodipine [37, 38]. Amiloride hydrochloride was found to be highly sensitive to alkaline treatment. Two unknown impurities which were believed to be the degradants of amiloride hydrochloride at the retention times of 0.913 and 1.013 (Fig 6a), respectively, were formed after alkaline degradation. The possible structure of the major unknown amiloride degradant was proposed and confirmed by LCMSMS studies.

The drug components in tablet sample were found to be stable when treated with 30 % hydrogen peroxide at 100° C. Overall degradation of 7 % including known and unknown impurities was achieved when drug product was exposed to peroxide oxidation. All compounds of the 1,4-dihydropyridine class are susceptible to photolytic decomposition and AB is no exception and undergoes oxidation when exposed to light [39–41] resulting in the formation of a pyridine analogue, lacking any therapeutic effect [42]. However all other drug compound selected in this study were unaffected during oxidative degradation. Baseline resolution was achieved between all the impurities and drug components. Typical chromatograms of sample solutions under acidic, basic and oxidative degradation are as presented in fig.3a, 3b and 3c, respectively.

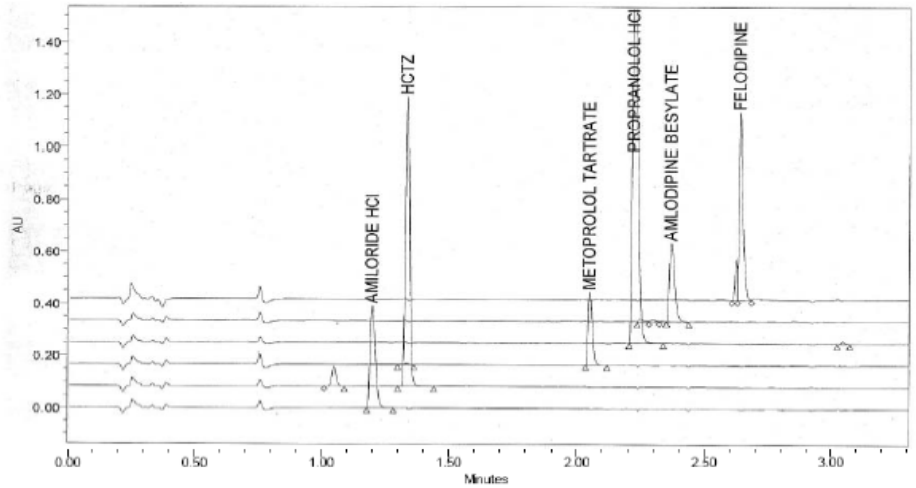


Fig 3a: Overlaid chromatograms - Acid degradation of Betablockers

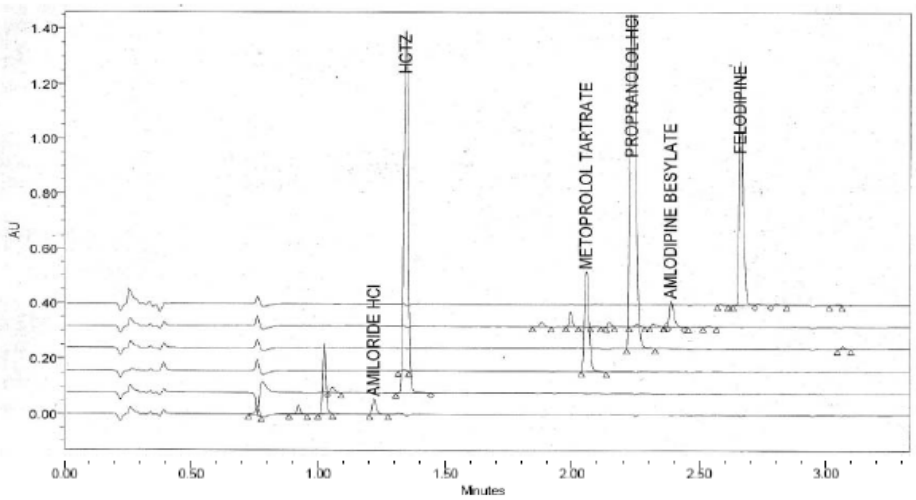


Fig 3b: Overlaid chromatograms - Base degradation of Betablockers

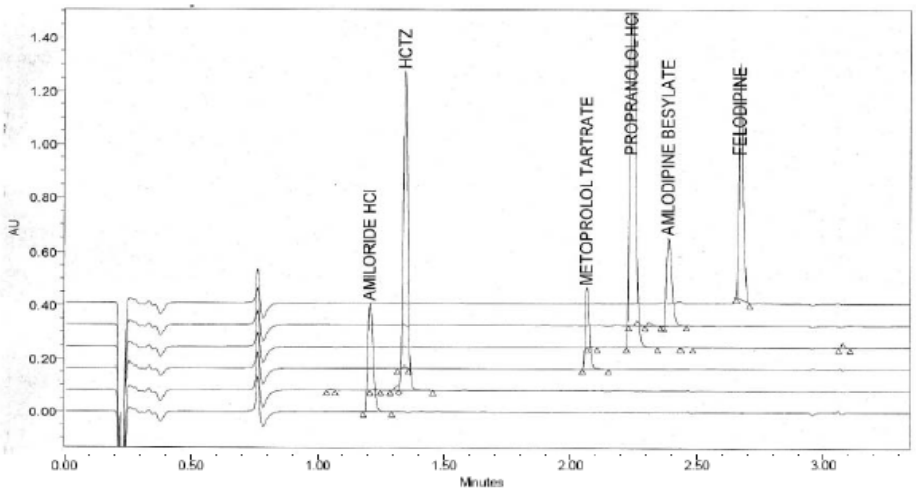
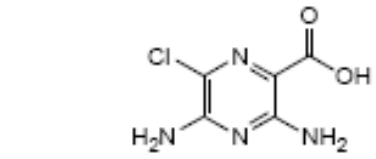


Fig 3c: Overlaid chromatograms - Oxidative degradation of Betablockers

3.3.2. Structural identification of degradants

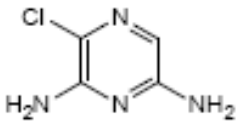
From the new degradants formed upon alkaline degradation of amiloride hydrochloride, we could identify these two impurities by LCMS, one with m/z 189 (Fig 4, impurity A) and other with m/z 145 (Fig 5, impurity B). Since both the impurities were found to be polar in nature, all the attempts to isolate these impurities either by reverse phase or normal phase preparative chromatography were not fruitful. Hence, LC/MS analysis alone provided the primary means for structure determination. The structure was confirmed mechanistically.



3,5-Diamino-6-chloro-pyrazine-2-carboxylic acid

Molecular wt. 188

Fig 4: Proposed structure of impurity A.



3-Chloro-pyrazine-2,6-diamine

Molecular wt. 144

Fig 5: Proposed structure of impurity B.

Fig. 6a and 6b represents the UPLC chromatogram and corresponding mass spectra of amiloride hydrochloride obtained from alkaline degradation, respectively. Since, the chromatographic conditions employed for both LC/MS and UPLC experiment were same, correlation of the peaks by both the methods were straightforward.

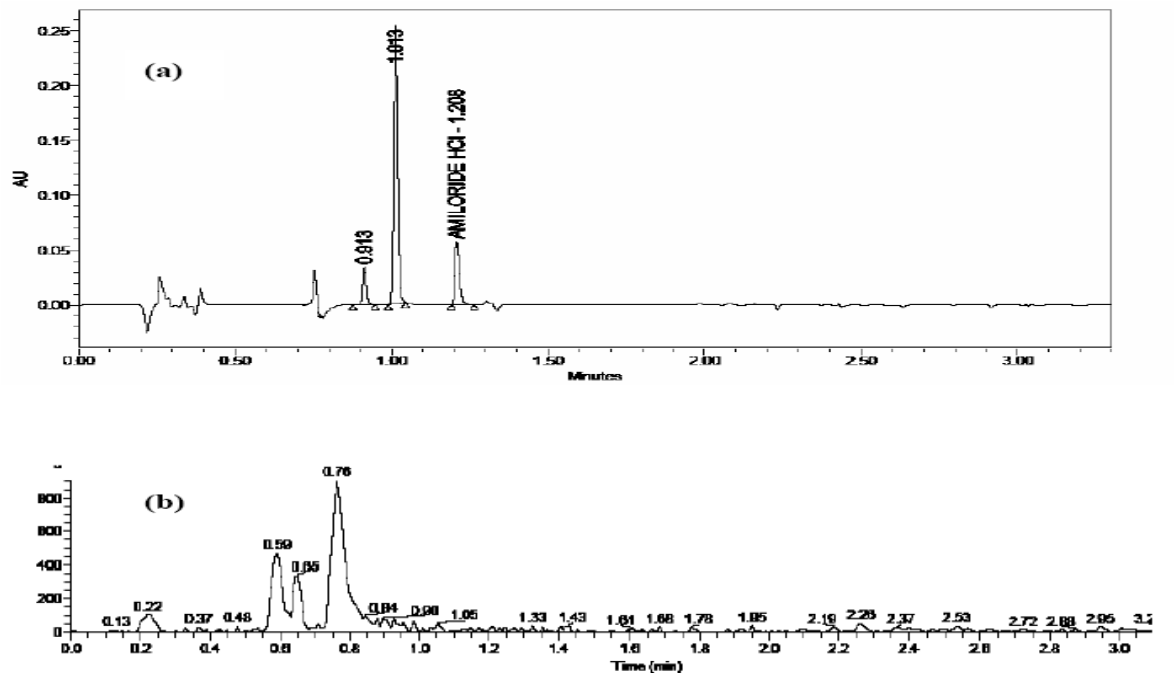


Fig 6a and 6b: UPLC and mass spectra of amiloride degradation profile by sodium hydroxide.

The positive ion mass spectrum of impurity A as shown in Fig. 7a possesses M+1 ion of 189. This *M*W requires the presence of an even number of nitrogens. In addition, the MS data indicated the presence of chlorine from this species. The only site which can be susceptible to hydrolysis is the guanidine moiety. Hence the proposed structure of impurity A must account for the loss of guanidine moiety and on hydrolysis to form an acid to bring the *M*W to 188. The formation of 187 (M-1) ion from negative ion mode confirms to the proposed structure as shown in Fig. 7b.

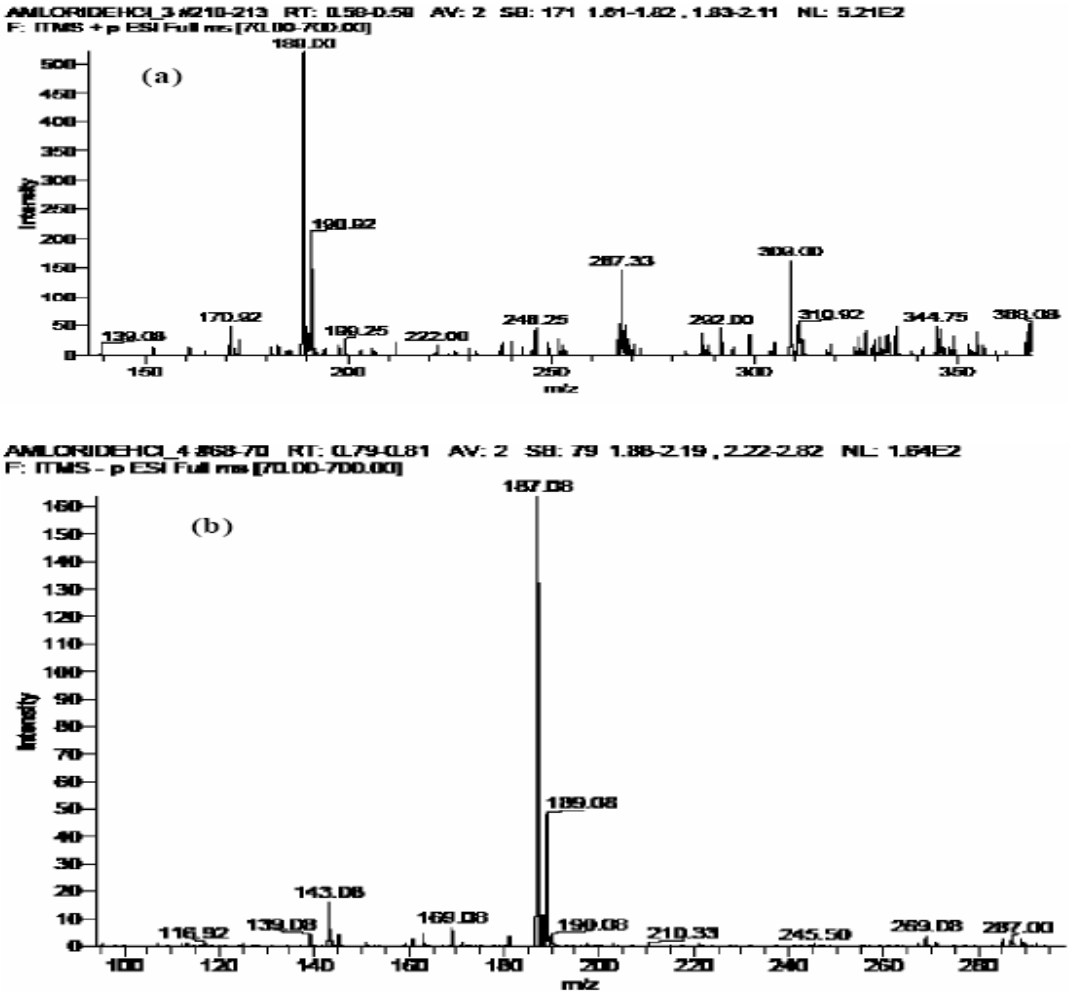


Fig 7: Positive and negative ion mode mass spectra of impurity A

Mechanism for the formation of impurity A is shown in Fig 8. Formation of fragmented ion of *m*/*z* 171 (M+1) by MS/MS experiment, as shown in Fig 9, indicates the loss of water from proposed structure of impurity A.

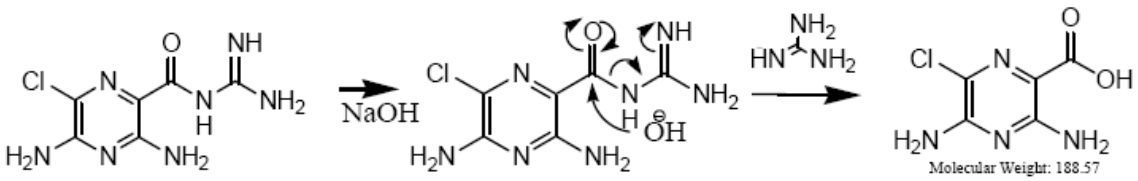


Fig 8: Mechanism of formation of impurity A.

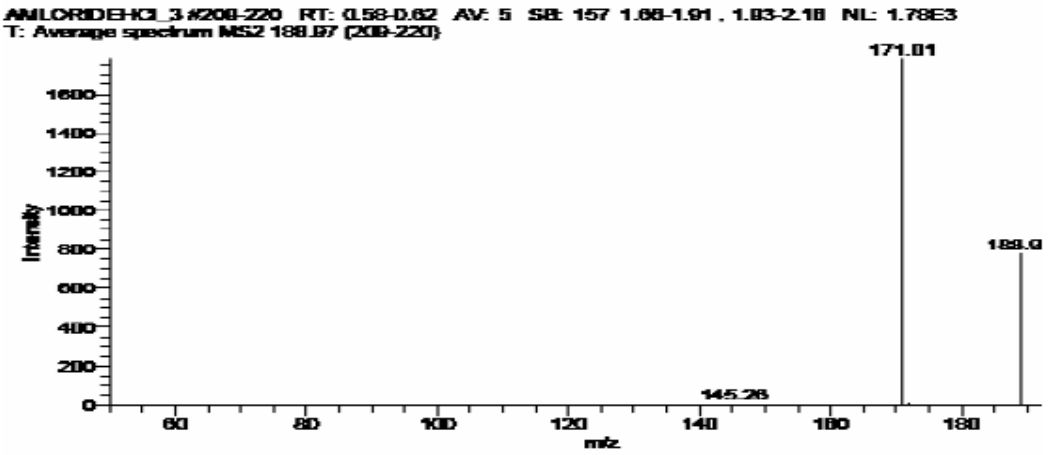


Fig 9: MS/MS spectra of impurity A

The positive ion mass spectrum of impurity B as shown in Fig 10 possesses M+1 ion of 145. The proposed structure for impurity B seems to be most reasonable possibility based on HPLC-MS data. However, in absence of additional data it is not clear whether impurity B is arising from amiloride or from impurity A.

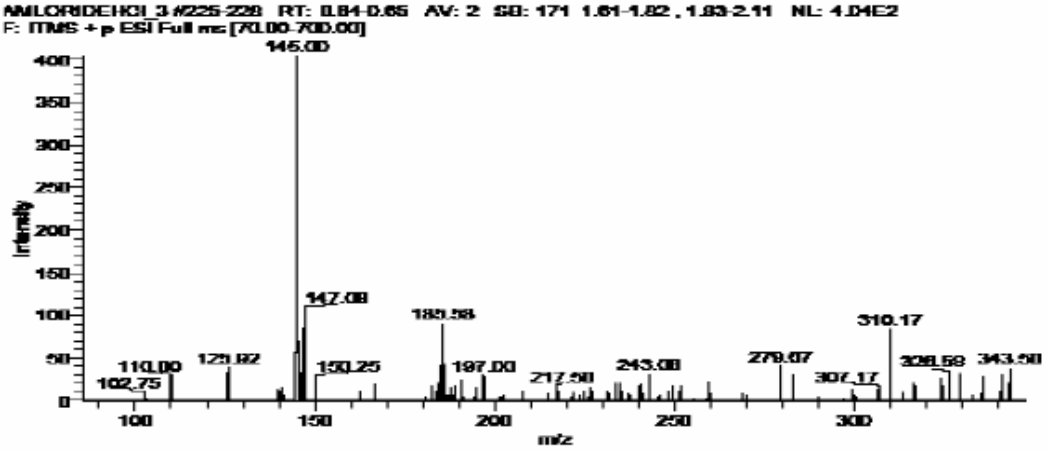


Fig 10: Positive ion mode mass spectra of impurity B

3.3.4. Linearity

The linearity was found in the range of 25 to 150 µg/mL, 250 to 1500 µg/mL, 125 to 750 µg/mL, 50 to 300 µg/mL, 25 to 150 µg/mL and 50 to 300 µg/mL for AML, HCTZ, MT, PRO, AB and FEL respectively. The slope, Y-intercept, coefficient of determination (r^2) and standard deviation on slope were calculated from linear regression analysis. Correlation coefficients (n=3) were found to be more than 0.995 for all the selected drugs with % RSD values ranging from 1.6 – 5.0% across the concentration range studied. The results are listed in the Table 2.

Table 2 Linear regression equations and correlation coefficient

Name of the drug component	Range (µg/mL)	Analyst 1				Analyst 2			
		Slope	Intercept	R	RSD on Slope	Slope	Intercept	R	RSD on Slope
Amiloride	25 - 150	5.32×10^2	-1.18×10^2	0.9951	5.0	5.85×10^2	-9.99×10^1	0.9958	4.62
Hydrochlorothiazide	250 - 1500	1.39×10^3	$+4.76 \times 10^4$	0.9994	1.6	1.40×10^3	$+7.83 \times 10^4$	0.9982	2.82
Metoprolol	125 - 750	3.02×10^2	$+6.04 \times 10^3$	0.9985	2.7	3.19×10^2	$+6.71 \times 10^3$	0.9973	3.51
Propranolol	50 - 300	2.07×10^3	-6.99×10^3	0.9983	2.9	2.33×10^3	-8.82×10^3	0.9992	2.03
Amlodipine	25 - 150	5.07×10^2	$+2.36 \times 10^3$	0.9981	2.9	5.99×10^2	-1.55×10^2	0.9991	2.16
Felodipine	50 - 300	1.08×10^3	-8.61×10^3	0.9981	3.2	1.18×10^3	-9.50×10^3	0.9975	2.63

3.3.5. Accuracy

The absolute recoveries were calculated by comparing the areas under the peaks obtained from standard working solution with the peak areas from standard samples. The mean recoveries for all the components were found to be in the range of 96.5-103 %. The % relative standard deviation at each level was found to be less than 2.0 for all the three drug components. Accuracy was also used to check the interference of the excipients. None of the excipients were found to interfere in the analysis of these drugs indicating that the method is specific for the simultaneous determination of AML, HCTZ, MT, PRO, AB and FEL in tablet dosage form. Recovery data presented in Table 3 indicate that the newly developed method is highly accurate.

Table 3 Accuracy data (analyte recovery)

Compound	Theoretical (% Target level)	Amount added (µg/mL)	Analyst 1			Analyst 2		
			Amount recovered (µg/mL)	Recovery (%)	% RSD	Amount recovered (µg/mL)	Recovery (%)	% RSD
Amiloride	50	50	50.7	101.3	1.2	49.3	101.3	1.1
	80	80	80.5	100.6	1.1	81.4	100.6	1.2
	100	100	99.7	99.7	0.9	100.3	99.7	0.9
	120	120	116.8	97.3	0.7	118.5	97.3	0.8
	150	150	152.2	101.8	0.4	150.4	101.8	0.1
HCTZ	50	400	398.9	99.4	1.3	381.4	99.4	1.1
	80	800	788.5	98.5	1.3	789.2	98.5	0.9
	100	1020	1030.2	101.1	0.9	1030.2	101.1	0.9
	120	1200	1207.7	100.1	0.7	1230.1	100.1	0.4
	150	2000	1994.8	99.7	0.3	1981.1	99.7	0.1
Metoprolol	50	220	212.1	96.4	1.3	215.6	96.4	0.9
	80	400	401.2	100.3	0.1	393.4	100.3	0.5
	100	500	508.4	101.7	1. 1	511.1	101.7	0.7
	120	630	642.1	101.7	1.2	645.3	101.7	0.7
	150	730	714.6	97.9	0.8	712.6	97.9	0.4
Propranolol	50	91	87.6	96.2	1.4	89.1	96.2	1.4
	80	166	165.2	99.5	1.8	162.5	99.5	0.4
	100	203	209.4	103.1	0.3	208.5	103.1	0.8
	120	245	249.2	101.4	1.5	249.1	101.4	0.9
	150	295	288.7	97.9	0.8	289.5	97.9	0.7
Amlodipine	50	50	50.1	100.2	0.9	49.3	100.2	0.6
	80	80	78.1	97.5	0.9	78.3	97.5	1.8
	100	100	101.1	101.1	0.8	100.4	101.1	1.9
	120	120	122.1	101.7	1.1	123.2	101.7	0.2
	150	150	148.1	98.9	1.1	147.8	98.9	0.5
Felodipine	50	100	98.1	98.1	1.1	98.2	98.1	1.1
	80	160	163.0	101.8	0.7	164.3	101.8	0.2
	100	200	201.4	100.7	1.3	199.5	100.7	1.1
	120	240	235.2	98.1	0.2	235.2	98.1	0.5
	150	300	301.8	100.6	1.8	302.2	100.6	0.7

3.3.6. Precision

The relative standard deviations (RSDs) were found to be 1.5%, 1.3%, 0.5%, 1.0%, 0.5% and 0.7% for AML, HCTZ, MT, PRO, AB and FEL, respectively, which are well within the acceptable limit of 2.0%. The RSDs for intermediate precision were found to be 1.2%, 0.9%, 0.7%, 0.9%, 0.7% and 0.8% for AML, HCTZ, MT, PRO, AB and FEL, respectively. The results for all the tested compounds are listed in Table 4, which indicates that the method has a good reproducibility and intermediate precision.

Table 4 Intermediate precision and ruggedness

	Analyst 1		**Analyst 2**	
Components	**Mean Assay (%)**	**RSD**	**Mean Assay (%)**	**RSD**
Amiloride HCl	99.6	1.5	98.1	1.2
HCTZ	98.1	1.3	98.8	0.9
Metoprolol	99.6	0.5	99.6	0.7
Propranolol	101.4	1.1	101.7	0.9
Amlodipine	100.2	0.5	98.4	0.7
Felodipine	99.9	0.7	98.8	0.8

3.3.7. Solution stability

Solutions of the studied compounds in the mobile phase exhibited no chromatographic changes during solution stability study and also the absolute difference in the assay value for all the drug components were found to be within the limit of 2.0 for 48 hours when studied at regular time intervals at room temperature.

3.3.8. Robustness

In all deliberately varied conditions, the RSD of peak areas of AML, HCTZ, MT, PRO, AB and FEL were found to be well within the acceptable limit of 2%. The tailing factor for all the peaks was found to be <2.0. The elution order and resolution of drug components and their impurities were not affected and only slight variations in retention times were observed.

3.3.9. Assay

The validated method was applied to the determination of AML, HCTZ, MT, PRO, AB and FEL in commercially available tablets. The result of the assays (n = 6) undertaken yielded 99.6% (%RSD = 1.50%), 98.1% (%RSD = 1.30%), 99.6% (%RSD = 0.50%), 101.4% (%RSD = 1.0%), 100.2% (%RSD = 0.50%) and 99.9% (%RSD = 0.7%) of label claim for AML, HCTZ, MT, PRO, AB and FEL respectively. The results for all the tested compounds are listed in Table 4, indicate that the method is selective for the analysis of all the selected drugs without interference from the excipients used to formulate and produce these tablets.

4. Conclusion

The newly developed UPLC method for the simultaneous determination of AML, HCTZ, MT, PRO, AB and FEL in pharmaceutical dosage forms was found to be capable of giving faster retention times, maintaining good resolution than that achieved with conventional HPLC. The method was completely validated showing satisfactory data for all the parameters tested. This method exhibited an excellent performance in terms of sensitivity and speed. It is a stability indicating method suitable for routine analysis and quality control of pharmaceutical preparations containing these drugs either as such or in combination

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