ORIGINAL ARTICLE

SIMULTANEOUS CHIRAL SEPARATION OF PERINDOPRIL ERBUMINE AND INDAPAMIDE ENANTIOMERS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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Abstract

Simultaneous enantioseparation of perindopril tert-butylamine (PER) and indapamide (IMP) enantiomers was developed successfully by high performance liquid chromatography using ovomucoid as chiral selector. The chiral separation was performed on an Agilent 1100 Series HPLC system (Agilent Technologies, USA) equipped with UV-VIS detector using an Ultron ES OVM, 150 x 4.6 mm, 5 µm (Shinwa Chemical Industries LTD, Agilent Technologies) column. The effects of organic modifier, the buffer pHs, the column temperature and the injection volume have been investigated considering resolution and selectivity. The analytical results of the proposed method showed that optimal experimental conditions were: a mobile phase consisting of 93% KH_2PO_4 20 mM pH 3.75 and 7% acetonitrile, under an isocratic elution (flow rate = 1 mL/min) with an injection volume of 5 µL and a temperature of 25°C in a less than 15 minutes analysis time. The method was validated for linearity, limits of detection (LOQ) and quantification (LOD), recovery and precision of retention time and peak-area response. The linearity of the method has been established in the range of 20 - 200 µg/mL for PER and 6.25 - 62.5 µg/mL for IMP respectively. Good results were obtained from the analysis of tablets indicating that the proposed method is suitable for the simultaneous analysis of PER and IMP in pharmaceutical fixed-dose combinationS.

Rezumat

Enantioseparareа simultană a perindoprilului terţ-butilamină (PER) şi a indapamidului (IMP) a fost realizată cu succes, folosind ovomucoidul drept selector chiral, prin cromatografie de lichide de înaltă performanţă. Separarea chirală a fost efectuată folosind un sistem HPLC Agilent 1100 Series (Agilent Technologies, USA) dotat cu un detector UV-VIS folosind o coloană chirală de tip Ultron ES OVM, 150 x 4,6 mm, 5 µm (Shinwa Chemical Industries LTD, Agilent Technologies). Au fost investigate efectele modificatorului organic, a pH-ului componentei apose a fazei mobile, a temperaturii de eluţie, a volumului probei injectate asupra rezoluţiei şi selectivităţii. Condiţiile experimentale optime ale metodei dezvoltate s-au dovedit a fi: o fază mobilă formată din 93% KH_2PO_4 20 mM pH 3,75 şi 7% acetonitril, eluţie izocratică (debit = 1 mL/min), un volum de injectare de 5 µL şi o temperatură de eluţie de 25°C într-un timp de analiză mai scurt de 15 minute. Metoda dezvoltată a fost validată stabilindu-se domeniul de liniaritate, limita de detecţie (LOQ) şi cea de cuantificare (LOD), regăsirea, respectiv precizia, luând în considerare atât timpul de retenţie al analiţilor, cât şi aria acestora. Liniaritatea metodei a fost stabilită în domeniul 20 - 200 µg/mL pentru PER respectiv 6.25 - 62.5 µg/mL pentru IMP. Rezultatele obţinute la analiza compuşilor analizaţi din tablete ne indică faptul că metoda propusă este adecvată pentru analiza simultană a PER şi IMP ca ingrediente farmaceutice active în combinaţiile fixe existente.

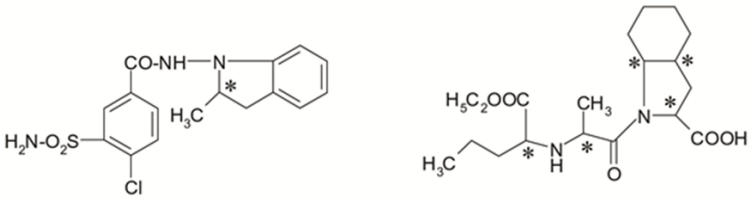
Keywords: perindopril, indapamide, chiral separation, ovomucoid, HPLC

Introduction

It is well known that the stereochemistry of the drugs influences the interaction with the receptors and also the metabolic biotransformation pathway of the drug. The problem involving chiral separations for pharmaceutical compounds is extremely important for the pharmaceutical industry since it is recognized that the enantiomers of chiral compounds may exhibit different pharmacological, toxicological and pharmacokinetic properties [1, 10]. Usually, the desired pharmacological effect is often restricted to one of the enantiomers, called eutomer, while the other enantiomer, called distomer, can have an increased or diminished pharmacological activity or sometimes be responsible for the adverse effects [18].
Fixed-dose combinations are nowadays used routinely for the present day treatment of high blood-pressure. One of these combinations may consist in the association of an angiotensin converting enzyme (ACE) inhibitor (perindopril) and a thiazide-like diuretic (indapamide), together being responsible of a synergistic antihypertensive pharmacological

effect [14]. In the present study, a new simple and direct chiral HPLC method was developed and validated for the simultaneous enantiomeric separation of perindopril erbumine (2S,3aS,7aS)-1-[(2S)-2-[[(2S)-1-ethoxy-1-oxopentan-2-yl]amino]propanoyl]-2,3,3a,4,5,6,7,7a-octahydroindole-2-carboxylic acid; 2-methylpropan-2-amine and indapamide (4-chloro-N-(2-methyl-2,3-dihydroindol-1-yl)-3-sulfamoylbenzamide).

The chemical structures of the two studied substances are presented in Figure 1.



Indapamide Perindopril

Figure 1.
Chemical structures of the two analytes (*Asymmetric carbon atoms)

In the published literature, there are various analytical methods approaches dealing with the problems of chiral separation of a single active pharmaceutical ingredient, but only a few studies regarding the simultaneous chiral determination of optically active analytes from complex mixtures [3-5, 16]. For this reason, we aimed to develop a direct HPLC analysis method applicable for the simultaneous chiral separation of the two studied pharmaceutical substances, indapamide (IMP) and perindopril (PER), using a chiral protein-type selector, the Ultron ES OVM, ovomucoid chiral column. The two compounds investigated in this study, PER and IMP, exhibit at least one chiral centre in their chemical structure nonetheless, in therapy they are used as racemic mixtures [4, 17]. In the case of PER it is known that its pharmacological action is related to one of the enantiomers, S-Perindoril (2S,3aS,7aS)-1-[(S)-N-[(S)-1-carbethoxybutyl]alanyl]hexahydro-2-indole carboxylic acid, while in the case of IMP no concluding studies have been published regarding the differences between the pharmacological activity of the two enantiomers [11, 17].

Only a few chiral methods have been reported for the enantioseparation of PER mentioning here an HPLC-UV method with a ChiraDex chiral column and a capillary electrophoresis method using 2-hydroxypropyl-β-cyclodextrin (HP- β-CD) as chiral selector [5].

Enantiomeric separations with satisfactory resolutions of IMP were achieved using a Chiralpak IC column, filled with cellulose tris (3,5-dichloro-phenylcarbamate) as stationary phase, Chiralpak AD-H column with amylose tris-(3,5-dimethylphenyl-carbamate) as chiral selector or an Ultron ES OVM column with ovomucoid chiral selector.[3, 15] Capillary electro-chromatography methods were also reported using cellulose-based stationary phase and capillary electrophoresis methods (CE) using sulpho-buthyl ether-β-cyclodextrin (SBE-β-CD) as chiral selector [12, 16].

Due to the higher compliance of patients to treatment, the attention of drug developers has recently been focused on fixed-dose combinations, thus the development of analytical methods which allow the simultaneous separation and quantification of the studied substances in mixtures, is a necessity and also a challenge [9].

Materials and Methods

Chemicals and Reagents

The racemic mixtures of perindopril tert-butyl-amine and indapamide were purchased from Sigma-Aldrich (Germany). For the analytical dosages from commercial pharmaceutical preparations, we used a pharmaceutical product, each tablet containing 4 mg PER (perindopril tert-butylamine) and 1.25 mg IMP. Other reagents were used as follows: methanol-MeOH (LC Grade, Merck, Germany), acetonitrile-ACN (LC Grade, Merck, Germany), sodium hydroxide (Lach-Ner, Czech Republic), orto-phosphoric acid (Merck, Germany), potassium dihydrogenphosphate (Merck, Germany). A Millipore Direct-QTM system (Millipore, USA) water purifier dispensing system was used for supplying the purified water, for the preparation of buffer and other aqueous solutions. For pH buffer adjustments we used a Terminal 740 (Inolab, Germany) pHmeter.

Equipment and Instruments

Analyses were performed on an Agilent 1100 Series LC (AgilentTechnologies USA) chromatographic system, equipped with an autosampler, a thermostated column compartment and an UV-VIS detector. The enantioseparation was performed on an ULTRON ES OVM (150 x 4.6 mm, Shinwa Chemical Industries LTD., Agilent Technologies USA) column with 5 µm particle size containing an ovomucoid stationary

phase as chiral selector. The data was processed using Chemstation 7.01 (Agilent, Germany) software.
Detection was accomplished at 210 nm. The mobile phase consisted in a mixture of phosphate buffer, 20 mM KH_2PO_4 and an organic modifier (methanol or acetonitrile) in various proportions. Selectivity and resolutions were optimised function of the type of the organic modifier, buffer pH, and column compartment temperature.

Preparation procedure of working or stocks solutions and mobile phase

PER stock solution was prepared by weighing a suitable amount of powder and dissolving it in methanol in order to give a concentration of 400 µg/mL in a volumetric flask. IMP stock solution was prepared also in methanol, at a concentration of 125 µg/mL. Both stock solutions were later diluted to the appropriate concentration with the mobile phase mixture. All samples and buffers were filtered through a 0.45 µm syringe filter and degassed by ultrasound for 5 minutes before use.
Twenty tablets (each containing 4 mg PRD and 1.25 mg IMP) were individually weighed and the mean net *per* tablet was calculated together with the uniformity of the mass. The tablets were powdered in a mortar and the suitable amount of powder was transferred in volumetric flasks (100 mL) in order to give a theoretical concentration of 400 µg/mL PRD and 125 µg/mL IMP, using methanol as extraction solvent. Further on, the samples were sonicated using an ultrasound bath Transsonic T700H (Elma) for 10 minutes. Sample solutions were centrifuged at 3500 rpm for 10 minutes and the supernatant was diluted using the same procedure as for the preparation of standard solutions, before HPLC analysis.
The 20 mM potassium dihydrogenphosphate KH_2PO_4 buffer used as aqueous component of the mobile phase was prepared by weighing and dissolving the necessary amount of salt in the suitable volume of water in a volumetric flask, in order to give the above mentioned concentration.

Results and Discussion

Chiral HPLC method development and optimization

PER and IMP are co-formulated in antihypertensive dosage forms, consequently it is important to develop simple, rapid and direct stereo-selective HPLC methods for their simultaneous analysis.
The optimization of the HPLC method separation was performed taking into account the nature of the stationary phase and the physicochemical properties of PER and IMP (Table I). The UV spectra of the two analytes have been recorded previously in order to select the optimum detection wavelength.
PER, due to the lack of chromophores, displays a low absorption in the UV domain, consequently the chromatographic determinations were performed at 210 nm, a non-selective wavelength. Nevertheless, indapamide presents an absorption maximum at 242 nm.

Table I

Physicochemical properties

Analyte	M (g/mol)	LogP	logS	pKa
Perindopril	368.468	0.63	-2.5	3.79
Indapamide	365.84	2.52	-4.0	8.85

The effect of mobile phase composition and pH of the aqueous component on the enantioseparation

Ovomucoid along with several other proteins have been used as chiral selectors, including here human α1-acid glycoprotein (AGP), human serum albumin (HSA) and bovine serum albumin (BSA). Their chiral recognition mechanism involves a complex process, as proteins have specific chiral interaction sites for a pair of enantiomers, thus the mechanism being based on a unique combination of hydrophobic and polar interactions. Hermansson was the first who described the enantioselective discriminations based on natural proteins immobilized into a silica support in 1983 [6, 13].
Diastereomeric interaction with the two chiral analytes, PER and IMP, enables the chiral selector, ovomucoid, which is immobilized on the surface of the stationary phase, to distinguish the enantiomers of the studied substances.
To optimize the chromatographic conditions, several different combinations of mobile phases were used and the influence of the nature and the proportion of organic modifier have been studied. The type of the organic modifier in the mobile phase may strongly influence the resolution (R) and selectivity (α) values in enantioseparations using proteins like the ovomucoid, as chiral selector. As starting organic modifier, methanol was chosen, due to the intermediate elution power, in order to ensure chiral discrimination of the racemic mixture.
The methanol percentage in the composition of the mobile phase varied between 5% and 35%. Methanol seemed to allow the stereospecific interactions for the two analytes – PER, IMP - and the chiral selector. Nevertheless, the retention time of PER enantiomers was very close to the "tee zero" or dead time: ($t_{R\ PRD\ 1}$ = 2.5 min, $t_{R\ PRD\ 2}$ = 2.9 min) when using high percentages of methanol (20 - 35%) in the mobile phase. The use of lower percentages of methanol (5 - 15%) provided the desired enantio-resolution but caused peaks broadening, which is why we chose to change the nature of the organic modifier. The use of acetonitrile as organic modifier gave an optimum enantiomeric chiral resolution and selectivity for PER and IMP respectively, shorter analysis time and well defined peaks without any peak tailing effect. The influence of acetonitrile concentration in the mobile phase was studied between 5% and 30% and showed that decreasing the

acetonitrile percentage from 12% to 5% improved the baseline separation for both analytes. The resolution on the other hand, increased from 0.76 to 1.98 for PER enantiomers and from 1.76 to 2.55 for IMP enantiomers. However, with this resolution improvement, the analysis time increased from 10 minutes to 15 minutes and a peak broadening effect was observed. In order to obtain a high resolution, a proper peak shape and to avoid prolonged analysis time, a percentage of 7% acetonitrile in the composition of the mobile phase was chosen.

The influence of the mobile phase flow rate was also examined, being varied between 0.8 - 1.2 mL/min. Good baseline separation was obtained by using a 1 mL/min flow rate.

The retention time for PER and IMP enantiomers was established by independent injections of the two standard solutions in the same chromatographic conditions.

One of the most important chromatographic method parameters for enantiomeric separation on protein columns is the pH of the mobile phase, since the effective charges on both ionisable enantiomers and protein stationary phases are influenced by pH [2].

Research has shown that the immobilized ovomucoid on the silica gel column shows high stability over a broad spectrum of organic solvents and a high pH range. The ovomucoid (OVM) contains three homologous domains, that is why the column retention on a OVM-type protein stationary phase is considered to occur due to the two types of interactions: electrostatic and hydrophobic [19].

OVM, a chiral polymer, is able to present stereo-selective interactions with a large number of pharmacologically active compounds, having an isoelectric point (pI) of 4.1, therefore, it is expected to exhibit a negative charge at the level of the stationary phases when the mobile phase pH is above the pI values. These aspects have also been confirmed in our previous studies [3, 8]. The reduction of the mobile phase pH towards the p*I* points reduces the number of negative charges at the level of stationary phase, resulting in decreased electrostatic interactions for the basic-positively charged compounds, while increasing the mobile phase pH towards the pI reduces the number of positive charges at the level of stationary phase, resulting in decreased electro-static interactions for the acidic-negatively charged compounds [2, 19].

Taking into account the different values of the pKa of the two analytes and pI of the ovomucoid, it is difficult to establish the optimal pH for a simultaneous enantioseparation. The effect of the pH of the aqueous component of the mobile phase was studied over a range of three to seven pH units, recommended by the manufacturer of the column.

Considering all the chromatographic determinations performed at different pH values (Table II), it turned out that the optimal pH value suitable for the simultaneous separation of the two analytes, is pH = 3.75. At this pH value, the following chiral resolutions were obtained, under an analysis time of less than 10 minutes: $R_{PRD} = 2.76$; $R_{IMP} = 1.56$.

Table II

The influence of the pH of the aqueous on the separation parameters of the analysed enantiomers

	Perindopril				Indapamide			
pH	**t_{R1}**	**t_{R2}**	**R**	**A**	**t_{R1}**	**t_{R2}**	**R**	**α**
3.00	1.82	2.09	1.17	3.03	5.63	6.54	1.10	1.23
3.50	3.00	3.65	1.44	1.49	7.45	9.95	2.05	1.44
3.75	**3.05**	**4.44**	**2.76**	**2.03**	**6.63**	**8.42**	**1.56**	**1.36**
4.00	3.10	4.98	3.64	2.34	6.27	7.70	1.52	1.31
4.75	3.52	10.25	5.27	2.99	7.16	8.93	1.63	1.32

Another important aspect to be noticed is that the use of an aqueous phase with a certain pH value can modify the elution order of the enantiomers. Therefore, it was found that at the pH value of 4.75, the experimental elution order proved to be as follows: **PRD_1 - $t_{R\ PRD1}$ – 3.52 min**, followed by **IMP_1 - $t_{R\ IMP1}$ – 7.16 min** and **IMP_2 - $t_{R\ IMP2}$ – 8.93**, the last eluted enantiomer being **PRD_2 - $t_{R\ PRD2}$ – 10.25 min**.

The influence of the column compartment temperature and injection volume

After establishing the optimal composition of the mobile phase, 93% KH_2PO_4 20 mM buffer pH 3.75, 7% ACN, a further optimization was performed by varying the column temperature and the injection volume.

Temperature has an important influence on all chromatographic techniques, because the thermos-dynamics and kinetics of the chromatographic processes are dependent on temperature, with a direct effect on chromatographic parameters such as retention time, peak shape, selectivity and enantiomeric resolution. Selectivity (α) is governed by all types of enantioselective interactions such as ionic interaction, hydrogen bonding, π□π complexation, dipole stacking, steric interaction and inclusion, which are functions of temperature, between an analyte and chiral stationary phase (CSP) [1, 18].

For this reason, in this study, the effect of temperature was investigated between 10°C and 35°C with an increment of 5°C on the retention time, chiral resolution (R) and selectivity (α).

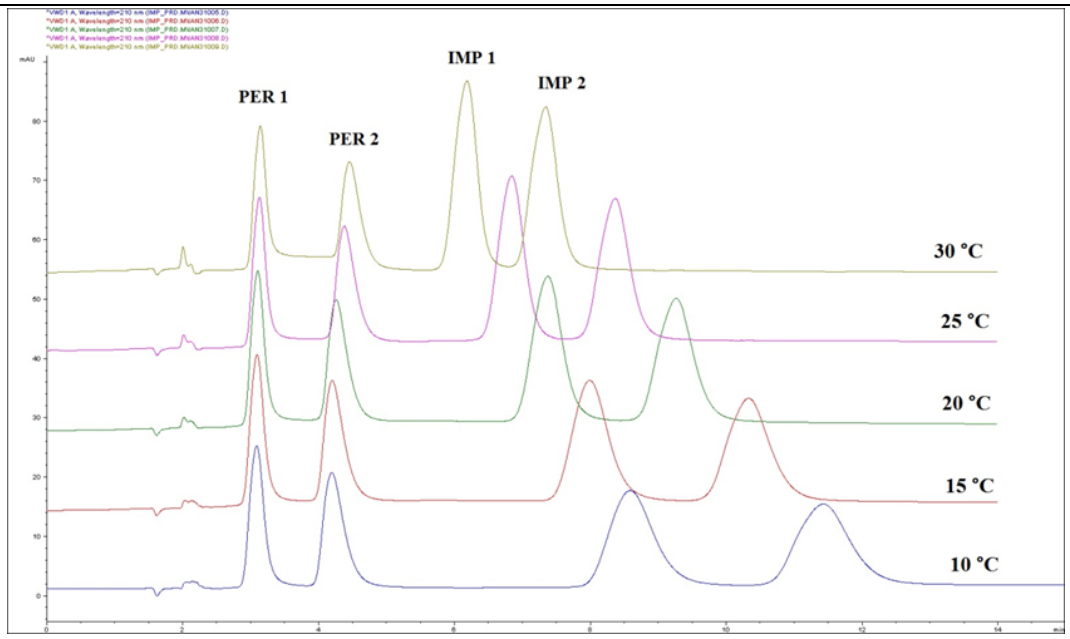


Figure 2.
Effect of temperature on retention time and peak shape (mobile phase: 95% KH_2PO_4 20 mM buffer, 5% ACN; pH 3.75; flow 1 mL/min; different column temperature)

Table III
The variation of the separation parameters of the analysed enantiomers according to the temperature

The elution temperature (°C)	Perindopril				Indapamide			
	t_{R1}	t_{R2}	R	A	t_{R1}	t_{R2}	R	A
10°C	3.08	4.19	2.31	1.79	8.58	11.43	2.13	1.41
15°C	3.09	4.20	2.45	1.80	7.99	10.33	2.19	1.37
20°C	3.10	4.26	2.64	1.82	7.37	9.25	2.12	1.33
25°C	3.13	4.37	2.95	1.87	6.84	8.36	1.99	1.30
30°C	3.14	4.45	3.14	1.91	6.18	7.34	1.70	1.26

As can be seen in the recorded chromatograms, ranging the temperature between 10 - 30°C (Figure 2), selectivity and resolution decrease when temperature increases. Also, the temperature increase is responsible for a decrease in retention times and improvement of peaks shapes, due to the fact that elevated temperatures decrease viscosity of the mobile phase and increase solubility and diffusivity [19]. As can be seen, in our study, decreasing the elution temperature improves only the resolution of IMP, while the resolution of the PER enantiomers separation decreases.
In order to keep a high resolution, the optimal temperature for the enantioseparation of the mixture was set at 25°C, a value at which the resolution and selectivity correspond to our premises, both for the two investigated analytes ($R > 1.5$; $\alpha > 1.2$).
The sample injection volume had an important effect on the peak intensity and shape; in order to achieve a reasonable signal/noise ratio and to avoid peaks broadening, we investigated the effect of injection volume against resolution in a range comprised between 1 µL and 10 µL. A 5 µL injection volume has proved to be optimal for this particular chiral separation.
Taking in consideration the aspects presented above the optimum chromatographic conditions for the simultaneous enantioseparation of the studied substances were obtained when using a mobile phase containing 7% organic modifier (acetonitrile) and 93% KH_2PO_4 20 mM buffer, pH = 3.75, under an isocratic elution (flow = 1 mL/min) and a column compartment temperature of 25°C (Figure 3). The proposed HPLC method, due to its simplicity and high efficiency turned to be an ideal method for the separation of enantiomers of the two analytes in a single analysis.

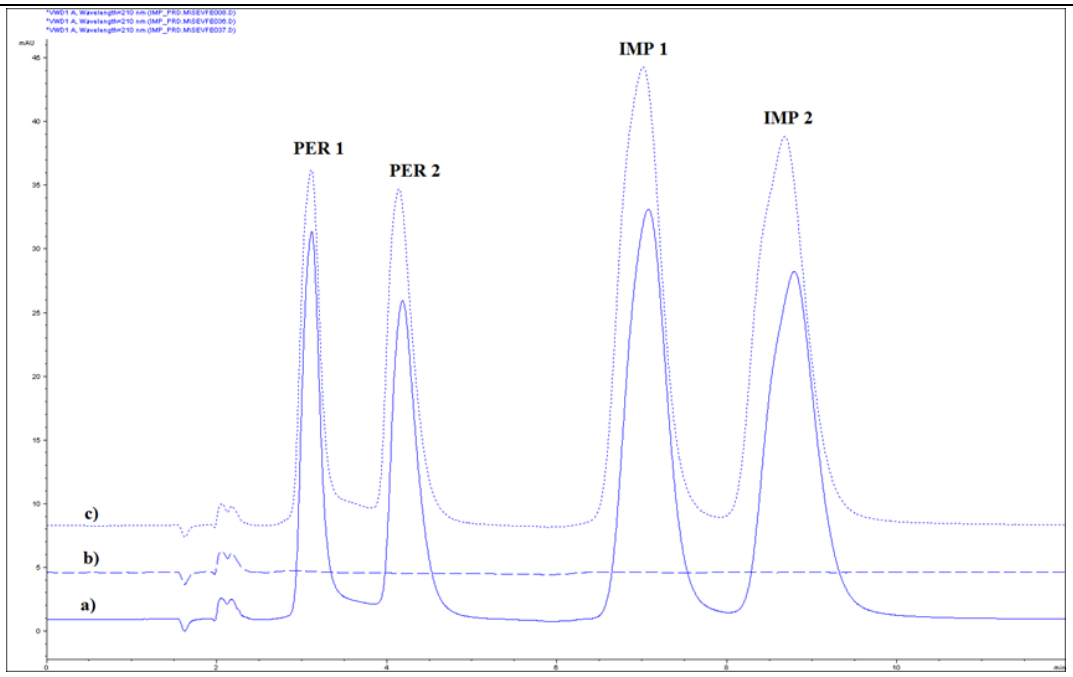


Figure 3.
Chiral separation of 20 µg/mL (±)-PER and 5 µg/mL (±)-IMP in finalized condition (mobile phase: 93% KH_2PO_4 20 mM buffer, 7% ACN; pH 3.75; flow 1 mL/min; column temperature 25°C) (a) Standard solution, (b) Blank, (c) Marketed formulation

Validation of the stereo-selective HPLC method
The main analytical validation parameters were investigated for the developed method as follows: LOD and LOQ, linearity, specificity, trueness, precision and robustness [7].
Specificity. Specificity was investigated for the interference of any other peak at the retention time of the principal peaks in blank solution. No peaks were detected from blank solution at the retention time of the main peaks. PER and IMP could be quantified from tablets without any interference from tablet excipients.

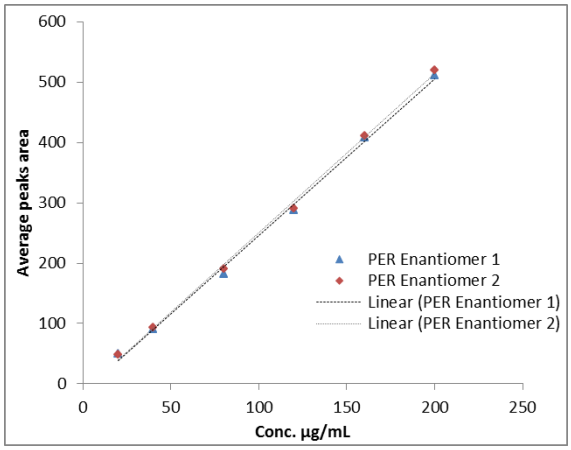


Figure 4.
The calibration curve for **PER** enantiomers in the range comprised between **20 - 200 µg/mL**

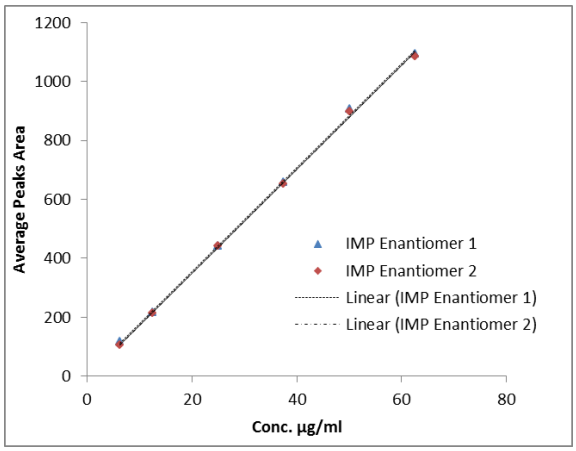


Figure 5.
The calibration curve for **IMP** enantiomers in the range comprised between **5 - 50 µg/mL**

Linearity. The linear regression equations (Table IV) were calculated for each enantiomer using six concentration levels and three replicates per concentration level. The calibration curve was plotted in the range comprised between **20 - 200 µg/mL** for **PER** and was found to be linear with a **R = 0.998** for each enantiomer. Similarly, the calibration curve for **IMP** was plotted in the range comprised between **6.25 - 62.5 µg/mL** with a correlation coefficient higher than **R = 0.999** for both **IMP** enantiomers.
Limit of detection (LOD) and quantification (LOQ). The limit of detection was established as being the signal in terms of peak height, not less than 3 times

the intensity generated by the noise signal. The limit of quantification was chosen as being the lowest concentration where the peak's height is 10 times more intense than the noise signal intensity (Table IV).

Trueness. The trueness of the investigated methods was verified by analysing neat samples containing both analytes at four different levels of concentration (Table V).

The concentrations of the active pharmaceutical ingredients were calculated from the previously established regression equations.

Table V

Linearity parameters for the chiral simultaneous determination of PER and IMP

Analyte	Regression equation	Correlation coefficient	LOD (mg/mL)	LOQ (µg/mL)
PRD Enantiomer 1	y = 2.6323x - 13.034	R = 0.9980	5	15.5
PRD Enantiomer 2	y = 2.5962x - 13.629	R = 0.9972	5	15.5
IMP Enantiomer 1	y = 17.665x + 2.1761	R = 0.9991	1	3.33
IMP Enantiomer 2	y = 17.659x - 3.6134	R = 0.9992	1	3.33

Table V

Trueness parameters for the chiral simultaneous determination of PER and IMP

Amount (µg/mL)	Amount (µg/mL)	Recovery %	Mean ± RSD	Amount (µg/mL)	Recovery %	Mean ± RSD
	Perindopril Enantiomer 1			**Perindopril Enantiomer 2**		
200	194.48	97.24		195.91	97.95	
160	154.25	96.40	97.78% ± 1.19	157.02	98.12	97.08 ± 1.30
120	118.73	98.94		114.22	95.18	
80	78.84	98.55		77.67	97.30	
Amount (µg/mL)	**Amount (µg/mL)**	**Recovery %**	**Mean ± RSD**	**Amount (µg/mL)**	**Recovery %**	**Mean ± RSD**
	Indapamide Enantiomer 1			**Indapamide Enantiomer 2**		
62.5	64.53	103.24		63.45	101.53	
50	52.37	104.75	103.49% ± 0.82	51.41	102.83	101.02 ± 1.65
25	25.78	103.11		25.21	100.86	
12.5	12.85	102.86		12.35	98.83	

Precision. The precision of the method was evaluated for both intra-day precision at three different concentrations of sample solutions from tablets and inter-day precision at three different concentrations over three successive days, using the developed chromatographic methods and calculating the recovery percentages and relative standard deviations (RSD).

Table VI

Inter- and intra- day precision for the chiral simultaneous determination of PER and IMP from sample solutions

Conc. (µg/mL)	Relative standard deviation %							
	RSD % t_R		RSD % Area peak		RSD % t_R		RSD % Area peak	
	Intra-day precision (n = 6)				Inter-day precision (n = 18)			
	PER 1	**PER 2**	**PER 1**	**PER 2**	**PER 1**	**PER 2**	**PER 1**	**PER 2**
200	0.19	0.59	1.67	1.31	0.45	0.74	1.92	1.56
120	0.24	0.51	2.54	2.11	0.62	0.71	1.68	1.57
80	0.23	0.92	2.12	2.89	0.67	0.91	1.72	1.62
Conc. (µg/mL)	**IMP 1**	**IMP 2**	**IMP 1**	**IMP 2**	**IMP 1**	**IMP 2**	**IMP 1**	**IMP 2**
50	0.43	0.87	1.33	1.78	0.61	0.78	1.83	1.97
37.5	0.31	0.43	1.45	1.58	0.56	0.58	1.65	1.73
25	0.22	0.35	0.83	0.76	0.43	0.66	1.56	1.61

The robustness. To demonstrate the robustness of the method, minor changes in terms of experimental conditions were made; like the pH values of the phosphate buffer, pH being varied in the range ± 0.2 pH units around the optimum pH value, elution temperature in the range ± 2°C and flow rate of mobile phase in the range ± 0.2 mL/min, relative to the optimum chromatographic parameters. None of the modifications caused significant changes in the resolution with RSD (%) for retention times shifts and peak areas of under 2%.

Conclusions

Simultaneous enantiomeric resolution of PER and IMP was achieved directly on an ovomucoid protein column Ultron ES-OVM, under an isocratic elution (1 mL/min) with a mobile phase consisting of a phosphate buffer KH_2PO_4 20 mM:ACN = 93:7, (v/v) with UV detection at 210 nm and 5 µL injection volume.

The proposed stereo-selective HPLC method was found to be suitable for the enantioseparation of PER and IMP, offering a relatively short time of analysis, less than 15 minutes ($t_{R\ PRD1}$ = 3.11 min;

$t_{R\ PRD2}$ = 4.20 min; $t_{R\ IMP1}$ = 7.10 min; $t_{R\ IMP2}$ = 8.84 min) and suitable resolutions (R_{PRD} = 2.32; R_{IMP} = 1.76) and selectivity (α_{PRD} = 2.32; α_{IMP} = 1.76).

The stereo-selective high performance liquid chromatographic method was validated with good results regarding linearity, precision, accuracy, specificity and robustness being suitable for the quality control assessment of oral dosage forms containing the two active pharmaceutical ingredients.

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