

Validated Chromatographic Methods for Determination of Perindopril and Amlodipine in Pharmaceutical Formulation in the Presence of their Degradation Products

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Two specific, sensitive, and precise stability-indicating chromatographic methods have been developed, optimized and validated for determination of perindopril arginin (PER) and amlodipine besylate (AML) in their mixtures and in the presence of their degradation products. The first method was based on thin-layer chromatography (TLC) combined with densitometric determination of the separated bands. Adequate separation was achieved using silica gel 60 F254 TLC plates and ethyl acetate–methanol–toluene–ammonia solution, 33% (6.5:2:1:0.5 by volume), as a developing system. The second method was based on high-performance liquid chromatography, by which the proposed components were separated on a reversed-phase C18 analytical column using a mobile phase consisting of phosphate buffer (pH 2.5, 0.01 M)–acetonitrile–tetrahydrofuran (60:40:0.1% by volume) with ultraviolet detection at 218 nm. Different parameters affecting the suggested methods were optimized for maximum separation of the cited components. System suitability parameters of the two developed methods were also tested. The suggested methods were validated in compliance with the ICH guidelines and were successfully applied for the quantification of PER and AML in their commercial tablets. Both methods were also statistically compared to each other and to the reference methods with no significant differences in performance.

Introduction

Perindopril (PER), (2*S*,3*a*,7*a**S*)-1-[(2*S*)-2-[[1*S*]-1-(ethoxycarbonyl)butyl] amino]-1-oxopropyl] octahydro-1*H*-indole-2-carboxylic acid, is an angiotensin-converting enzyme inhibitor that is used in the treatment of hypertension and heart failure. It is also used to reduce the risk of cardiovascular events in patients with stable ischaemic heart disease (1). PER is converted in the body into its active metabolite, perindoprilate. Amlodipine (AML), 2-[(2-aminoethoxy) methyl]-4-(2-chlorophenyl)-1,4-dihydro-6-methyl-3,5-pyridinedicarboxylic acid 3-ethyl 5-methyl ester, is a dihydropyridine derivative with calcium antagonist activity (1). It is used in the management of hypertension, chronic stable angina pectoris and Prinzmetal variant angina (2).

Recently, perindopril arginin has been marketed in combination with amlodipine besylate in tablets for the treatment of essential hypertension. This combination is advised for patients whose blood pressure is not adequately controlled by either drug alone, because it has been shown to be superior in lowering systolic and diastolic blood pressures when compared with either of the monotherapy regimens. The combination, thereby also has significantly fewer dose-dependent adverse experiences than high-dose calcium antagonist monotherapy (3).

PER is formulated in dosage form in two salt forms, perindopril erbumine and perindopril arginin. The advantage of arginin salt over erbumine is that it imparts a certain stability to perindopril and inhibits the cyclization process in open containers or at high temperatures (4).

A literature survey revealed that both perindopril erbumine salt and amlodipine besylate are official in the British Pharmacopoeia (5). However, neither perindopril arginin nor its pharmaceutical combination with amlodipine besylate (Coveram tablet) is official in any pharmacopoeia. A detailed literature survey found that perindopril erbumine has been determined in biological fluids and pharmaceutical formulations either in binary mixtures, enantiomeric mixtures or in the presence of its metabolite with different techniques such as thin-layer chromatography (TLC) (6–8), high-performance liquid chromatography (HPLC) (9, 10), LC (11, 12), gas chromatography (GC) (13–15), potentiometric selective electrodes (16–18), capillary zone electrophoresis (CZE) (19–21), selective biosensors (22–24), spectrophotometry (25, 26) and radioimmunoassay (RIA) (27), but no method has been reported for the analysis of perindopril arginin.

Amlodipine besylate has been assayed using HPLC (28–30), TLC (31–33), capillary electrophoresis (CE) (34–36), LC–mass spectrometry (MS)–nuclear magnetic resonance (NMR), electrospray ionization (ESI) (37, 38) and voltammetric techniques (39, 40) either in binary or enantiomeric mixtures. Although perindopril erbumine and amlodipine besylate have been analyzed by two methods in binary mixtures (41, 42), none of the reported methods have described a stability-indicating method for the simultaneous determination of PER and AML in tablet dosage form. Therefore, the objective of this manuscript is to establish and validate stability-indicating methods for the determination of both active compounds in the presence of their degradation products. To establish the stability-indicating nature of the methods, forced degradation of PER and AML was performed under stress conditions, and stressed samples were analyzed by use of the proposed TLC and reversed-phase (RP)-HPLC methods, which were able to separate the drugs from compounds produced during forced degradation studies.

Experimental

Instruments

The liquid chromatography system consisted of an isocratic pump, Model G1310A (Agilent Technologies, Santa Clara, CA), an ultraviolet (UV) variable wavelength detector (Model G1314A, Agilent 1100 Series), a Rheodyne injector, Model

7725 I (Sigma-Aldrich, Taufkirchen bei München, Germany) equipped with a 20 μ L injector loop (Agilent Technologies). The stationary phase was a 250 \times 4.6 mm i.d. Nucleosil C18 analytical column from Alltech Associates (Deerfield, IL). The mobile phase was a phosphate buffer solution (1.74 g of dipotassium hydrogen phosphate anhydrous was dissolved and diluted to a volume of 900 mL with deionized water, and adjusted to a pH of 2.5 with 85% phosphoric acid)–acetonitrile–tetrahydrofuran (60:40:0.1% by volume). The mobile phase was filtered through a 0.45 μ m Millipore membrane filter and was degassed for 15 min in an ultrasonic bath before use. UV detection was conducted at 218 nm. The samples were filtered also through a 0.45 μ m membrane filter, and were injected by the aid of a 20 μ L Agilent analytical syringe.

Also used were a Camag Linomat 5 autosampler (MuttENZ, Switzerland), Camag microsyringe (100 μ L) and a Camag TLC scanner 3 S/N 130319 with winCATS software. The following requirements are taken into consideration: slit dimensions, 5 \times 0.2 mm; scanning speed, 20 mm/s; spraying rate, 10 s/ μ L; data resolution, 100 μ m/step.

Additional instruments included precoated TLC plates, silica gel 60 F254 (20 \times 20 cm, 0.25 mm) (Merck, Darmstadt, Germany). The mass spectrometer was a Shimadzu Qp-2010 (Kyoto, Japan), which was operated on electron impact (EI) mode at 70 eV. The infrared (IR) spectrophotometer was a Shimadzu 435; samplings were taken as potassium bromide discs. The pH meter was a digital pH/MV/TEMP/ATC meter, Model 5005 (Jenco Instruments, San Diego, CA).

Materials and reagents

Pure standard

Standard PER (99.68%) and AML (100.14%) were supplied by Servier Egypt Industries (Cairo, Egypt). Their purities were assessed according to the manufacturer method and official (5) HPLC methods, respectively.

Pharmaceutical formulation

Coveram tablets are available in several different strength combinations, including 5/5, 10/10, 5/10 and 10/5 mg of PER and AML, respectively. They are manufactured by Servier (Ireland) Industries Limited for Les Laboratoires Servier (France). Batch numbers 77597, 376112, 73935 and 59423 were purchased from the Egyptian markets.

Degraded sample

Both PER and AML were stressed for alkali degradation studies. Solutions were prepared by dissolving, separately, 25 mg of pure PER and AML powders in 25 mL of 0.1 M and 1 M NaOH, respectively, and the solutions were refluxed for 2 h in the case of PER and 1 h for AML. The degradation process was followed by TLC using 33% ethyl acetate–methanol–toluene–ammonia solution (6.5:2:1:0.5 by volume) as the developing system. Degradation products were precipitated at specific pH for each degradation product by controlling the pH using hydrochloric acid, washed three times each with 10 mL of distilled water and re-crystallized from methanol. The degradation products were elucidated by IR and MS.

Chemicals and reagents

All chemicals used throughout this work were of analytical grade, and the solvents were of spectroscopic grade. These included hydrochloric acid, 33% ammonia solution, toluene, ethyl acetate, sodium hydroxide, methanol and chloroform (El-Nasr Pharmaceutical Chemicals Cairo, Egypt); tetrahydrofuran (THF) and acetonitrile; HiPerSolv, HPLC grade (Merck); o-phosphoric acid (85%) (BDH, Poole, England); de-ionized water, bi-distilled from an Aquatron Automatic Water Still A4000 (Bibby Sterillin Staffordshire, UK).

Standard solutions

Stock standard solutions of both perindopril arginin and amlodipine besylate (2 mg/mL) were prepared in methanol (for the TLC densitometric method) and 0.1 mg/mL in the mobile phase for the HPLC method.

Stock standard solutions of the alkaline degradation products derived from the complete degradation of standard solutions of both PER and AML (1 mg/mL) were prepared in methanol (for the TLC densitometric method) and 0.1 mg/mL in the mobile phase for the HPLC method.

All stock standard solutions were freshly prepared on the day of analysis and stored in the refrigerator to be used within 24 h.

Procedure

Chromatographic conditions

TLC was performed on 20 \times 10 cm TLC aluminum sheets precoated with 0.25 mm silica gel 60 F254; the samples were applied as bands (bandwidth: 4 mm; spacing: 13.8 mm; 15 mm from the bottom edge of the plate). Linear ascending development was conducted in a chromatographic tank, previously saturated with 33% ethyl acetate–methanol–toluene–ammonia solution (6.5:2:1:0.5, by volume) for 1 h at room temperature. The developed plates were air dried and scanned at 218 nm for PER and 237 nm for AML on a Camag TLC scanner 3 operated in the absorbance mode, with a deuterium lamp as a source of radiation.

HPLC was conducted on an RP-C18 column (250 \times 4.6 mm i.d., 5 μ m p.s.). The mobile phase consisted of phosphate buffer (pH 2.5, 0.01 M)–acetonitrile–tetrahydrofuran (60:40:0.1% by volume). The mobile phase was filtered through a 0.45 μ m Millipore membrane filter (Billerica, MA) and was degassed for 15 min in an ultrasonic bath before use. UV detection was conducted at 218 nm. The system was operated at ambient temperature. The flow rate was isocratic at 1 mL/min. The samples were also filtered through a 0.45 μ m membrane filter, and were injected by the aid of a 20 μ L Hamilton analytical syringe.

Linearity

For TLC–densitometry, aliquots equivalent to 2–14 mg of PER and 1–4 mg of AML were transferred from their respective standard solutions (2 mg/mL in methanol) into two separate series of 10 mL measuring flasks, and then the volume of each flask was completed with methanol. Twenty microliters of each solution were applied in triplicate to TLC plates in the form of bands using a Camag Linomat IV applicator, then chromatographed

and scanned. The calibration curves relating the integrated area under the peak to the corresponding concentrations of PER and AML, as $\mu\text{g}/\text{band}$, were constructed.

For the RP-HPLC method, standard solutions (0.1 mg/mL) of PER and AML were further diluted with the mobile phase to obtain dilutions of PER and AML in the ranges of (10–100 and 5–45 $\mu\text{g}/\text{mL}$), respectively. Triplicate 20 μL injections were made and chromatographed for each solution. To reach good equilibrium, the analysis was usually performed after passing 50–60 mL of the mobile phase, for conditioning and pre-washing of the stationary phase. The relative peak area ratios (drug/external standard), using either 80 $\mu\text{g}/\text{mL}$ of PER or 25 $\mu\text{g}/\text{mL}$ of AML as external standards for PER and AML, respectively, were calculated and plotted against the corresponding concentrations to obtain the calibration graph for each component.

Analysis of laboratory prepared mixtures containing different ratios of the drugs

Aliquots of intact PER, AML and the degraded drugs were mixed to prepare mixtures containing different ratios of PER and AML, including the market ratios and different ratios of their degradation products down to 5%, and then procedures were undertaken as described under each method. The concentrations were calculated from the corresponding regression equations.

Assay of pharmaceutical formulations (Coveram tablets)

Ten tablets of Coveram (5/5, 10/10, 5/10 and 10/5 mg of PER and AML, respectively) were weighed, finely powdered and

thoroughly mixed. An accurately weighted portion of the powder equivalent to the weight of one tablet was transferred into four separate 100 mL volumetric flasks, 50 mL of appropriate solvent (methanol for TLC or acetonitrile for HPLC) was added, the mixture was sonicated for 10 min, and then 25 mL solvent was added and the mixture was sonicated for a further 10 min to affect complete dissolution. Volumes were completed with appropriate solvent and then filtered. Suitable dilution was achieved using the appropriate solvent to obtain concentrations of each of the two drugs in the previously described range of linearity. The previously detailed procedure was followed.

Application of standard addition technique

When conducting the standard addition technique, different known concentrations of pure standards of PER and AML were added to the pharmaceutical formulation before proceeding with the previously mentioned methods.

Results and Discussion

Stability testing forms is an important part of the process of drug product development. The purpose of stability testing is to provide evidence about how the quality of a drug substance or drug product varies with time under the influence of a variety of environmental factors such as temperature, humidity and light, and allows recommendations to be established for storage conditions, retest periods and shelf lives. The two primary aspects of drug products that play an important role in

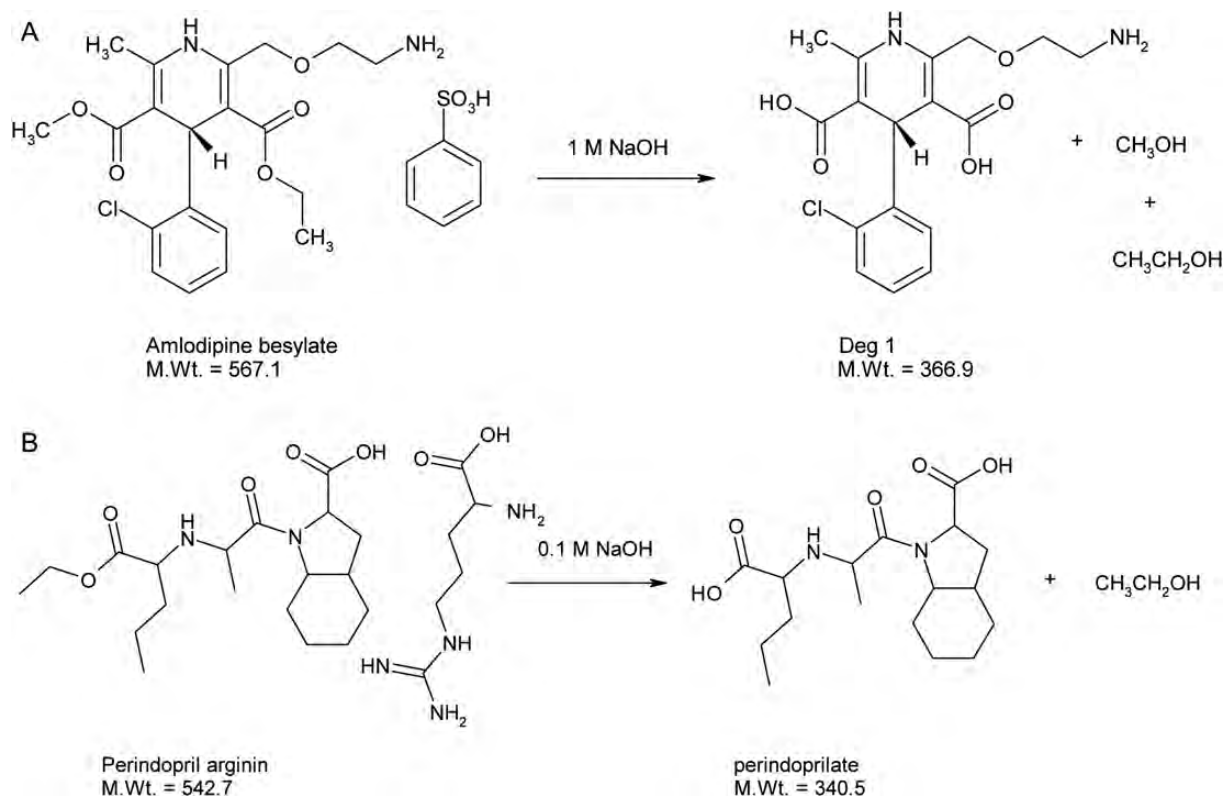


Figure 1. Suggested alkaline degradation pathways: AML (A); PER (B).

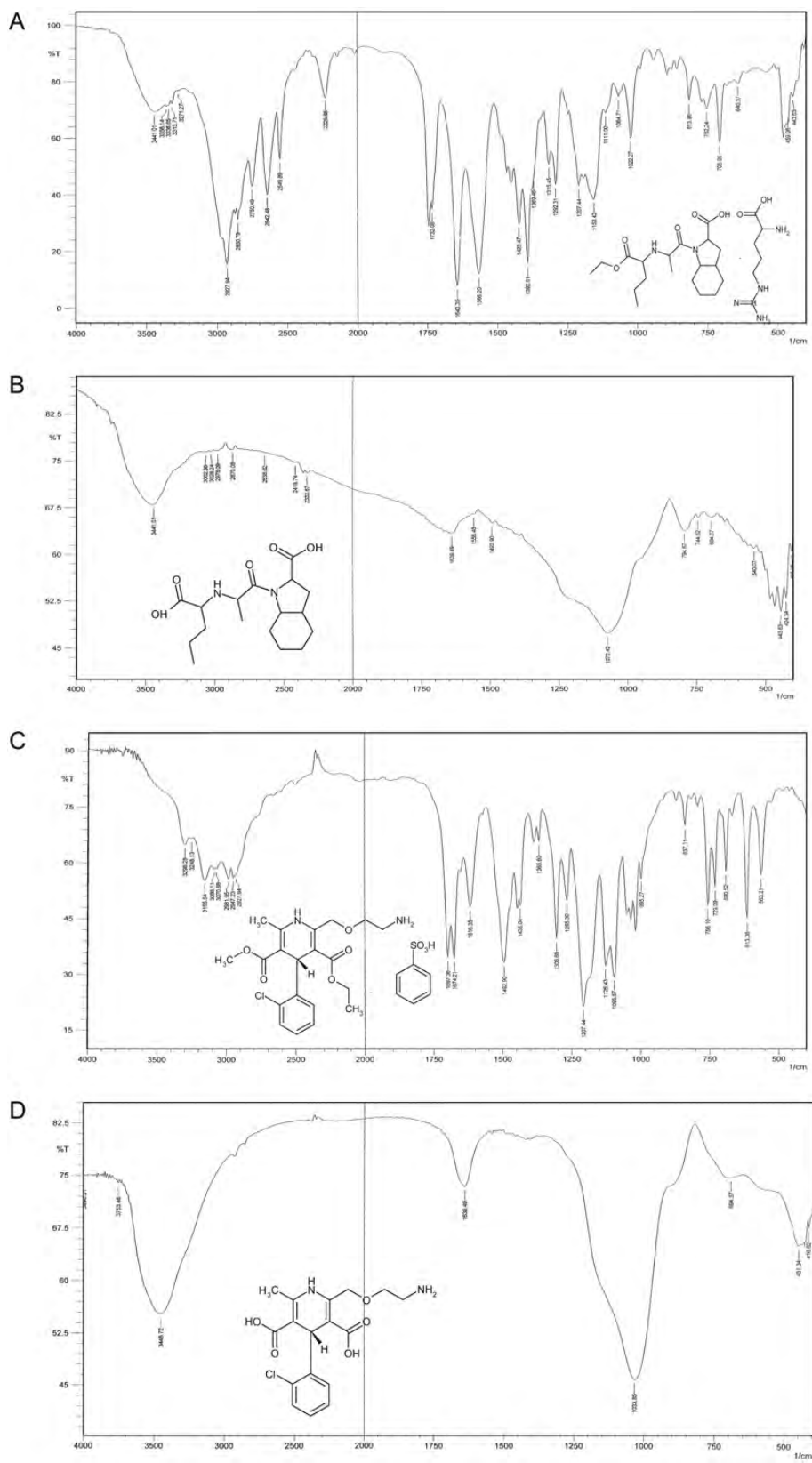


Figure 2. IR spectra: perindopril arginin (A); perindoprilate (B); amlodipine besylate (C); Deg 1 (D).

shelf-life determination are the assay of the active drug and the degradation product generated during the stability study. The assay of drug products in stability test samples needs to be determined using a stability-indicating method, as recommended by the International Conference on Harmonization (ICH) guidelines (43) and USP (3). The published methods of analysis for PER and AML in formulations have not been validated for specificity and degradation studies (41, 42). Therefore, this work, which describes stability-indicating methods for the selective determination of PER and AML in the presence of their degradation products and formulation excipients, is considered to be a prime requirement. The two drugs were subjected to alkaline forced degradation studies, which are considered to be the least drastic conditions for the degradation of both PER and AML (6, 44). The two aforementioned drugs were hydrolyzed through cleavage of ester linkage, giving the corresponding acid and alcohol. PER was hydrolyzed into the diacid active form (perindoprilate), which decreases the bioavailability of the drug from 65 to 25% due to its poor absorption from gastrointestinal tract (GIT). AML was hydrolyzed into a pharmacologically inactive degradation product (Deg 1). The suggested pathways for degradation process are shown in Figure 1.

The structures of alkaline degradation products were elucidated by IR and MS, in which the IR spectrum of PER arginin showed a characteristic peak at $1,732.08\text{ cm}^{-1}$ due to presence of $\text{C}=\text{O}^-$ of ester linkage, while its degradation product showed peaks at $1,639.49$ and $3,441.01\text{ cm}^{-1}$ due to presence of carboxylic $\text{C}=\text{O}$ and OH , respectively, indicating the cleavage of the ester link into the corresponding acid and alcohol (Figures 2A and 2B). The same changes were observed in the IR spectrum of AML besylate, as the $\text{C}=\text{O}^-$ of the ester appeared at $1,697.36\text{ cm}^{-1}$ in the AML spectrum and disap-

peared in its degradation product spectrum with the appearance of two characteristic peaks at $1,639.49$ and $3,448.72\text{ cm}^{-1}$ of carboxylic $\text{C}=\text{O}$ and OH , respectively (Figures 2C and 2D). Moreover, mass spectra of the alkaline degradation products confirm these claims, in which the parent peak for each degradation product coincided with its molecular weight and the other principle peaks correspond to their fragmentation (Figures 3A and 3B).

Because of the importance of separating multiple components during the analysis of stability samples, chromatographic methods have taken precedence over the conventional methods of analysis. Other than the separation of multiple components, the advantage of chromatographic methods is that these possess greater accuracy and sensitivity, even for small quantities of degradation products (45).

TLC–densitometry

A sensitive, stability-indicating TLC method was suggested for the determination of PER and AML in the presence of their degradation products. The method was based on the difference in the migration rates of the four compounds [PER retention factor (R_f) = 0.27, AML R_f = 0.68, perindoprilate R_f = 0.08 and Deg 1 R_f = 0.04] using 33% ethyl acetate–methanol–toluene–ammonia solution (6.5:2:1:0.5 by volume) as a developing system, with a total time of 20 min for the analysis of each run. It was necessary to test the effects of different variables to optimize this method. Different developing systems of different composition and ratios were tried for separation, e.g., methanol–ethyl acetate (8:2, v/v), chloroform–methanol–ethyl acetate (2:2:6 by volume) and 33% chloroform–methanol–ethyl acetate–ammonia solution (1:2: 6.5: 0.5 by volume). The

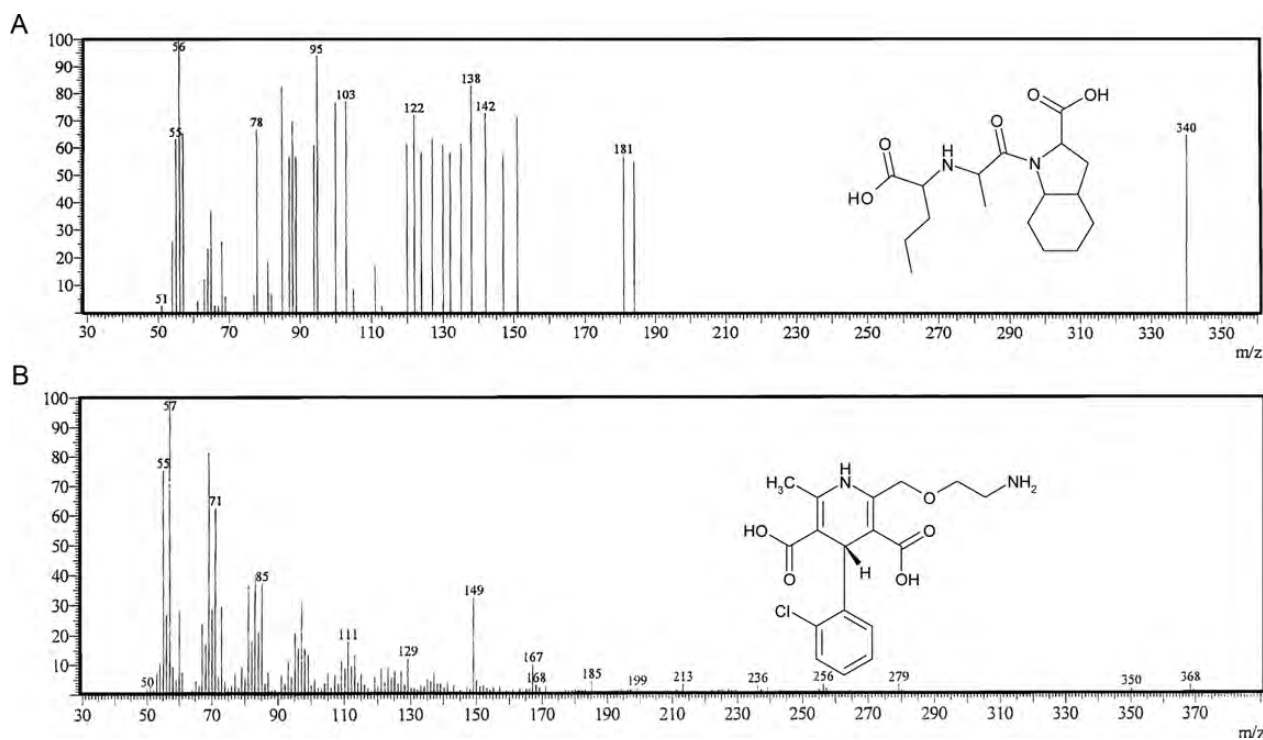


Figure 3. Mass spectra: perindoprilate (A); Deg 1 (B).

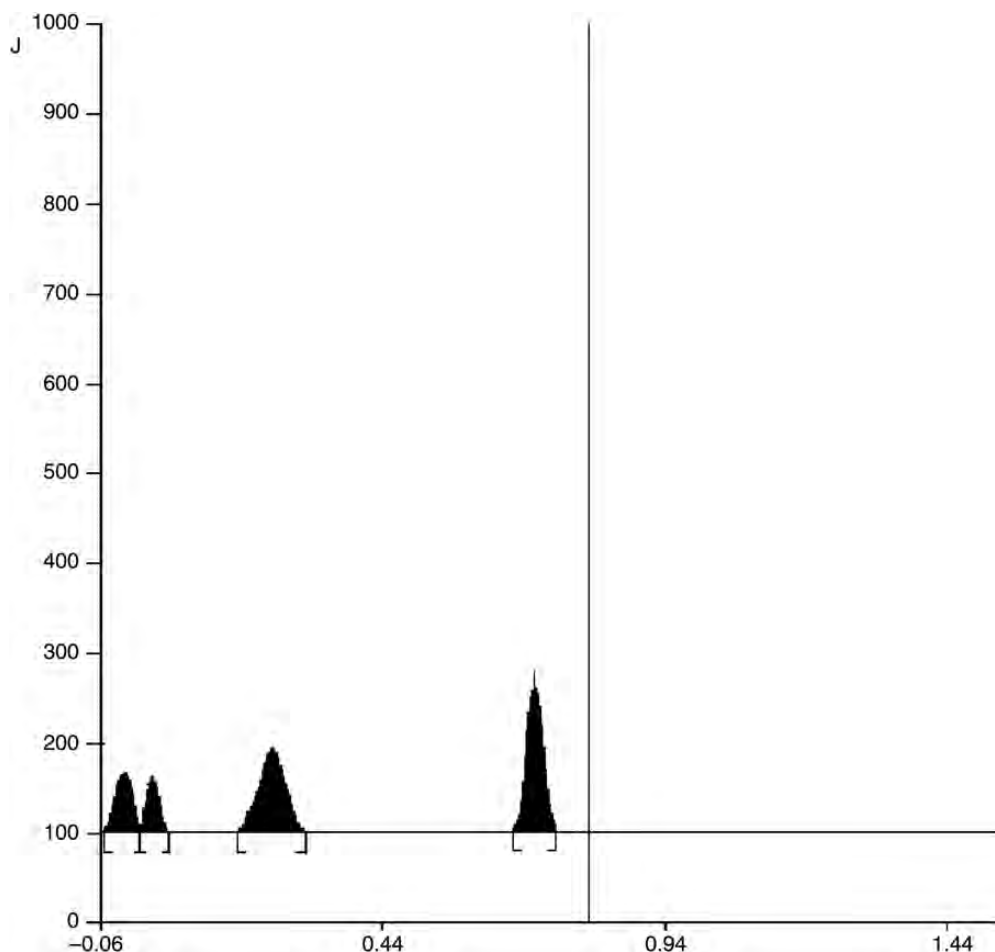


Figure 4. TLC chromatograms of PER ($R_f = 0.27$), AML ($R_f = 0.68$), perindoprilate ($R_f = 0.04$) and Deg 1 ($R_f = 0.08$) using ethyl acetate–methanol–toluene–ammonia solution (6.5:2:1:0.5, by volume) as a mobile phase.

Table I
Statistical Analysis of Parameters Required for System Suitability Testing of Chromatographic Methods

Parameters		Deg 1	Perindoprilate	PER	AML
HPLC	Resolution (R)	1.58	2.09		2.19
	Selectivity (α)	1.13	1.20		1.12
	Tailing factor (T)	1.20	0.9	0.9	1.08
	Capacity factor (K')	2.13	1.85	3.82	6.03
	Column efficiency (N)	7027	4776	5113	4967
	HETP*	3.56×10^{-3}	5.23×10^{-3}	4.90×10^{-3}	5.03×10^{-3}
TLC	Resolution (R)	0.55	5.41		3.00
	Selectivity (α)	2.43	4.51		14.38
	Tailing factor (T)	1.00	1.06	1.00	0.92
	Capacity factor (K')	1.87	0.13	8.44	20.50

*Height equivalent to theoretical plate (cm/plate).

best mobile phase was 33% ethyl acetate–methanol–toluene–ammonia solution (6.5:2:1:0.5 by volume). This selected mobile phase allows the determination of PER and AML without interference from their degradation products and without tailing of the separated bands (Figure 4).

Different band dimensions were tested to obtain sharp and symmetrical separated peaks. The optimum bandwidth chosen was 4 mm and the interspace between bands was 14 mm. Different scanning wavelengths were tested: scanning at

218 nm was suitable, providing good sensitivity for PER, but distorted peaks for AML, so 237 nm was the wavelength chosen for AML quantitation, because the peaks were more sharp and symmetrical with minimum noise. Also, the slit dimensions of the scanning light beam should ensure complete coverage of band dimensions on the scanned track without interference of adjacent bands. Different slit dimensions were tried, and 5×0.2 mm proved to be the slit dimension of choice, which provided the highest sensitivity.

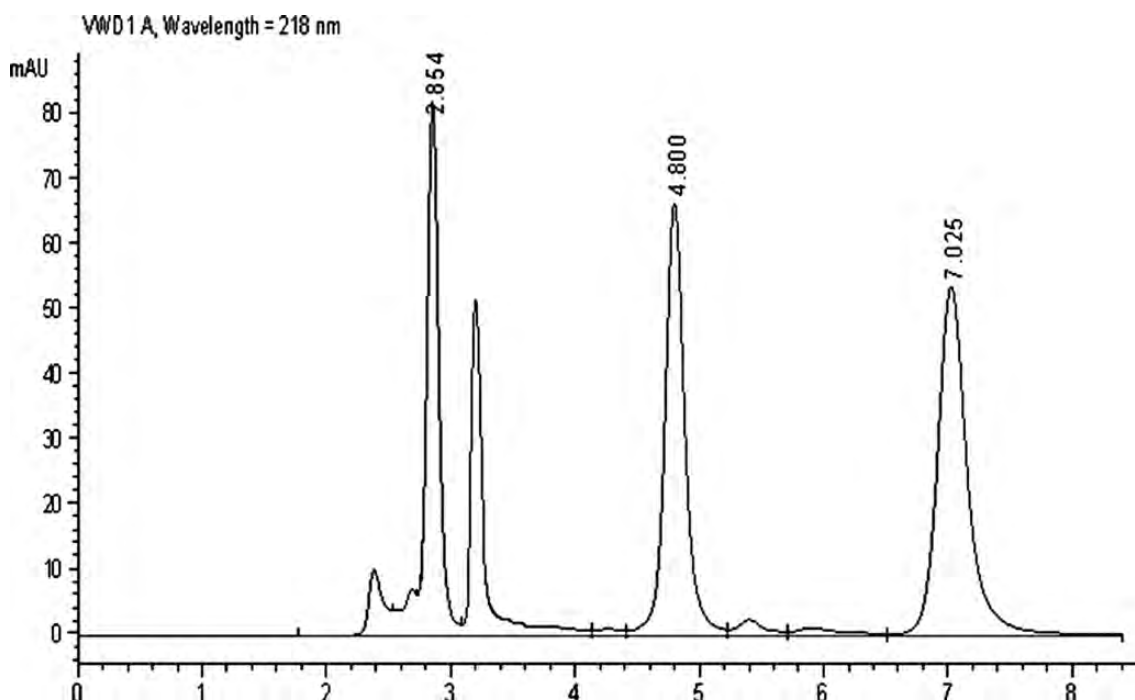


Figure 5. Liquid chromatographic separation of PER (4.80 ± 0.2 min.), AML (7.025 ± 0.2 min.), perindoprilate (3.125 ± 0.2 min.) and Deg 1 (2.854 ± 0.2 min.) using phosphate buffer (pH 2.5, 0.01 M)–acetonitrile–THF (60:40:0.1%, by volume) as mobile phase.

Table II
Analytical Parameters and Validation Results of the Determination of PER and AML by the Proposed Methods

Method parameter	TLC method		HPLC method	
	PER	AML	PER	AML
Wavelength (nm)	218	237	218	218
Linearity range	4–28 $\mu\text{g}/\text{band}$	2–8 $\mu\text{g}/\text{band}$	10–100 $\mu\text{g}/\text{mL}$	5–45 $\mu\text{g}/\text{mL}$
Time of analysis (min/run)	20	7.5		
Regression equation ($A = bC + a$)*				
Intercept (a)	0.1635	-0.1023	0.1901	0.0239
Slope (b)	0.0328	0.2087	0.0160	0.0324
Correlation coefficient (r)	1.0000	0.9999	0.9999	0.9999
Accuracy (mean \pm RSD)				
80%	100.05 \pm 0.53	100.52 \pm 0.44	99.68 \pm 0.41	99.56 \pm 0.42
100%	100.86 \pm 0.62	101.06 \pm 0.59	100.25 \pm 0.54	100.66 \pm 0.69
120%	98.66 \pm 0.47	100.89 \pm 0.67	99.77 \pm 0.32	99.32 \pm 0.84
Specificity and selectivity [†]	100.30 \pm 0.62	99.68 \pm 0.91	99.86 \pm 1.12	100.05 \pm 0.96
Precision				
RSD [‡]	\pm 0.66	\pm 0.53	\pm 0.64	\pm 0.47
RSD [§]	\pm 0.94	\pm 1.04	\pm 1.00	\pm 0.96
Robustness	100.85 \pm 1.10	100.32 \pm 0.98	99.47 \pm 0.81	100.07 \pm 0.99
LOD	0.14 $\mu\text{g}/\text{band}$	0.09 $\mu\text{g}/\text{band}$	0.92 $\mu\text{g}/\text{mL}$	0.58 $\mu\text{g}/\text{mL}$
LOQ	0.41 $\mu\text{g}/\text{band}$	0.26 $\mu\text{g}/\text{band}$	2.78 $\mu\text{g}/\text{mL}$	1.77 $\mu\text{g}/\text{mL}$

*A is the analytical signal and C is the concentration.

[†]Recovery of PER and AML in laboratory-prepared mixtures containing different ratios of the degradation products.

[‡]Intra-day precision [average of three different concentrations of three replicates each ($n = 9$) within the same day].

[§]Inter-day precision [average of three different concentrations of three replicates each ($n = 9$) repeated on three successive days].

Two-dimensional chromatography was applied to calculate system suitability parameters, including resolution (R_s), peak symmetry, capacity factor (k') and selectivity factor (α). For PER and AML, the R_s is always above two, the selectivity more than one and an accepted value for symmetry factor was obtained, as shown in Table I (3).

RP-HPLC method

A simple, accurate, and selective RP-HPLC method was investigated and validated for quantitative analysis of PER and AML. The LC procedure was optimized with a view to develop a quantitative and stability-indicating method in a convenient time and with high-quality separation of the four proposed

components. The chromatographic operational conditions were selected by considering the peak resolution and retention times of the first and last eluted components. Parameters affecting the efficiency of the chromatographic separation were tested and optimized in a trial to obtain the maximum separation of the cited components. In the first attempt, an isocratic mobile phase of phosphate buffer (pH 3, 0.01 M)–acetonitrile (70:30, v/v) was used to separate the four compounds; unfortunately, the degradation products of PER and AML co-eluted as one peak without separation. Therefore, several systems were tried by the addition of different surface active agents, tetrahydrofuran, triethylamine and combinations of different mobile phases. The best resolution was achieved when using a mobile phase consisting of K phosphate buffer (pH 2.5, 0.01 M)–acetonitrile–tetrahydrofuran (60:40:0.1% by volume). The pH of the mobile phase has a great effect on the peak shapes of the separated components. Variation of pH of the mobile phase resulted in a k' value where at $3.5 < \text{pH} < 2$, loss of peak symmetry was obtained.

Scanning the effluent at 218 nm showed reasonable sensitivity for the active drugs. Reasonable separation with good resolution and suitable analysis time was obtained upon using a flow rate of 1 mL/min. Under the optimum chromatographic conditions, PER, AML, perindoprilate and Deg 1 were eluted at 4.8, 7.025, 3.125 and 2.85 min \pm 0.2, respectively, as shown in Figure 5. To validate the suggested chromatographic method, an overall system suitability test was conducted to determine whether the operating systems were performing properly (Table I) (3).

Table III

Determination of PER and AML in the Presence of their Alkaline Degradation Products in Laboratory Prepared Mixtures by the Proposed Methods

Degradation products	Ratio of PER:AML	TLC method		HPLC method	
		PER	AML	PER	AML
5%	1: 1	100.92	100.36	100.11	100.48
25%	1:2	100.96	100.17	99.64	101.18
50%	2:1	100.17	100.49	98.05	98.79
75%	1.5:1	99.61	98.73	100.92	100.46
90%	2.5:1	99.84	98.66	100.57	99.34
Mean \pm RSD		100.30 \pm 0.62	99.68 \pm 0.91	99.86 \pm 1.12	100.05 \pm 0.96

Table IV

Determination of PER and AML in Tablets by the Proposed and Manufacturer Methods

Pharmaceutical formulations	PER			AML		
	TLC	HPLC	Manufacturer method	TLC	HPLC	Manufacturer method
Coveram tablets, 5(PER)/5(AML) mg, batch 77597	99.13 \pm 0.74	100.55 \pm 0.47	100.06 \pm 0.77	100.61 \pm 0.62	99.65 \pm 1.17	99.95 \pm 0.58
F-value (5.05)	1.11	2.68	—	1.12	4.03	—
Student's <i>t</i> -test (2.228)	2.153	1.332	—	1.902	0.561	—
Coveram tablets, 10(PER)/10(AML)mg, batch 376112	99.18 \pm 0.89	99.46 \pm 1.08	100.66 \pm 0.80	99.25 \pm 0.71	98.22 \pm 0.70	99.09 \pm 0.79
F-value (5.05)	1.20	1.78	—	1.24	1.27	—
Student's <i>t</i> -test (2.228)	1.762	1.200	—	0.373	2.037	—
Coveram tablets, 5(PER)/10(AML) mg, batch 73935	100.44 \pm 0.75	100.08 \pm 0.42	100.19 \pm 0.70	101.48 \pm 0.87	100.59 \pm 0.58	100.82 \pm 0.49
F-value (5.05)	1.14	2.72	—	3.21	1.42	—
Student's <i>t</i> -test (2.228)	0.597	0.328	—	1.606	0.737	—
Coveram tablets, 10(PER)/5(AML) mg, batch 59423	100.50 \pm 0.93	99.04 \pm 0.62	99.61 \pm 0.55	100.25 \pm 0.92	99.48 \pm 0.59	99.50 \pm 0.71
F-value (5.05)	2.87	1.23	—	1.70	1.43	—
Student's <i>t</i> -test (2.228)	2.023	1.701	—	1.579	0.053	—

Method Validation

ICH guidelines for method validation (43) were followed for validation of the suggested methods.

Linearity and ranges

Under the previously described experimental conditions, linear relationships were obtained by plotting the drug concentrations against integrated peak areas or relative peak areas for each drug, for TLC and HPLC, respectively. The corresponding concentration ranges, calibration equations, limit of detection (LOD) and limit of quantification (LOQ) and other statistical parameters are listed in Table II.

Accuracy

The accuracy of the investigated methods was validated by analyzing pure samples of both PER and AML at three stages of the assay amount: 80, 100 and 120%. The concentrations of the active drugs were calculated from the calculated regression equations. Good results are shown in Table II.

Precision

Precision was evaluated by calculating intra-day and inter-day precision by repeating the assay of three different concentrations at three levels, 50, 100 and 150%, three times in the same day. The same samples were assayed in triplicate on three successive days, using the developed chromatographic methods and calculating the recovery percentage and relative standard deviation (RSD). The results in Table II indicate satisfactory precision of the proposed methods.

Specificity

Specificity was ascertained by analyzing different mixtures containing the four proposed components in different ratios. The bands of the active drugs in the prepared mixtures were confirmed by comparing their R_f values with those of a standard solution (in the TLC method). Table III illustrates that the proposed chromatographic methods showed good selectivity in the determination of PER and AML in the presence of different ratios of their degradation products.

Robustness

To determine the robustness of the developed LTC and HPLC methods, experimental conditions were deliberately altered,

Table V

Statistical Comparison between Proposed, Manufacturer and Official Methods for the Determination of PER and AML in Pure Powder Form*

Item	Perindopril			Amlodipine		
	TLC method	HPLC method	Manufacturer method [†]	TLC method	HPLC method	Official method [‡]
Mean \pm RSD	99.87 \pm 0.34	100.20 \pm 0.62	99.68 \pm 0.69	100.09 \pm 0.78	99.83 \pm 0.87	100.14 \pm 1.14
<i>n</i>	7	7	6	7	7	6
Variance	0.12	0.38	0.48	0.61	0.76	1.30
<i>F</i> -value (4.39)	4.00	1.26			2.13	1.71
Student's <i>t</i> -test (2.201)	0.633	1.405		0.093	0.310	

*Note: The figures in parentheses are the corresponding tabulated values at $P = 0.05$.[†]Manufacturer method: HPLC using a C18 column and a mobile phase consisting of (66:34, v/v) acetonitrile–aqueous phase (water + 0.3% triethylamine adjusted to pH 2.5 with 35% perchloric acid) and UV detection at 215 nm.[‡]Official method: HPLC method using a C18 column, 2.3 g/L ammonium acetate–methanol (30:70, v/v) as a mobile phase and UV detection at 237 nm.**Table VI**

Results of ANOVA to Compare the Proposed Methods for the Determination of PER and AML in Pure Powder Forms

Source of variation		Sum of squares	Degrees of freedom	Mean square	F	P- value	d_{Crit}^*
Perindopril	Between groups	0.346992	1	0.346992	1.369303	0.264647	4.747225
	Within groups	3.040897	12	0.253408			
Amlodipine	Between groups	0.210503	1	0.210503	0.297666	0.595346	4.747225
	Within groups	8.486132	12	0.707178			

* d_{Crit} = critical corresponding to theoretical value.

then tailing factor and the resolution between the studied drugs were recorded. Small changes were allowed in the ethyl acetate–toluene ratio (6.5:1 to 6:1.5), travelled distance (8 to 13 cm) the pH (2.5 ± 0.3), percent of modifier ($0.1 \pm 0.05\%$ THF) and buffer–acetonitrile ratio (from 60:40 to 65:35) in the mobile phase. While studying each factor, other factors were held constant, as mentioned previously.

Application to commercial tablets

The suggested methods were successfully applied for the determination of both drugs in Coveram tablets. The results, shown in Table IV, were satisfactory and agreed with the labeled amounts. Statistical comparison of the results obtained by the proposed techniques and those of the manufacturer and official methods proved the validity of the methods, and proved that excipients do not adversely affect the results, as shown in Table IV.

Results obtained by the proposed methods for the determination of pure samples of PER and AML were statistically compared to those obtained by the manufacturer's HPLC method [using a C18 column and mobile phase consisting of (66: 34, v/v) acetonitrile–aqueous phase (water + 0.3% triethylamine adjusted to pH 2.5 with 35% perchloric acid)] and the official method (4) of both drugs, respectively; they were also compared to each other using one-way analysis of variance (ANOVA) test and no significant difference was observed (Tables V and VI).

Conclusion

Perindopril arginin and amlodipine besylate are co-formulated in antihypertensive formulations, and because they are widely

used drugs, it is important to find simple, rapid and inexpensive methods for their analysis, especially in quality control laboratories. The suggested chromatographic methods provide simple, accurate and reproducible stability-indicating methods for their quantitative analysis in the presence of their degradation products. The developed TLC method is sensitive and has the advantages of short time for analysis of different concentrations in one run, large sample capacity and use of a minimal volume of solvents. The HPLC method offers good resolution between the four proposed components within a suitable analysis time. It is highly specific, but more expensive. The proposed methods have advantages over other published methods for analyzing the binary mixture in the presence of their degradation products. Therefore, the applied methods may be useful for stability investigation of the active drugs and for checking the extent of degradation in pharmaceutical formulations.

References

- Sweetman, S.C. (ed.). Martindale: The complete drug reference, 36th edition. Pharmaceutical Press, London, UK, (2009).
- Goodman, A.G., Gilman, L.S.; The pharmacological basis of therapeutics, 8th edition. Gilman, A. G., Rall, T. W., Nies, A.S., Taylor, P. (eds.), Pergamon Press, Oxford, UK, (1990).
- United States Pharmacopeia, 30th edition. U.S. Pharmacopeial Convention Rockville, MD, (2007).
- Telejko, E.; Perindopril arginine: Benefits of a new salt of the ACE inhibitor perindopril; *Current Medical Research and Opinion*, (2007); 23: 953–960.
- British Pharmacopoeia, Volume II, The Stationery Office on Behalf of the MHRA, London, UK, (2010), pp. 138.
- Csermely, T., Kalasz, H., Deak, K., Hasan, M.Y., Darvas, F., Petroianu, G.; Lipophilicity determination of some ACE inhibitors by TLC; *Journal of Liquid Chromatography and Related Technologies*, (2008); 31: 2019–2034.

7. Kowalczyk, D., Pietras, R., Hopkala, H.; Development and validation of an HPTLC-densitometric method for determination of ACE inhibitors; *Chromatographia*, (2004); 60: 245–249.
8. Czerwinska, K., Wyszomirska, E., Kaniewska, T.; Identification and determination of selected medicines reducing hypertension by densitometric and gas-chromatographic methods; *Acta Poloniae Pharmaceutica*, (2001); 58: 331–338.
9. Nirogi, R.V.S., Kandikere, V.N., Shukla, M., Mudigonda, K., Maurya, S., Komarneni, P.; High-throughput quantification of perindopril in human plasma by liquid chromatography/ tandem mass spectrometry: application to a bioequivalence study; *Rapid Communications in Mass Spectrometry*, (2006); 20: 1864–1870.
10. Medenica, M., Ivanovic, D., Maskovic, M., Jancic, B., Malenovic, A.; Evaluation of impurities level of perindopril tert-butylamine in tablets; *Journal of Pharmaceutical and Biomedical Analysis*, (2007); 44: 1087–1094.
11. Elshanawane, A.A., Mostafa, S.M., Elgawish, M.S.; Development and validation of LC method for simultaneous determination of two binary mixtures containing indapamide; *Chromatographia*, (2008); 67: 837–840.
12. Jain, D.S., Subbaiah, G., Sanyal, M., Pande, U.C., Shrivastav, P.; First LC-MS/MS electrospray ionization validated method for the quantification of perindopril and its metabolite perindoprilat in human plasma and its application to bioequivalence study; *Journal of Chromatography B*, (2006); 837: 92–100.
13. Lin, S.J., Wu, H.L., Chen, S.H., Wen, Y.H.; Derivatization–gas-chromatographic determination of perindopril; *Analytical Letters*, (1996); 29: 1751–1762.
14. Sereida, K.M., Hardman, T.C., Dilloway, M.R., Lant, A.F.; Development of a method for the detection of angiotensin-converting enzyme [peptidyl-dipeptidase A] inhibitors using electron-capture gas chromatography detection; *Analytical Proceedings*, (1993); 30: 371–372.
15. Tsacostas, C., Devissaguet, M., Padieu, P.; Gas chromatography–mass spectrometry of perindopril and its active free metabolite, an angiotensin convertase inhibitor: pChoice of derivatives and ionization modes; *Journal of Chromatographic and Biomedical Applications*, (1989); 80: 249–265.
16. Stefan, R., van Staden, J.F., Aboul-Enein, H.Y.; S-Perindopril assay using a potentiometric, enantioselective membrane electrode; *Chirality*, (1999); 11: 631–634.
17. Ozoemena, K.I., Stefan, R.-I., van Staden, J.F., Aboul-Enein, H.Y.; Utilization of maltodextrin based enantioselective, potentiometric membrane electrodes for the enantioselective assay of S-perindopril; *Talanta*, (2004); 62: 681–685.
18. Ozoemena, K.I., Stefan, R.-I., van Staden, J.F., Aboul-Enein, H.Y.; Enantioselective, potentiometric membrane electrode, based on vancomycin as chiral selector, for the assay of S-perindopril; *Instrumentation Science and Technology*, (2004); 32: 371–378.
19. Hillaert, S., Vander Heyden, Y., van den Bossche, W.; Optimization by experimental design of a capillary electrophoretic method for the separation of several inhibitors of angiotensin-converting enzyme using alkylsulfonates; *Chromatographia*, (2002); 978: 231–242.
20. Hillaert, S., van den Bossche, W.; The quantitative determination of several inhibitors of the angiotensin-converting enzyme by capillary electrophoresis (CE); *Journal of Pharmaceutical and Biomedical Analysis*, (2001); 25: 775–783.
21. Hillaert, S., van den Bossche, W.; Optimization of capillary electrophoretic separation of several inhibitors of the angiotensin-converting enzyme; *Chromatographia*, (2000); 895: 33–42.
22. van Staden, J.F., Stefan, R.-I., Aboul-Enein, H.Y.; Amperometric biosensor based on D-amino-acid oxidase for the R-perindopril assay; *Fresenius' Journal of Analytical Chemistry*, (2000); 367: 178–180.
23. Stefan, R.-I., van Staden, J.K.F., Aboul-Enein, H.Y.; Determination of S-perindopril using a flow-injection system with an amperometric biosensor; *Sensors and Actuators B: Chemical B*, (1999); 54: 261–265.
24. Stefan, R.-I., van Staden, J.F., Mulaudzi, L.V., Aboul-Enein, H.Y.; On-line simultaneous determination of S- and R-perindopril using amperometric biosensors as detectors in flow systems; *Analytica Chimica Acta*, (2002); 467: 189–195.
25. Erk, N.; Comparison of spectrophotometric and an LC method for the determination of perindopril and indapamide in pharmaceutical formulations; *Journal of Pharmaceutical and Biomedical Analysis*, (2001); 26: 43–52.
26. Abdellatif, H.E., Ayad, M.M., Taha, E.A.; Spectrophotometric and atomic absorption spectrometric determination of ramipril and perindopril through ternary complex formation with eosin and copper(II) ions; *Journal of Pharmaceutical and Biomedical Analysis*, (1999); 18: 1021–1027.
27. Van den Berg, H., Resplandy, G., De Bie, A.T.H.J., Floor, W., Bertrand, M., Arts, C.J.M.; New radio-immunoassay for the determination of the angiotensin-converting enzyme inhibitor perindopril and its active metabolite in plasma and urine: padvantages of a lysine derivative as immunogen to improve the assay specificity; *Journal of Pharmaceutical and Biomedical Analysis*, (1991); 9: 517–524.
28. Ramadan, N.K., Mohamed, H.M., Moustafa, A.A.; Rapid and highly sensitive HPLC and TLC methods for quantitation of amlodipine besilate and valsartan in bulk powder and in pharmaceutical dosage forms and in human plasma; *Analytical Letters*, (2010); 43: 570–581.
29. Kamble, A.Y., Mahadik, M.V., Khatal, L., Dhaneshwar, S.R.; Validated HPLC and HPTLC method for simultaneous quantitation of amlodipine besylate and olmesartan medoxomil in bulk drug and formulation; *Analytical Letters*, (2010); 43: 251–258.
30. Lu, D.Q., Dang, A.W., Wang, J., Ling, X.Q., Li, Y., Jia, J., et al.; NP-HPLC separation, determination of amlodipine enantiomers; *Fenxi Cesbi Xuebao*, (2009); 28: 1291–1294.
31. Kakde, R., Bawane, N.; High-performance thin-layer chromatographic method for simultaneous analysis of metoprolol succinate and amlodipine besylate in pharmaceutical preparations; *Journal of Planar Chromatography—Modern TLC*, (2009); 22: 115–119.
32. Dhaneshwar, S.R., Patre, N.G., Mahadik, M.V.; Validated TLC method for simultaneous quantitation of amlodipine besylate and valsartan in bulk drug and formulation; *Chromatographia*, (2009); 69: 157–161.
33. Meyyanathan, S.N., Suresh, B.; HPTLC method for the simultaneous determination of amlodipine and benazepril in their formulations; *Journal of Chromatographic Science*, (2005); 43: 73–75.
34. Quaglia, M.G., Barbato, F., Fanali, S., Santucci, E., Donati, E., Carafa, M., et al.; Direct determination by capillary electrophoresis of cardiovascular drugs previously included in liposomes; *Journal of Pharmaceutical and Biomedical Analysis*, (2005); 37: 73–79.
35. Wang, R., Jia, Z.-P., Fan, J.-J., Chen, L.-R., Xie, H., Ma, J., et al.; CE, with hydroxypropyl- β -cyclodextrin as chiral selector, for separation and determination of the enantiomers of amlodipine in the serum of hypertension patients; *Chromatographia*, (2007); 65: 575–579.
36. Jankovics, P., Nemeth, T., Nemeth-Palotas, J., Koszegi-Szalai, H.; Amlodipine besilate screening in pharmaceutical preparations by CE; *Chromatographia*, (2008); 68: S43–S48.
37. Kesting, J.R., Huang, J.Q., Sorensen, D.; Identification of adulterants in a Chinese herbal medicine by LC-HRMS and LC-MS-SPE/NMR and comparative in vivo study with standards in a hypertensive rat model; *Journal of Pharmaceutical and Biomedical Analysis*, (2010); 51: 705–711.
38. Pereira, A.S., Bicalho, B., Ilha, J.O., de Nucci, G.; Analysis of dihydropyridine calcium channel blockers using negative ion photoionization mass spectrometry; *Journal of Chromatographic Science*, (2008); 46: 35–41.
39. Dogan-Topal, B., Bozal, B., Demircigil, B.T., Uslu, B., Ozkan, S.A.; Electroanalytical studies and simultaneous determination of amlodipine besylate and atorvastatin calcium in binary mixtures using first derivative of the ratio-voltammetric methods; *Electroanalysis*, (2009); 21: 2427–2439.
40. Kazempour, M., Ansari, M., Mohammadi, A., Beitollahi, H., Ahmadi, R.; Use of adsorptive square-wave anodic stripping voltammetry at carbon paste electrode for the determination of amlodipine

- besylate in pharmaceutical preparations; *Journal of Analytical Chemistry*; (2009); 64: 65–70.
41. Prajapati, J., Patel, A., Patel, M.B., Prajapati, N., Prajapati, R.; Analytical method development and validation of Amlodipine besylate and Perindopril erbumine in combined dosage form by RP-HPLC; *International Journal of PharmTech Research*, (2011); 3: 801–808.
42. Prajapati, J., Patel, M.B., Prajapati, R.J., Prajapati, N.A.; Simultaneous determination of perindopril erbumine and amlodipine besylate by absorption factor method; *International Journal of Applied Biology and Pharmaceutical Technology*; (2011); 2: 230–233.
43. ICH, Q1A (R2), Stability testing of new drug substances and products. International Conference on Harmonization, IFPMA, Geneva, Switzerland, (2003).
44. Naidu, K.R., Kale, U.N., Shingare, M.S.; Stability indicating RP-HPLC method for simultaneous determination of amlodipine and benazepril hydrochloride from their combination drug product; *Journal of Pharmaceutical and Biomedical Analysis*, (2005); 39: 147–155.
45. Bakshi, M., Singh, S.; Development of validated stability-indicating assay methods—Critical review; *Journal of Pharmaceutical and Biomedical Analysis*, (2002); 28: 1011–1040.