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Original Research Article

FACILE AND MANIFEST RPHPLC METHOD FOR THE DETERMINATION OF CAPTPRIL, LISIOPRIL , ENALPRIL AND DICLOFENAC SODIUM: ITS APPLICATIONS IN DOSAGE FORMULATIONS AND IN HUMAN SERUM

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ABSTRACT

A simple, rapid, isocratic, high-performance liquid chromatography (RP-HPLC) method has been developed for the first time for simultaneous determination of ACE inhibitors (captopril, lisinopril and enalapril) and diclofenac sodium in bulk drugs, pharmaceutical products and human serum. The separation was performed on a Purospher Start C_{18} (250cm x 4.6mm, 5µm) and Hypersil ODS (25cm x 4.6mm 5µm) column using methanol–water as mobile phase 80:20 (v/v) and 60:40 (v/v) as diluent. The pH of mobile phase was adjusted to 3.0 with ortho-phosphoric acid, flow rate was adjusted to 1 $mLmin^{-1}$ at room temperature and analytes peaks were observed using UV detector at 220 nm. The retention times of captopril, lisinopril, enalapril and diclofenac sodium were 2.5, 3.8, 4.5 and 5 min and LLOD, LLOQ were 1, 4, 2, 6, 3 and 12, 8, and 14 $ngmL^{-1}$ respectively. The method was validated according to ICH guidelines. The linearity of the method was studied over the concentration range of 2.5–50 μgmL^{-1} for ACE inhibitors and diclofenac sodium, where it demonstrated good linearity with r = 0.9998, 0.9999, 0.9997, and 0.9998 (n = 6), respectively. The HPLC method presented here shows an easy but reliable and precise analysis of the antihypertensive drugs captopril, lisinopril, enalapril, and diclofenac sodium. The values for LLOD, precision of RT, precision of area and linearity shows good performance of the analysis. The developed method was successfully applied to quantitate, the three ACE inhibitors and diclofenac sodium in pharmaceutical formulations and human serum.

Keywords : Captopril, lisinopril, enalapril, diclofenac sodium and RP-HPLC

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INTRODUCTION

Antihypertensive drugs are given to patients to lower the blood pressure. The compounds captopril, lisinopril and enalipril, inhibit the enzyme, which converts angiotensin I into angiotensin II. Angiotensin II is one of the strongest blood pressure decreasing substance.

Captopril 1-[(2S)-3-mercapto-2-methyl-1-oxo-proptonyl]-L-proline is the first orally active and specific inhibitor of angiotensin-converting enzyme and is used therapeutically as an antihypertensive agent. (Fig 1)

Several methods have been reported for the quantitative determination of captopril in formulations and biological fluids by spectrophotometry [1-6], GC-MS [7, 8] HPLC [9-16] and capillary electrophoresis [17].

Lisinopril, a synthetic peptide derivative, is an oral long-acting an- giotensin-converting enzyme inhibitor, chemically named as (S)-1-[N-(1-carboxy-3-phenylpropyl)-L-lysyl]-L-proline dihydrate. It may be used to treat hypertension and symptomatic congestive heart failure, to improve survival in certain individuals following myocardial infarction and to prevent progression of renal disease in hypertensive patients with diabetes mellitus and micro albuminuria or overt nephropathy. Literature reports a number of methods for the determination of lisinopril [18- 21].

Enalapril(S)-1-[N-[1-(ethoxycarbonyl)-3-phenylpropyl]-L-alanyl]-L-proline,(Z)-2- butenedioate is the maleate salt of enalapril a derivative of 2-amino acid, L-alanine and L-proline. Enalapril is a pro-drug, is bioactivated by hydrolysis of the ethyl ester to enalaprilate which acts as an inhibitor of angiotensin-converting enzyme (ACE) in the renin-angiotensin aldosterone system. It is used to treat high blood pressure and heart failure. In treating high blood pressure or heart failure it is desirable to maintain a certain level of enalapril in the blood. Therefore, when investigating the efficacy of enalapril or its synthetic equivalent, it is necessary to determine the

concentration of residual enalapril in the plasma. Analytical methods for enalapril in plasma reported in the literature include high performance liquid chromatography with UV detection [22], capillary electrophoresis, [23] and HPLC/MS [24] in bulk.

Hypertension and co-existing musculoskeletal problems are two of the frequent conditions [25]. Both antihypertensive and nonsteroidal anti-inflammatory drugs are frequently prescribed together, the concomitant use of NSAIDs would counteract the effects of antihypertensive therapy in patients with hypertension or even cause hypertension in normotensive persons [26].

Our research group has worked on the simultaneous determination of a number of co-adminstered drugs [27-38] but there is no single method reported for determination of these ACE inhibitors with diclofenac sodium. So in continuation of our previous work and recently published articles, we have attempted to develop a simultaneous method for the determination three ACE inhibitors (captoprl, lisinopril and enalipril with diclofenac sodium.

The main purpose of our study was to develop a simple, reliable and economical method for the simultaneous determination of these coadminstered drugs: captopril, lisinopril, enalapril and diclofenac sodium in a relatively short time with high linearity. Therefore, this study is focused on the development of simple and rapid isocratic RP-HPLC method which may be employed for the routine analysis of these drugs in bulk, pharmaceutical formulations and in serum.

MATERIALS AND METHODS

Pharmaceutical grade enalapril was gifted by MSD Laboratories Limited, Karachi, Pakistan. Captopril, lisinopril and diclofenac sodium were gifted from Bristol Meyers Pvt Ltd, Atco Laboratories Ltd Yung Shin Pharmaceutical Ind. Co. respectively. Formulations of captopril namely (capoten 25 mg), lisinopril (lisinopril 10 mg), enalapril (renitec 10 mg) and diclofenac sodium (voren 50 mg) were purchased from retail pharmacies. Methanol and acetonitrile used were of HPLC grade from E. Merck, Germany. Orthophosphoric acid 85 % used were of analytical grade (Merck, Germany). HPLC grade water was used to prepare mobile phase. Stock solutions and working solutions were prepared daily. All solutions were filtered through 0.45 μm filter and degassed using sonicator.

Instruments

Two HPLC systems Shimadzu-10A and 20A provided with LC-20-AT HPLC pump, a SPD-20A Shimadzu UV visible detector in both the systems were used. CBM-102 communication

Bus Module Shimadzu was attached to record the chromatographic and integrated data. Data acquisition was performed by Shimadzu Class-GC 10 software (version 5.03). Elma Ultrasonic LC 30 H sonicator was used for sonication and degassing of mobile phase. Chromatographic separation was carried out on a Purospher STAR RP-and Hypersil ODS column C-18 column (250 X 4.6 mm, with a particle size of 10μ) and DGU-14 AM on-line degasser. Shimadzu 1800 UV–visible spectrophotometer was used for the determination of isosbestic point of drug analytes.

Chromatographic conditions

The mobile phase used was methanol: water (80:20 v/v) whose pH was adjusted to 3.0 with o-phosphoric acid. It was filtered through 0.45 μm pore-size membrane filter paper and degassed through sonicator. Separation was carried out under isocratic conditions at flow rate 1.0 mL min^{-1}. Chromatographic elution was monitored at 220 nm. Sample was injected into the system through 20 μL loop fitted with rheodyne manual injector.

Standard and working solutions

Stock standard solutions of API of 100 μg mL^{-1} o f CAP, LSP, ENP and DS were prepared by transferring 10 mg of each drug in 100 mL volumetric flasks and volumes were brought to the mark with diluents as solvent. Working solutions were prepared by serial dilutions from 2.5–50 μgmL^{-1}. Inter-day variations of the method were studied daily for 3 days by storing the working solutions prepared on day.

Pharmaceutical formulations and serum

Twenty tablets of each dosage formulation were crushed separately. Calculated amount of powder corresponding to 10 mg of each API were transferred into separate conical flasks with sufficient amount of diluents and left for 1 h with intermittent sonication for complete extraction and solubility of drugs. These solutions were then filtered through Whatman filter paper to separate insoluble excepients into 100 mL volumetric flasks and made up to the mark with the same diluent to obtain the stock solution of 100 μgmL^{-1}. Serial dilutions ranging from 2.5–50 μgmL^{-1} of each CAP, LSP, ENP and DS were prepared for working solutions. The solutions were filtered through 0.45 μm Millipore filter paper before injection into the system. Three milliliter blood sample of a healthy volunteer (aged 24 years) was collected in an evacuated glass tube through an indwelling cannula placed on forearm vein at Fatmid foundation. The volunteer was not involved in any medication, smoking, and strenuous activity. The blood was shaken and centrifuged at 10,000 rpm for 10 min to separate out plasma. 9 mL ACN was added to 1 mL plasma and centrifuged at 10,000 rpm for 10 mins to de proteinate it. The supernatant serum thus obtained was filtered and used for the analysis and stored at -20◦C. Working solutions of various concentrations were prepared by spiking stock solutions into the serum maintaining the ratio 1:1 (drug stock diluted by diluent:serum v/v). Triplicate injections were made for each working solution for the analysis in serum.

RESULTS AND DISCUSSION

Method optimization and chromatographic conditions

A reversed-phase high performance liquid chromatography was used for the development of simultaneous determination of diclofenac sodium and ACE inhibitors. Initial method development was conducted on a Purospher Start C_{18} (250 x 4.6mm, 5μm) column for separation at ambient temperature. Mobile phase included methanol: water (80:20, v/v) which is non-toxic, cheap and commonly used solvent for RP-HPLC.

pH 3 adjusted with phosphoric acid then degassed by sonicator and filtered by 0.45-micron membrane filter. Flow rate was adjusted at 1.0 ml min^{-1} with isocratic elution. The work was carried out at 225nm, which was selected by isosbestic points. The samples were injected by a 20 µl sample loop.

System suitability

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

Table 1-System suitability parameters

Parameters →	Retention time (Rt)	Capacity factors (K')	Theoretical plates (N)	Separation factor	Resolution (R)	Tailing factor (T)
Captopril	3.8	5.95	4915	1.23	2.49	1.32
Lisinopril	4.5	6.82	5092	2.3	2.52	1.32
Enalapril	2.5	5.12	4253	1.25	2.37	1.28
Diclofenac sodium	5	2.9	3914	2.3	2.5	1.22

Linearity

The reason of the check for linearity was to demonstrate that the entire analytical system (including detector and data acquisition) presents a linear response and it is directly proportional over the relevant concentration range of analytes. Calibration curves were constructed in the concentrations range 2.5-50 µgml^{-1}. Concentration of analytes versus peak area was subjected to least square linear regression analysis. Excellent linearity was obtained in all cases with correlation coefficient (r^2) > 0.999. The standard curve, slope and intercept were determined by statistical software. Statistical analysis of the data gave high values of the correlation coefficients r of the regression equation, small values of the standard deviation of residuals (S), of intercept (Sa) and of slope (Sb) and small value of the percentage relative standard deviation and the percentage relative error (table 2). These data proved the linearity of the calibration graphs.

Table 2-Regression characteristics

Analytes	Conc. range (µg ml^{-1})	Regression equation	r^2	LLOD	LLOQ
				ngml^{-1}	ngml^{-1}
Captopril	25-2.5	y = 18370x + 5919	0.999	2	8
Lisinopril	25-2.5	y = 76778x + 4611	0.999	6	14
Enalapril	25-2.5	y = 15290x + 3329.	0.999	4	12
Diclofenac sodium	25-2.5	y = 19975x + 2066	0.999	1	3

Accuracy

To prove the accuracy of the proposed method, the results of the assay of the studied drugs were evaluated as the percentage of recovery of known amounts of analytes in the pharmaceutical formulations and human serum (figs. 2, 3). Each sample was injected five times and accuracy was determined in range of 80%, 100% and 120% for all investigated analytes. The results are presented in tables 3; high recovery indicated that the method has a high degree of accuracy.

Analytes	Assay (spiking method)			Assay in serum	
	Conc. (μgml^{-1})	%RSD	%Recovery	%RSD	%Recovery
Captopril	8	0.36	103.1	0.36	103.61
	10	0.35	98.13	0.26	98.93
	12	0.003	98.81	0.36	99.41
Lisinopril	8	0.003	102.43	0.36	102.21
	10	0.09	98.42	0.26	98.42
	12	0.089	103.1	0.36	103.1
Enalapril	8	0.003	102.75	0.36	103.32
	10	0.05	103.81	0.63	102.6
	12	0.08	98.42	0.36	99.49
Diclofenac sodium	8	0.3	102.5	0.36	103.72
	10	0.31	102.69	0.08	102.19
	12	0.013	102.19	0.23	102.62

Table 3-Accuracy of ACE inhibitors and diclofenac sodium

Precision

Precision of the proposed method was determined by repeatability (intra-day precision) and intermediate precision (inter-day precision). It is expressed as relative standard deviation (RSD). The intraday precision was assessed by analyzing three concentrations and three replicates of each concentration of analytes in the linear range on the same day (intra-day precision). Also the inter-day precision was assessed by analyzing three concentrations and three replicates each concentration over three successive days; every sample was injected five times. The relative standard deviations were found to be very small (0.001 to 1.88 %) indicating reasonable repeatability and intermediate precision of the proposed method (table 4).

Limit of detection (LLOD) and quantification (LLOQ)

Limit of detection (LLOD) and quantification (LLOQ) were calculated according to ICH Q2R1 recommendations (REF) using the following equations: LOQ= 10Sa/b and LOD = 3.3 Sa/b

Where Sa = standard deviation of the intercept of the calibration curve and b = slope of the calibration curve. LOD was expressed as a concentration that gives a signal to noise ratio of 3:1. Quantification limit (LOQ) is the lowest amount of analyte in a sample that can be determined with acceptable precision and accuracy under the stated experimental conditions. LOQ is measured in terms of signal to noise ratio of 10:1 [39]. The LLOD and LLOQ are calculated as: LOD = 3.3μ/*S* and LOQ =10 μ/*S;* where μ is the

standard deviation of the lowest standard concentration and *S* is the slope of the standard curve. The limits of detection (LLOD) and quantification (LLOQ) were determined from the calibration curve for captopril, lisinopril enalapril, and diclofenac sodium 2, 6, 4, 1, and 8,14, 8,14 respectively.

Analytes	Conc. (μgmL^{-1})	(%RSD)		
		API	Formulation	Serum
Captopril	25	0.013	0.004	0.002
	15	0.017	0.005	0.019
	10	0.013	0.018	0.016
	5	0.015	0.003	0.014
	2.5	0.159	0.088	0.015
Lisinopril	25	0.544	0.44	0.499
	15	0.626	0.066	0.611
	10	0.153	0.674	0.122
	5	0.0928	0.335	0.124
	2.5	1.048	1.336	1.112
Enalapril	25	0.002	0.012	0.019
	15	0.001	0.017	0.011
	10	0.016	0.07	0.014
	5	0.01184	0.012	0.011
	2.5	0.011	0.199	0.012
Diclofenac sodium	25	0.231	1.418	0.24
	15	1.677	1.885	1.59
	10	1.324	0.3414	1.32
	5	0.389	0.323	0.032
	2.5	0.374	0.445	0.36

Table 4-Precision of ACE inhibitors and diclofenac sodium

Specificity and selectivity

The selectivity and specificity of the method was established by studying resolution factor of the peak of ACE inhibitors from that of diclofenac sodium. The selectivity of the method was investigated by observing any interference encountered from common excipients in different formulations . The method confirmed good resolutions and was found to be free of interference from the excipients used in pharmaceutical formulations. Additionally, no intereference was observed from human plasma matrix although no prior extraction procedure was performed.

Robustness

The robustness of the proposed method was indicated by the constancy of the peak area ratio with deliberate changes in the experimental parameters. These parameters included minor variations in the

ratio of methanol and water of the mobile phase, pH of the mobile phase and flow rate, which proved quite stable. When a parameter was intentionally changed ±2% (in mobile phase), ±0.1% (in flow rate) and ±0.05% (pH 3) from its optimum condition, the shifting in retention time of ±0.1% was less. These inor changes did not greatly affect the peak area ratio of all the drugs. These results indicated better robustness of the developed method.

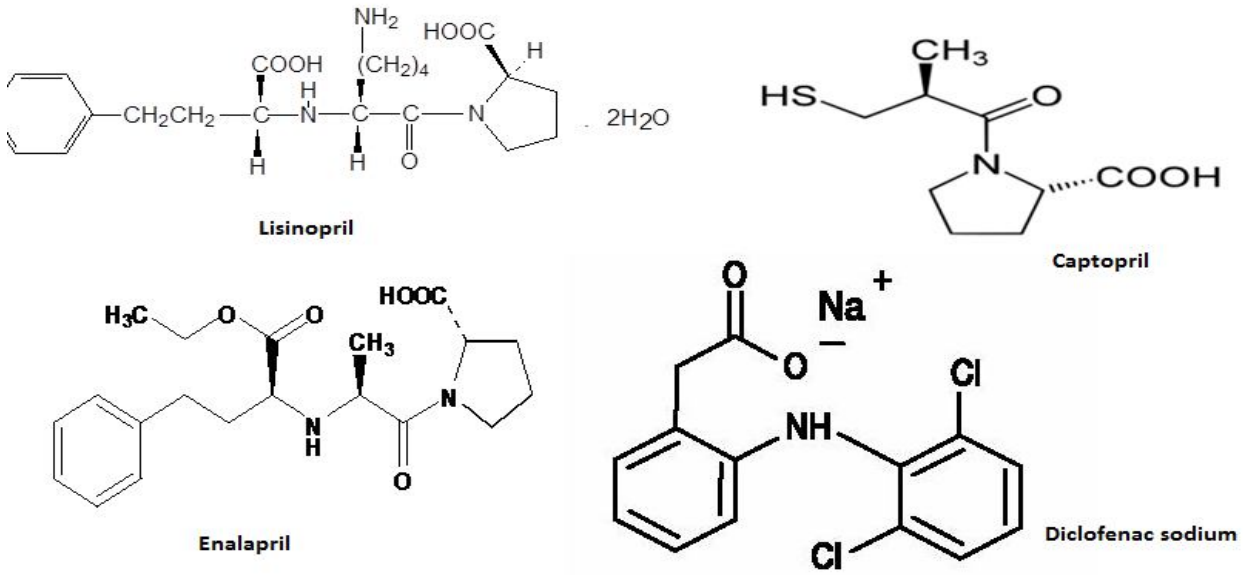

Figure 1: ACE inhibitors and diclofenac sodium

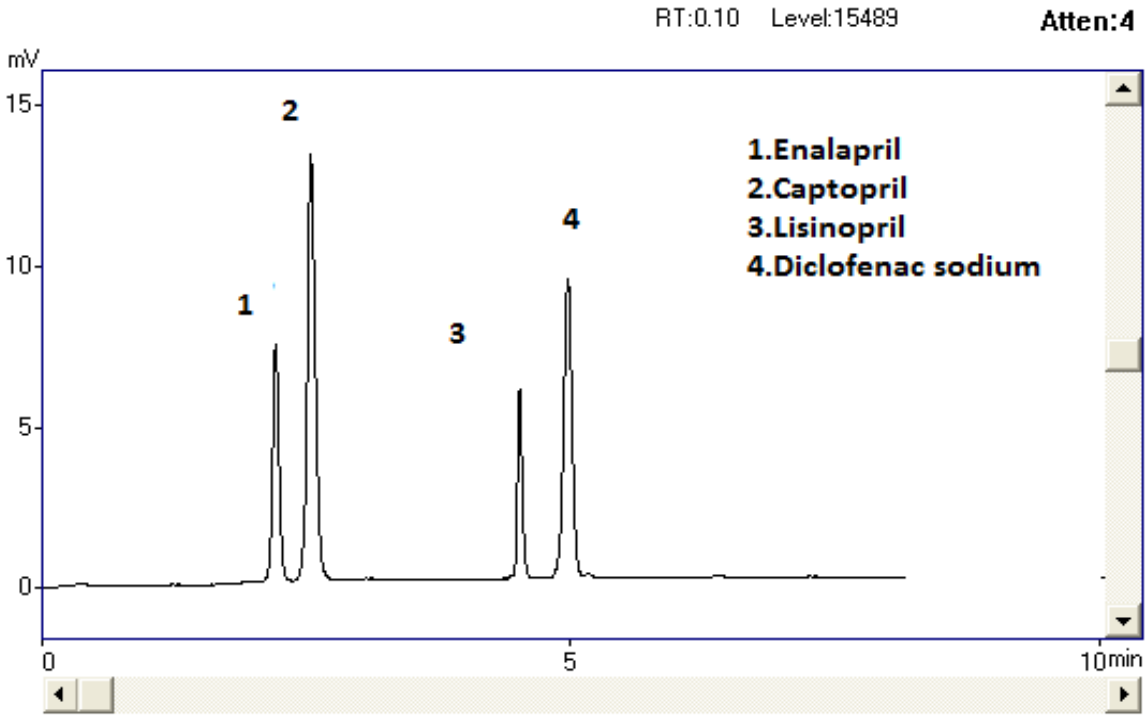

Figure 2: Representative chromatogram of ACE inhibitors and diclofenac sodium in formulations

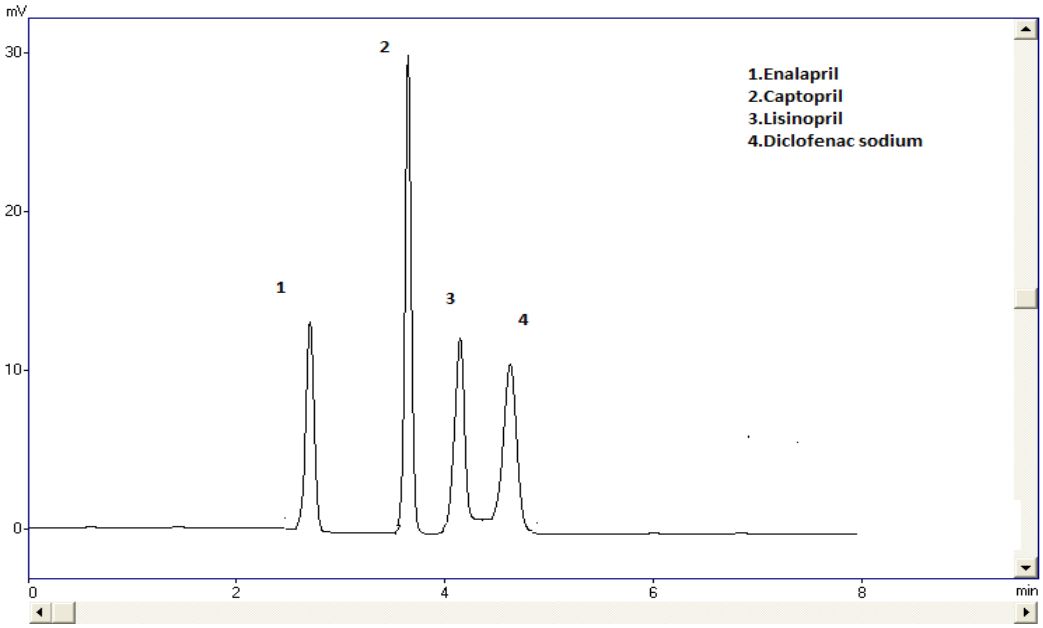

Figure 3: Representative chromatogram of ACE inhibitors and diclofenac sodium in serum

Ruggedness

The ruggedness of an analytical method is the degree of reproducibility of test results obtained by the analysis of the same samples under a variety of normal test conditions, such as different laboratories, different analysis different instruments, different days, etc . Ruggedness is an older term that has been replaced by intermediate precision (degree of reproducibility) obtained under a variety of circumstances. Different concentrations of analytes were used in two different days and two different systems of same configuration indicate suitability of method for all analytes.

Analysis of spiked human plasma

Having established the validity of the analytical method, the LC-UV method was applied to determination of these drugs in the plasma. The LC-UV chromatograms obtained from the spiked drugs were practically identical with that shown in figure 2 from spiked plasma. The percent recovery values ranged between 98-103% both by spiking in the serum as well as by analysis of the drugs in serum. No intereference of exciepients or endogenous materials was observed.

CONCLUSION

The proposed method for the simultaneous determination of captopril, lisinopril enalapril, and diclofenac sodium, is shown to be reliable simple accurate and precise. The short run time with high linearity, low limit of quantification, small sample volume. makes the method allowable for use in quality control laboratories and for drug analysis in API, formulations and serum. This method is further applicable for studies of interaction studies of ACE inhibitors and diclofenac sodium .

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