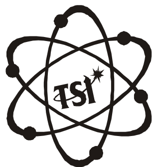

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Development and validation of a stability indicatimg HPLC method for the simultaneous determination of captopril and indapamide

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ABSTRACT

A simple, selective, precise and stability indicating reversed phase HPLC method was developed and validated for the simultaneous determination of captopril (CAP) and indapamide (IN) in presence of their degradation products, using hydrochlorothiazide (HZ) as internal standard, in powder and tablets.

The separation was achieved on an Agilent Zorbax C_{18} column, with isocratic flow. The mobile phase consisted of methanol:water:triethylamine (42.5:57.5:0.028, v/v/v), adjusted with *o*-phosphoric acid to pH 2.5. The flow rate was maintained at 2 ml min^{-1}. The UV detection was carried out at 220 nm for CAP, CAP oxidation product and IN degradation product (I) and 242 nm for IN and 272 nm for HZ. Linearity was demonstrated with good correlation coefficients (0.9998), over the concentration range of 27.5–550 µg.ml^{-1} and 2.5– 50 µg.ml^{-1}, in case of CAP and IN, respectively.

The method was successfully validated in accordance to ICH guidelines. Individual drugs (CAP and IN) were exposed to hydrolytic and oxidative stress conditions. The method gave high resolution among the degradation products and the analytes.

The proposed method is accurate, stability indicating and successfully applied for the determination of CAP and IN in synthetic mixtures and tablets. © 2012 Trade Science Inc. - INDIA

KEYWORDS

Captopril;
Degradation;
Indapamide;
HPLC;
Stability indicating method.

INTRODUCTION

Captopril ((*S*)-1-(3-mercapto-2-methyl-1-oxopropyl) *L*–proline), (CAP), figure 1A, is an orally active ACE inhibitor[1,2], its combination with the diuretic indapamide (1-(4-chloro-3-sulfamoylbenzamido)-2-methylindoline), (IN), figure 1B, is of synergistic value in counteracting hypertension[3,4].

Literature studies show various analytical methods reported for the estimation of CAP in biological fluids and for pharmaceutical formulations, such as titrimetric[5], electrochemical[6], UV spectrophotometric[7], colorimetric[8], fluorimetric[9], atomic absorption spectroscopic[10], chromatographic methods[11]. In addition, volumetric and HPLC methods were described by U.S.P, B.P. and Eur. P.[12-14]. Several methods such as titrimetric[15], electrochemical[16], UV spectrophotometric[17], colorimetric[18], chemiluminescence[19], chromatographic methods[20] were reported for the determination of IN in plasma, and pharmaceutical dosage form. Furthermore, HPLC methods were reported in U.S.P,

B.P. and Eur.P.[12-14].

The International conference on harmonization of technical requirements for registration of pharmaceuticals for human use (ICH) guideline states that the degradation products formed under a variety of conditions should be identified and degradation pathways established[21,22]. Moreover, according to current good manufacturing practices, all drugs must be tested with a stability indicating assay method before release[23]. From the above considerations, it is clear that the investigation of drug stability represents an important issue in drug quality evaluation.

CAP and IN are drugs of clinical and analytical interest; two HPLC methods have been reported for their simultaneous determination in binary mixtures[24,25]. In a previous work of the authors, derivative spectophotometry, first-derivative ratio spectra and TLC densitometry were investigated[26]. Literature survey reveals that no methods were reported as stability indicating assay for the analysis of CAP and IN, in binary mixture. Hence, the authors deemed useful to propose a stability indicating and accurate HPLC method for the simultaneous determination of CAP and IN in admixture either in laboratory samples or in dosage form of these drugs.

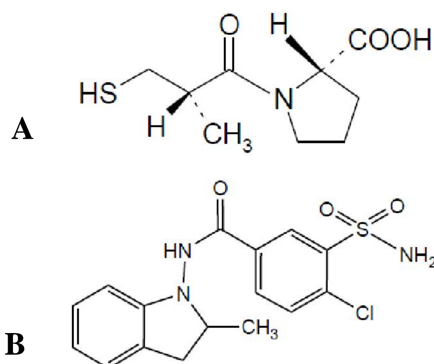

Figure 1 : Structures of captopril (1A) and indapamide (1B).

EXPERIMENTAL

Instrument and chromatographic conditions

Integrated HPLC system, Agilent 1100 series, was used for method development and method validation. This system was equipped with an isocratic pump G1310A, a manual injector G1328B with a 20 µl loop and a UV – visible variable wavelength detector.

Isocratic mobile phase consisted of methanol: water: triethylamine (42.5:57.5:0.028, v/v/v), adjusted with *o*-phosphoric acid to pH 2.5, using a pH meter, Jenway, 3505, Essex, U.K. The flow rate was maintained at 2 $ml.min^{-1}$. A membrane filter of 0.45µm porosity was used to filter the mobile phase and an ultrasonic processor, Soniclean, Thebarton SA, Australia, was used to degas the mobile phase.

Agilent Zorbax C_{18} column, 5 µm particle size (4.6 X 250 mm) Japan was used as a stationary phase and hydrochlorothiazide (HZ) was used as an internal standard. The samples were injected (20 µl) with a 25 µl Agilent analytical syringe. The detector was set at 220 nm for CAP, CAP oxidation product and IN degradation product (I) and at 242 nm and 272 nm for IN and HZ, respectively. The analysis was carried out at ambient temperature.

IR charts were obtained using Burker FT-IR spectrophotometer Vector 22, Schimadzu 435, Perkin-Elmer 457 and Jasco FT-IR plus 460 Japan, using potassium bromide discs. NMR charts were obtained using Varian Gemini 200 MHZ, Joel Fx 90Q, 90 MHz FT spectrophotometer and Joel Ex 270 MHz spectrophotometer. The chemical shifts were expressed in δ ppm units, using trimethylsilane as the internal standard. Mass spectra were obtained using Hewlett Packard 5988 mass spectrometer at 70 eV.

Materials and reagents

CAP and IN pure samples were obtained from Rameda (The tenth of Ramadan) Co. for Pharmaceutical Industries and Diagnostic Reagents, 6th of October city, Egypt. CAP and IN were analyzed and found to be 99.6 ± 0.826 and 100.45 ± 0.383, respectively, applying RP-HPLC method[27]. Pharmaceutical preparations were obtained from the local market. Normaten® tablets, Batch No. 03642, were labelled to contain 3 mg of IN and 33 mg of CAP per tablet. HZ was kindly supplied from National Organization for Drug Control and Research in Egypt.

All chemicals were of analytical grade and the solvents were of HPLC grade. Aquatron double distillator was used to prepare distilled water.

Standard solutions

(a) Standard stock solutions

CAP standard solution (1.1 $mg.ml^{-1}$), IN standard

solution (0.1 mg.ml^{-1}) and HZ standard solution (0.2 mg.ml^{-1}) were prepared in the mobile phase. In addition, CAP oxidation product solution (1.1 mg.ml^{-1}) and IN degradation product (I) solution (0.1 mg.ml^{-1}) were also prepared in the mobile phase.

(b) Sample stock solution

Twenty tablets were weighed, powdered and mixed well. An amount of the powdered tablets equivalent to IN (2.5mg) and CAP (27.5 mg) was weighed accurately and transferred into a 25 ml volumetric flask. Methanol (15 ml) was added and the flask was shaken mechanically for 30 min, then completed to volume with the mobile phase, mixed well and filtered on dry funnel and dry filter paper, discarding the first few milliliters.

Laboratory prepared mixtures

(a) In absence of degradation products

Different mixtures containing CAP and IN in the same ratio as that of the dosage form (1:11) were prepared from intact drug working standard solutions. One ml of HZ solution (0.2 mg.ml^{-1}) was added as an IS to each flask.

(b) In presence of degradation products

Different mixtures containing 10 – 90 % of the degradation product and CAP and IN in the same ratio as that of the dosage form (1:11) were prepared from intact drug working standard solutions. One ml of HZ solution (0.2 mg.ml^{-1}) was added as an IS to each flask.

Method development

A variety of mobile phases were investigated in the development of a stability indicating HPLC method for the analysis of CAP and IN in tablet dosage form. The suitability of mobile phase was decided on the basis of selectivity and sensitivity of the assay and separation among impurities formed during forced degradation studies.

Forced degradation study

Forced degradation study was conducted on samples containing individual drugs.

(a) Preparation of captopril oxidation product

An accurate weight (0.5 gm) of CAP was dissolved in 20 ml water, 0.05 M iodine was added till a faint yellow color was obtained and then few drops of 0.1 M sodium thiosulphate were added till colorless. A white precipitate was formed which is the oxidation product of CAP[14,28]. The precipitate was filtered, washed with water (15 ml x 3) and left to dry overnight in an oven at 50^{0}C. A small amount of the precipitate was dissolved in ethanol, spotted on a TLC plate next to a spot of CAP and the plate was allowed to develop using chloroform : glacial acetic acid (6.5:1, v/v) as developing system. The spots were detected using iodine vapors. Two different spots were obtained, one for CAP (R_f = 0.53) and the other for its oxidation product (R_f = 0.26) confirming that the obtained precipitate was the oxidation product, figure 2A.

(b) Preparation of indapamide acid degradation product

An accurate weight (0.3 gm) of IN was dissolved in the least amount of methanol and refluxed with 35 ml 3 N hydrochloric acid for 3 hours at 100^{0}C, wrapping the round bottom flask with aluminum foil. After cooling, the obtained precipitate was filtered, washed with water (15 ml x 3) and left to dry overnight in an oven at 50^{0}C. This precipitate was the acid degradation product (I) of IN[17,29]. A small amount of the precipitate was dissolved in methanol and spotted on a TLC plate next to a spot of IN and the plate was allowed to develop using chloroform:glacial acetic acid (6.5:1, v/v) as developing system. Two spots were detected under UV lamp, at 254 nm, the first corresponding to IN (R_f = 0.37) and the second to its degradation product (I) (R_f = 0.24), confirming that the obtained precipitate was the degradation product (I), figure 2B.

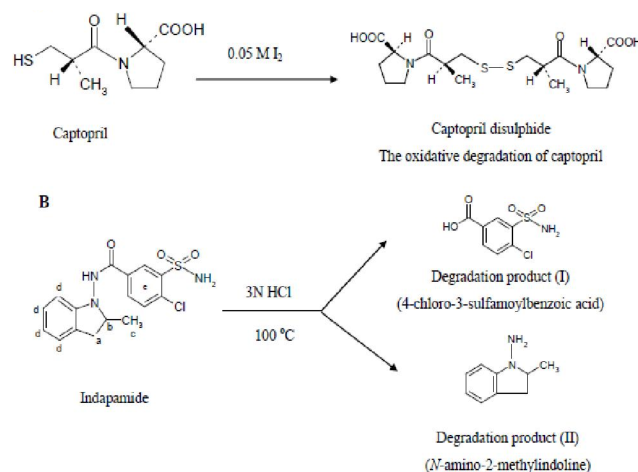

Figure 2 : Degradation pathways of captopril (1A) and indapamide (1B).

The degradation products of CAP and IN were identified using IR, ^{1}H-NMR and mass spectroscopy.

Procedures

(a) Linearity and construction of calibration curve

Aliquots equivalent to either (275 – 5500 µg) of CAP standard solution (1.1 mg.ml^{-1}) or (25 – 500 µg) of IN standard solution (0.1 mg.ml^{-1}) were transferred into two separate series of 10 ml volumetric flasks, 1 ml of HZ standard solution (IS) (0.2 mg.ml^{-1}) was added as an IS to each flask and the volumes were completed with the mobile phase. Triplicate 20 µl of each solution were injected onto the column, using a 25 µl analytical syringe. Chromatograms were recorded under the following instrumental parameters; flow rate was 2 ml.min^{-1} at ambient temperature. The separation was achieved on C_{18} column using methanol:water:triethylamine (42.5:57.5:0.028, v/v/v), adjusted with *o*-phosphoric acid to pH 2.5, as a mobile phase. The wavelength was 220 nm for CAP, CAP oxidation product and IN degradation product (I) and 242 nm and 272 nm for IN and HZ, respectively. The peak area ratios of each drug to that of the internal standard were calculated and used for the construction of the calibration curves of CAP and IN. The corresponding regression equations were then computed.

(b) Analysis of laboratory prepared mixtures

The chromatographic conditions used under linearity and construction of calibration curve were applied for different laboratory prepared mixtures, in absence and in presence of the degradation products. The peak area ratios of CAP and IN were calculated for each mixture, and their concentrations were calculated by substituting in the regression equations.

(c) Application to pharmaceutical formulation

Aliquots of appropriate volume were transferred into 10 ml volumetric flasks and diluted to volume with the mobile phase to furnish the concentration range listed in TABLE 1.

RESULTS AND DISCUSSION

In recent years, two LC methods have been published for simultaneous analysis of CAP and IN in tablet dosage form[24,25]. One of the reported methods[25] involved the use of cyanopropyl column and also stability indicating nature was not explored. System suitability tests were not studied for these methods.

Therefore, the proposed method was found superior in terms of easily available laboratory columns, reagents, greater linearity range and separation of degradation products.

Results of forced degradation studies

(a) Identification of the oxidation product of captopril

The literature shows that CAP, in aqueous solution, undergoes oxidative degradation at its thiol function to yield CAP disulphide[30,31], figure 2A. Oxidation was carried out using 0.05 M iodine and excess iodine was reduced using sodium thiosulphate[14,28]. A white precipitate was formed which, when dissolved in ethanol and spotted on a TLC plate, showed a spot at R_f = 0.26, while that of intact CAP was at R_f = 0.53, using chloroform : glacial acetic acid (6.5:1, v/v) as developing system. The spots were detected using iodine vapors.

The structure of the oxidation product was confirmed by measuring the melting point which was found to be 242 °C, ^{1}H-NMR and mass spectroscopy.

^{1}H-NMR spectrum of CAP in $CDCl_3$ was characterized by the appearance of the protons of methyl group as doublet at δ 1.163 ppm, SH as triplet at 1.523 – 1.625 ppm, (β,-CH_2-) and (γ, -CH_2-) as multiplet at δ 1.947 – 2.286 ppm, (-CH-C=O) as multiplet at 2.363 – 2.528 ppm, (-CH_2-SH) as multiplet at 2.716 – 2.979 ppm, (δ, -CH_2-) as multiplet at 3.562 – 3.753 ppm, (α, -CH-COOH) as triplet at 4.569 – 4.624 ppm and OH group as singlet at 11.302 ppm, figure 3A. The disulphide formation led to the disappearance of the SH signal, as revealed in the ^{1}H-NMR spectrum of CAP oxidation product (DMSO-d_6), figure 3B.

Moreover, mass spectrum showed the molecular ion peak of the oxidation product (M^+ +1) at 433 which is equivalent to its expected molecular weight.

(b) Identification of the acid degradation product of indapamide

IN is photolabile and undergoes amide hydrolysis under acidic as well as basic conditions. However, the yield of acid hydrolysis is more than that of alkaline hydrolysis[17,29,32]. Therefore, in this study, complete

degradation was done by refluxing IN with 3N HCl for three hours at 100 °C, protected from light; yielding two degradation products. The degradation pathway of IN[17,29] is presented in figure 2B. The degradation product (I) was obtained after precipitation and cooling of the solution.

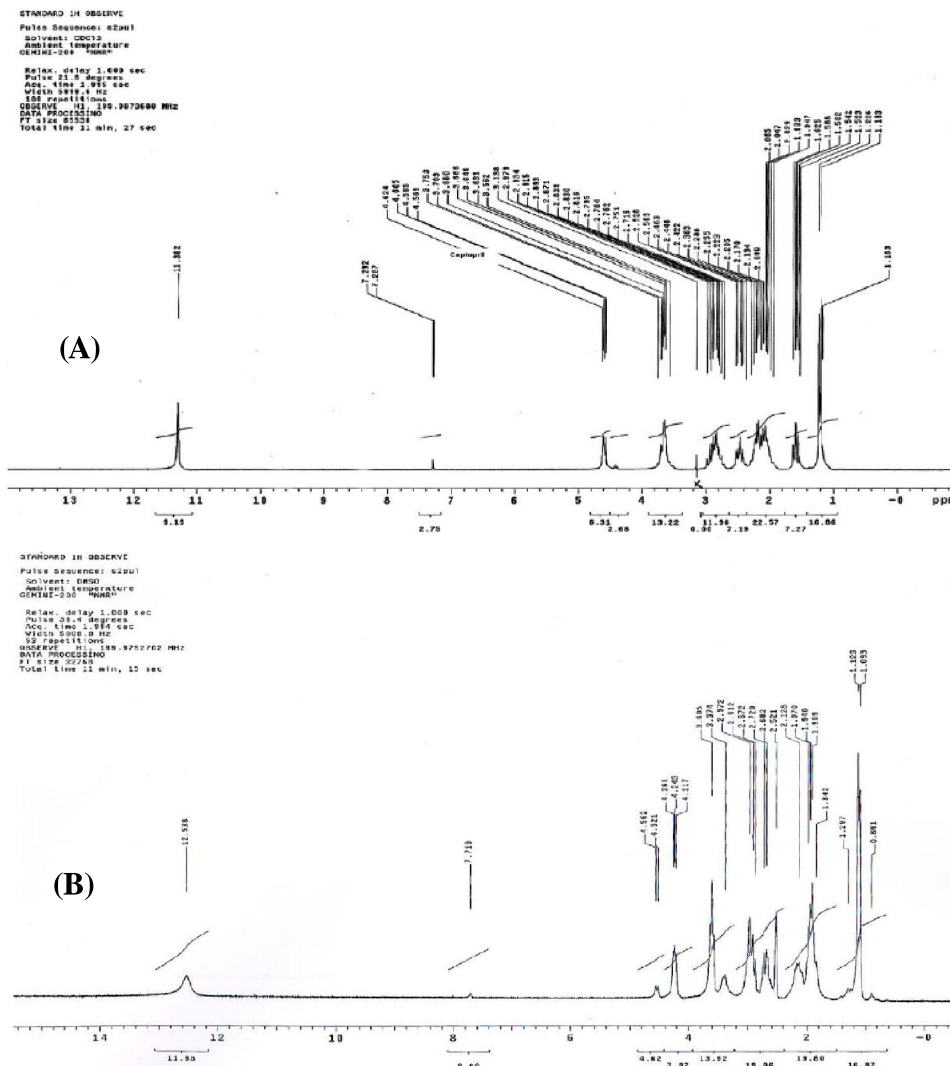

Figure 3 : ^{1}H-NMR spectra of intact captopril in chloroform ($CDCl_3$), (3A); captopril oxidation product in DMSO-d_6, (3B).

Both the intact drug and the acid degradation product (I) were dissolved separately in methanol and spotted on a TLC plate. Two different spots were detected under the UV lamp, with R_f = 0.37 and 0.24 for IN and the degradation product (I), respectively, using chloroform : glacial acetic acid (6.5 : 1, v/v) as developing system.

The melting point of the degradation product (I) was tested and found to be 254 – 256 °C. Furthermore, its structure was confirmed by IR, ^{1}H-NMR and mass spectroscopy.

IR spectrum (KBr) of intact IN showed three stretching bands in the range of 3430.5 – 3220.9 cm^{-1} corresponding to the primary NH_2 and the secondary amide, the absorption frequency of C=O at 1658.5 cm^{-1} and that of SO_2 at 1341.5 and 1172.8 cm^{-1}, figure 4A. However, IR spectrum (KBr) of IN degradation product (I) revealed two stretching bands characteristic of primary amine in the range of 3403 – 3282.8 cm^{-1}, a broad band of carboxylic OH stretching vibration which extends in the range of 3088–2578.3 cm^{-1}, the absorption frequency of C=O at 1694.7 cm^{-1} and that of SO_2 at 1342.5 and 1169.7 cm^{-1}, figure 4B.

^{1}H-NMR spectrum of intact IN in DMSO-d_6 was characterized by the appearance of the signals of the protons of the methyl group (c) as a doublet at δ 1.297

– 1.326 ppm, methylene group of the indole ring (a) as a doublet at δ 2.515 – 3.233 ppm, proton of the indole ring (b) as a triplet at δ 3.967 ppm, aromatic protons of the indole ring (d) and benzene ring (e) as a multiplet at δ 6.487 – 7.135 and 7.765 - 8.504 ppm, respectively and amine group as a singlet at 10.554 ppm, figure 5A.

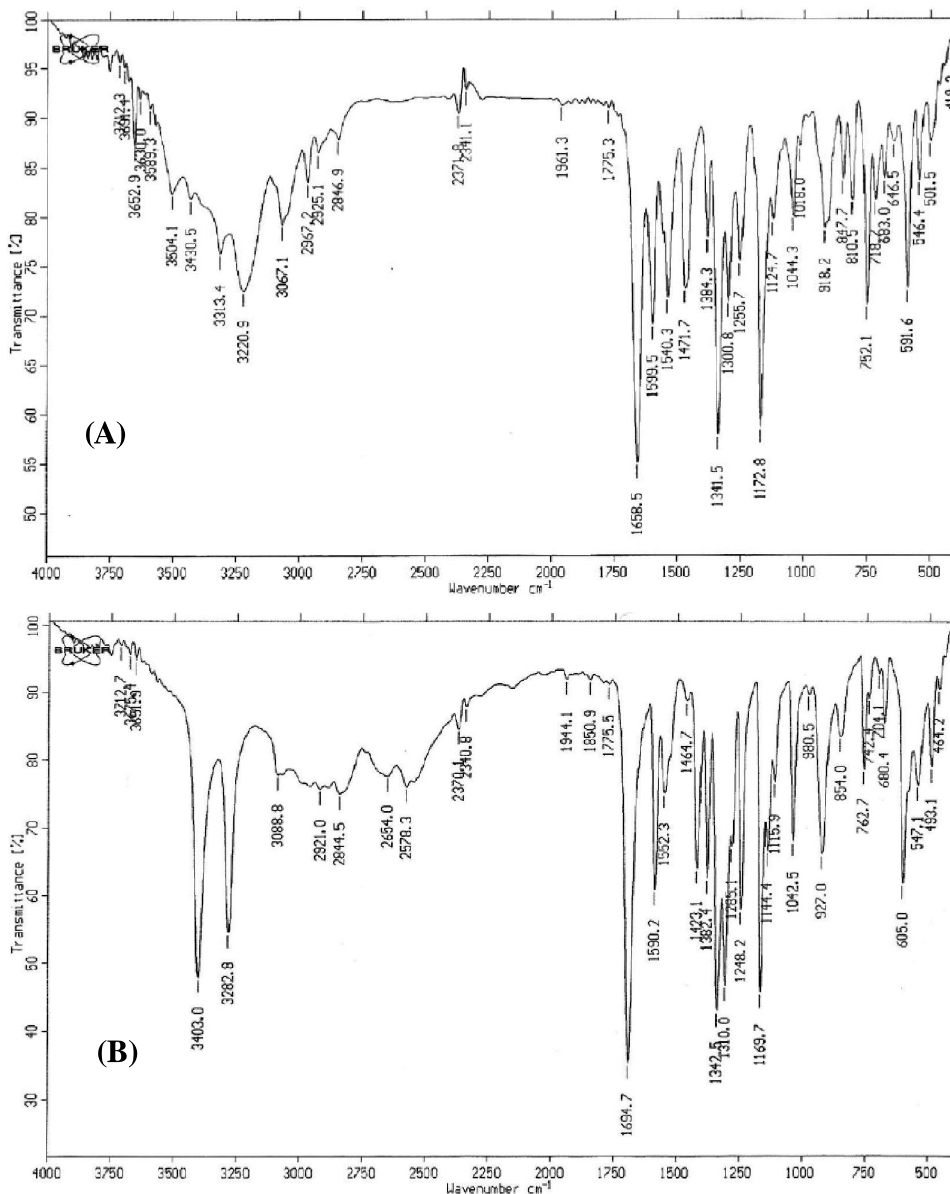

Figure 4 : IR spectra of intact indapamide, (5A); indapamide degradation product (I), (5B).

By contrast, ^{1}H-NMR spectrum IN degradation product (I) in the same solvent lacked the characteristic protons signals of the indole ring and showed the signals of aromatic protons as a multiplet at δ 7.816 – 8.522 ppm, NH_2 as a singlet at δ 8.823 ppm and OH as a singlet at δ 13.542 ppm which disappears by deuteration, figure 5B. This indicates that the amide group suffered cleavage by 3N HCl leading to the formation of the degradation products.

In addition, mass spectroscopy revealed M^+ at 235 and $M^+ + 2$ at 237 due to the presence of Cl and which is identical to the molecular weight of IN degradation product (I).

HPLC method development

Preliminary tests were performed with the objective to select adequate and optimum conditions for the separation under isocratic conditions. Parameters such as column type, mobile phase composition, pH, flow rate and detection wavelengths were thoroughly investigated.

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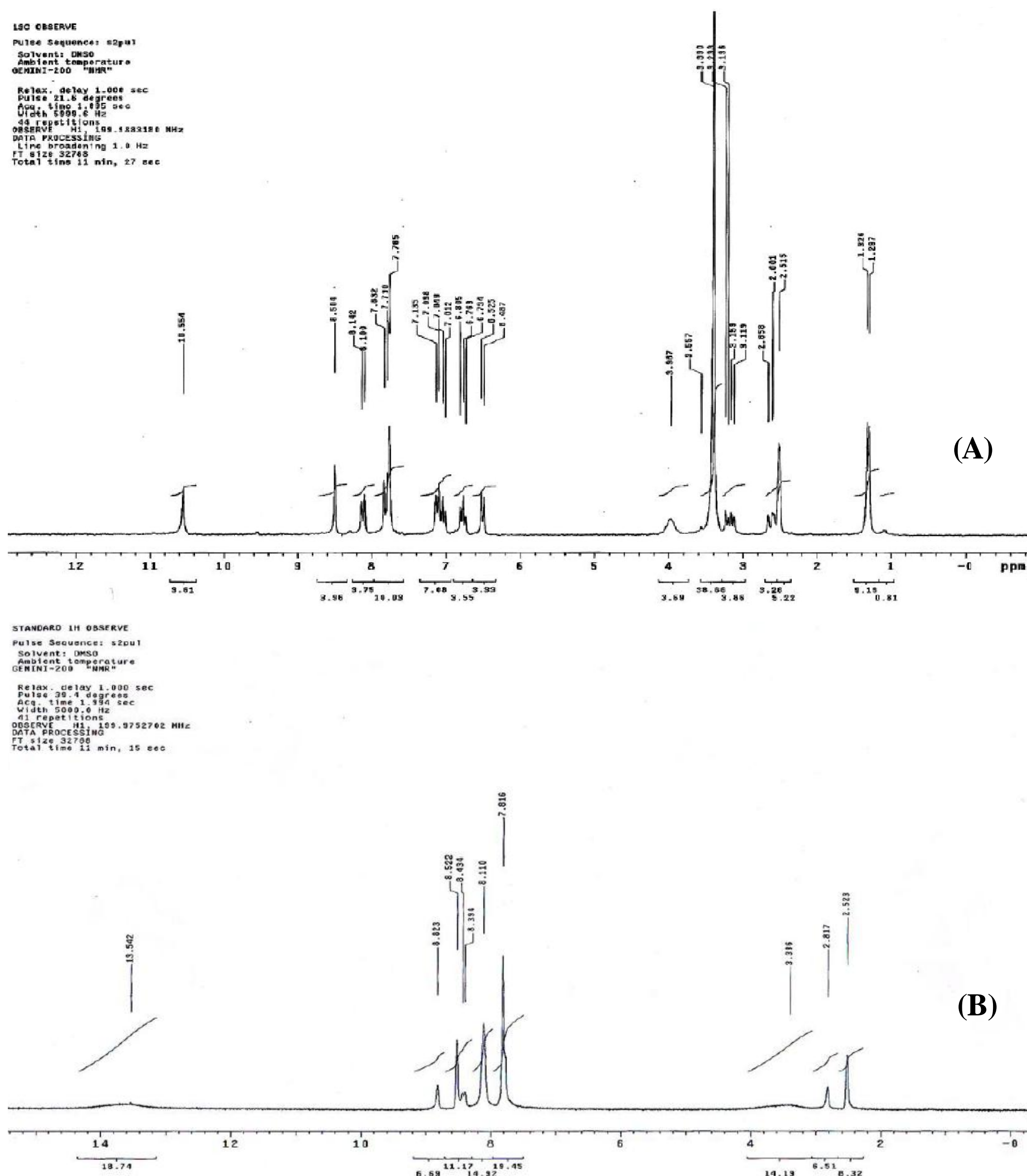

Figure 5 : ^{1}H-NMR spectrum of intact indapamide in DMSO-d_6, (6A); indapamide degradation product (I) in DMSO-d_6, (6B).

Attempts were made using different stationary phases, such as C_8 and C_{18} columns. The use of C_8 column reduced the hydrophobicity of the column and decreased the retention time of the drugs but poor resolution was obtained.

Several binary or ternary eluents were tested using different proportions of solvents, such as, acetonitrile, methanol and water. The separation improved with the decrease of the percentage of methanol to water. The use of ion – pair reagent, triethylamine, resulted in less peak broadening and less peak asymmetry[33,34]. The pH of the mobile phase was found to be critical in achieving the separation between the drugs of choice[34,35]. A pH, higher than 2.5, showed poor resolution of the studied drugs and their degradation products. To improve the precision of the method and to compensate for small variabilities in the instrumentation performance[35] HZ was used as an IS.

A satisfactory separation was obtained using C_{18} column with a mobile phase consisting of methanol:water: triethylamine (42.5:57.5:0.028, v/v/v), adjusted with *o*-phosphoric acid to pH 2.5, at a flow rate of 2 ml.min^{-1}. Detection was carried out at 220 nm for IN degradation product (I), CAP and CAP oxidation product and at 242 nm and 272 nm for IN and HZ (IS), respectively. A representative chromatogram is

shown in figure 6, where the peaks obtained are sharp and well resolved and have a clear baseline separation. The average retention time ± S.D.[24] and the elution order were found to be 1.363 ± 0.002 for HZ, 1.997 ± 0.026 for IN degradation product (I), 2.969 ± 0.031 for CAP, 7.951 ± 0.029 for IN and 10.517 ± 0.091 for CAP oxidation product, with an overall analysis time of about 13 min.

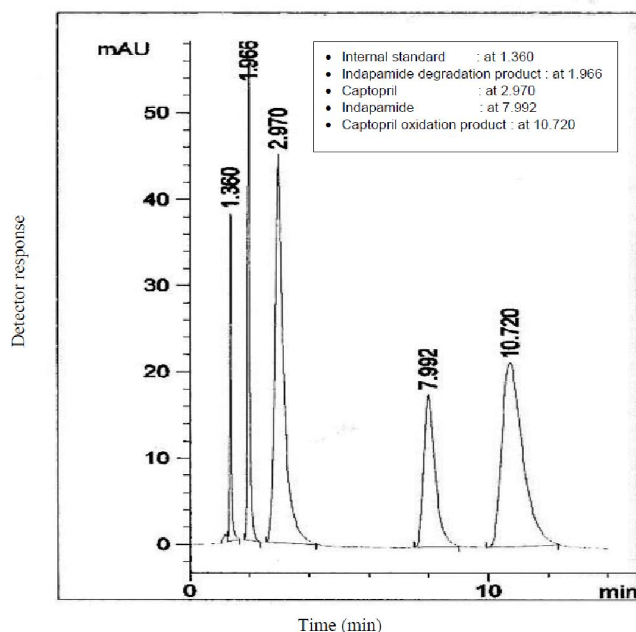

Figure 6 : HPLC chromatogram of a mixture of hydrchlorothiazide solution as an IS (200 µg.ml-1), indapamide degradation product (I) solution (12.5 µg.ml-1), captopril standard solution (137.5 µg.ml-1), indapamide standard solution (12.5 µg.ml-1) and captopril oxidation product solution (137.5 µg.ml-1) in mobile phase.

Method validation

The developed chromatographic method was validated for linearity, precision, accuracy, limit of detection (LOD), limit of quantification (LOQ) and system suitability in accordance with the ICH guideline Q2 (R1)[22].

(a) Linearity

A linear relationship was obtained between the peak area ratios (drug/IS) and the drug concentrations in the ranges of 27.5 – 550 µg.ml^{-1} and 2.5 – 50 µg.ml^{-1}, in case of CAP and IN, respectively. Linear regression analysis was performed to calculate slope, intercept and regression coefficient (r^2) of the calibration curves. The analytical data of the calibration curves including standard deviations for the slope and intercept (S_b, S_a) are summarized in TABLE 1.

TABLE 1 : Assay parameters and method validation obtained by applying HPLC method for the simultaneous determination of CAP and IN in mixture.

Item	HPLC method	
	CAP	IN
Retention time	2.969 ± 0.031min	7.951 ± 0.029 min
Wavelength of detection	220 nm	242 nm
Range of linearity	27.5 – 550 µg. ml^{-1}	2.5 – 50 µg.ml^{-1}
Regression equation	y=0.0402 x – 0.027	y=0.2178 x – 0.0037
Correlation coefficient (r)	0.9998	0.9998
S_b	0.0001	0.001
S_a	0.055	0.028
LOD	2.75 µg. ml^{-1}	0.14 µg. ml^{-1}
LOQ	8.35 µg. ml^{-1}	0.41 µg. ml^{-1}
Intra day[a]		
%RSD	0.890-0.147	0.316-0.273
Inter day[b]		
%RSD	0.983-0.882	0.703-0.650
Drug in dosage form	100.38±0.953	100.43±0.958

[a]The intraday (n = 3), average of two concentrations (88, 396 µg.ml^{-1}), (8, 36 µg.ml^{-1}) for CAP and IN, respectively, repeated three times within the day; [b]The interday (n = 3), average of two concentrations (88, 396 µg.ml^{-1}), (8, 36 µg.ml^{-1}) for CAP and IN, respectively, repeated three times in three successive days.

(b) Accuracy

Accuracy of the method was checked by analyzing synthetic mixtures of both drugs at various concentrations. The mean percentage recoveries were 99.56 ± 0.831 and 99.84 ± 0.755 (mean ± RSD), in case of CAP and IN, respectively, TABLE 2.

(c) Selectivity

The selectivity of the proposed procedure was established by analyzing different laboratory prepared mixtures of intact drugs in presence of varying concentrations of degradation products. The mean percentage recoveries were found to be 99.02 ± 0.733 and 99.62 ± 0.859, in case of CAP and IN, respectively, indicating that the method is not affected by the presence of up to 90 % of the degradation products, TABLE 2.

(d) Precision

The precision of the method was determined using parameters of repeatability (intraday) and intermediate precision (interday), analyzing 2 sample solutions of (88, 396 µg.ml^{-1}) and (8, 36 µg.ml^{-1}) for CAP and IN, re-

spectively, in triplicate, on the same day. The values of RSD were calculated to determine intraday precision. This study was also repeated on three days to determine interday precision, TABLE 1.

TABLE 2 : RP-HPLC method for the simultaneous determination of CAP and IN in laboratory prepared mixtures.

Mixture No.	In absence of degradation products				In presence of degradation products						
	Taken (μg.ml^{-1})		% Recovery		% Degradtion products	Intact Taken (μg.ml^{-1})		Degradation product taken (μg.ml^{-1})		% Recovery	
	CAP	IN	CAP	IN		CAP	IN	CAP	IN	CAP	IN
1	66	6	100.33	98.53	10	247.5	22.5	27.5	2.5	99.82	99.49
2	88	8	99.93	99.39	30	192.5	17.5	82.5	7.5	98.26	100.55
3	176	16	99.43	100.83	50	137.5	12.5	137.5	12.5	98.76	99.50
4	286	26	98.01	100.14	70	82.5	7.5	192.5	17.5	98.54	98.91
5	396	36	100.48	100.19	80	55.0	5.0	220.0	20.0	100.05	100.70
6	440	40	99.55	99.50	90	27.5	2.5	247.5	22.5	98.69	98.56
7	484	44	99.18	100.28							
X			99.56	99.84						99.02	99.62
± S.D.			0.831	0.755						0.733	0.859
RSD			0.835	0.756						0.740	0.862

TABLE 3 : Chromatographic parameters required for system suitability test in the simultaneous determination of CAP and IN in binary mixture and in presence of their degradation products by RP-HPLC method.

Parameter	Obtained value		Reference value[12,35]
	CAP	IN	
Tailing factor (T)	1.42	1.29	≤ 2
Column capacity (K)	1.97	6.992	1 – 10
Column efficiency (N)	1585.022	6445.081	The higher the value, the more efficient the column is.
Height equivalent to theoretical plate (H)	0.0158 cm/plate	0.0039 cm/plate	The smaller the value, the higher the column efficiency
Resolution (R)	5.11 (a_3)*	4.78 (a_1)* 14.42 (a_2)*	> 2
Relative retention (α)	2.04 (b_3)**	1.39 (b_1)** 3.55 (b_2)**	≥ 1

*a_1, a_2 and a_3 calculated for IN - CAP oxidation product, IN - CAP and CAP - IN degradation product (I), respectively; **b_1, b_2 and b_3 calculated for IN – CAP oxidation product, IN - CAPl and CAP – IN degradation product (I), respectively.

(e) Limit of detection and limit of quantification

In accordance with ICH recommendations, the approach based on the standard deviation of the response and the slope of the calibration plots was used to determine detection and quantification limits. The values are given in TABLE 1.

(f) System suitability

System suitability tests are an integral part of chromatographic methods and are used to verify that the resolution and precision of the system are adequate for the analysis to be performed[13]. Good agreement was found when the results were compared with the recommended values[12,35], where the system was found to be suitable, TABLE 3.

Tablet analysis

The proposed method was successfully applied for the analysis of the cited drugs in Normaten® tablet. Applying the standard addition technique assessed the validity of the method, TABLE 4. The results of analysis of the commercial tablets and the recovery study (standard addition method) suggested that there is no interference from any excipients which are normally present in tablets.

TABLE 4 : Application of the standard addition technique to the simultaneous determination of CAP and IN in Normaten® tablets by RP-HPLC method.

Claimed Taken (μg.ml^{-1})		Pure Added (μg.ml^{-1})		% Recovery of added	
CAP	IN	CAP	IN	CAP	IN
220	20	110	10	99.28	99.82
220	20	165	15	99.06	100.55
220	20	220	20	99.33	101.61
220	20	275	25	98.69	100.18
220	20	330	30	99.63	99.47
X				99.20	100.33
± S.D.				0.349	0.823
RSD				0.352	0.820

TABLE 5 : Statistical comparison between the results obtained by applying the proposed method for the analysis of CAP and IN by the proposed RP-HPLC method and the reference method.

Preparation	Statistical Term	CAP		IN	
		RP-HPLC	Reference Method▲	RP-HPLC	Reference Method▲
Laboratory	Mean±S.D.	99.56^ab^±0.831	99.60^ab^±0.826	99.84±0.755	100.45±0.383
Prepared	N	7	5	7	5
Mixtures	Variance	0.691	0.682	0.570	0.147
	S.E.	0.314	0.369	0.285	0.171
	Student's t	0.083 (2.228)*		1.835 (2.228)*	
	F ratio	1.013(6.16)*		3.878(6.16)*	
	F value	2.75		0.73	
	p-value	0.035**		0.607	
Normaten®	Mean±S.D.	100.38±0.953	99.97±0.856	100.43±0.958	99.69±0.784
Tablet	N	3	5	3	5
	Variance	0.908	0.733	0.918	0.615
	S.E.	0.550	0.383	0.553	0.351
	Student's t	0.612(2.447)*		1.130(2.447)*	
	F ratio	1.239(6.94)*		1.493(6.94)*	
	F value	0.71		1.57	
	p-value	0.627		0.229	

***The values in the parenthesis are the corresponding values of t and F at (p=0.05); **= There is a significant difference between methods by using one way ANOVA at p<0.05; The same letter means that no significant difference by using Duncan t-test at p< 0.05; ▲Reference method for pure CAP and pure IN in binary mixture is RP-HPLC method, using C_{18} column, water : acetonitrile : methanol : glacial acetic acid (650:175:175:1, v/v/v/v) as mobile phase and UV detection at 220 nm[27].**

TABLE 6 : Comparison between the results obtained by applying the proposed RP-HPLC method and the reference method for the analysis of CAP and IN.

Methods	CAP				IN			
	Laboratory prepared mixtures		Normaten® tablet		Laboratory prepared mixtures		Normaten® tablet	
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
RP-HPLC method	99.56^ab^	0.831	100.38	0.953	99.84	0.755	100.43	0.958
Reference method	99.60^ab^	0.826	99.97	0.856	100.45	0.383	99.69	0.784
F-value	2.75		0.71		0.73		1.57	
p-value	0.035*		0.627		0.607		0.229	

*** = There is a significant difference between methods by using one way ANOVA at p <0.05; The same letter means that no significant difference by using Duncan t-test at p < 0.05.**

Statistical analysis

Statistical comparison of the results was performed with regard to accuracy and precision using Student's *t*-test, the *F*-ratio and *P*-value at 95% confidence level, by using One-way ANOVA. The calculated Student's *t*-test and *F*-ratio values were found to be less than the theoretical values, and the *P*-value of the *F*-test is always greater than 0.05, revealing that there is no significant difference between the suggested and the reference methods with regard to accuracy and precision, TABLE 5.

CONCLUSION

The developed and validated LC method is stability indicating and enables specific, accurate and precise simultaneous analysis of CAP and IN in tablet formulation. The method is sensitive enough for quantitative detection of the analytes in pharmaceutical preparation. Therefore, the proposed method can be used for routine analysis, quality control and for studies of the stability of pharmaceutical tablet containing these drugs.

REFERENCES

[1] P.Kumar, M.Clark; Clinical medicine, 6th Edition, Elsevier limited, **(2005)**.

[2] R.D.Howland, M.J.Mycek, R.A.Harvey, P.C.Champe; Lippincott's illustrated reviews: Pharmacology, 3rd Edition, Lippincott williams & wilkins, Philadelphia, **(2006)**.

[3] P.P.Gerbino; Remington the science and practice of pharmacy, 21st Edition, Lippincott williams & wilkins, by the university of the sciences in philadelphia, **(2006)**.

[4] M.D.Randal, K.E.Neil; Disease management, Pharmaceutical press, London, Chicago, **(2004)**.

[5] N.Rahman, M.Singh, M.N.Hoda; Farmaco, **60(6-7)**, 569-574 **(2005)**.

[6] H.Parham, B.Zargar; Talanta, **65(3)**, 776-780 **(2005)**.

[7] N.Ghasemi, A.Niazi; Chin.Chem.Lett., **18(4)**, 427-430 **(2007)**.

[8] K.M.Emara, I.H.Refaat, H.M.Abdel-Wadood, M.G.Hassan; Bull.Pharm.Sci, Assiut.Univ., **28(2)**, 225-236 **(2005)**.

[9] D.Liu, C.Z.Huang, Yz. F.Li, Y.F.Long; Anal.Chem.Acta., **577(2)**, 244-249 **(2006)**.

[10] M.A.El Reis, F.M.Abou Attia, I.M.M.Kenawy; J.Pharm.Biomed.Anal., **23(2-3)**, 249-254 **(2000)**.

[11] S.Ahmed, M.Rizk, F.Belal, F.Ibrahim, Z.Sheribah; J.of Liq.Chromatogr.Relat.Technol., **29** (**4**), 521-532 (**2006**).

[12] The united states pharmacopeia (USP 30), The national formulary (NF 25), Asian Edition, (**2007**).

[13] The british pharmacopoeia, Her majesty's stationary office, London, UK, (**2007**).

[14] The european pharmacopoeia, 5th Edition, Council of europe, Strasbourg, (**2005**).

[15] M.Y.Ebeid, B.A.Moussa, A.A-Malek, F.M.Ashour; Egypt.J.Pharm.Sci., **38(1-3)**, 171-182 (**1997**).

[16] A.Radi; J.Pharm.Biomed.Anal., **24**(**3**), 413-419 (**2001**).

[17] B.El-Zeiny, M.AbdelKaway, D.El-Zeihery; Bull.Fac.Pharm.Cairo.Univ., **41**(**3**), 269-283 (**2003**).

[18] N.F.Youssef; J.AOAC Int., **86(5)**, 935-946 (**2003**).

[19] M.Ciborowski, M.C.Icardo, J.V.G.Mateo, J.M.Calatayud; J.Pharm.Biomed.Anal., **36(4)**, 693-700 (**2004**).

[20] W.D.Chen, Y.Liang, H.Zhang, H.Li, Y.Xiong, G.J.Wang, L.Xie; J.Chromatogr.B, **842(1)**, 58-63 (**2006**).

[21] A.K.S.Ahmad, M.Abdel Kawy, M.Nebsen; J.Pharm.Biomed.Anal., **30**, 479-489 (**2002**).

[22] International conference on harmonization of technical requirements for registration of pharmaceuticals for human use (ICH), Q1A (R2) : Stability testing of new drug substances and products, Geneva, (**2003**).

[23] B.M.Rao, A.Chakraborty, M.K.Srinivasu, M.L.Devi, P.R.Kumar, K.B.Chandrasekhar, A.K.Srinivasan, A.S.Prasad, J.Ramanatham; J.Pharm.Biomed.Anal., **41**, 676-681 (**2006**).

[24] A.El-Gindy, S.Emara, G.M.Hadad; Farmaco, **59(9)**, 703-712 (**2004**).

[25] A.Elshanawane, S.M.Mostafa, M.S.Elgawish; Chromatographia, **67**, 837-840 (**2008**).

[26] B.A.Moussa, A.H.Abadi, H.E.Abou-Youssef, M.A.Mahrouse; Bull.Fac.of Pharm., Cairo.Univ., **44(2)**, 63-76 (**2006**).

[27] Through rameda co. for Pharmaceutical industries and diagnostic reagents 6th of october City, Egypt, by personal communication.

[28] U.Rose; J.Pharm.Biomed.Anal., **18**, 1-14 (**1998**).

[29] M.V.Padval, H.N.Bhargava; J.Pharm.Biomed.Anal., **11(10)**, 1033 – 1036 (**1993**).

[30] H.Kadin; Analytical profiles of drug substances and excipients, Academic Press, Inc., 11 (**1982**).

[31] S.Hillaert, W.Van den Bossche; J.Pharm.Biomed.Anal., **21(1)**, 65-73 (**1999**).

[32] R.Davis, C.H.J.Wells, A.R.Taylor; J.Pharm.Sci., **68(8)**, 1063-1064 (**1979**).

[33] J.Lambropoulos, G.A.Spanos, N.V.Lazaridis, T.S.Ingallinera, V.K.Rodriguez; J.Pharm.Biomed.Anal., **20**, 705-716 (**1999**).

[34] D.Zendelovska, T.Stafilov; J.Pharm.Biomed.Anal., **33**, 165-173 (**2003**).

[35] P.D.Sethi; High performance liquid chromatography, Quantitative analysis of pharmaceutical formulations, 1st Edition, CBS Publishers & distributors, New Delhi, India, (**2001**).