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Development and Validation of Novel RP-HPLC Method for Simultaneous Determination of Ramipril, Hydrochlorothiazide and Bisoprolol in Ternary Combinations

Shoeb Alahmad*, Hamed M Elfatatry, Mokhtar M Mabrouk, Sherin F Hammad, Fotouh R Mansour

Department of Pharmaceutical Analytical Chemistry, Faculty of Pharmacy, Tanta University, Tanta-31111, Egypt

ABSTRACT

The present work describes a novel reversed phase high performance liquid chromatographic method for the determination of Bisoprolol Fumarate (BIS), Hydrochlorothiazide (HCT) and Ramipril (RAM) in their synthetic mixtures and dosage forms. The developed method performed on a Hypersil® C18 reversed phase column (250 mm × 4.6 mm, particle size 5 µm) using a mobile phase consisting of acetonitrile: methanol: buffer (0.01 M potassium dihydrogen ortho phosphate; pH 3.5) in a ratio of 65:15:20 (v/v/v). The flow rate was 1 ml/min and detection was carried out at 210 nm. The retention times of BIS, HCT and RAM were 2.2, 2.6 and 4 min, respectively. The method showed linearity over the concentration range of 5-40 µg/ml for each drug. The described High performance Liquid Chromatography (HPLC) method was successfully applied for simultaneous determination of those drugs in their combined dosage form. The mean percentage recoveries were found to be 99.37%, 98.75% and 99.48% for BIS, HCT and RAM, respectively. Validation of the method was carried out according to the guidelines of the International Conference on Harmonization (ICH).

Keywords: HPLC, Simultaneous determination, Bisoprolol fumarate, Hydrochlorothiazide, Ramipril

INTRODUCTION

Bisoprolol fumarate (BIS) (±)-1-[4-[[2-(1-Methylethoxy) ethoxy] methyl] phenoxy]-3[(1-methylethyl) amino]-2-propenol (E)-2-butenedioate (Figure 1a), a USP official drug [1], is a selective cardio beta blocker. It is absorbed completely from the gastrointestinal tract and undergoes first pass metabolism resulting in an oral bioavailability of about 90% [2].

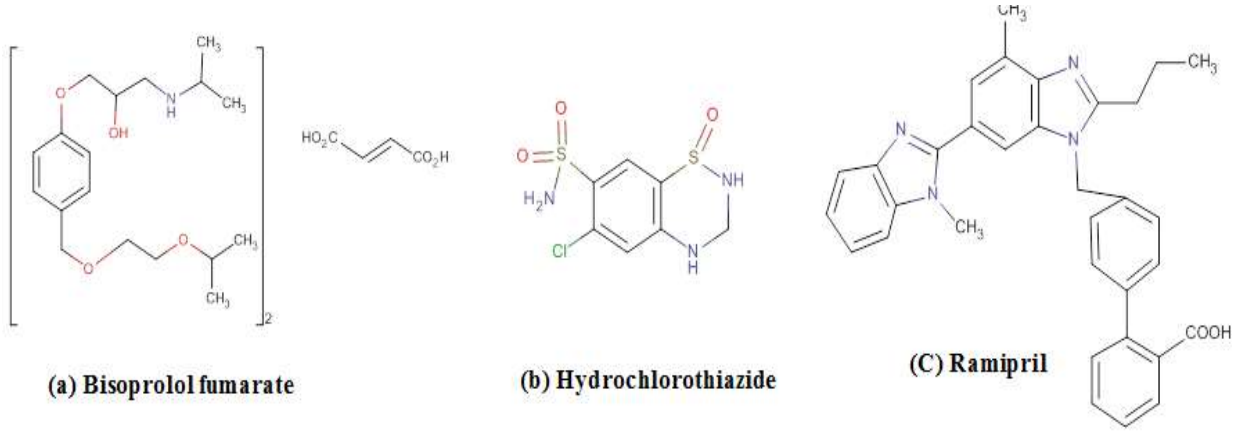

Figure 1: Chemical structure of (a) Bisoprolol fumarate, (b) Hydrochlorothiazide, (c) Ramipril

Hydrochlorothiazide (HCT), 6-chloro-3,4-dihydro-2*H*-1,2,4-benzothiazine-7-sulfonamide,1,1-dioxide (Figure 1b), is one of the oldest thiazide diuretics and is used in the treatment of hypertension patients either alone or with other antihypertensive drugs such as angiotensin-converting-enzyme inhibitors and beta blockers. It is a modestly intense diuretic and applies its diuretic impact by lessening the reabsorption of electrolytes from the renal tubules, along these lines expanding the discharge of sodium and chloride particles and, consequently, of water [2].

Ramipril (RAM) 2-[N-[(S)-1-(ethoxycarbonyl)-3-phenylpropyl)]-L-alanyl] (1S,3S,5S)-2-azabicyclo[3-3-0] octane carboxylic acid (Figure 1c), is an Angiotensin-converting Enzyme (ACE) inhibitor. It acts on the renin–angiotensin aldosterone system. It represses the transformation of the dormant angiotensin I to the exceedingly intense vasoconstrictor, angiotensin II and furthermore lessens the corruption of bradykinin [3]. RAM is an effective agent for the treatment of hypertension, management of heart failure, treatment of myocardial infarction and prophylaxis of cardiovascular events in high risk patients. It is a white powder that is sparingly soluble in water, freely soluble in acetonitrile and methanol [4].

Different analytical methods were reported for the determination of RAM, HCT and BIS either individually or in combination with other drugs. However there is no reported method till now for the determination of these three drugs in ternary mixtures. These methods include spectrophotometry [5], chemo metrics [6], high Performance Liquid Chromatography (HPLC) [7-9] and thin layer chromatography (HPTLC) [10]. A ternary mixture of bisoprolol, hydrochlorothiazide and ramipril has been lunched in the Egyptian market scince 2017. The aim of the present research is to build up a simple, precise, reliable, sensitive and cost effective RP-HPLC method for the simultaneous determination of, bisoprolol, hydrochlorothiazide and ramipril in ternary combinations and dosage form.

EXPERIMENTAL SECTION

Apparatus and software

A Dionex UltiMate 3000 RS system was used, (Thermo Scientific™, Dionex™, Sunnyvale, CA, USA), equipped with a quaternary RS pump, an RS auto-sampler injector, a thermostated RS column compartment and an RS Diode Array Detector (DAD). The instrument was connected to a Dell compatible PC, bundled with Chromeleon® 7.1 Chromatography Data System software. Hanna HI 8314 pH Meter was used to adjust the pH of the buffer used in the mobile phase.

Materials

Pure drugs

Ramipril was manufactured by Sigma-Aldrich Co., Schnelldorf, Germany with a purity of 99.8%. Hydrochlorothiazide was manufactured by Santic with a purity of 100.2% and Bisoprolol fumarate was manufactured by Smaart Pharmaceuticals with purity 99.23%. These drug substances were kindly supplied by Pharmadar Pharmaceutical CO. Elsadat, Menofia, Egypt.

The pharmaceutical dosage form Combitust® containing 2.5 mg BIS, 12.5 mg HCT and 2.5 mg RAM, was kindly supplied by Pharmadar Pharmaceutical CO. Elsadat, Menofia, Egypt.

Reagents

Acetonitrile, methanol HPLC grade were used. Potassium dihydrogen phosphate, orthophosphoric acid analytical grade were used.

Chromatographic conditions

The separation between the three drugs was accomplished utilizing Hypersil® C18 Column 5 µm (4.6 × 250 mm) and a mobile phase containing acetonitrile:methanol:buffer (0.01 M potassium dihydrogen ortho phosphate); pH 3.5 in a ratio of 65:15:20 (v/v/v), at flow rate of 1 ml/min at ambient temperature. Quantitation based on peak area was achieved using DAD at 210 nm. The phosphate buffer was prepared 0.01 M potassium dihydrogen phosphate using distilled water, adjusted to pH 3.5 using orthophosphoric acid then filtered through a membrane filter 0.22 µm and degassed using sonication.

Standard solutions and calibrations

Stock standard solutions: A stock standard solution (400 µg/ml) of each drug was prepared in methanol.

Working standard solutions: A 50 µg/ml working standard solution of each drug was prepared by diluting. 12.5 ml of each stock solution to 100 ml with the mobile phase.

Construction of calibration curves: Different aliquots of the working standard solution were taken and diluted with the mobile phase to obtain concentrations of each drug in the range 5-40 µg/ml. 20 µl of each solution was injected under the optimum chromatographic conditions. The calibration curves were acquired by plotting peak area of each drug against the corresponding concentration and the regression equations were calculated.

Assay of dosage form: The contents of ten capsules of Combitrust®, were weighed and mixed together. An exact amount of the powder that equivalent to one capsule was transferred into a 100 ml volumetric flask, dissolved in methanol. The solution was completed to volume with methanol, sonicated for 10 min. The solution was filtered using 0.22 µm membrane filter paper. 5.0 ml of this filtrate was transferred to 25 ml and finally completed to volume with the mobile phase to give a final concentration of 5, 25 and 5 µg/ml, ramipril, hydrochlorothiazide and bisoprolol, respectively.

RESULTS AND DISCUSSION

Method development and optimization

Several trials were carried out using reversed-phase (250 mm × 4.6 mm (i.d.)) Hypersil® C18 column (5 µm particle size) and different mobile phase composition to attain optimum condition of these 3 drugs one factor at a time concerning higher resolution and good symmetry. Initially a mobile phase consisting of 0.1 M potassium dihydrogen ortho phosphate (pH=2.5) acetonitrile [50:50, v/v] was used, at these conditions a severe overlap occurs between BIS and HCT in addition to the asymmetry peak shapes. The symmetry of peak increased by increasing of acetonitrile ratio and the peaks shape became better, but BIS and HCT peaks extensively overlapped. By using methanol instead of acetonitrile [25:75, v/v] at a flow rate of 1 ml/min, good resolution and symmetry were achieved with run time less than 3 min, but with forked HCT peak (Figure 2).

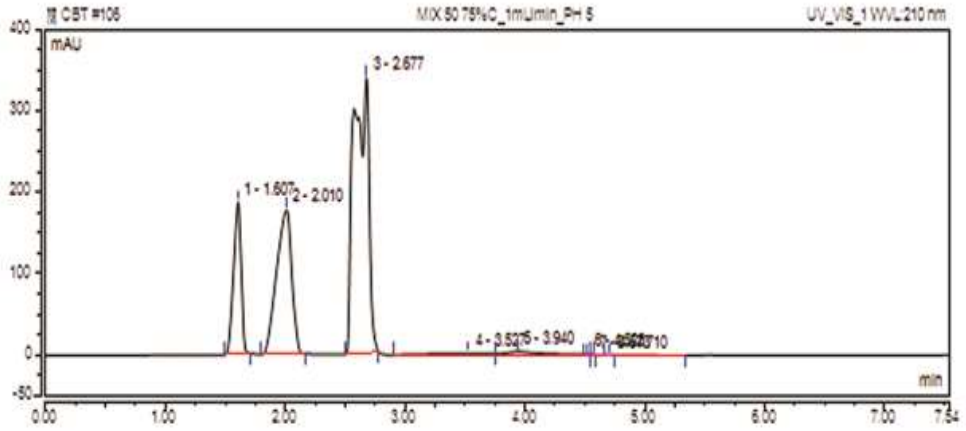

Figure 2: HPLC chromatograms of 20 µl injection of a ternary mixture containing 25 µg/ml of each drug with mobile phase water and methanol (25:75, v/v)

By using phosphate, acetonitrile and methanol different ratio were tested, it was noticed that ratio lower than 60% of acetonitrile led to tailing of ramipril peak whereas higher than 70% led to peak overlap between BIS and HCT. Thus, 65% acetonitrile was selected. Different pH were tried, it was found lower than pH 3.0 led to the peak overlap between BIS and HCT whereas higher than pH 4.0 led to forked HCT peak. It was noticed that decreasing in phosphate concentration led to better symmetry. A mobile phase consisting of 0.01 M potassium dihydrogen ortho phosphate (pH=3.5) acetonitrile, methanol [20:65:15, v/v/v] led to the best conditions for separation and suitable for determination of BIS, HCT and RAM in their ternary mixture with short run time. Figure 3 shows chromatographic separation for the ternary mixture under optimum conditions. The retention times at a flow rate of 1 ml/min were found to be 2.2 ± 0.021 min for BIS, 2.61 ± 0.016 min for HCT and 4.00 ± 0.03 for RAM for ten replicates. Run time was found to be less than 5 min. The wavelength was tested between 210-300 nm by diode array detection, 210 nm give the best results. Different trials were carried out and optimum conditions were selected to give higher resolution and higher symmetry. System suitability parameters are shown in Table 1.

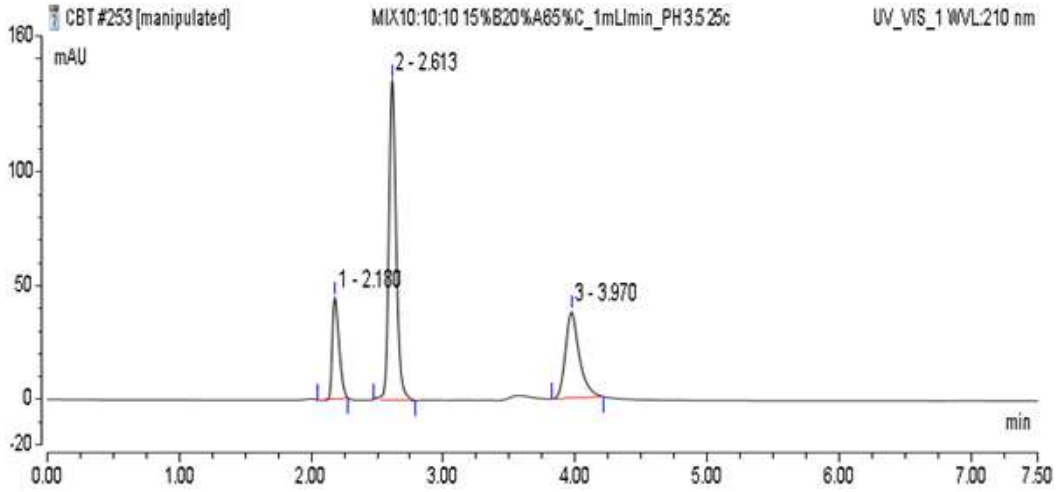

Figure 3: HPLC chromatograms of 20 µl injection of a ternary mixture containing 10 µg/ml of each drug under optimum conditions

Table 1: Results of system suitability tests for the determination of BIS, HCT and RAM by the proposed RP-HPLC method'

Drug	Retention time (min)	Resolution (RS)	Asymmetry factor (AS)	Plat count (N)
BIS	2.20		1.28	15354
HCT	2.61	4.96	1.76	12908
RAM	4.00	10.11	1.7	7487

Method validation

The method was validated as per ICH guidelines [11].

Linearity and range: A series of standard solutions of eight different concentrations were analyzed to evaluate the linearity of HPLC detector and analyses were performed in triplicates. The method was found to be linear over a concentration range of 5-40 µg/ml for each drug. Regression data of the calibration curves are given in Table 2. The linearity of the calibration curves is clearly evident by the values of the correlation coefficients and the standard deviations around the slope and the intercept (Table 2).

Table 2: Characteristic parameters of the calibration equations for BIS, HCT and RAM by the proposed HPLC method

Parameter	BIS	HCT	RAM
Calibration range (µg/ml)	5-40	5-40	5-40
Detection limit (µg/ml)	1.6759	2.3320	1.7310
Quantitation limit (µg/ml)	5	5	5
Slope (b)	0.2524	0.7225	0.5182
Standard deviation of the slope (Sb)	0.0056	0.0222	0.0119
Intercept (a)	0.1788	1.8660	-0.2154
Standard deviation of the intercept (Sa)	0.1410	0.5616	0.2996
Residual standard deviation S Y/X	0.1965	3.1172	0.8869
Correlation coefficient	0.9971	0.9943	0.9969

Y=a+bC, where *C* is the concentration of compound in µg/ml and *Y* is the peak area

Accuracy: It was carried out by calculating the mean percentage recovery of triplicate determination for BIS, HCT and RAM at three concentrations within the linearity range. The mean percentage recoveries were found to be 99.37 ± 0.59, 98.75 ± 0.39 and 99.48 ± 1.68 for BIS, HCT and RAM, respectively. The results obtained are summarized in Table 3.

Table 3: Evaluation of accuracy for the determination of BIS, HCT and RAM

drug	Concentration taken (µ/ml)	Concentration found (µ/ml)	Recovery %	Mean Rec ± S.D.
BIS	5	4.99	99.8	99.37 ± 0.59
	10	9.87	98.7	
	20	19.92	99.6	
HCT	5	4.96	99.2	98.75 ± 0.39
	10	9.85	98.5	
	25	24.64	98.56	
RAM	5	5.07	101.4	99.48 ± 1.68
	10	9.88	98.8	
	20	19.65	98.25	

Specificity: The method specificity was evaluated by the value of mean percentage recovery acquired from analyses of dosage form. The mean percentage recovery obtained by the developed method was found to be 97.82 ± 1.21% for BIS, 98.97 ± 1.25% for HCT and 99.66 ± 1.61% for RAM. This indicates that there are no interferences from the excipients.

Precision: Repeatability (intra-day precision) was evaluated by calculating the relative standard deviations (%RSD) for triplicate determinations of three different test concentrations of BIS, HCT and RAM within the linearity range in the same day. Intermediate (inter-day) precision was evaluated by calculating the Relative Standard Deviations (%RSD) of three different test concentrations of BIS, HCT and RAM within the linearity range on three different days. The relative standard deviations were found to be less than 2% for BIS, HCT and RAM (Table 4).

Table 4: Evaluation of the precision of the proposed HPLC method for the determination of BIS, HCT and RAM combinations in their ternary mixtures

Drug	Intra day				Inter day			
	Concentration taken (µg/ml)	Concentration found (µg/ml)	Recovery %	R.S.D %	Concentration taken (µ/ml)	Concentration found (µ/ml)	Recovery %	R.S.D %
BIS	5.00	5.00	99.94	0.79	5.00	4.97	99.41	0.92
	10.0	9.87	98.7	0.4	10.0	9.83	98.3	1.07
	20.0	19.92	99.6	1.89	20.0	19.76	98.81	0.83
HCT	5.00	4.97	99.4	0.7	5.00	4.99	99.7	0.33
	10.0	9.86	98.56	0.53	10.0	9.89	98.93	0.34
	25.0	24.77	99.07	0.87	25.0	25.1	100.41	1.23
RAM	5.00	5.07	101.46	0.79	5.00	5.01	100.17	1.02
	10.0	9.88	98.84	1.24	10.0	10.03	100.26	1.45
	20.0	19.66	98.28	0.8	20.0	19.73	98.66	0.78

Detection and quantitation limits: For determining the detection and quantitation limits, the S.D. of the response and the slope of calibration curve was used. The theoretical values were assessed practically and are given in Table 2.

Robustness: From preliminary experiments three factors were found to greatly affect HPLC method performances which are pH of phosphate buffer, ratio of acetonitrile in the mobile phase and temperature. A set of preliminary studies was performed to establish the low and high levels of each factor. Full factorial design was carried out for optimization of chromatographic conditions. Two responses were selected, tailing of peaks and resolution between HCT and BIS. Optimum chromatographic conditions got from factorial design are shown in Figure 4. The results obtained from the assay of synthetic mixtures (simulated to dosage form) using the conditions got from factorial design were compared to those gotten by the optimum condition of HPLC method (one factor at a time). The assay results were statistically analyzed and compared using t-test and F-test. The difference in the mean percentage recovery (t-test) or in variance (F-test) was not statistically significant (Table 5).

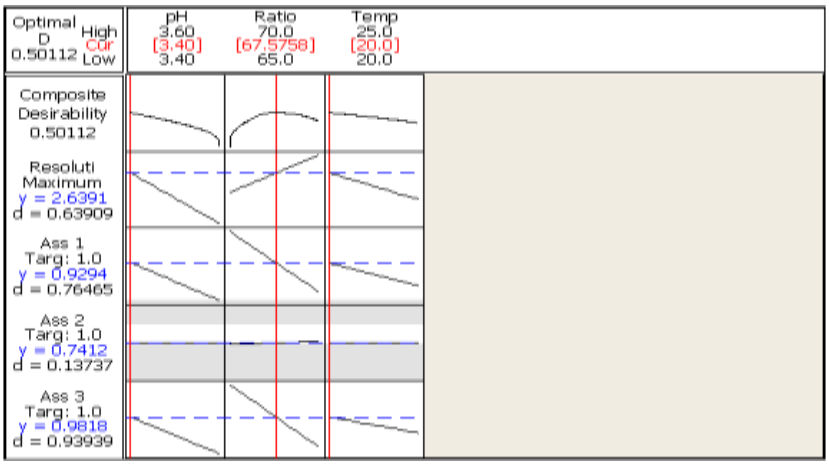

Figure 4: Response optimizer showing optimum chromatographic conditions by factorial design

Table 5: Comparison of the results of analysis of the synthetic mixture using the two methods of analysis factorial and proposed HPLC

		Recovery%	S.D	RSD%	F	t
BIS	factorial	96.77	0.79	0.82	6.33	0.21
	HPLC	97.82	1.21	1.24		
HCT	factorial	95.23	0.89	0.93	10.96	2.93
	HPLC	98.97	1.25	1.26		
RAM	factorial	100.30	0.80	0.80	4.00	0.62
	HPLC	99.66	1.61	1.61		

n=3; F-tabulated=19; t-tabulated=4.3

Analysis of dosage form: The developed HPLC method was applied for the simultaneous determination of BIS, HCT and RAM in their mixtures prepared from Combitrust® capsules. Three replicates were determined. Satisfactory results were obtained for each drug (Table 6).

Table 6: The results obtained from the analysis of dosage form

Drug	Mean ± S.D %Recovery	R.S.D %
BIS	97.82 ± 1.21	1.24
HCT	98.97 ± 1.25	1.26
RAM	99.66 ± 1.61	1.61

CONCLUSION

In this study a novel, simple, accurate, robust, sensitive and precise HPLC method was developed and applied for simultaneous determination of bisoprolol, hydrochlorothiazide and ramipril in their pharmaceutical dosage forms. The result of the study followed the protocol of ICH guidelines and it can be used for the routine quality control analysis in the combined formulation either in authentic samples or in dosage forms. Development of chemometric- assisted spectrophotometric methods for simultaneous determination of bisoprolol, hydrochlorothiazide and ramipril is the focus of our future researches.

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