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VALIDATION OF AN ANALYTICAL PROCEDURE FOR SIMULTANEOUS DETERMINATION OF HYDROCHLOROTHIAZIDE, LISINOPRIL, AND THEIR IMPURITIES

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SUMMARY

The main objective of the work discussed in this paper was evaluation of a chromatographic method for simultaneous determination of hydrochlorothiazide (HCTZ), lisinopril (L), and their impurities in pharmaceuticals. Chlorothiazide (CTZ) and disulfonamide (DSA), as potential impurities in hydrochlorothiazide, and diketopiperazine (DKP), as an impurity of lisinopril, were analyzed. The chromatographic behaviour of these substances on different columns was studied using mobile phases of different polarity. The optimum separations were achieved by gradient elution on a 4.6 mm × 20 mm, 3.5 μm particle size, C_{18} column. The mobile phase was a gradient prepared by mixing 7:93 (*v*/*v*) acetonitrile–25 mM potassium dihydrogen phosphate, pH 5, and 50:50 (*v*/*v*) acetonitrile–25 mM potassium dihydrogen phosphate pH 5 in different ratios. The flow rate was 1.0 mL min^{-1}. UV detection was performed at 215 nm. Methylparaben was used as internal standard. The method was validated for selectivity, linearity, precision, and accuracy. The limits of detection, LOD, and quantification, LOQ, were determined experimentally. Because of its speed and accuracy the method can be used for quality-control analysis.

INTRODUCTION

Lisinopril, a synthetic peptide derivative, is an oral long-acting an-

giotensin-converting enzyme inhibitor. Its chemical name is (*S*)-1-[N^2-(1-carboxy-3-phenylpropyl)-L-lysyl]-L-proline dihydrate. Hydrochlorothiazide is 6-chloro-3,4-dihydro-2*H*-1,2,4-benzothiadiazine-7-sulfonamide 1,1-dioxide. Combinations of inhibitors of angiotensin-converting enzyme and diuretics, usually hydrothiazide class, are very common in antihypertensive therapy. By reducing the blood pressure, the combination of lisinopril and hydrochlorothiazide can help reduce the risk of damage to the kidneys, heart, or other organs.

Pharmaceutical analysis of such a combination is a challenge because of their extremely different physicochemical properties and their impurities. Methods proposed for analytical purposes must be as economical as possible, to enable their use in routine quality control. Many spectroscopic and chromatographic methods have been proposed for analysis of similar combinations.

Spectrophotometric methods after derivatisation of lisinopril have been described [1,2]. Hydrochlorothiazide, in combination with different drugs, for example amiloride, cilazapril, valsartane, and fosinopril, has been determined by use of derivative spectrophotometric methods [3–7]. Liquid chromatographic methods with UV detection have been described for determination of lisinopril in pharmaceutical products [8–10]. Fluorimetric detection of lisinopril in human plasma has also been described in the literature [11]. Many chromatographic methods for simultaneous determination of hydrochlorothiazide and angiotensin-converting enzyme inhibitors (benzapril, fosinopril, ramipril, and captopril) have been reported [12–15]. In combination with antagonists of angiotensin receptors, for example losartan and valsartan, hydrochlorothiazide has been analyzed by use of liquid chromatography [16–19]. Amiloride and hydrochlorothiazide in tablets have been assayed by use of RP–HPLC [20]. Hydrochlorothiazide and its two impurities have been determined in tablets by use of LC–MS–MS with gradient elution [21]. Liquid chromatographic methods with UV or electrochemical detection have been used for determination of hydrochlorothiazide in biological samples [22–24]. Capillary electrophoresis (CE) has been used for optimizing the separation of several ACE inhibitors [25].

Our objective in this investigation was to develop and validate an RP-HPLC method for simultaneous determination of hydrochlorothiazide (HCTZ) and lisinopril (L), of chlorothiazide (CTZ) and disulfonamide (DSA) (potential impurities of hydrochlorothiazide), and of diketopiperazine (DKP) (an impurity of lisinopril), in tablets. Having in mind the large difference between the amounts of the active substances and the impurities, their

structural similarity, and the absence of literature data on the analysis of these substances in tablets, the method proposed is a significant advance in pharmaceutical analysis.

EXPERIMENTAL

Reagents and Samples

All reagents used were of analytical grade. Acetonitrile (gradient grade; Lab Scan, Ireland), water (HPLC grade), potassium dihydrogen phosphate (Merck, Darmstadt, Germany), and 85% orthophosphoric acid (Carlo Erba, Milan, Italy) were used to prepare the mobile phase. Two samples of tablets (manufactured by Srbolek, Belgrade, Serbia) were analyzed; sample 1 contained 20 mg lisinopril as the dihydrate and 25 mg hydrochlorothiazide, and sample 2 contained 5 mg lisinopril as the dihydrate and 12.5 mg hydrochlorothiazide. According to the pharmacopoeias [26,27] the maximum amounts of impurities should be 1% for chlorothiazide and disulfonamide and 1.5% for diketopiparazine, both calculated relative to the declared content of active substances.

Mobile Phase Components

The mobile phase components were 7:93 (*v*/*v*) acetonitrile–25 mM potassium dihydrogen phosphate, pH 5 (component A), and 50:50 (*v*/*v*) acetonitrile–25 mM potassium dihydrogen phosphate pH 5 (component B). Before use these solutions were filtered through a 0.2-μm pore size Millipore filter and degassed in an ultrasonic bath.

Stock Solutions

Stock solutions were prepared by dissolving the respective working standard substances in mobile phase component A at concentrations of 4 mg mL^{-1} for L, 5 mg mL^{-1} for HCTZ, 50 μg mL^{-1} for CTZ, 50 μg mL^{-1} for DSA, 60 μg mL^{-1} for DKP, and 0.4. mg mL^{-1} for the internal standard (methylparaben).

Solutions for Determination of the Linearity of the Method

To construct calibration plots, a series of seven solutions in the concentration range 0.08 to 1.0 mg mL^{-1} for L, 0.1 to 1.25 mg mL^{-1} for HCTZ, 1.0 to 12.5 μg mL^{-1} for CTZ and DSA, and 1.2 to 15 μg mL^{-1} for

DKP was prepared from stock solutions. Methyl paraben was added as internal standard (20 μg mL^{-1}).

Solutions for Estimation of Method Precision

To prove the validity and applicability of the method, a laboratory mixture of L, HCTZ, CTZ, DSA, and DKP was prepared from the stock solutions in the ratio corresponding to amounts in the tablets analyzed. For quantitative analysis of the mixture three series (0.2, 0.4, and 0.6 mg mL^{-1} for L; 0.25, 0.50, and 0.75 mg mL^{-1} for HCTZ; 2.5, 5.0, and 7.5 μg mL^{-1} for CTZ and DSA; 3.0, 6.0, and 9.0 μg mL^{-1} for DKP) were prepared, with ten solutions for each concentration. Methylparaben, at a concentration of 20 μg mL^{-1}, was used as internal standard.

Solutions for Estimation of Method Accuracy

For testing accuracy, a laboratory mixture containing placebo components (mannitol, starch, magnesium stearate, microcrystalline cellulose, iron oxide red E172, and talc) and lisinopril, hydrochlorothiazide, and their impurities were prepared in the ratio corresponding to the amounts in the tablets investigated. This laboratory mixture was treated in the same manner as the tablets used for preparation of sample solutions. For quantitative analysis of the laboratory mixture three series of dilutions, calculated as 80, 100, and 120% of the amounts in the tablets, were prepared, with five solutions for each concentration. The concentrations obtained were 0.32, 0.4, and 0.48 mg mL^{-1} for L; 0.40, 0.50, and 0.60 mg mL^{-1} for HCTZ; 4.0, 5.0, and 6.0 μg mL^{-1} for CTZ and DSA; and 4.8, 6.0, and 7.2 μg mL^{-1} for DKP. Methylparaben was used as internal standard at a concentration of 20 μg mL^{-1}.

Solutions of Sample 1

Ten tablets (each of which contained 20 mg L and 25 mg HCTZ) were accurately weighed and finely powdered. A quantity of the powder containing 80 mg L and 100 mg HCTZ was transferred to a 100-mL volumetric flask. Mobile phase component A (70 mL) was then added and the powder was dissolved by immersion in an ultrasonic bath for 15 min. The solution was then diluted to volume with the same solvent mixture and filtered. From that solution, ten solutions were prepared containing final concentrations of 0.4 mg mL^{-1} L and 0.5 mg mL^{-1} HCTZ. Each solution contained methylparaben as internal standard (20 μg mL^{-1}). These solutions were used for analysis.

Solutions of Sample 2

Ten tablets (each of which contained 5 mg L and 12.5 mg HCTZ) were accurately weighed and finely powdered. A quantity of the powder containing 40 mg L and 100 mg HCTZ was transferred to a 50-mL volumetric flask. Mobile phase component A (30 mL) was then added and the powder was dissolved by immersion in an ultrasonic bath for 15 min. The solution was then diluted to volume with the same solvent mixture and filtered. From that solution, ten solutions were prepared containing final concentrations of 0.4 mg mL^{-1} L and 1.0 mg mL^{-1} HCTZ. Each solution contained methylparaben as internal standard (20 μg mL^{-1}). These solutions were used for analysis.

Chromatographic Conditions

HPLC was performed with a Hewlett–Packard 1100 system comprising an HP 1100 pump, HP 1100 UV–visible detector, and HP ChemStation integrator. Separations were performed at 25°C on a 4.6 mm × 20 mm, 3.5 μm particle size, XTerra column. The samples were introduced through a Rheodyne injection valve with 20-μL sample loop.

Simultaneous separation and quantification of HCTZ, CTZ, DSA, L, and DKP were performed by use of a gradient prepared from 7:93 (*v*/*v*) acetonitrile–25 mM potassium dihydrogen phosphate, pH 5 (component A), and 50:50 (*v*/*v*) acetonitrile–25 mM potassium dihydrogen phosphate, pH 5 (component B). The gradient program is presented in Table I. All changes in the gradient were linear, and the re-equilibration time was 15 min. The mobile phase flow rate was 1.0 mL min^{-1}.

UV detection was performed at 215 nm.

Table I

The gradient elution program

Time (min.)	Component A (%)	Component B (%)
0	100	0
8	100	0
15	0	100
20	100	0

RESULTS AND DISCUSSION

Chromatographic analysis of process-related impurities and of de-

gradation products are very important in the pharmaceutical industry. The possibility of side and toxic effects, and reduced activity of the active substances must be reduced to a minimum. For this reason pharmacopoeias and the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) have established very restrictive requirements for levels of impurities in pharmaceutical products. One of the main analytical problems is the large difference between the amounts of active substances and impurities, so a method for their simultaneous identification and quantification must be sufficiently selective. A second important problem is the structural similarity of the components analyzed, which lead to similar chromatographic behaviour. This paper reports analysis of L and HCTZ and their impurities CTZ, DSA, and DKP (Fig. 1).

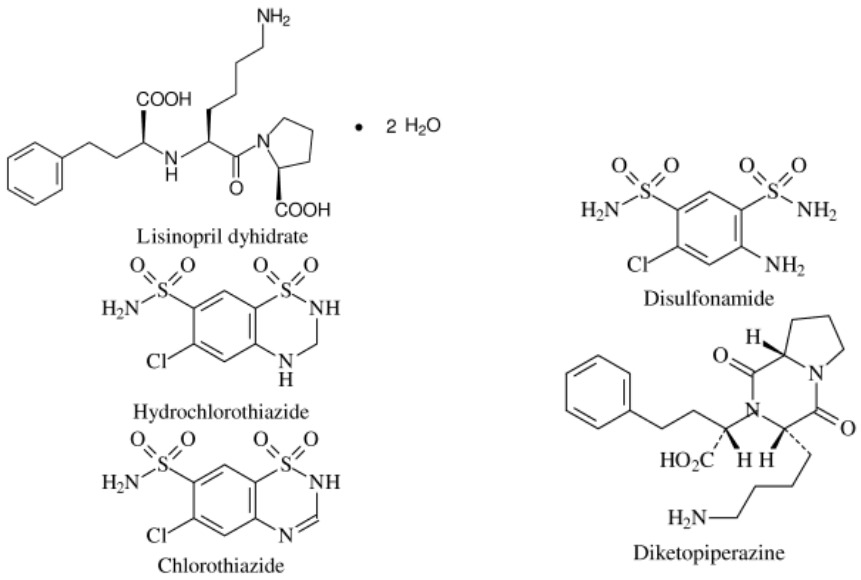

Fig. 1

Structures of lisinopril dihydrate (L), hydrochlorothiazide (HCTZ), chlorothiazide (CTZ), disulfonamide (DSA), and diketopiperazine (DKP)

Because of the structures and the physical and chemical properties of the substances analyzed, non-polar columns were chosen for preliminary study. The retention behaviour of the compounds was studied using different columns, for example 4.6 mm × 20 mm, 3.5-μm particle, XTerra

RP18, 4.6 mm × 20 mm, 3.0-μm particle, Atlantis dC_{18}, 4.6 mm × 150 mm, 5-μm particle, Symmetry C_{18}, and 4.6 mm × 150 mm, 5-μm particle, Zorbax. The first stage of the investigation was to determine chromatographic retention under different conditions. Conditions such as organic solvent content, pH, and buffer concentration, were investigated. The critical pairs in the separation were L from DSA and DSA from CTZ. It was concluded some kind of buffer should be used as the aqueous phase. Solutions of potassium dihydrogen phosphate and sodium dihydrogen phosphate of different ionic strength were prepared. The effect of addition of TEA was also investigated. It was noticed that increasing the ionic strength of the buffer solution reduced the retention times of the compounds, especially DKP. For further investigations potassium dihydrogen phosphate was selected as the aqueous phase. Addition of TEA had no important effect on separation and peak shape so it was excluded from further investigation. The problem of non-retention of L occurred when DKP was eluted in 15 min, i.e. when the concentration of buffer solution was >25 mM. It was noticeable that although the other four compounds were eluted in 5 min, with acceptable retention of L, under the same chromatographic conditions DKP was retained for almost 40 min.

Successful separation of such complex combinations can be achieved only by simultaneous investigation of different factors that may affect retention. In all HPLC analysis the amount of organic modifier can have a large effect on retention behaviour. Optimum separation of the critical pairs was achieved by use of 5 to 12% (*v*/*v*) acetonitrile in potassium hydrogen phosphate buffer as aqueous phase, but DKP was retained. The acetonitrile content should be >35% (*v*/*v*) to achieve elution of DKP in 20 min. This led to the conclusion that isocratic separation might not be possible. Another way of affecting chromatographic retention in isocratic separations is to change the pH of the mobile phase by adding 0.1 M sodium hydroxide. At pH below 3.0 co-elution of L, HCTZ, and DSA was observed. In contrast, pH > 6.0 enabled good separation of the critical pairs and acceptable retention of L, but DKP was retained. It was not possible to achieve separation by simultaneous varying acetonitrile content from 5 to 12% (*v*/*v*) and pH from 3.0 to 7.0. Changing the composition or pH of the mobile phase, or the polarity of the stationary phase, did not enable use of isocratic elution, and so the need for gradient elution was established. Aqueous phase of pH 5.0 resulted in optimum peak symmetry. Optimum run times were achieved by use of a 4.6 mm × 20 mm, 3.5-μm particle, XTerra column in gradient mode (Table I). The XTerra column contains a specific packing

on which free silanol groups are protected by methyl groups; this enables use over a wide pH range. The small particles enable rapid, efficient separations.

After establishing the optimum conditions for the separation, the method was validated for selectivity, linearity, precision, accuracy, and limits of quantification and detection.

Representative chromatograms obtained from the placebo, laboratory mixture, sample 1, and sample 2 are presented in Fig. 2.

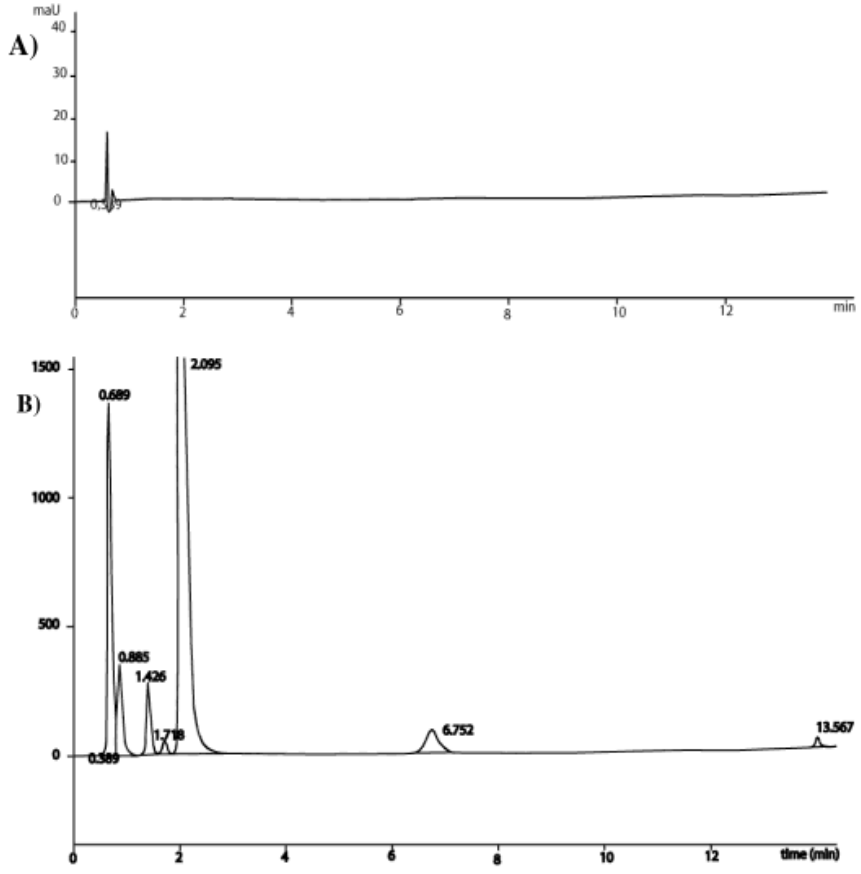

Fig. 2

Representative chromatograms obtained from: (A) placebo (t_R = 0.369 min); (B) laboratory mixture of L (t_R = 0.689 min), DSA (t_R = 1.426 min), CTZ (t_R = 1.718 min), HCTZ (t_R = 2.095 min), internal standard (t_R = 6.752 min), and DKP (t_R = 13.567 min)

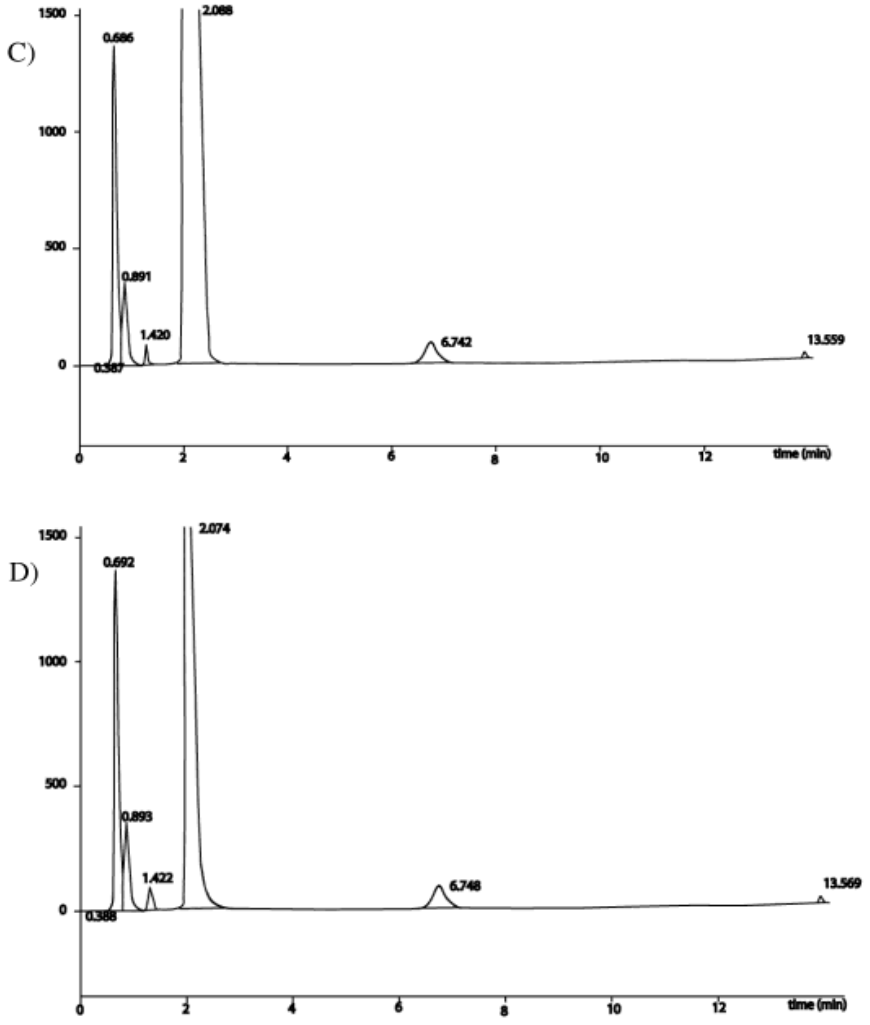

Fig. 2 (continued)

Representative chromatograms obtained from: (C) sample 1 (L (t_R = 0.686 min), DSA (t_R = 1.420 min), HCTZ (t_R = 2.088 min), internal standard (t_R = 6.742 min), and DKP (t_R = 13.559 min); and (D) sample 2 (L (t_R = 0.692 min), DSA (t_R = 1.422 min), HCTZ (t_R = 2.074 min), internal standard (t_R = 6.748 min), and DKP (t_R = 13.569 min)

The method is selective, because no significant interfering peaks were observed at the retention times of L, HCTZ, CTZ, DSA, DKP, or the internal standard. All excipients were eluted at different times and did not interfere with the compounds analysed.

Linear relationships were obtained between peak area and concentration over the range investigated. The important regression equation data slope (a), intercept (b), correlation coefficient (r), and standard deviation of the intercept (S_b) are listed in Table II.

Table II

Important calibration data

Substance	Concentration range	a	b	r	S_b	t_b
HCTZ	0.1–1.0 (mg mL^{-1})	17.522	10.904	0.9993	14.51	2.159
L	0.08–1.0 (mg mL^{-1})	11.545	0.842	0.9990	1.909	0.238
CTZ	1.0–12.5 (μg mL^{-1})	0.0597	0.0033	0.9999	0.003	1.184
DSA	1.0–12.5 (μg mL^{-1})	0.0761	0.0151	0.9999	0.148	2.125
DKP	3.0–15.0 (μg mL^{-1})	0.0183	−0.0056	0.9995	0.003	1.635

For the regression equation $y = ax + b$, r is the correlation coefficient and S_b is the standard deviation of the intercept; $t_{tab.} = 3.707$

Table III

Intermediate precision of the RP–HPLC method

Compound (concn)	Injected	Found	R (%)	CV (%)
HCTZ (mg mL^{-1})	0.25	0.252 ± 0.003*	100.6	1.0
	0.50	0.507 ± 0.005	101.5	0.9
	0.75	0.742 ± 0.007	98.9	1.0
L (mg mL^{-1})	0.20	0.207 ± 0.001	103.5	0.5
	0.40	0.406 ± 0.004	101.6	0.9
	0.60	0.598 ± 0.003	99.7	0.6
CTZ (μg mL^{-1})	2.5	2.51 ± 0.01	100.4	0.5
	5.0	5.08 ± 0.01	101.6	0.5
	7.5	7.40 ± 0.07	98.73	0.2
DSA (μg mL^{-1})	2.5	2.49 ± 0.01	99.6	0.2
	5.0	5.04 ± 0.01	100.7	0.4
	7.5	7.60 ± 0.03	101.4	0.2
DKP (μg mL^{-1})	3.0	3.007 ± 0.003	100.2	1.0
	6.0	5.96 ± 0.05	99.3	0.5
	9.0	9.06 ± 0.05	100.7	0.9

R is the recovery and CV the coefficient of variation
* Standard deviation ($n = 10$)

The results obtained from determination of the precision of the method are listed in Table III. The values of the standard deviation (*S*) and coefficient of variation (CV), and the good recoveries indicate the assay is precise.

The results obtained from determination of the accuracy of the method are listed in Table IV. The values obtained for the standard deviation (*S*) and the coefficient of variation (CV), and the good recoveries, again indicate the method is accurate.

Table IV

Accuracy of the method

Compound (concn)	Injected	Found	*R* (%)	CV (%)
HCTZ (mg mL^{-1})	0.40	0.393 ± 0.003*	98.3	0.7
	0.50	0.499 ± 0.005	99.8	1.0
	0.60	0.593 ± 0.005	98.9	0.8
L (mg mL^{-1})	0.32	0.316 ± 0.003	98.6	0.8
	0.40	0.398 ± 0.002	99.5	0.6
	0.48	0.476 ± 0.001	99.1	0.2
CTZ (μg mL^{-1})	4.0	3.99 ± 0.02	99.8	0.6
	5.0	4.93 ± 0.02	98.7	0.5
	6.0	5.97 ± 0.03	99.5	0.6
DSA (μg mL^{-1})	4.0	3.99 ± 0.01	99.7	0.3
	5.0	4.97 ± 0.03	99.5	0.7
	6.0	6.03 ± 0.04	100.6	0.7
DKP (μg mL^{-1})	4.8	4.97 ± 0.04	99.9	0.8
	6.0	5.98 ± 0.06	99.7	0.9
	7.2	7.24 ± 0.06	100.5	0.8

R is the recovery and CV the coefficient of variation
* Standard deviation ($n = 10$)

Table V

Experimentally determined limits of quantification (LOQ) and detection (LOD) (μg mL^{-1})

Compound	LOQ	LOD
HCTZ	0.05	0.005
L	0.05	0.005
CTZ	0.1	0.01
DSA	0.1	0.01
DKP	1.0	0.04

Experimentally determined limits of detection and quantification are listed in Table V. The LOD and LOQ were regarded as the amounts of the compounds for which the signal-to-noise ratios were 3:1 and 10:1, respectively. The results were further confirmed by diluting the secondary stock solution until the peak areas obtained were 3 (for LOD) and 10 (for LOQ) times the standard deviations of six determinations.

Table VI

Results from quantitative analysis of hydrochlorothiazide (HCTZ), lisinopril (L), and their impurities in tablets (Sample 1, containing 25 mg HCTZ and 20 mg L)

Compound	Amount taken (mg mL^{-1})	Amount found (mg mL^{-1})	Amount found (mg $tablet^{-1}$)	CV (%)	*R* (%)
HCTZ	0.5	0.501 ± 0.004*	25.06	0.8	100.2
L	0.4	0.401 ± 0.003	20.08	0.8	100.1
Impurity	MAC (μg mL^{-1})	Amount found (μg mL^{-1})	Amount found (%)	CV (%)	
CTZ	5.0	Less than LOD	Less than 0.01	–	
DSA	5.0	1.25 ± 0.13	0.12	0.1	
DKP	6.0	4.59 ± 0.07	0.57	1.5	

MAC is the maximum allowed content, LOD the limit of detection for the impurity, *R* the recovery, and CV the coefficient of variation

* Standard deviation (n = 10)

Table VII

Results from quantitative analysis of hydrochlorothiazide (HCTZ), lisinopril (L), and their impurities in tablets (Sample 2, containing 12.5 mg HCTZ and 5 mg L)

Compound	Amount taken (mg mL^{-1})	Amount found (mg mL^{-1})	Amount found (mg $tablet^{-1}$)	CV (%)	*R* (%)
HCTZ	1.0	1.01 ± 0.01*	12.69	0.9	100.7
L	0.4	0.398 ± 0.004	4.98	0.9	99.5
Impurity	MAC (μg mL^{-1})	Amount found (μg mL^{-1})	Amount found (%)	CV (%)	
CTZ	5.0	Less than LOD	Less than 0.01	5.0	
DSA	5.0	3.24 ± 0.15	0.16	4.5	
DKP	6.0	4.41 ± 0.12	0.55	2.8	

MAC is the maximum allowed content, LOD the limit of detection for the impurity, *R* the recovery, and CV the coefficient of variation

* Standard deviation (n = 10)

Results from determination of the L, DSA, CTZ, HCTZ, and DKP content of Samples 1 and 2 are given in Tables VI and VII, respectively. The results obtained are in good agreement with the amounts declared.

CONCLUSIONS

The proposed RP–HPLC method enables simultaneous determination of lisinopril dihydrate, hydrochlorothiazide, and their impurities, enabling good separation and resolution of the chromatographic peaks. This is the first reported method for simultaneous quantitative analysis of lisinopril dihydrate, hydrochlorothiazide, chlorothiazide, disulfonamide, and diketopiperazine, and is a significant advance in chromatographic analysis of such pharmaceutical mixtures. The method is suitable for qualitative and quantitative analysis of these pharmaceutical products. The results obtained are in a good agreement with the declared contents. Statistical analysis showed the method is accurate and precise. There was no interference from excipients in the tablets.

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