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Short communication

Liquid chromatographic separation of pregabalin and its possible impurities with fluorescence detection after postcolumn derivatization with o-phtaldialdehyde

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ABSTRACT

A rapid procedure based on direct extraction and RP-HPLC separation of pregabalin and its possible impurities with fluorescence detection has been developed. The separation conditions and parameters of derivatization reaction for postcolumn derivatization of pregabalin with *o*-phtaldialdehyde/2-mercaptoethanol were studied. Purospher STAR RP-8e column with isocratic elution was employed. Fluorescence detection was performed at excitation and emission wavelength of 345 nm and 450 nm, respectively. The proposed method has an advantage of a simple sample pre-treatment and a quick and very sensitive HPLC method. The applicability of developed method was successfully verified during analysis of commercial samples of tablets of Lyrica (Pfizer, USA).

1. Introduction

The drug pregabalin (PGB; 3-(aminomethyl)-5-methyl-hexanoic acid; CAS = 148553-50-8) is related to the endogenous inhibitory neurotransmitter γ-aminobutric acid (GABA), which is involved in the regulation of brain neuronal activity. Pregabalin exhibits anti-seizure activity and is thought to be useful for treating, among other conditions, pain, physiological conditions associated with psychomotor stimulants, inflammation, gastrointestinal damage, alcoholism, insomnia, and various psychiatric disorders, including mania and bipolar disorder [1,2]. The possible impurities, 3-(aminomethyl)-5-methyl-hex-4-enoic acid (PG157-I) and 3-(aminomethyl)-5-methyl-hex-5-enoic acid (PG157-II) (Fig. 1), can originate during synthesis of pregabalin by the Hofmann rearrangement of a primary amides to a primary amines or they can be present as degradation products in pharmaceutical preparation [3].

The separation and detection systems employed today offer a number of alternatives for analysis of amino acids. The separation of underivatized amino acids on synthetic ion-exchange resins was first described by Moore and Stein more than 50 years ago [4]. Amino acids are zwitterionic compounds with the pK_a of the acid function less than 5 and the pK_a of the amino function greater than 8 [5]. Buffered mobile phases of pH 2–3 suppress carboxylic acid dissociation and leave the amino functions protonized. Chromatography of these positively charged free amino acids on reversed-phase supports typically yields poor retention or deteriorated peak-shape [6]. Mobile phase containing ion-pairing reagents (e.g. alkylsulfonic acid) [7,8] can improve retention of such positively charged species but separation generally suffers insufficient selectivity and reproducibility for routine analyses of complex amino acid mixtures. The improvement of selectivity and reproducibility of separation on octyl and octadecylsilica supports was accomplished using perfluorinated carboxylic acids as ion interaction reagents [9,10]. Tridecafluoroheptanoic acid was tested as a relatively volatile ion interaction reagent but the equilibration time for such system exceeded 1 h under isocratic conditions [9]. Thus, perfluorinated carboxylic acids with shorter carbon chains (pentafluoropropionic acid and heptafluoropropionic acid) were tested in order to find a suitable chromatographic system with shorter equilibration time [11].

The volatile mobile phase allows the use of direct detection, mass spectrometry [12,13] or evaporative light scattering detection [11,14]. The direct detection by refractive index, electrochemical detection [15,16] or ultraviolet absorbance in the low UV region is possible but generally lacks adequate sensitivity or specificity for

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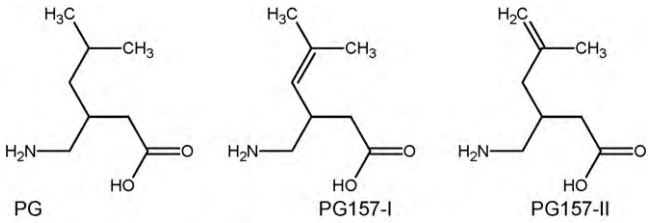

Fig. 1. Structural formula of PG, PG157-I and PG157-II.

most needs in amino acid analysis. Due to this reason, the postcolumn derivatization is often used for enhancement of selectivity and sensitivity. The derivatization agent, e.g. *o*-phtaldialdehyde (OPA) [17], ninhydrin [18] or fluorescamine [19], reacts with amino acids and enable their detection. Therefore, the most successful approach to amino acid analysis by RP-HPLC is to employ the techniques of precolumn derivatization leading to hydrophobic and highly absorbing UV or fluorescence derivates which can be effectively separated and sensitively quantified.

The reaction of OPA in presence of 2-mercaptoethanol with primary amino group is displayed in Fig. 2 [20]. The reaction is relatively rapid, it proceeds at laboratory temperature but the stability of 1-alkyl-2-thioindoles products is not sufficient due to the spontaneous rearrangements that have been observed [21]. For that reason, the reaction is often applied at postcolumn derivatization [22].

Currently available quantitative methods, as reviewed in a recent publication [23] determine only the amount of pregabalin in human serum by liquid chromatography with MS/MS detection [24] or with fluorescence detection using precolumn derivatization with *o*-phtaldialdehyde (OPA)/2-mercaptoethanol [25]. In this paper [23], a method for the simultaneous analysis of pregabalin, vigabatrin and gabapentin which are structural analogues of GABA is described. The precolumn derivatization of pregabalin with picryl sulfonic acid has been used for separation of pregabalin on a C_8 column with UV detection at 340 nm [26]. The other papers are focused on chiral separation of pregabalin in the bulk drug. The use of precolumn derivatization of pregabalin with Marfey's chiral reagent, sodium-2,4-dinitro-5-fluorophenyl-L-alanine amide, makes possible resolution of enantiomers on an ordinary chromatography reversed-phase ODS column with phosphoric acid buffer and acetonitrile [27,28].

The present study was designed to find optimum separation of pregabalin and its possible impurities using reversed-phase system and optimal reaction conditions for postcolumn derivatization of pregabalin with *o*-phtaldialdehyde/2-mercaptoethanol. The other aim of the presented work was to develop a quick and sensitive quantitative chromatographic method for the determination of PGB in pharmaceutical formulations. The applicability of new method was successfully verified by analysis of commercial samples of tablets of Lyrica (Pfizer, USA).

2. Experimental

2.1. Chemicals and materials

Acetonitrile of HPLC grade (Merck, Germany) and water purified on Milli-Q system (Millipore, USA) were used. Other chemicals were of analytical grade. Extraction solvent was prepared by mixing 950 ml water and 50 ml acetonitrile.

2.2. Instrumentation

Sample extraction was performed on a laboratory horizontal shaker and ultrasonic bath. All chromatographic experiments were carried out using a liquid chromatograph system consisting of Alliance 2695 separation module and fluorescence detector W2475 (all Waters, USA). The system was controlled by data station using Empower software (Waters, USA). Postcolumn addition of a derivatization mixture was performed in postcolumn reaction system PCRS-100 (volume 1000 μl; ID 0.42 mm) (Science Instruments and Software, Czech Republic), placed between a chromatographic column and the detector and connected to the column with a low-dead-volume PEEK mixing tee (Valco Instruments Company, USA). The reaction temperature was 35 °C. As the postcolumn reagent, 0.06 mol/l *o*-phtaldialdehyde/0.001 mol/l 2-mercaptoethanol in 0.05 mol/l sodium tetraborate pH 10.5 was used and delivered at a flow rate of 0.2 ml min^{-1}.

2.3. Chromatographic conditions

HPLC separation was performed on a Purospher STAR RP-8e column (250 mm × 4.0 mm, 5 μm; Merck, Germany). The mobile phase consisted of methanol (solvent A) and 0.010 mol/l acetate buffer at pH 5.0 (solvent B) in ratio 15:85 delivered isocratically. The flow rate was 0.8 ml min^{-1}, the injection volume was 10 μl, the column was thermostated at 30 °C and the run time was 15 min. The fluorescence detection was carried out at excitation wavelength of 345 nm and emission wavelength of 450 nm.

2.4. Standard preparation

The standard of pregabalin (in house standard, purity 99.5%), PG157-I (in house standard, purity 95.5%) and PG157-II (in house standard, purity 93.5%), respectively, was dissolved in extraction solvent at a concentration of 500 mg l^{-1} to obtain the standard stock solution. The standard stock solution was dissolved in extraction solvent at a concentration of 40 mg l^{-1} to obtain working stock solution. The working stock solution was prepared daily.

2.5. Sample preparation

Ten tablets were powdered and an aliquot corresponding to 100 mg of PGB was weighed into a 50 ml volumetric flask. Extraction solvent (40 ml) was added and sample was extracted for 10 min in an ultrasonic bath. After cooling to the laboratory temperature,

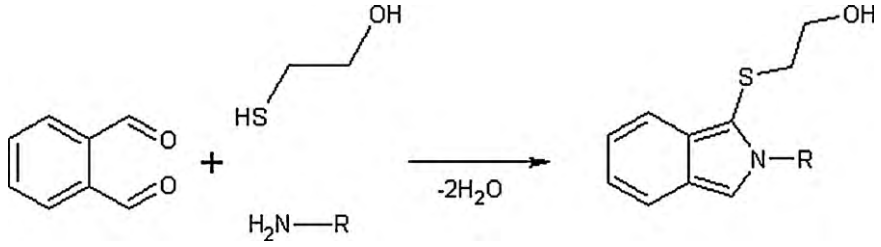

Fig. 2. Derivatization reaction of amino compounds with OPA/2-mercaptoethanol.

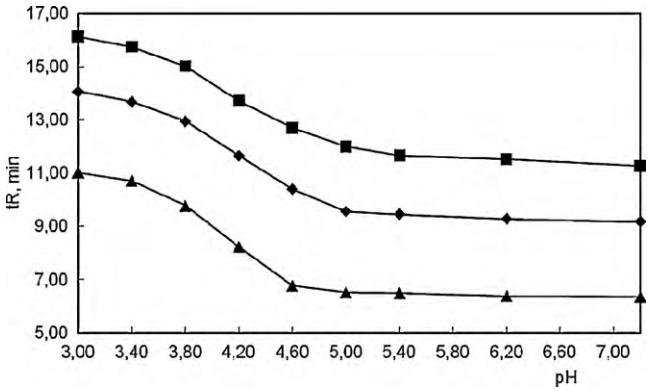

Fig. 3. Dependence of retention time of PG (■), PG157-I (♦) and PG157-II (▲) on pH of aqueous component of mobile phase.

the flask was filled in by extraction solvent. The obtained extract was filtered through a membrane filter of 0.45 μm. The solution was diluted with extraction solvent at a concentration of 40 mg l^{-1} and injected into the liquid chromatograph.

3. Results and discussion

3.1. Optimization of the HPLC method

The final mobile phase was optimized so as to the capacity factor was $k \geq 1.0$, asymmetry factor $t_a \leq 1.3$, selectivity ≥ 1.5 and resolution between PG157-I and PG157-II ≥ 2.0. The following parameter of optimized chromatographic method was determined using calibration solutions of standard. The resolution, as critical parameter, was applied as system suitability test. The minimum resolution in all experiments was 2.97.

The retention of PGB in a reverse phase system is weak due to high polarity of ionized form. The increase in retention can be achieved using an ion-pairing agent or it can be utilized by amphoteric properties of PGB ($pK_1 = 4.2$ and $pK_2 = 10.6$; isoelectric point pI = 7.4). PGB can be converted to its most hydrophobic form by tuning of mobile phase pH (molecule appears outwards as neutral). These pH variations should not be underestimated when optimizing experimental conditions for chromatographic analysis of ionisable analytes, especially amino acids. Fig. 3 shows the measured chromatographic times of PG157-I, PG157-II and PGB as a function of the aqueous pH of the mobile phase. PGB and its impurities showed a change in retention time with the change in pH of the mobile phase (for investigated range of pH). The retention of PGB and its impurities increased with the decrease of pH of the mobile phase. This behaviour indicates that secondary interactions of PGB and its impurities with free silanol groups of silica bonded stationary phase occur. The secondary interactions of PGB with free silanol groups were verified using addition of 15 mM triethylamine (TEA) into the mobile phase (at the same pH) to reduce the availability of stationary phase silanols and interaction of the analytes with the silanols. The result of addition of TEA was a decrease of retention time of PGB and its impurities and an increase of the efficiency of separation.

It has been shown that the retention times of PGB decrease with increasing pH values of the mobile phase due to the amphoteric properties of PGB. The optimum pH value for the separation of these analytes has been determined to be 5 (small change of capacity factor with change of pH of the mobile phase). The pH of the mobile phase has no influence on selectivity but it has significant influence on retention of all investigated analytes.

Table 1
The calculated coefficients of Eq. (1a).

Coefficient	Analytes		
	PG157/I	PG157/II	PG157
$\log k_a$	0.4518	0.7003	0.9479
m	−2.7058	−2.9990	−3.3484
Correlation coefficient r	−0.9988	−0.9988	−0.9992

To evaluate the influence of organic solvent fraction in mobile phase Eq. (1a) can be used:

$$\log\ k = \log\ k_a - m\varphi \tag{1a}$$

where k_a is the (extrapolated) value of k for $\varphi = 0$ (in this case it corresponds to retention in 10 mM acetate buffer) and m is a constant for each solute [29,30]. The calculated coefficients of Eq. (1a) for volume fraction $\varphi = 0.075$–0.175 are mentioned in Table 1. The effect of organic solvent fraction in mobile phase on separation selectivity (α) is as follows:

$$\alpha = \frac{k_{a2}}{k_{a1}} 10^{(m_1 - m_2)\varphi} \tag{1b}$$

where the coefficients are the same as in Eq. (1a). Due to the close values of constants m in Eq. (1b) the volume fraction of methanol has no significant influence on selectivity of separation. The above-mentioned equations allow prediction of retention and selectivity of pregabalin and its impurities in studied chromatographic system (for mentioned ranges of volume fraction of methanol). The optimal mobile phase contains 150 volumes of methanol and 850 volumes of aqueous solution of 10 mM acetate buffer adjusted to pH 5.0 with acetic acid. The effect of temperature on the retention in RP-HPLC has been previously examined e.g. by Melander et al. [31]. The expected temperature dependence of retention can be expressed using van't Hoff's equation [32]:

$$\ln\ k = -\frac{\Delta H^\circ}{RT} + \frac{\Delta S^\circ}{R} + \ln\frac{V_S}{V_M} = A + \frac{B}{T} \tag{2a}$$

where ΔH°, ΔS° is standard enthalpy resp. standard entropy in chromatographic system, R is gas constant, V_S is stationary phase volume, V_M is mobile phase volume and A, B are constants dependent on chromatographic system. In this study linear van't Hoff plot was obtained over narrow temperature range (20–40 °C). The calculated coefficients of Eq. (2a) for temperature range 20–40 °C are mentioned in Table 2. The influence of temperature on selectivity (α) of separation can be expressed using following equation:

$$\ln\ \alpha = \ln\ k_2 - \ln\ k_1 = A_2 - A_1 + \frac{B_2 - B_1}{T} \tag{2b}$$

where the coefficients are the same as in Eq. (2a). The temperature has significant influence on separation selectivity of both impurities due to large difference of constants B in Eq. (2b). The above-mentioned equation allows prediction of retention of PGB and its impurities in studied chromatographic system (for investigated ranges of temperature). The temperature has no significant influence on separation and the most suitable temperature for separation is 30 °C.

Table 2
The calculated constants of Eq. (2a).

Coefficient	Analytes		
	PG157/I	PG157/II	PG157
A	−1.402	−2.778	−1.832
B	452.1	1012.0	861.1
Correlation coefficient r	0.9963	0.9981	0.9964

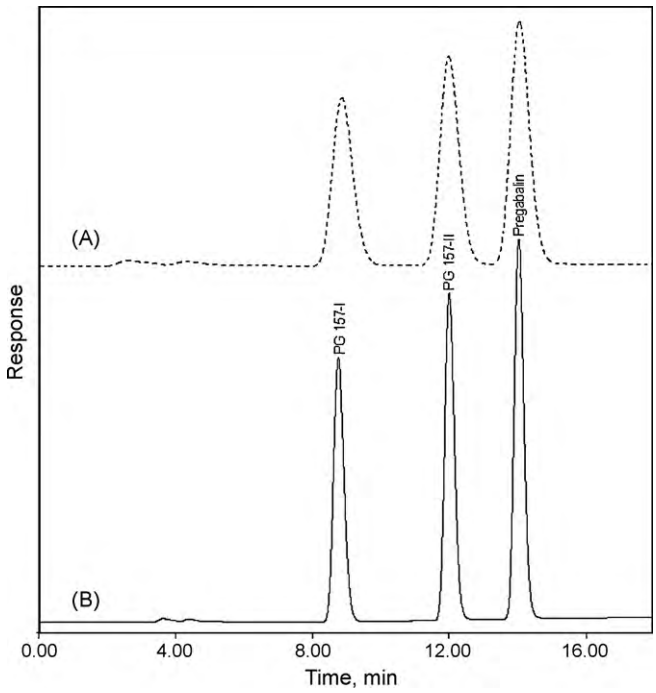

Fig. 4. Influence of internal diameter of tube of reaction coil at derivatization reaction on performance of HPLC system. A—tube: 0.5 mm internal diameter, length 510cm; B—tube: 0.25 mm internal diameter, length 2040 cm.

3.2. Optimization of derivatization reaction

The formation of the OPA/thiol derivates of amino acids is simple and fast. Reactions proceed to completion at room temperature in 1 min using mercaptoethanol as the reducing agent. For such fast reactions, capillary coil reactors show the best performance. That is why this type was used. The peak broadening due to extracolumn volume (expressed as standard deviation σ_t where the subscript t means tube band spreading) of the reaction tube depends on proportion of its internal diameter (d_t) and flow rate of effluent (F); $\sigma t \approx (d_t^4/F)$. The standard deviation (in μl) of a PGB peak for tube with 0.25 mm internal diameter and 2040 cm was about 210 μl and with 0.5 mm internal diameter × 510 cm was about 780 μl (Fig. 4).

The decrease of baseline-noise was accomplished using postcolumn pump with active pulse dampening controlled by software. Comparing a tee and static mixer with inside volume of 50 μl showed the baseline-noise for the tee to be 3 times smaller than that for the static mixer in the mixer part. The baseline-noise was measured using chromatographic software. This is demonstrated in Fig. 5.

The residence time in the postcolumn reactor had to be at least 60 s to achieve a substantial enhancement of the detector response. The increase of residence time was achieved by linear decrease of flow of derivatization reagent. The linear dependence between residence time (s) and relative detector response (%) was calculated using linear regression analysis, it showed slope 1.57, y-intercept 17.71 and correlation coefficient of 0.9996. The optimal flow is of 0.2 ml min^{-1}.

The derivatization reaction proceeds at room temperature, the highest response was obtained at temperature ranging from 25 to 30 °C (Fig. 6).

The derivatization reaction depends on pH of reaction medium and it should be kept above pH 9.0. The buffer capacity (β) of derivatization reagent has to be high enough to maintain pH of medium and it depends on buffer capacity of mobile phase too. The influence of buffer capacity of mobile phase on relative detector response is

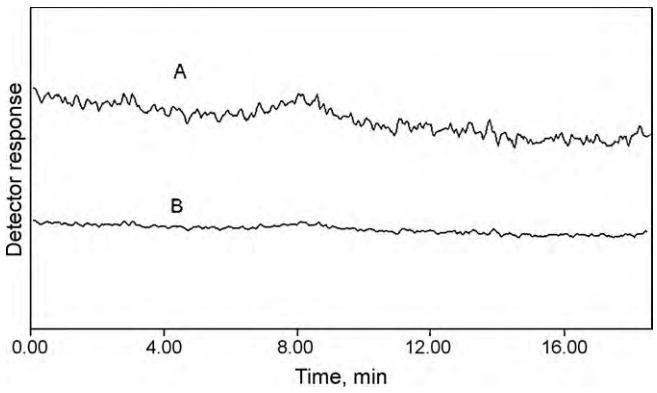

Fig. 5. Effect of tee piece and static mixer in postcolumn reactor on size of baseline-noise. A—static mixer (50 μl); B—tee piece.

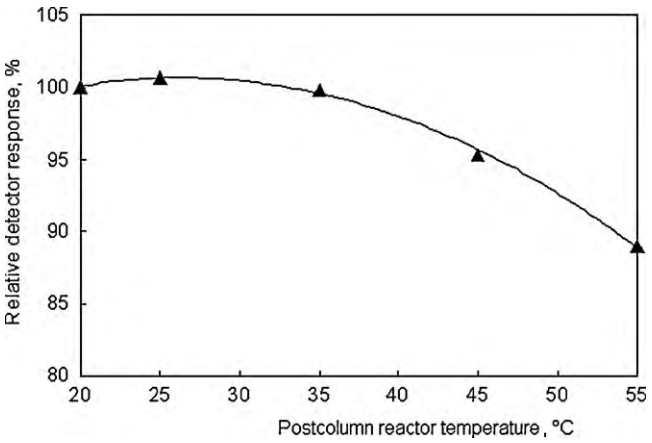

Fig. 6. Effect of postcolumn temperature on PGB fluorescence intensity. Detector response is expressed as a percentage of the maximum PG peak area.

shown in Fig. 7. The buffer capacity of mobile phase has to be lower than 0.004 (corresponding to concentration of 0.01 mol/l acetate buffer in mobile phase).

The repeatability of derivatization reaction and retention time was evaluated by relative standard deviation of eight repetitive injections of working standard solution. The retention time repeatability (relative standard deviation) of PGB was 0.06% and area repeatability of PGB was 0.51%.

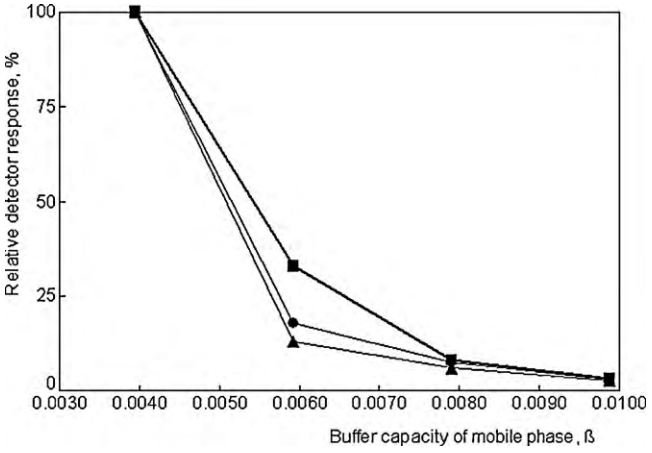

Fig. 7. Detector response to analytes as a function of buffer capacity of mobile phase (PG (●); PG157-I (■); PG157-II (▲)). Detector response is expressed as a percentage of the maximum PG peak area.

3.3. Validation parameters

The method was validated according to ICH Q2(R1) [33].

3.3.1. Limit of detection (LOD) and limit of quantitation (LOQ)

LOD and LOQ were calculated for possible impurities of pregabalin, PG157-I and PG 157-II, based on a detector signal-to-noise ratio and the slope of the calibration curve according to the equations:

$$\mathrm{LOD} = 3\frac{ND}{S} \qquad \mathrm{LOQ} = 10\frac{ND}{S}$$

where *ND* is baseline-noise of chromatograms of the extraction solvent and *S* is the slope of the calibration curve (expressing as calibration function of peak height) in the low concentration range of analyte. The baseline-noise was measured in a blank experiment in the region of retention time of PGB using chromatographic software. The LOD was 4.8 $\mu g\,l^{-1}$ and the LOQ was 16 $\mu g\,l^{-1}$ which corresponding 0.012% and 0.04% to relative active, respectively, in a real sample calculated according to the treatment described in Section 2. The calibration curve in the low concentration range of 0.015–1.0 $mg\,l^{-1}$ of PG157-I and PG157-II was constructed by plotting the peak area against the concentration and the calibration equation was calculated using linear regression analysis. It showed slope 1,747,541, *y*-intercept −91.2 and correlation coefficient 0.9994 for PG157-I and slope 1,843,141, *y*-intercept −32.3 and correlation coefficient 0.9998 for PG157-II that indicates a good linearity. The LOD and LOQ of pregabalin have not been investigated.

3.3.2. Linearity

A set of five standard solutions at the following concentration range (30.0, 35.0, 40.0, 45.0 and 50.0 $mg\,l^{-1}$) including analytical range was prepared. Each of them was analyzed in duplicate. The calibration curve was constructed by plotting the peak area against the concentration and the calibration equation was calculated using linear regression analysis. It showed slope 8,534,081, *y*-intercept 419,847 and correlation coefficient of 0.9999 that indicates an excellent linearity. The linearity of calibration curve was investigated using the other statistical tests like the quality coefficient QC [34]. If the quality coefficient QC fulfils the criterion QC < 5%, the linearity of calibration model is demonstrated. The calculated QC was 0.69.

3.3.3. Specificity and selectivity

The specificity of the method was tested by analyzing PGB in presence of the interfering ingredients (excipients such as lactose, starch and silica) showed good separation of the target compounds. No interference due to excipients was detected in the produced chromatogram as shown in Fig. 8. This demonstrates the suitability of the method for routine analysis and quality control of the PGB in pharmaceutical formulations.

3.3.4. Precision and accuracy

The precision of the assay was evaluated by determining real samples of tablets in six repetitive analyses. The sample was prepared under the same conditions as described above (Section 2.5).

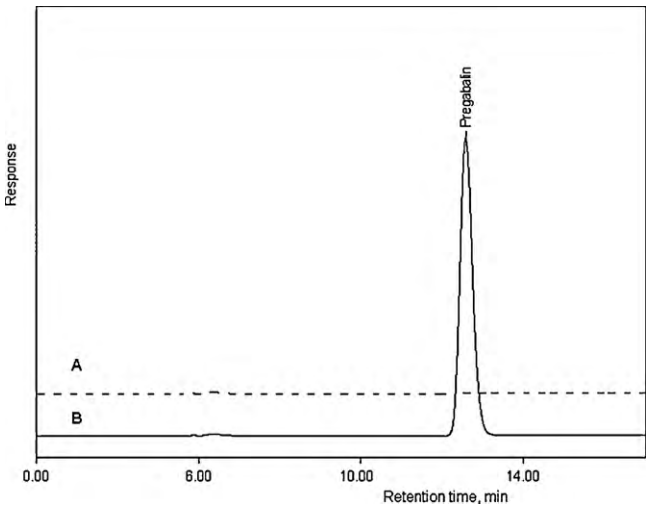

Fig. 8. HPLC chromatograms of PG in real sample of Lyrica tablets (content 25 mg per tablet); A—blank; B—extract of real sample.

Table 3
Analysis of real samples Lyrica 25 mg (Pfizer, USA) (tablets with declared amount of PG 25 mg per tablet).

Sample preparation no.	Assay (mg per tablet)
1	24.93
2	25.01
3	25.03
4	24.96
5	24.96
6	25.03
Average	24.99
Relative standard deviation (%)	0.17

The results are shown in Table 3. The intermediate precision of the method was not studied. The traditional accuracy of the proposed method was not studied but the accuracy of method was verified using analysis of real samples of different commercial pharmaceuticals formulations. Table 4 shows a comparison of assay values with declared contents in commercial samples. The obtained results are according to declared value of PGB (as recovery) in commercial samples.

4. Conclusion

The article describes the first reversed-phase HPLC determination of pregabalin and its possible impurities in pharmaceutical preparations in which postcolumn *o*-phtaldialdehyde OPA/2-mercaptoethanol reaction was used for derivatizing PGB. The high sensitivity, combined with high robustness of postcolumn derivatization, good reproducibility, and high sample throughput, makes this method ideal for the routine pharmaceutical analysis of PGB.

Table 4
Assay of pregabalin in four different commercial samples (tablets with different content of active substance).

Sample	Declared amount (mg per tablet)	Found amount (mg per tablet)	Recovery (%)	Confidence interval (±mg)
Lyrica tablets (Pfizer, USA)	25	24.99	100.0	0.18
	50	50.02	100.0	0.31
	100	100.10	100.1	0.85
	200	198.95	99.5	0.92

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