Available online at www.sciencedirect.com
ScienceDirect
ELSEVIER
Journal of Pharmaceutical and Biomedical Analysis 44 (2007) 1087–1094
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Evaluation of impurities level of perindopril *tert*-butylamine in tablets

M. Medenica [a,*], D. Ivanović [b], M. Mašković [c], B. Jančić [b], A. Malenović [b]

[a] *Faculty of Pharmacy, Department of Physical Chemistry, Vojvode Stepe 450, 11000 Belgrade, Serbia*
[b] *Faculty of Pharmacy, Department of Drug Analysis, Vojvode Stepe 450, 11000 Belgrade, Serbia*
[c] *Medicines and Medical Devices Agency of Serbia, Vojvode Stepe 458, 11000 Belgrade, Serbia*

Received 12 November 2006; received in revised form 6 May 2007; accepted 9 May 2007
Available online 13 May 2007

Abstract

Perindopril *tert*-butylamine is a new member of angiotensin-converting enzyme inhibitors group used in the treatment of hypertension and heart failure. In this paper, the evaluation of reversed-phase high-performance liquid chromatographic method (RP-HPLC) for the determination of impurities level of perindopril *tert*-butylamine in tablets was done. The chromatograms were recorded using a Hewlett Packard 1100 chromatographic system with DAD detector. Separations were performed on a YMC-Pack C8 column (250 mm × 4.6 mm; 5 μm particle size) at 50 °C column temperature. Mobile phase was a mixture of acetonitrile–potassium phosphate buffer (0.05 M) (37:63, v/v) (pH 2.5). pH of the mobile phase was adjusted with ortophosphoric acid. Mixture of acetonitrile–water (40:60, v/v) was used as a solvent. Injection volume was 50 μl, flow rate 1.7 ml min^{-1} and UV-detection was performed at 215 nm. The developed method subjected to method validation and parameters in terms of selectivity, linearity, precision, accuracy, limit of detection, limit of quantitation and robustness were defined. The validated method is suitable for the simultaneous determination of perindopril *tert*-butylamine as well as its impurities in pharmaceuticals.
© 2007 Elsevier B.V. All rights reserved.

Keywords: Perindopril *tert*-butylamine; Perindoprilat; Impurities; High-performance liquid chromatography; Validation

1. Introduction

In the past few years the identification and quantification of impurities in totally synthesized drugs became obligatory in drug analysis. Simultaneous identification and determination of analyzed drug and its impurities usually represents analytical challenge because of their structural similarity. The proposed methods have to be economical as much as possible offering the possibility to apply the method in the routine drug analysis.

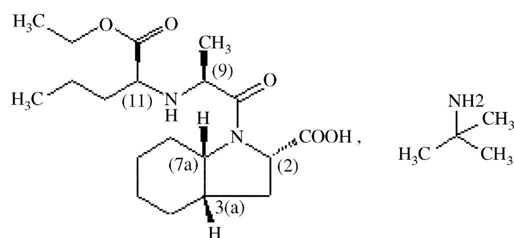
Perindopril *tert*-butylamine (in following text perindopril) is a new member of angiotensin-converting enzyme inhibitors group, which acts through its active metabolite diacid perindoprilat. Following oral administration, perindopril is rapidly absorbed and metabolized, with only 4–12% of the dose recovered unchanged in the urine. Six metabolites resulting from hydrolysis, glucuronidation and cyclization via dehydration have been identified. These include the active form perindoprilat (hydrolyzed perindopril), perindopril and perindoprilat glucuronides, dehydrated perindopril and the diastereoisomers of dehydrated perindoprilat. In humans, hepatic esterase appears to be responsible for the hydrolysis of perindopril. Molecule having five asymmetric carbon atoms of (S) configuration is used as antihypertension agent. Perindoprilat, Y31, Y32 and Y33 are the degradation products of perindopril *tert*-butylamine, but they can also be present as impurities from the route of synthesis.

Perindoprilat is lactam derivative arised from perindopril intramolecular cyclisation. Compound Y33 is a result of hydrolytical decomposition of Y31 compound ester group. Y32 is epimer of Y33 having the opposite configuration at *4a* stereogenic centre. Structural formulas and IUPAC names of perindopril *tert*-butylamine and its impurities perindoprilat, Y31, Y32 and Y33 are presented in the following Scheme 1.

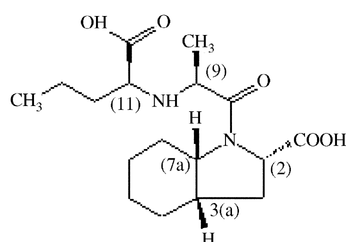
Perindopril *tert*-butylamine monograph is presented in the European Pharmacopoeia, Fifth Edition [1]. The official method proposes HPLC analysis of related substances in bulk drug using gradient elution in 45 min. Being a new member of the angiotensin-converting enzyme inhibitors group, there is not a large number of references referring its analysis in pharmaceutical dosage forms. The most recent efforts were aimed at

* Corresponding author. Tel.: +381 11 3970 379; fax: +381 11 3972 840.
E-mail address: medenica@pharmacy.bg.ac.yu (M. Medenica).

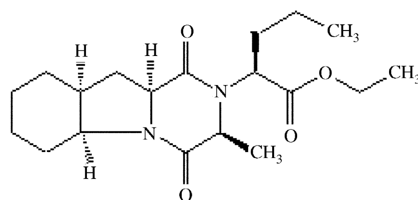
0731-7085/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2007.05.008



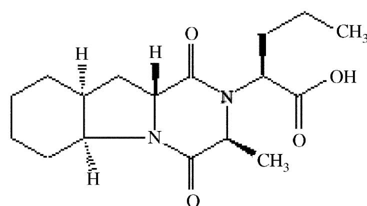
Perindopril *tert*-butylamine
(2a,3aS,7aS)-1-((S)-N-((S)-1-(ethoxycarbonyl)alanyl)octahydro-lH-indole-2-carboxylic acid, tert-butylamine salt



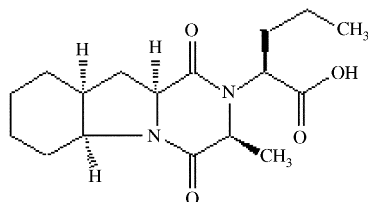
Perindopril
(2S,3aS,7aS)-1-((S)-N-((S)-1-carboxylbutyl)alanyl)octahydro-lH-indole-2-carboxylic acid



Impurity Yl
((2S)-ethyl(2-((2S,4aS,5aS,9aS)-2-methyl-1,4-dioxooctahydro-lH-indolo(1,2-a)piperazin-3yl))pentanoate



Impurity Y 32
((2S)-2-((2S,4aR,5aS,9aS)-2-methyl-l,4-dioxooctahydro-lH-indolo(1,2-a)piperazin-3yl) pentanoic acid



Impurity Y 33
((2S)-2-((2S,4aS,5aS,9aS)-2-methyl-l,4-dioxooctahydro-lH-indolo(1,2-a)piperazin-3-yl) pentanoic acid

Scheme 1. Structures of perindopril *tert*-butylamine and its impurities.

enantioselective assay of (S) perindopril using electrochemical detection which employed enantioselective, potentiometric membrane electrodes impregnated with chiral selectors [2,3]. Simultaneous assay of (S) – and (R) – perindopril using amperometric biosensors as detectors in flow systems was also described in some papers [4–6]. Procedures based on formation of copper and eosin complexes with some angiotensin-converting enzyme inhibitors were developed for their spectrofotometric determination and atomic absorption spectrometric investigations [7,8]. Optimization of a capillary electrophoresis method for the separation and quantitative determination of several angiotensin-converting enzyme inhibitors was described in some publications [9–11]. There are some papers describing densitometry and gas-chromatographic methods for the identification and determination of perindopril and some other angiotensin-converting enzyme inhibitors [12–14]. Gas-chromatography/mass spectrometry as well as radio-immunoassay methods were developed for the determination of perindopril and its active metabolite in biological fluids [15,16]. LC determination only perindopril as active substance in some pharmaceutical dosage forms was described in some papers [17,18], using RP C_{18} column with isocratic elution or perindopril was used as an internal standard.

In the present literature there are no references concerning simultaneous quantitative analysis of perindopril and its impurities in pharmaceuticals. The aim of this paper was to present the development of suitable HPLC method for the simultaneous quantitative analysis of perindopril *tert*-butylamine and its impurities perindoprilat, Y31, Y32 and Y33. After the evaluation through the process of method validation it was proved that the proposed method could be considered as the improvement in chromatographic separation and determination of the mentioned substances.

2. Experimental

2.1. Reagents and samples

All the reagents used in the experimental work were of analytical grade. Acetonitrile-gradient grade (Sigma–Aldrich, Germany), water-HPLC grade, potassium dihydrogen phosphate (Reanal, Hungary) and ortophosphoric acid (Carlo Erba, Italy) were used for preparing a mobile phase and solvent. Tablets which contain 4 mg of perindopril *tert*-butylamine were manufactured by Les Laboratories Servier, France and contain not more than 2% of perindoprilat, 2% of Y31, 1% of Y32 and 1% of Y33 impurities, calculated to the content of perindopril *tert*-butylamine. Working standards of perindopril *tert*-butylamine and its impurities perindoprilat, Y31, Y32 and Y33 were purchased from the Les Laboratories Servier, France. Ph.Eur. reference standard of perindopril *tert*-butylamine was purchased from the European Directorate for the Quality of Medicines, Strasbourg, France.

Lactose monohydrate, microcrystalline cellulose, magnesium stearate and silica, colloidal, anhydrous are substances used for placebo mixture preparing. All of them are of Ph. Eur. quality, too.

2.2. Standard solutions

Stock solutions were prepared by dissolving the respective working standard substances in solvent (mixture of acetonitrile–water (40:60, v/v) to obtain the concentration of 2 mg ml^{-1} for perindopril *tert*-butylamine, 200 μg ml^{-1} for perindoprilat and impurity Y31, 100 μg ml^{-1} for impurity Y32 and impurity Y33.

2.3. Solutions for estimating the selectivity

To prove selectivity of the proposed RP-HPLC method, placebo mixture which consisted lactose monohydrate, microcrystalline cellulose, magnesium stearate and silica and colloidal, anhydrous was prepared in solvent in the concentration ratio corresponded to the content in tablets. It was treated in the same manner as tablet mass used for the preparation of sample solution. Standard solution mixture (2) for precision evaluation was used as a standard solution mix for selectivity proving.

2.4. Solutions for estimating the linearity

For the calibration curve, nine solutions containing perindopril *tert*-butylamine as well as nine solutions for each of its impurities were prepared in solvent in the concentration range from 0.01 to 1 mg ml^{-1} for perindopril *tert*-butylamine; from 0.2 to 20 μg ml^{-1} for perindoprilat and impurity Y31; from 0.1 to 10 μg ml^{-1} for impurity Y32 and impurity Y33.

2.5. Solutions for estimating the precision

To check the precision of the proposed RP-HPLC method, three series of standard solutions in solvent were prepared with 10 solutions for each concentration mixtures:

(a) Mixture (1) contained 0.6 mg ml^{-1} of perindopril *tert*-butylamine, 12 μg ml^{-1} of perindoprilat and impurity Y31, 6 μg ml^{-1} of impurity Y32 and impurity Y33;
(b) Mixture (2) contained 0.4 mg ml^{-1} of perindopril *tert*-butylamine, 8 μg ml^{-1} of perindoprilat and impurity Y31, 4 μg ml^{-1} of impurity Y32 and impurity Y33;
(c) Mixture (3) contained 0.2 mg ml^{-1} of perindopril *tert*-butylamine, 4 μg ml^{-1} of perindoprilat and impurity Y31, 2 μg ml^{-1} of impurity Y32 and impurity Y33.

2.6. Solutions for estimating the accuracy

Accuracy is another important parameter for method validation. Therefore, the laboratory mixture containing the above mentioned placebo components as well as perindopril *tert*-butylamine and its impurities were prepared in solvent in the ratio corresponding to the investigated tablets. Laboratory mixture was treated in the same manner as tablet mass used for the preparation of sample solution. For the quantitative analysis of the laboratory mixture, three series of dilutions calculated as 80%, 100% and 120% at concentrations correspond to those in

tablets were prepared, with five solutions for each concentration mixtures:

(a) Mixture (1) contained 0.48 mg ml^{-1} of perindopril *tert*-butylamine, 9.6 μg ml^{-1} of perindoprilat and impurity Y31, 4.8 μg ml^{-1} of impurity Y32 and impurity Y33;
(b) Mixture (2) contained 0.4 mg ml^{-1} of perindopril *tert*-butylamine, 8 μg ml^{-1} of perindoprilat and impurity Y31, 4 μg ml^{-1} of impurity Y32 and impurity Y33;
(c) Mixture (3) contained 0.32 mg ml^{-1} of perindopril *tert*-butylamine, 6.4 μg ml^{-1} of perindoprilat and impurity Y31, 3.2 μg ml^{-1} of impurity Y32 and impurity Y33.

2.7. Sample solutions

The quantity of the pulverised tablet mass corresponding to 50 mg of perindopril *tert*-butylamine was placed into 50 ml volumetric flask and extracted with the solvent using ultrasonic bath and mechanical stirrer, during 20 min each, and then centrifugated. Supernatant was used to prepare a series of 10 solutions containing 0.4 mg ml^{-1} of perindopril *tert*-butylamine.

2.8. Chromatographic conditions

The chromatographic system Hewlett Packard 1100 Series (Agilent Technologies, Germany) consisted of HP 1100 binary pump, HP 1100 DAD detector, HP 1100 column and auto sampler thermostat, HP 1100 degasser and HP ChemStation integrator. Separations were performed using YMC-Pack C8 4.6 mm × 250 mm, 5 μm particle size column at 50 °C column temperature. Injection volume was 50 μl. Solutions were filtered through 0.45 μm Nylon pre-cut membrane filters. Separations and simultaneous determination of perindopril *tert*-butylamine and its impurities perindoprilat, Y31, Y32 and Y33 were performed using the mixture of acetonitrile–potassium phosphate buffer (0.05 M) (37:63, v/v) (pH 2.5) as a mobile phase. Mobile phase was filtered through a 0.2 μm Millipore filter. The flow rate was 1.7 ml min^{-1} and the UV detection was performed at 215 nm.

3. Results and discussion

Monitoring of impurities level in bulk substances and pharmaceutical dosage forms presents an important fact in pharmaceutical analysis. Identification and quantification of impurities are very difficult because of small quantities present in the investigated sample and structural similarity between impurities as well as the drug they originate from. Chemical structure similarities of the analyzed substances are the important facts having the influence on selection of the optimal chromatographic conditions. As already mentioned in the paper, perindoprilat is hydrolized perindopril, arising from a hydrolytical decomposition of perindopril ester group. Impurities Y31, Y32 and Y33 are perindopril lactam derivatives arised from its intramolecular cyclisation. Y33 is a result of a hydrolytical decomposition of Y31 compound ester group. Furthermore, Y32 and Y33 are epimers having the biggest chemical structure similarity.

From the preliminary investigation C_8 column 4.6 mm × 250 mm, 5 μm particle size (YMC-Pack C_8) was selected as appropriate stationary phase. As all the investigated substances are acids (except Y31), the investigation commenced with the phosphate buffer at acidic pH. As water phase solution of potassium dyhidrogen phosphate was chosen. Acetonitrile as organic solvent was selected because the shape of resulting peaks was improved compared to the chromatograms obtained with methanol. Content of acetonitrile was varied between 30% and 40%. With certain mobile phases, amongst critical pair, active substance/Y 32, one unknown impurity was eluted with no satisfactory resolution. The amount of unknown impurity could significantly influence the quantification of the nearest impurity peak. Therefore, it was concluded that the further investigations concerning satisfactory separation of the above mentioned unknown impurity was important. Acetonitrile content of 37% enabled acceptable separation of all the components in reasonable analysis time. Therefore, the content of 37% of acetonitrile was selected as optimal. The influence of buffer concentration was estimated as well. As the effect on the separation was negligible, 0.05 M potassium dihydrogen phosphate was chosen. pH of the mobile phase mixture was adjusted to 2.5 with ortophosphoric acid. The temperature as the important chromatographic factor was investigated, too. The peak shapes of perindoprilat and perindopril *tert*-butylamine were not acceptable when ambient column temperature was applied. Temperature of 40 °C appeared to be suitable for perindopril *tert*-butylamine peak shape, but still not acceptable symmetry of perindoprilat peak was achieved; 50 °C was found to be suitable as one of the chromatographic conditions.

The representative chromatogram of perindopril *tert*-butylamine and its impurities under established chromatographic conditions is presented in Fig. 1. The retention times for perindoprilat, perindopril *tert*-butylamine, unknown impurity,

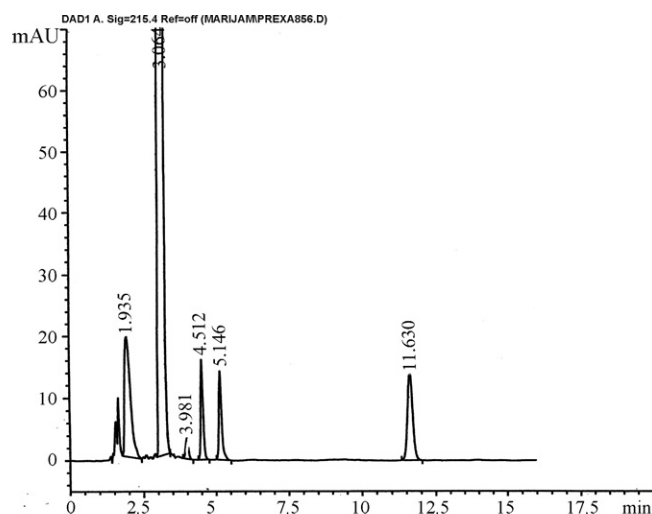


Fig. 1. The representative chromatogram of laboratory mixture: t_r = 1.935 min (perindoprilat), t_r = 3.064 min (perindopril), t_r = 3.981 min (unknown impurity), t_r = 4.512 min (Y32), t_r = 5.146 min (Y33) and t_r = 11.630 min (Y31) (mobile phase: acetonitrile–potassium phosphate buffer (0.05 M) (37:63, v/v) (pH 2.5); flow rate 1.7 ml min^{-1}; λ = 215 nm and 40 °C column temperature).

Table 1
The important parameters for the calibration curves

Parameter	Perindopril *tert*-butylamine	Perindoprilat	Y31	Y32	Y33
Concentration range	0.01–1 mg ml^{-1}	0.2–20 μg ml^{-1}	0.2–20 μg ml^{-1}	0.1–10 μg ml^{-1}	0.1–10 μg ml^{-1}
$y = ax + b$	$21\,232x + 651.61$	$29.914x + 9.843$	$19.395x + 4.507$	$22.403x + 5.577$	$23.778x + 0.272$
r	0.9973	0.9989	0.9996	0.9969	1.0000
S_b	294.434	5.2559	1.892	3.3065	0.2064
S_a	593.77	0.530	0.191	0.667	0.042
t_b	2.213	1.8728	2.382	1.6865	1.3165

r, Correlation coefficient; S_b, standard deviation of the intercept; t_b, statistical parameter having the lower value than the tabular one ($t_{tab.}$); t_{tab}, 2.998 ($p = 0.02$, $n = 7$).

Table 2
Precision of the RP-HPLC method

Compound	Injected	Found	RSD (%)	Recovery (%)
Perindopril *tert*-butylamine (mg ml^{-1})	0.2	0.212 ± 0.001[a]	0.638	105.8
	0.4	0.409 ± 0.002	0.545	102.48
	0.6	0.628 ± 0.003	0.399	104.60
Perindoprilat (μg ml^{-1})	4	4.274 ± 0.071	1.663	106.85
	8	8.296 ± 0.124	1.493	103.70
	12	12.175 ± 0.145	1.187	101.46
Y31 (μg ml^{-1})	4	4.05 ± 0.046	1.122	101.28
	8	8.052 ± 0.102	1.268	100.65
	12	11.915 ± 0.103	0.863	99.29
Y32 (μg ml^{-1})	2	2.039 ± 0.021	1.045	101.95
	4	4.016 ± 0.035	0.872	100.40
	6	6.121 ± 0.0028	0.452	102.02
Y33 (μg ml^{-1})	2	2.029 ± 0.035	1.714	101.47
	4	3.959 ± 0.024	0.601	98.98
	6	5.935 ± 0.077	1.296	98.92

[a] Standard deviation, S.D. ($n = 10$).

Y31, Y32 and Y33 are 1.94, 3.06, 3.98, 11.63, 4.51 and 5.15 min, respectively. Relative standard deviation of the retention times were lower than 2%.

After establishing the optimal separation conditions, method was subjected to method validation and parameters in terms of selectivity, linearity, precision, accuracy, limit of detection, limit of quantitation and robustness were defined.

Placebo mixture chromatogram indicates no significant interfering peaks at the analyzed compounds retention times. Therefore selectivity was proved.

Table 3
Accuracy of the RP-HPLC method

Compound	Injected	Found	RSD (%)	Recovery (%)
Perindopril *tert*-butylamine (mg ml^{-1})	0.32	0.332 ± 0.001[a]	0.222	103.78
	0.4	0.406 ± 0.002	0.566	101.55
	0.48	0.484 ± 0.001	0.308	100.83
Perindoprilat (μg ml^{-1})	6.4	6.547 ± 0.103	1.579	102.29
	8	7.953 ± 0.083	1.049	99.54
	9.6	9.824 ± 0.060	0.611	102.33
Y31 (μg ml^{-1})	6.4	6.827 ± 0.067	0.974	106.67
	8	8.421 ± 0.123	1.466	105.27
	9.6	9.917 ± 0.038	0.386	103.30
Y32 (μg ml^{-1})	3.2	3.125 ± 0.017	0.549	97.66
	4	3.897 ± 0.023	0.581	97.43
	4.8	4.68 ± 0.032	0.676	97.50
Y33 (μg ml^{-1})	3.2	3.235 ± 0.038	1.186	101.41
	4	4.033 ± 0.033	0.827	100.83
	4.8	4.836 ± 0.032	0.669	100.75

[a] Standard deviation, S.D. ($n = 5$).

Table 4
Limit of detection (LOD) and limit of quantification (LOQ)

Compound	LOQ[a] (μg ml^{-1})	LOD[a] (μg ml^{-1})
Perindopril *tert*-butylamine	0.04	0.02
Perindoprilat	0.03	0.01
Y31	0.15	0.05
Y32	0.06	0.03
Y33	0.03	0.01

[a] Experimentally determined values.

Linear relationships of the peak area over the mentioned concentration range for perindopril *tert*-butylamine and impurities perindoprilat, Y31, Y32 and Y33 were obtained. All linearity parameters values are within the linearity acceptable criteria. The calculated regression parameters are given in Table 1.

The important statistical parameters for the method precision evaluation were also being considered. Relative standard deviation (RSD) were determined and their values are fulfilling the required criteria (Table 2).

To evaluate the method accuracy, the recovery values for laboratory mixtures were calculated. They are all within the acceptable criteria (Table 3).

Being important for the quantitative analysis, limit of detection and limit of quantification were experimentally determined and their values are given in Table 4. The signal-to-noise ratio of 3:1 and 10:1 were taken as LOD and LOQ, respectively, and further confirmed by taking dilutions from the secondary stock solution till the peak area obtained has 3- (for LOD) and 10- (for LOQ) fold then the standard deviation of six determination.

As defined by the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH), the robustness of an analytical procedure refers to its capability to remain unaffected by small and deliberate variations in method parameters [19]. The robustness test starts with the selection of the factors that might influence the performance of the method [20–22].

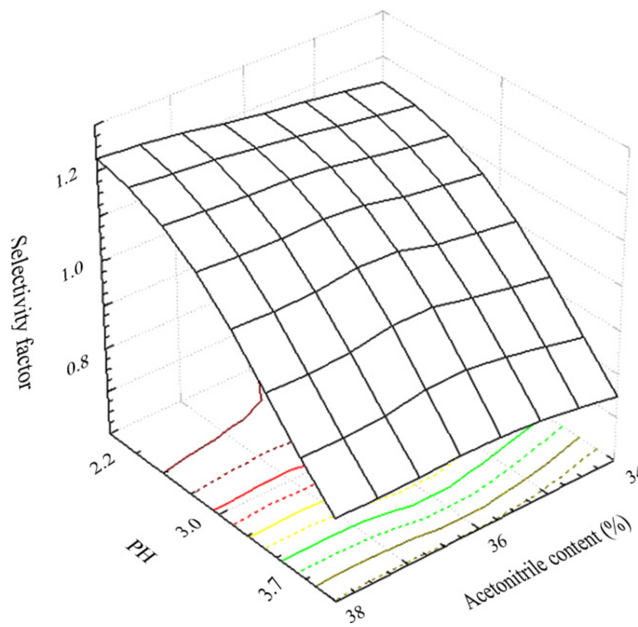


Fig. 3. Three-dimensional plot of $\alpha_2 = f(\%ACN, pH)$, α_2 is the selectivity factor for Y32 and unknown impurity.

In this case the content of acetonitrile (from 34%, v/v, to 38%, v/v) and pH (from 2.2 to 4.0) of the mobile phase are the factors important for chromatographic separation. Selectivity factors were calculated for the critical pairs unknown impurity/perindopril *tert*-butylamine (α_1), Y32/unknown impurity (α_2) and Y33/Y32 (α_3). Important factors (acetonitrile content and pH) were simultaneously changed in given range and obtained results were estimate using Response Surface

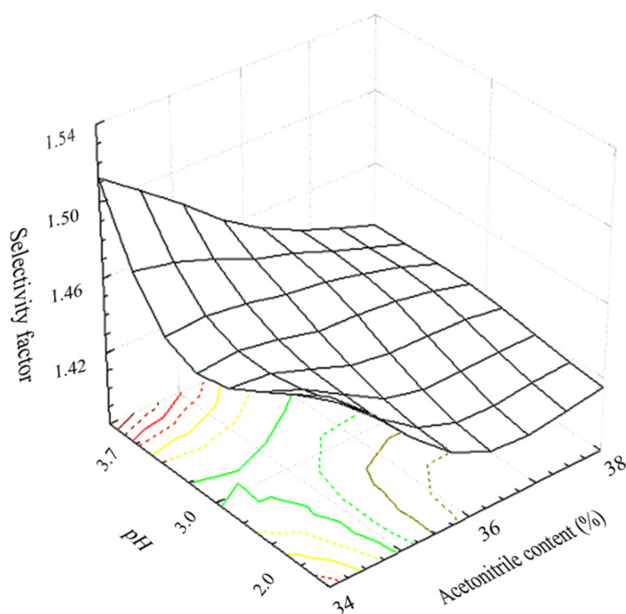


Fig. 2. Three-dimensional plot of $\alpha_1 = f(\%ACN, pH)$, α_1 is the selectivity factor for perindopril *tert*-butylamine and unknown impurity.

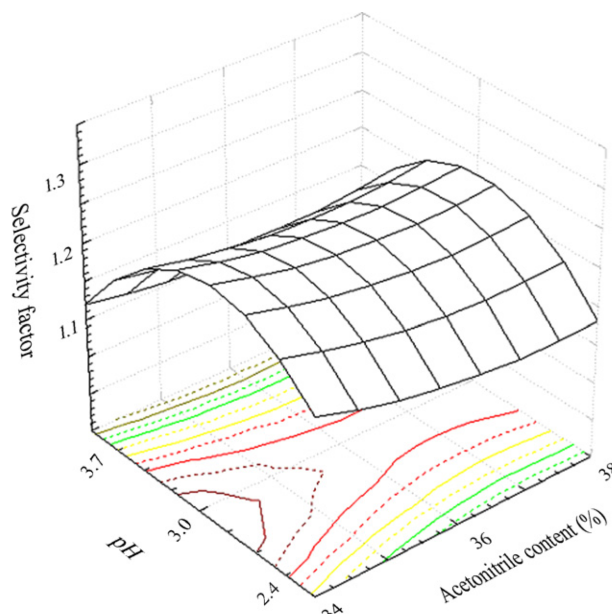


Fig. 4. Three-dimensional plot of $\alpha_3 = f(\%ACN, pH)$, α_3 is the selectivity factor for Y33 and Y32.

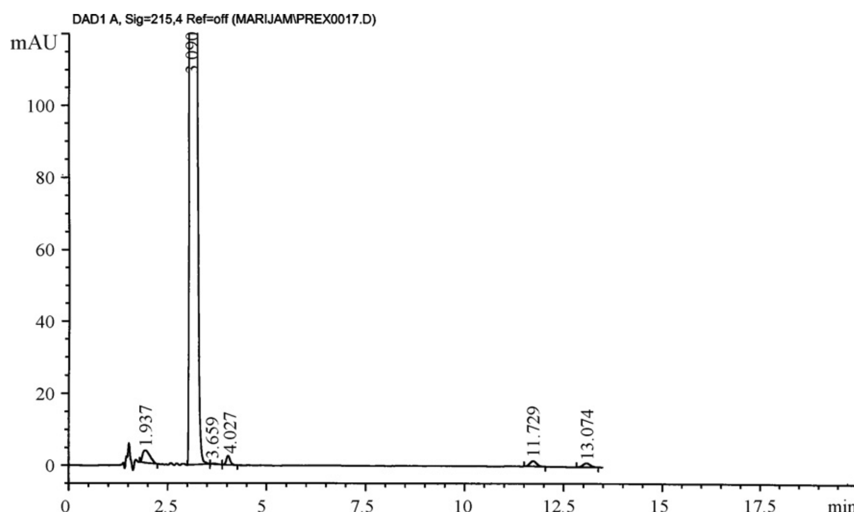


Fig. 5. The chromatogram of Prexanil® tablets.

Table 5
Quantitative analyses

Compound	Injected (mg ml^{-1})	Found (mg ml^{-1})	Found (mg tbl^{-1})	RSD (%)	Recovery (%)
Perindopril *tert*-butylamine	0.4	0.414 ± 0.001	4.138 ± 0.009[a]	0.220	103.45

Compound	MAC[b] (μg ml^{-1})	Found (μg ml^{-1})	Found (%)
Perindoprilat	8	1.219 ± 0.018[a]	0.305
Y31	8	0.681 ± 0.024	0.17
Y32	4	Not detected	
Y33	4	Not detected	
Unknown impurity	1	0.351 ± 0.026	0.088

[a] Standard deviation, S.D. ($n = 10$).
[b] MAC, maximum allowed content.

Methodology (RSM). This is a collection of mathematical and statistical techniques useful for analyzing problems where several independent variables influence a dependent variable or response [23]. Based on the performed experiments three-D graphs were constructed and presented in Figs. 2–4.

Analyzing the mentioned three-D graphs for selectivity factors of critical pairs, it can be concluded that, method is robust in pH range from 2.2 to 2.5, with content of acetonitrile from 36% to 38%. In those ranges of investigated factors, the proposed method can be changed with no effect on the separation.

The proposed RP-HPLC method was then applied for perindopril *tert*-butylamine and its impurities assay in Prexanil® tablets. Appropriate chromatogram is presented in Fig. 5. The content of perindopril *tert*-butylamine is between 103.05% and 103.83%. Impurity Y31 level of 0.17% and 0.30% of perindoprilat were within the required criteria, as being less than determined 2 % each. Unknown impurity level of 0.088% was in accordance with the requirement for other impurities, single, of not more than 0.25% (Table 5).

4. Conclusions

The RP-HPLC method for the determination of impurities level of perindopril *tert*-butylamine in tablets was developed, validated and applied in pharmaceutical analysis. As there are no references, in the present literature, concerning to simultaneous quantitative analysis of perindopril *tert*-butylamine and its impurities the proposed method is quite novel. The method was evaluated through the method validation and proved to be selective, precise and accurate, so it can be used for separation, identification and simultaneous determination of perindopril *tert*-butylamine and its impurities. Therefore, the proposed method as rapid and sensitive, presents the significant improvement in chromatographic analysis and routine application of this technique in drug analysis.

Acknowledgement

The authors thank to Ministry of Science for supporting these investigations in Project 142077G.

References

[1] European Pharmacopoeia, Fifth ed., 2005.
[2] K.I. Ozoemena, R.I. Stefan, J.F. van-Staden, H.Y. Aboul-Enein, Talanta 62 (2004) 681–685.
[3] R.I. Stefan, J.F. van-Staden, H.Y. Aboul-Enein, Chirality 11 (1999) 631–634.

[4] R.I. Stefan, J.F. van-Staden, L.V. Mulaudzi, H.Y. Aboul-Enein, Anal. Chim. Acta 467 (2002) 189–195.
[5] J.F. van-Staden, R.I. Stefan, H.Y. Aboul-Enein, Fresenius' J. Anal. Chem. 367 (2000) 178–180.
[6] R.I. Stefan, J.F. van-Staden, H.Y. Aboul-Enein, Sens. Actuators B B54 (1999) 261–265.
[7] S. Emara, A. El-Gindy, A.N. El-Shorbagi, G. Hadad, Anal. Chim. Acta 489 (2003) 115–123.
[8] H.E. Abdellatef, M.M. Ayad, E.A. Taha, J. Pharm. Biomed. Anal. 18 (1999) 1021–1027.
[9] S. Hillaert, Y. Vander-Heyden, W.J. van-den-Bossche, J. Chromatogr. A 978 (2002) 231–242.
[10] S. Hillaert, W. van-den-Bossche, J. Pharm. Biomed. Anal. 25 (2001) 775–783.
[11] S. Hillaert, W. van-den-Bossche, J. Chromatogr. A 895 (2000) 33–42.
[12] K. Czerwinska, E. Wyszomirska, T. Kaniewska, Acta Pol. Pharm. 58 (2001) 331–338.
[13] S.J. Lin, H.L. Wu, Y.H. Wen, Anal. Lett. 29 (1996) 1751–1762.
[14] K.M. Sereda, T.C. Hardman, M.R. Dilloway, A.F. Lant, Anal. Proc. (Lond.) 30 (1993) 371–372.
[15] C. Tsaconas, M. Devissaguet, P. Padieu, J. Chromatogr. Biomed. Appl. 80 (1989) 249–265.
[16] H. Van-den-Berg, G. Resplandy, A.T.H.J. De-Bie, W. Floor, M. Bertrand, C.J.M. Arts, J. Pharm. Biomed. Anal. 9 (1991) 517–524.
[17] N. Erk, J. Pharm. Biomed. Anal. 26 (2001) 43–52.
[18] A. Gumieniczek, H. Hopkala, Chem. Anal. (Warsaw) 43 (1998) 951–954.
[19] International Conference on Harmonization, Topic Q2B, validation of analytical methods: methodology, in: The Third International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human use (ICH), Yokohama, Japan, 1996.
[20] E. Hund, Y. Vander Heyden, M. Haustein, D.L. Massart, J. Smeyers-Verbeke, J Chromatogr. A 874 (2000) 167–185.
[21] Y. Vander Heyden, A. Nijhuis, J. Smeyers-Verbeke, B.G.M. Vandeginste, D.L. Massart, J. Pharm. Biomed. Anal. 24 (2001) 723–753.
[22] S. Furlanetto, S. Orlandini, P. Mura, M. Sergent, S. Pinzauti, Anal. Bioanal. Chem. 377 (2003) 937–944.
[23] D.C. Montgomery, Design and Analysis of Experiments, John Wiley & Sons, Inc., New York, USA, 1976.