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A Validated Reverse Phase Liquid Chromatographic Method for Simultaneous Analysis of Enalapril Maleate, Hydrochlorothiazide and Furosemide in Active Pharmaceutical Ingredients, Pharmaceutical Dosage Forms and Human Serum

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Abstract

A sensitive, reproducible isocratic reversed phase method has been developed and validated for the simultaneous quantification of enalapril and diuretics (hydrochlorothiazide and furosemide) in active pharmaceutical ingredients, dosage formulations and human serum using high-performance liquid chromatography (RP-HPLC). The method was validated according to ICH guidelines for the parameters: specificity, stability, limits of detection (LLOD), limits of quantification (LLOQ), linearity, accuracy, precision and recovery. Chromatographic separation was performed on Hypersil ODS C18 (150×4.6mm, 5micron) and Purospher Start C18 (250 mm×4.6 mm, 5 µm) columns using gradient elution, when methanol: water (75:25 v/v) was used as the mobile phase and pH adjusted to 3 with orthophosphoric acid having flow rate of 1.0 mL min^{-1} at ambient temperature. The lower limit of quantitation (LLOQ) and detection (LLOD) were 5,4.6,12.6 and 1.6,1.53,4.1 ngL^{-1} for HCT, ENP and FRS respectively. Calibration curves were linear in the concentration range of 2.5-100 µg mL^{-1} with a correlation coefficient ± 0.999 for all three drugs. Intra-day and inter-day precisions were less than 2 %. The accuracies were in the range of precision 98.0–102%. The retention time of HCT, ENP and FRS were found 3,3.5 and 4 mins respectively which shows the rapidness. This is the first full report of a method for the simultaneous determination of these three key drugs. The newly developed method is useful for future routine analysis of these drugs enalapril and diuretics in active pharmaceutical ingredients, pharmaceutical preparations, serum and could be used in therapeutic drug monitoring, clinical, laboratories and adherence to medicine studies, which would be helpful in decision making regarding treatment change in combination therapies.

Keywords: Enalapril; Hydrochlorothiazide; Furosemide; Diuretics; simultaneous determination; RP-HPLC

Introduction

Enalapril (S)-1-[N-[1-(ethoxycarbonyl)-3-phenylpropyl]-L-alanyl]-L-proline,(Z)-2-butenedioate [1] is the maleate salt of enalapril a derivative of 2-amino acid, L-alanine and L-proline. Enalapril after conversion to enalaprilate inhibits angiotensin-converting enzyme (ACE) in human and animals. ACE is a peptidyl dipeptidase that catalyzes the conversion of angiotensin I to the vasoconstrictor substance, angiotensin II which also stimulates aldosterone secretion by the adrenal cortex. Effects of enalapril in hypertension and heart failure appears primarily from suppression of the renin-angiotensin-aldosterone system. ACE inhibitor drugs do not respond sufficiently to reduce hypertension. Hence, these are used as combined dosage forms with other specific classes of drug compounds such as diuretics, calcium channel blocker antihypertensives, etc. Similarly, hydrochlorothiazide (diuretics) combined with ramipril increased the antihypertensive effect of the drug [2]. Hydrochlorothiazide (HCT) and furosemide (FRS) have widely been used in the treatment of congestive heart failure and hypertension [3,4] (Figure 1). The acute effect of furosemide (40 mg i.v.) and hydrochlorothiazide (100 mg p.o.) on diuresis, natriuresis and renal kallikrein and kinin excretion was investigated [5]. Our research group has worked on method development of a number of class of drugs among one of these are the ACE inhibitors as captopril [6] and enalapril [7]. Moreover further work on simultaneous method development and validation has also been reported on co-adminstered drugs especially with ACE inhibitors this include enalapril with statins [8], H_2-receptor antagonists [9], lisinopril and H_2 antagonists [10], NSAIDs [11] and with pravasatin, atorvastatin and rosuvastatin [12] in API, formulations and in serum. Sultana et al., have simultaneously determined captopril with statins, NSAIDs and H_2 receptor antagonists [13-15] and rosuvastatin with lisinopril, captopril and enalapril [16] and with hypoglycemic agents [17].

There are very few methods reported in literature for the simultaneous estimation of angiotensin II receptor antagonist and hydrochlorothiazide in tablets [18-20], though a simultaneous method for captopril and diuretics has also been developed and validated by Sultana et al. (2011) [21]. Sultana et al. (2007) have also studied the in vitro interactions of captopril H_2-receptor antagonists [22]. However, no HPLC method for separation and simultaneous determination of enalapril and two different diuretics has been reported. ACE inhibitor enalapril maleate combined with diuretics (hydrochlorothiazide and furosemide) is used for the treatment of hypertension. Diuretics

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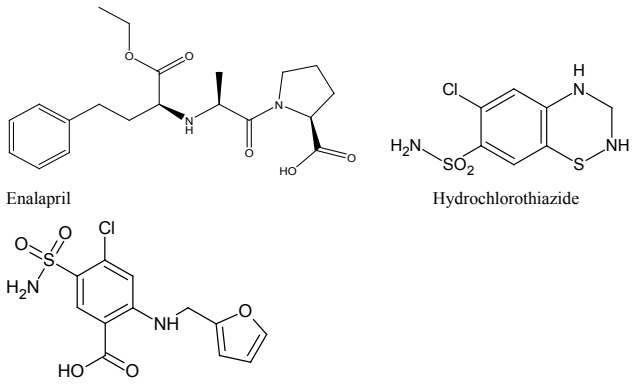

Figure 1: Chemical structures of enalapril, hydrochlorothiazide and furosemide.

combined with ACE inhibitors increased the antihypertensive effects. Therefore, in this article, we report a simple, easy, quick, and inexpensive isocratic reversed phase chromatographic method with ultraviolet detection at 225 nm for the simultaneous determination of ENP and two diuretics, i.e., HCT and FUR. The method is equally valid for the determination in bulk materials, pharmaceutical dosage formulations and human serum. The short analysis time (less than 5 min) enables its application in routine and quality control analysis. The low LLOD and LLOQ values merit the method for the determination of these drugs in clinical samples.

Experimental

Materials and reagents

All chemicals and reagents were of analytical grade. Enalapril was a kind gift from BMS (Pvt) Limited, Pakistan. Hydrochlorothiazide and furosemide were gifts from Zafa Pharmaceutical Laboratories (Pvt) Ltd and Sanofi Aventis (Pvt) Ltd Pakistan. HPLC grade methanol and phosphoric acid were obtained from Tedia (USA) and Merck Darmstadt, Germany.

Pharmaceutical dosage form

Renitec™ (Enalapril 10 mg tablets by BMS Pharmaceuticals (Pvt) Ltd), Diuza™ (Hydrochlorothiazide 25 mg tablets by Zafa Pharmaceutical Laboratories (Pvt) Ltd) and Lasix™ (40 mg tablets from Sanofi Aventis Pakistan Limited), were purchased from the local pharmacies (Figure 2). All these drugs had an expiry of not less than 1 year at the time of study.

Instrumentation

HPLC system consisted of an LC-10 AT VP Shimadzu pump, SPD-10AV VP Shimadzu UV visible detector, a Purospher Start C_{18} (250×4.6 mm, 5 µm) and Hypersil ODS C18 columns were used for separation. The chromatographic system was integrated using a CBM-102 communication Bus Module Shimadzu with a Pentium™ IV PC loaded with Class GC software for data acquisition. Separation was carried out under isocratic elution with methanol:water (75:25) as mobile phase, pH of which was adjusted to 3.0 with ortho phosphoric acid (85%), sonicated by DGU-14 AM on-line degasser, and filtered through 0.45-micron membrane filter. The flow rate was 1.0 mL min^{-1}, elution was monitored at 225 nm, and the injection volume was 20 µL.

Preparation of standard and sample solutions

Standard preparation: Various ratios (50:50, 60:40, 70:30, 80:20 v/v) of methanol:water were tested as starting solvent for system suitability study. The variation in the mobile phase led to considerable changes in the chromatographic parameters, like peak symmetry, capacity factor, and retention time. Stock standard solutions 100 ppm of ENP, HCT and FRS were prepared in 100 ml mobile phase solvent. Working solutions were prepared separately by making serial dilutions from the standard solution to obtain concentrations of 2.5, 5, 10, 15, 25, 50 and 100 for ENP, HCT and FRS. These solutions were stored at 20°C. Once prepared, analyzed daily for inter- and intraday variations of the method. 20 µL of these solutions were injected into LC system and chromatographed.

Procedure for tablets

Twenty tablets of each formulation were powdered finely and an amount equivalent to 10 mg of ENP, HCT and FRS was weighed and then dissolved in the mobile phase. Solutions were then filtered through ordinary filter paper. The desired concentrations 2.5, 5, 10, 25, and 50 for ENP, HCT and FRS were obtained by accurate dilution. Finally, all the solutions were filtered through a 45- µm Millipore filter.

Procedure for human serum

Plasma samples, obtained from healthy volunteers, were collected and stored. To 1.0 ml of plasma, 9.0 ml of acetonitrile was added; the mixture was vortexed for 1 min, then centrifuged for 10 min at 10,000 rpm, and the supernatant was filtered by a 0.45-lm filter. An aliquot of serum sample was fortified with ENP, HCT and FRS to achieve final concentration.

Results and Discussion

Development and optimization of isocratic HPLC conditions

Various methods have been developed for the quantitation of enalipril maleate alone [7] as well as along with co-administered drugs [8,9]. No simultaneous method for the quantitation of these diuretics with enalapril has been reported; though we have developed a simultaneous method of another ACE inhibitor i.e. captopril with diuretics [21]. In continuation of our work we have made an attempt to develop a simple, isocratic, accurate, sensitive, economical and less time consuming reversed phase chromatographic method for the simultaneous determination of ENP, HCT and FRS. UV scan showed a maximal absorbance at 225 nm. Initial method development was conducted on two columns a Purospher Start C_{18} (250×4.6 mm, 5 µm) and Hypersil ODS C18 columns were used for separation at ambient temperature. Initially various mobile phases in variable ratios were tested to obtain the best separation and resolution. The mobile phase consisting of methanol and water in the ratio of 75:25 v/v was found to have good resolution. The chromatographic conditions were optimized to achieve best separation and to get best resolution between analytes and to optimize chromatographic parameters like tailing factor, resolution and retention time these all parameters are done according to ICH (International Conference on Hormonisation) [23] guideline. The optimized conditions were reached at pH 3.0, producing well resolved and sharp peaks for all drugs. Purospher Start C_{18} (250×4.6 mm, 5 µm) column provided efficient and reproducible separations of non-polar compounds while minimizing solvent usage For validation of analytical methods, the guidelines of the International Conference on the Harmonization of Technical Requirements for the Registration of Pharmaceuticals for Human Use and USP 2002 were followed

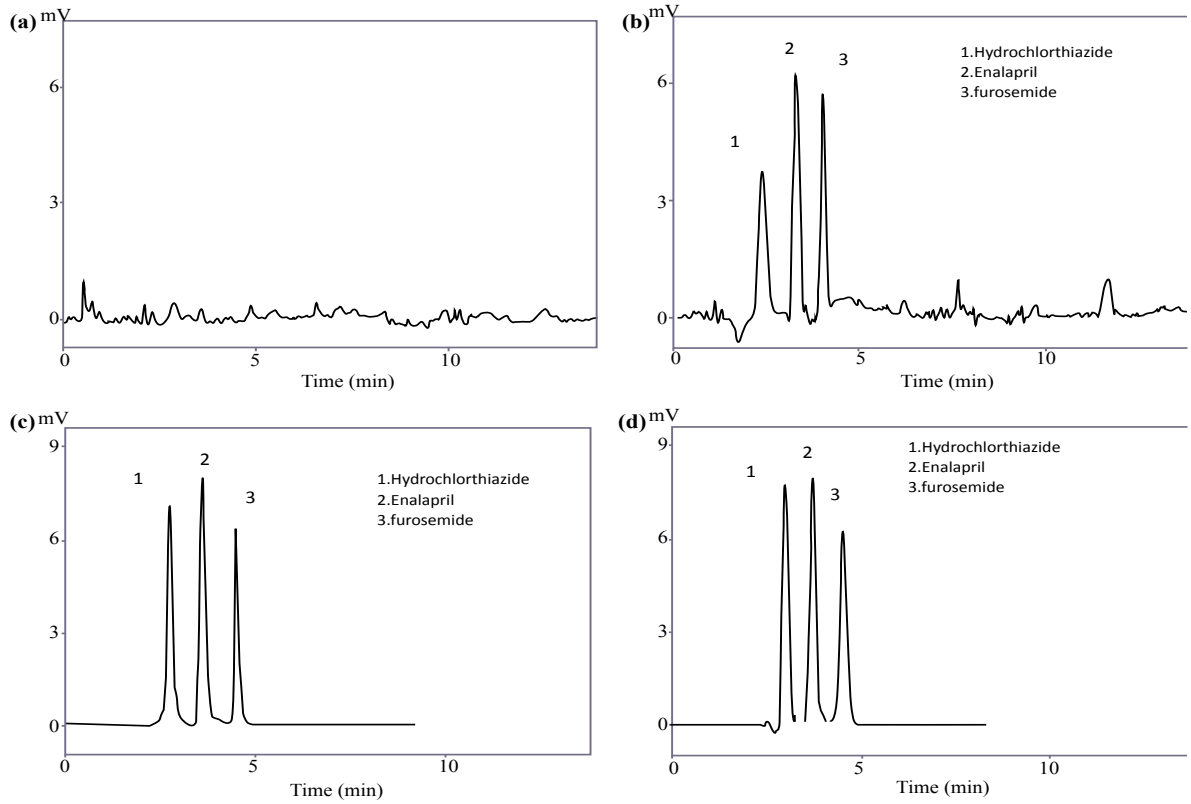

Figure 2: Chromatograms of (a) Blank serum (b) HCT, ENP and FRS in serum(c) formulation and (d) in API at 225 nm.

for the accuracy tests, precision, specificity, linearity, work strip and robustness of the method [23,24]. Retention time of HCT 3.0 min, ENP was 3.5 min and FRS was 4.0 min, at a flow rate of 1.0 mL min^{-1}.

System suitability

The HPLC system was equilibrated with the initial mobile phase composition, followed by 6 injections of the same standard. These 6 consecutive injections were used to evaluate the system suitability on each day of method validation. Parameter of system suitability, peaks symmetry (symmetry factor), theoretical plates of the column, mass distribution ratio (capacity factor), and resolution are summarized in Table 1.

Linearity

Linearity is generally reported as the variance of the slope of the regression line. Linearity was tested with known concentrations of ENP, HCT and FRS i.e.2.5, 5, 10, 15, 25, 50 and 100 µg mL^{-1}, respectively. Results from linear regression analysis of response and concentration data are given in Table 2 which shows that the method is linear. LLOD and LLOQ for HCT, ENP and FRS were 1.6, 1.53 and 4.1 $ngmL^{-1}$ and 5, 4.6 and 12.6 $ngmL^{-1}$ respectively, suggesting that nanogram quantities of all the compounds can be estimated accurately (Table 4).

Accuracy

The accuracy of an analytical procedure measures the closeness of measured values to the true values. It was evaluated as percentage relative error between the measured mean concentrations and taken concentrations. Minimal of 3 concentration levels covering the specified ranges were selected and five runs were performed for every concentration and then peak area was calculated as given in Table 3.

Precision

For intra-day and inter-day precision, ten samples of five concentrations were analyzed on the same day and after one day (Table 4). Generally acceptable repeatability of the results within one day and day-to-day were observed. The precision of the method was analyzed as % RSD throughout the linear range of concentrations.

Ruggedness

Ruggedness was established by determining ENP, HCT and FRS in dosage formulation and in human serum using same and different chromatographic systems one was in the Research Institute of pharmaceutical Sciences and the second one was in Lab 9 of the Chemistry Department and two different columns on different days. The assay results indicated that the method was capable with high precision (Table 4).

Robustness

Robustness of the method was accomplished by designed modifications made to the method parameters such as mobile phase composition, flow rate, pH and detection wavelength and it was found that the % R.S.D values did not exceed more than 2% (Table 5).

Conclusion

A simple and reliable HPLC method for monitoring ENP, HCT and FRS in raw material human serum and pharmaceutical dosage

Analytes	Retention time (T_R) (min)	Capacity factors (K')	Theoretical plates (N)	Tailing factor (T)	Resolution (R)	Separation factor
HCT	3	6.46	6000	1.93	4.4	0.0
ENP	3.5	17.7	8000	1.73	5.5	1.6
FRS	4	14.34	11200	1.36	3.6	1.9

HCT: hydrochlorothiazide; ENP: enalapril; FRS: furosemide

Table 1: System suitability parameters of the proposed method for hydrochlorothiazide, enalapril and furosemide.

Drugs	Conc. (µgmL^{-1})	Regression Equation	r^2	LLOD(ngmL$^{-1)}$	LLOQ(ngmL^{-1})
Bulk					
HCT	2.5-100	y = 2489.4x + 3255.5	0.9996	1.6	5
ENP	2.5-100	y = 1970.7x + 2357.9	0.9995	1.53	4.6
FRS	2.5-100	y = 1982.5x + 1478.4	0.9994	4.1	12.6
serum					
HCT	2.5-100	2489.4x + 3254.9	0.9996	3.4	10.5
ENP	2.5-100	y = 1970.5x + 2372.9	0.9995	4.7	14.4
FRS	2.5-100	y = 1982.5x + 1481.1	0.9994	6.9	20.1

HCT: hydrochlorothiazide; ENP: enalapril; FRS: furosemide; LLOD: Lower limit of detection; LLOQ: Lower limit of quantification

Table 2: Regression characteristics of the proposed method for hydrochlorothiazide, enalapril and furosemide.

Conc. µgmL-1	HCT		ENP		FRS	
	% Recovery	%RSD	% Recovery	%RSD	% Recovery	%RSD
2.5	100	0	100	0	100	0
5	100	0.1	100	0.1	100	0.5
10	101	0.2	101	0.4	101	0.2
15	11.2	0.01	11.2	0	11.2	0
25	100.02	0.4	100.02	0.1	100.02	0.7
50	100	0.2	100	0.6	100	0.2
100	101	0.5	101	0.2	101	0.3
Assay (spiking method)						
80	100	0.1	100	0.1	100	0.1
100	100.2	0.2	100.2	0.2	100.2	0.1
120	100.3	0.1	100.3	0.1	100.3	0.1
Assay in serum						
80	100.2	0.1	100	0.1	100	0.1
100	100	0.2	100.2	0.2	100.2	0.1
120	100.2	0.2	100.3	0.1	100.3	0.2

HCT: hydrochlorothiazide; ENP: enalapril; FRS: furosemide

Table 3: Accuracy of the proposed method for hydrochlorothiazide, enalapril and furosemide.

Conc.µgmL^{-1}	Inter-day			Intra-day		
	HCT	ENP	FRS	HCT	ENP	FRS
	%RSD	%RSD	%RSD	%RSD	%RSD	%RSD
2.5	0	0	0	0	0	0
5	0.1	0.1	0.1	0.1	0.1	0.1
10	6	0.2	0.2	0.2	0.2	0.2
15	0	0	0	0	0	0
25	6	0.4	0.4	0.2	0.4	0.4
50	0.2	0.2	0.2	0.2	0.2	0.2
100	0.5	0.2	0.5	0.2	0.5	0.5
Assay in serum						
80	0.3	0.1	0.2	0.2	0.1	0.2
100	0.2	0.2	0.2	0.7	0.2	0.2
120	0.6	0.1	0.2	0.2	0.6	0.2

HCT: hydrochlorothiazide; ENP: enalapril; FRS: furosemide

Table 4: Inter and intra-day precision of the proposed method for hydrochlorothiazide, enalapril and furosemide (n=6).

formulation has been developed and validated for the first time. The intra-run and inter-run variability and accuracy results were also in acceptable limit. Simplicity of the separation procedure, low volume of injection and shorter run time makes this method suitable for quick and routine analysis in clinical labs. In addition, this method has the potential application to clinical research of drug combination and multidrug pharmacokinetics study.

	Level	K'	T	(R_s)
A: pH of mobile phase				
2.8	-0.2	17.3	1.71	5.3
3	0	17.7	1.73	5.5
3.2	0.2	17.2	1.78	5.35
B: Flow rate ($mLmin^{-1}$)				
0.8	-0.2	17.1	1.71	5.32
1	0	17.7	1.73	5.5
1.2	0.2	17.5	1.78	5.37
C: Percentage of water in mobile phase (V/V)				
80/20	-5	17.1	1.70	5.39
75/25	0	17.7	1.73	5.5
70/30	+5	17.5	1.70	5.32
D: Wavelength (nm)				
220	-5	17.7	1.72	5.39
225	0	17.7	1.73	5.5
230	5	17.1	1.79	5.32
K'= Capacity factors, T = Tailing factor, R_s = Resolution				

Table 5: Robustness of the proposed method n=6.

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