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Simultaneous determination of Atenolol, Amiloride hydrochloride and Hydrochlorothiazide using reversed phase liquid chromatography

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ABSTRACT

New, simple, rapid and precise reversed phase liquid chromatographic method has been developed and validated for the simultaneous determination of atenolol, amiloride hydrochloride and hydrochlorothiazide. Chromatographic separation was carried out on a C18 column with a mobile phase consisting of 25 mM sodium acetate anhydrous: acetonitrile: triethylamine (95:5:0.1%, v/v/v) at a flow rate 0.9 ml min^{-1} at ambient temperature. Quantitation was achieved with UV detection at 280 nm. Linearity, accuracy and precision were found to be acceptable over the concentration range of (5–50 μg ml^{-1}) for atenolol, (0.25–2.5 μg ml^{-1}) for amiloride hydrochloride and (1.25–25 μg ml^{-1}) for hydrochlorothiazide. The optimized method proved to be specific, robust and accurate for the quality control of the cited drugs in pharmaceutical preparation.

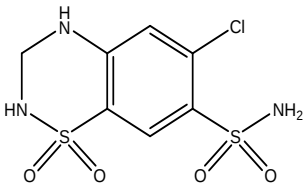
Keywords: Atenolol; Amiloride hydrochloride; hydrochlorothiazide; Reversed phase liquid chromatography; pharmaceutical preparation.

INTRODUCTION

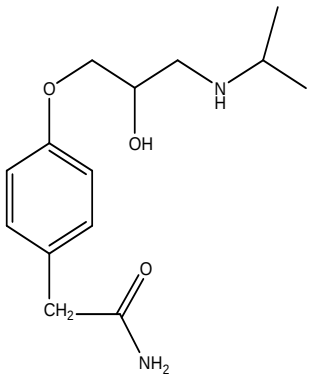
Atenolol (AT); (RS)-2-{4-[2-hydroxy-3-(propan-2-ylamino) propoxy]phenyl} acetamide (Fig.1A) is a β1-selective (cardioselective) adrenoreceptor antagonist drug commonly used for management of hypertension, prevention of heart diseases as angina pectoris and control of some forms of cardiac arrhythmia [1]. Amiloride hydrochloride (AM); 3,5-diamino-6-chloro-N-(diaminomethylene) pyrazine-2-carboxamide (Fig.1B) is a mild diuretic that acts by blocking sodium channels in the late distal tubules and collecting ducts. By increasing the loss of sodium and chloride ions while reducing the excretion of potassium, AM. adds to the natriuretic effects of other diuretics, while diminishing their kaliuretic effects [2].

Hydrochlorothiazide (HT); 6-chloro-1,1-dioxo-3,4-dihydro-2H-1,2,4-benzothia-zine-7-sulphonamide (Fig.1C) is a thiazide diuretic that increases sodium and chloride excretion by distal convoluted tubule [1].

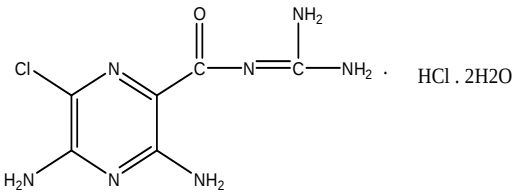
Literature survey reveals that several analytical methods have been reported for the determination of AT alone or in combination including spectrophotometry[6-13], spectrofluorimetry [14-17], HPLC [18-26]. AT,AM and chlorothalidone were simultaneously determined by HPLC and chemometric-assisted spectrophotometry [27], capillary zone electrophoresis[28-32], Voltametry [33] and TLC [34]. Besides, several analytical methods have been reported for the determination of AM alone or in combination with other drugs including spectrophotometry [35], spectroflourometry [36,37], gas chromatography [38] and HPLC [39] & [40,41]. Also several analytical methods have been reported for the determination of HT alone or in combination with other drugs, including spectrophotometry [42-62], TLC-densitometry and spectrofluorimetry [63], HPLC [64-74], HPTLC [75-77], LC [78-80] spectrophotometry, HPLC and HPTLC [81]. The ternary mixture of AT, AM, and HT has only been determined by derivative spectroscopy [3] and by chemometric-assisted spectrophotometric method with timolol [5]. Besides, a reversed-phase liquid chromatographic method has been described for the determination of AM, AT, HT in their combined mixtures but applying cyanopropyl column not C18 column [4]. Due to the wide use and availability of C18 columns, our aim was to develop and validate an alternative reversed-phase liquid chromatographic method for the determination of the ternary mixture under investigation applying C18 columns.



(Fig.1 C) Hydrochlorothiazide



(Fig.1 A) Atenolol



(Fig. 1 B) Amiloride

EXPERIMENTAL SECTION

2.1. Instrumentation

A chromatographic system consisting of Agilent 1100 series; interface equipped with an Agilent isocratic pump G1310A, Agilent UV-visible detector G1314A, an Agilent manual injector G1328B equipped with (20 µl) injector loop, and Agilent Zorbax C18 column (5µm, 4.6 x 250 mm) was used. Agilent syringe, LC 100 µl , CA, U.S.A. Ultrasonic processor; Soniclean 120T, 220/240v, 50/60Hz, 60W, Thebarton SA, Australia were used.

Pharmaceutical grade of AT and AM were supplied and certified by Ramida company (Cairo-Egypt) to contain 99.8, 99.9%, respectively. Pharmaceutical grade HT was supplied and certified by EPICO company (10^{th} of Ramadan- Egypt) to contain 99.85%. Atenoretic capsules (Manufactured by Sigma pharmaceutical Industries for Queen Pharm International) were labeled to contain 50 mg AT, 2.5 mg AM and 25 mg HT.

Acetonitrile used was HPLC grade (LAB-SCAN, Poland). Bi-distilled water was produced in-house (Aquatron Water Still, A4000D, UK). Membrane filters 0.45 µm from Teknokroma (Barcelona, Spain) were used. All other chemicals and reagents used were of analytical grade unless indicated otherwise.

2.3. Chromatographic conditions

Chromatographic separation was achieved on an Agilent Zorbax C18 column (5µm, 4.6 x 250 mm) applying an isocratic elution based on 25mM sodium acetate anhydrous buffer: acetonitrile: triethylamine (95:5:0.1%, v/v/v) as a mobile phase. The pH of the mobile phase was adjusted to 2.9 using acetic acid. The mobile phase was pumped through the column at a flow rate of 0.9 ml min^{-1}. The injection volume was 20 µl. Analyses were carried out at ambient temperature and detection was carried out at 280 nm for the three drugs.

2.4. Standard solutions

Standard solutions of each of AT, AM and HT were prepared by separately dissolving 50 mg, 25 mg & 25 mg respectively in 100 ml mobile phase then further dilutions were made to obtained (0.5 mg ml^{-1}) AT, (0.025 mg ml^{-1}) AM & (0.25 mg ml^{-1}) HT, within the concentration rang of 5–50 µg ml^{-1} for AT, 0.25–2.5 µg ml^{-1} for AM and 1.25–25 µg ml^{-1} for HT.

2.5. Sample preparation

The contents of twenty capsules were weighed and mixed in a mortar. An accurately weighed portion of the powder equivalent to about 50 mg of AT, 2.5 mg of AM and 25 mg HT was extracted and diluted to 100 ml with the mobile phase. The sample solution was filtered. Further dilution of the filtrate was carried out with the mobile phase to provide a solution of 50 µg ml^{-1} of AT, 2.5 µgml^{-1} of AM and 25 µg ml^{-1} of HT.

2.6. Procedure

2.6.1. Linearity and repeatability

Accurately measured aliquots of working standard solutions equivalent to 50–500 µg ml^{-1} for AT, 2.5–25 µg ml^{-1} for AM and 12.5–250 µg ml^{-1} for HT were transferred into three series of 10mL volumetric flasks. The volumes were completed with the mobile phase. A volume of 20 µl of each solution was injected in triplicates into the chromatograph under the specified chromatographic conditions described previously. A calibration curve for each compound was obtained by plotting area under the peak (AUP) against concentration (C).

The repeatability of the method was assessed by analyzing a laboratory prepared mixture containing 20 µg mL^{-1},10 µg $mL^{-1}$and 1.0 µg mL^{-1} for AT, HT and AM (n = 6). The precision (R.S.D %) for each compound was calculated.

2.6.2. Assay of laboratory prepared mixtures and Atenoretic ® capsules

The procedure mentioned under 2.6.1. was repeated using laboratory prepared mixtures equivalent to 5-50 µg mL^{-1}AT, 0.25-2.5 µg mL^{-1} AM and 1.25-25 µg mL^{-1} HT. For the determination of the examined drugs in Atenoretic ® capsules, the sample solution prepared under 2.5. was diluted to prepare different solutions equivalent to 7.5-40 µg mL^{-1}AT, 0.25-2 µg mL^{-1} AM and 1.25-20 µg mL^{-1} µg mL^{-1} HT and injected in triplicated into the chromatograph.

RESULTS AND DISCUSSION

A previous work [4] described the LC determination of the three drugs under investigation applying cyanopropyl column not C18 column. So, the aim of this work was to develop a new,

simple, accurate and reproducible LC method for the simultaneous determination of AT, AM and HT applying C18 columns due to its wide availability and versatility in all quality control laboratories.

Table 1: System suitability results of the proposed method.

Compound	N	R	T	% R.S.D. of	
				t_R	Peak Area
HT	3208	-	1.05	0.48	0.28
AT	2606	4.04	1.03	0.64	0.38
AM	2531	4.07	1.07	0.25	0.63

(N: Number of theoretical plates; R: resolution factor; T: Tailing factor; t_R: retention time)

3.1. Method development

For the separation of the examined drugs, various reversed-phase C18 columns, isocratic mobile phase systems were attempted. The mobile phase composition and pH were studied and optimized. A satisfactory separation was obtained with a mobile phase composed of 25mM sodium acetate anhydrous buffer, acetonitrile and triethylamine in the ratio of (95:5:0.1%, *v/v/v*), adjusted to pH 2.9 using acetic acid. At low acetonitrile concentration (<10%), separation was obtained but with excessive tailing for AM peak. At pH 2.9, optimum resolution with reasonable retention time was observed. Quantitation based on peak area achieved with UV detection at 280 nm where high sensitivity was obtained for the three drugs.

3.2. Validation of the method

3.2.1. Linearity

Linearity was studied for AT, AM and HT. A linear relationship between area under the peak (AUP) and component concentration (C) was obtained. The regression equation for each drug was also computed (Table 2) in this study, 6 concentrations for each compound were used. The linearity of the calibration curves were validated by the high value of correlation coefficients (Table 2) The analytical data of the calibration curves including standard deviations for the slope and intercept (S_b, S_a) are summarized in (Table 2)

3.2.2. Accuracy

Accuracy of the results was calculated by % recovery of laboratory prepared mixtures of 6 different concentrations of the AT, AM and HT and also by standard addition technique for Atenoretic® capsules. The results obtained including the mean of the recovery, standard deviation, relative standard deviation are displayed in (Table 2)

3.2.3. Precision

Precision was estimated by repeatability. The repeatability of the method was assessed by analyzing a mixture containing 20 µg mL^{-1},10 µg $mL^{-1}$and 1 µg mL^{-1} for AT, HT and AM (n = 6), respectively. The values of the repeatability (%R.S.D) and inter-day and intra-day precision (using 3 different concentrations in triplicates for three days) for the three drugs are displayed in (Table 2)

3.2.4. Specificity

Specificity is the ability of the analytical method to measure the analyte response in the presence of interferences. In the present work, the chromatograms of the samples were checked for the appearance of any extra peaks. No chromatographic interference from any of the excipients was found at the retention times of the examined drugs, Fig.2 In addition, the chromatogram of each drug in the sample solution is identical to the spectrum received by the standard solution at the

wavelengths applied. These results demonstrate that there was no interference from other materials in the pharmaceutical formulations and therefore confirm the specificity of the method.

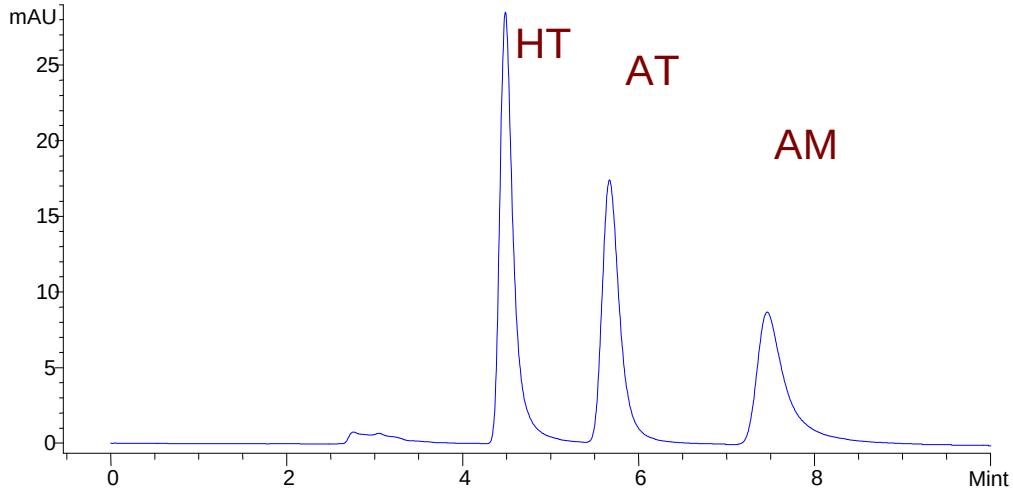


Figure 2: A typical LC chromatogram of 25µL injector of Atenoretic® sample solution.

Table 2: Assay parameters and method validation obtained by applying HPLC method for the simultaneous determination of atenolol, amiloride HCl and hydrochlorothiazide in mixture

Item	Atenolol	Hydrochlorothiazide	Amiloride HCl
Retention time	5.672 min	4.489 min	7.466 min
Wavelength of detection	280 nm	280 nm	280 nm
LOD	0.00138	1.012	1.41179×10^{-5}
LOQ	0.00419	3.067	4.278×10^{-5}
Range of linearity	5-50 µg ml^{-1}	1.25-25µg ml^{-1}	0.25-2.5 µg ml^{-1}
Regression equation	y = 4.3539x-1.2938	y = 47.566x + 4.8445	Y= 54.55x-0.0468
correlation coefficient (r^2)	0.9990	0.9992	0.9997
S_b	2.275	30.649	28.707
S_a	172.943	425.750	40.809
Confidence limit of the slope	4.3539±752.977	47.566±20251.225	54.55±2226.131
Confidence limit of the intercept	1.2938±2.943	4.844±148.464	0.0468±1.343
Standard error of the estimation	88.723	671.805	57.115
Intra day[a] %R.S.D.	0.921-1.01	0.54-1.23	0.35-1.4
Inter day[b] %R.S.D.	0.936-1.278	0.73 -1.68	0.48-1.49
Drug in dosage form	99.46±0.961	101.17±1.23	99.7±1.77

[a] *The intraday (n = 3), average of three concentrations (8, 10, 12 µg Ml^{-1}) for AT, (4, 5, 6 µg Ml^{-1})for HT and(0.4, 0.5, 0.6 µg Ml^{-1}) for AM repeated three times within the day.*

[b] *The interday (n = 3), average of three concentrations(8, 10, 12 µg Ml^{-1}) for AT, (4, 5, 6 µg Ml^{-1})for HT and(0.4, 0.5, 0.6 µg Ml^{-1}) for AM repeated three times within the day.*

3.2.5. Range

The calibration range was established through consideration of the practical range necessary, according to each compound concentration present in the pharmaceutical product, to give accurate, precise and linear results. The calibration range of the proposed HPLC method is given in (Table 2)

3.2.6. Limit of detection and limit of quantification

Limit of detection (LOD) which represents the concentration of analyte at S/N ratio of 3 and limit of quantification (LOQ) at which S/N is 10. According to ICH recommendations [82], the approach based on the S.D. of the response and the slope was used for determining the detection and quantitation limits. The theoretical values were assessed practically and given in Table (Table 2).

3.2.7. Robustness

Robustness is a measure of the method ability to remain unaffected by small variations in the method conditions and is an indication of the method reliability. Robustness was performed by deliberately changing the chromatographic conditions.

Variation of pH of the mobile phase by ± 0.2 and its organic strength by ± 2% did not have any significant effect on chromatogram Fig. 3.

The flow rate of the mobile phase was changed from 0.9 mL min^{-1} to 0.7 mL min^{-1} and 1.1 mL min^{-1}. The organic strength was varied by ± 2%, while pH was varied by ±0.1 units. Concentration of Sodium acetate anhydrous buffer was varied by ± 2%. The most important parameter to be studied was the resolution factor between the three peaks of AT, HT and AM. As can be seen in(Table 3-6), good values of the resolution factor were obtained for all these variations, indicating good robustness of the proposed LC method. It is worth noting that an increase in the organic modifier resulted in an increase in the retention time of AT., HT and vice versa. AM. was not much affected by the change of the organic modifier.

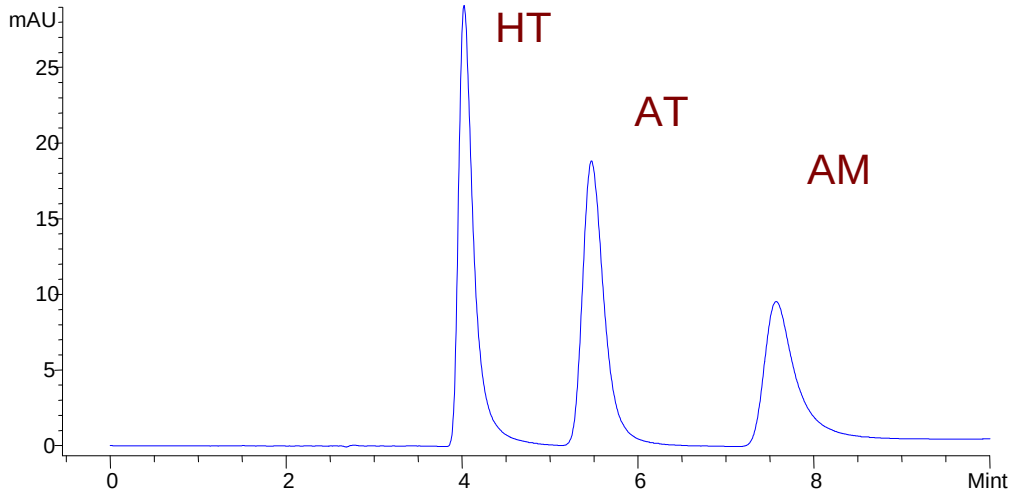


Figure 3: A typical LC chromatogram of 20µL injector of synthetic binary mixture of hydrochlorothiazide (HT) (25µg mL^{-1}), atenolol (AT) (50µg mL^{-1}) and amiloride hydrochloride (AM) (2.5µg mL^{-1}).

Table 3: Influence of flow rate of the mobile phase on resolution of peaks.

Item	0.7mLmin^{-1}	0.9 mLmin^{-1}	1.1 mLmin^{-1}
Resolution factor HT –AT	5.57	5.47	5.27
Resolution factor AT-AM	7.67	7.57	7.26

Table 4: Influence of organic strength of the mobile phase on resolution of peaks

Item	*3%*	5%	*7%*
Resolution factor HT –AT	5.54	5.47	5.31
Resolution factor AT-AM	7.46	7.57	7.32

Table 5: Influence of pH of the sodium acetate anhydrous buffer on resolution of peaks

Item	Ph 2.8	Ph 2.9	Ph 3.0
Resolution factor HT –AT	5.32	5.47	5.58
Resolution factor AT-AM	**7.47**	7.57	**7.66**

Table 6: Influence of concentration of Sodium acetate anhydrous buffer on resolution of peaks

Item	23Mm	25Mm	27Mm
Resolution factor HT –AT	5.42	5.47	5.38
Resolution factor AT-AM	**7.42**	7.57	**7.46**

3.3. Statistical analysis of the results

A statistical analysis of the results obtained by the proposed method and the reference methods was carried out by "SPSS statistical package version 11". The significant difference between groups was tested by (T-test) and (F-test) at p=0.05 as shown in (Table 7). The test ascertained that there was no significant difference among the methods.

Table 7: Statistical analysis of the results obtained by the proposed LC method and the reference methods

Item	AT		HT		AM	
	HPLC	**Reference method****	**HPLC**	**Reference method*****	**HPLC**	**Reference method******
Mean	100.45	100.26	99.93	99.73	100.052	100.00
S.D.	0.9731	0.493	1.3578	0.8801	0.7711	0.3688
R.S.D.	0.9687	0.492	1.3588	0.8824	0.7706	0.401
n	6	6	6	6	6	6
Variance	0.947	0.243	1.844	0.775	0.595	0.136
t-value	0.4266 (2.228)*		0.3027(2.228)*		0.1490(2.228)*	
F-value	3.897(5.05)*		2.3794(5.05)*		4.375(5.05)*	

** Figures in parentheses are the corresponding theoretical t -and F-values at P = 0.05.*

*** Reference method for AT using potentiometric method according to B.P [83]*

**** Reference method for HT using HPLC method according to U.S.P. [84]*

***** Reference method for AM using potentiometric method according to B.P. [83]*

CONCLUSION

The proposed HPLC method provides simple, accurate and reproducible quantitative analysis for the simultaneous determination of atenolol, hydrochlorothiazide and amiloride HCl in capsules. This method was validated as per ICH guidelines. The proposed method is suitable for the quality control determination of the cited drugs in ordinary laboratories.

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