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Rapid hydrophilic interaction chromatography determination of lysine in pharmaceutical preparations with fluorescence detection after postcolumn derivatization with *o*-phtaldialdehyde

Michal Douša[a,*], Jiří Břicháč[a], Petr Gibala[a], Petr Lehnert[a,b]

[a] Zentiva, a.s. Praha, U Kabelovny 130, 102 37 Praha 10, Czech Republic
[b] Department of Analytical Chemistry, Palacký University, Tř. Svobody 8, 771 46 Olomouc, Czech Republic

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ABSTRACT

A rapid procedure for the determination of lysine based on hydrophilic interaction chromatography (HILIC) separation of arginine and lysine with fluorescence detection has been developed. The separation conditions and parameters of lysine postcolumn derivatization with *o*-phtaldialdehyde (OPA)/2-mercaptoethanol were studied. The various HILIC columns were employed using isocratic elution. Fluorescence detection was performed at excitation and emission wavelength of 345 nm and 450 nm, respectively. An advantage of the reported method is a simple sample pre-treatment and a quick and very sensitive HPLC method. The developed method was successfully applied for analysis of commercial samples of Ibalgin Fast tablets (Zentiva, Czech Republic).

1. Introduction

Amino acids are the basic components of proteins, the principal material of skin, muscle, tendons, nerves, blood, enzymes, antibiotics, and many hormones. Lysine is an essential amino acid which has been recognized since 1889. Lysine is important for proper growth and it plays an essential role in the production of carnitine, a nutrient responsible for converting fatty acids into energy and helping to lower cholesterol. Lysine appears to help the body to absorb and conserve calcium and it plays an important role in the formation of collagen, a substance important for bones and connective tissues including skin, tendon, and cartilage [1–3].

Lysine is used as a fortifier in order to improve the nutritional values and correct possible deficiencies of this amino acid. The naturally occurring basic amino acid L-lysine has been used extensively to form crystalline salts of acids and basics such as acetylsalicylic acid, ibuprofen, ketoprofen, cephalexin and naproxen [4]. The lysine salts with active pharmaceutical ingredients (API) increase the absorption and bio-availability of relevant API [5–9].

The several methods for determination of lysine in pharmaceutical preparations have been described. The determination of lysine in its cephalexin salt was performed by separation of the two components by ion-exchange chromatography on Amberlite CG50 column followed by colorimetric or fluorimetric detection [10]. An RP-HPLC method using precolumn derivatization procedure with 2,4,6-trinitrobenzoic acid for the simultaneous determination of cephalexin and lysine was described later [11]. The applications of the oxidase biosensors for determination of lysine have been described using direct potentiometric method [12]. Moreover, micellar liquid chromatography method with potentiometric detection using a lysine biosensor was also reported [13]. Thin-layer chromatography [14] was also applied for detection and quantitation of lysine in pharmaceutical preparations. A kinetic spectrophotometric method based on catalytic activity of cobalt during the oxidation of purpurin by hydrogen peroxide in alkaline buffer solution was described for the determination of microquantities of lysine in pharmaceutical samples [15]. The disadvantage of the currently available quantitative methods is time-consuming analysis and using of the unusual instrumentations which might not be available in routine laboratory.

The currently available separation and detection systems 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 [16]. Amino acids are zwitterionic compounds with the pKa of the acid function less than 5 and the pKa of the amino function greater than 8 [17]. Buffered mobile phases of pH 2 bellow 3 suppress carboxylic acid dissociation and leave the amino functions pro-

* Corresponding author at: Zentiva, a.s.Praha, U Kabelovny130, 102 37 Prague, Czech Republic.
E-mail address: hplc@hplc.cz (M. Douša).

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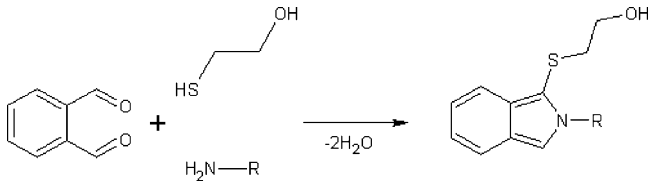

Fig. 1. Derivatization reaction of amino compounds with OPA/2-mercaptoethanol.

tonized. Chromatography of these positively charged free amino acids on reversed-phase supports typically yields poor retention or deteriorated peak-shape [18]. Mobile phase containing ion-pairing reagents (e.g. alkylsulfonic acid) [19,20] can improve retention of such positively charged species, but separation generally suffers insufficient selectivity and reproducibility for routine analyses of complex amino acid mixtures. Hydrophilic interaction chromatography was recently introduced as a useful alternative to RP-HPLC for applications involving polar compounds such as amino acids [21–23].

The reaction of OPA in the presence of 2-mercaptoethanol with primary amino group is displayed in Fig. 1 [24]. 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 [25]. For that reason, the reaction is often applied using postcolumn derivatization [26]. The procedure of precolumn derivatization using OPA with 2-mercaptoethanol at pH 9.5 was successfully described by Jones et al. [27]. The methods of precolumn derivatization with OPA/thiol were most widely used methods for HPLC analysis of amino acids [28,29], however the reproducibility is poor in comparison with postcolumn derivatization.

A major objective of the present study was to find optimum separation conditions for lysine determination using HILIC. Moreover, optimal reaction conditions for postcolumn derivatization of lysine with OPA/2-mercaptoethanol were studied. The other aim of the present work was to develop a quick and sensitive quantitative chromatographic method for the determination of lysine in pharmaceutical formulations. The applicability of the new method was successfully verified by analysis of commercial samples.

2. Experimental

2.1. Reagents

L-Lysine (Lys) and L-arginine (Arg) were obtained from Sigma–Aldrich® (Czech Republic, PhEur 6 grade quality). Acetonitrile of HPLC grade (Merck, Germany) and water purified on Milli-Q system (Millipore, USA) was used for preparing the solutions and mobile phases. All other used chemicals were of analytical grade quality (Sigma–Aldrich®, Czech Republic).

2.2. Apparatus

Sample extraction was performed on a laboratory horizontal shaker KS 130 (IKA Works, Wilmington, USA) and an ultrasonic bath UCC4 (TESON, Slovakia). 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 using postcolumn reaction system PCRS-100 (volume 1000 μl; 0.42 mm I.D.) (Science Instruments and Software, Czech Republic). The reaction system was 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 25 °C. As the postcolumn derivatization reagent, 0.06 mol l^{-1} OPA/0.001 mol l^{-1} 2-mercaptoethanol in 0.05 mol l^{-1} sodium tetraborate, pH 10.5 was used and delivered at a relevant flow rate.

2.3. Chromatographic conditions

HPLC separations were performed on an Atlantis HILIC Silica column (150 mm × 4.6 mm, 3 μm; Waters, Milford, USA), Luna 5μ HILIC (150 mm × 4.6 mm, 5 μm; Phenomenex, Torrance, CA, USA), TSKgel Amide-80 (250 mm × 4.6 mm, 5 μm; Tosoh Bioscience, Montgomeryville, PA, USA), Kromasil 60-5DIOL (250 mm × 4.6 mm, 5 μm; Bohus, Sweden), Nucleosil 100-5 N(CH3)2 (250 mm × 4.6 mm, 5 μm; Macherey-Nagel, Düren, Germany) and ZIC HILIC (250 mm × 4.6 mm, 5 μm; Merck, Germany). Mobile phases were prepared by mixing appropriate volumes of acetonitrile and stock salt solutions to reach desired organic modifier levels and salt concentrations. The buffer stock solution was made by dissolving appropriate amounts of various salts (potassium acetate, formate, citrate and phosphate) in water. The pH of the salt solution was adjusted before mixing with acetonitrile. The analyse were carried out at an appropriate flow rate (0.1–0.8 ml min^{-1}), the injection volume was 10 μl, the column was thermostated at a relevant temperature (20–45 °C). The fluorescence detection was carried out at excitation wavelength of 345 nm and emission wavelength of 450 nm.

2.4. Standard preparation

The extraction solvent was prepared by mixing 950 ml of water and 50 ml 36% hydrochloric acid, the dilution solvent was prepared by mixing 300 ml of water and 700 ml of acetonitrile. The standard of Lys and Arg was dissolved in extraction solvent to a concentration of 500 mg l^{-1} to obtain the standard stock solution. The standard stock solution was dissolved in dilution solvent to a concentration of 5 mg l^{-1} to obtain working stock solution.

2.5. Sample preparation

The samples were powdered and an aliquot corresponding to 100 mg of Lys was weighed into a 100 ml volumetric flask. 25 ml of water was added, mixed for 2 min and then 60 ml of extraction solvent was added and sample was extracted for 10 min in an ultrasonic bath. After cooling to the laboratory temperature, the flask was filled in by extraction solvent. The obtained extract was filtered through a membrane PVDF filter 0.45 μm (Millipore, USA). The solution was diluted with dilution solvent to a concentration of 5 mg L^{-1} and injected into the liquid chromatograph.

3. Results and discussion

The composition of the final mobile phase was optimized to achieve the capacity factor $k \geq 2.0$, symmetry factor $A_S \leq 1.3$, selectivity $\alpha \geq 1.1$ and resolution between Lys and Arg $R_S \geq 2.0$. The retention and chromatographic data were calculated in agreement with European Pharmacopoeia 6.4 and there was determined using calibration solutions of standard on column Luna 5μ HILIC. The resolution was a critical parameter and therefore it was applied as system suitability test. Chromatographic conditions such as mobile phase pH, organic modifier content, salt type and concentration and temperature have significant effects on the chromatography behavior of polar compounds under HILIC [30] and thus conditions were optimized.

Table 1
The effect of salt type on retention data of Lys.

10 mM potassium buffer	*N*	*k*	Symmetry factor A_S
Acetate	2117	3.68	2.00
Formate	2550	2.82	1.45
Citrate	2725	2.80	1.38
Phosphate	3503	3.38	1.31

3.1. The effect of salt type and buffer content

Many salts typically used in RP-HPLC are not suitable for HILIC due to poor solubility in the mobile phase containing high level of acetonitrile. This study investigated the effect of various potassium salts, namely, acetate, formate, citrate and phosphate on the retention of Lys. The mobile phase consisted of acetonitrile and relevant 10 mM potassium salt (275 + 725, v/v). The pH of the salt stock solution was 6.0 for all buffers and the temperature of separation was 35 °C. The type of different potassium salts had significant impact on the retention and on the column efficiency (expressed as *N*) (see Table 1). The potassium formate and acetate exhibit low column efficiency which can be explained by low buffer capacity of these buffers under the experimental conditions. In addition to salt type, the effect of salt concentration on the retention and column efficiency of Lys was also investigated by varying potassium phosphate concentration from 5 to 15 mM in the mobile phase acetonitrile/water (275 + 725, v/v). The temperature of the separation was hold at 35 °C. Further increase in the salt concentration was not possible due to solubility limitation in the mobile phase. It was observed that the higher salt concentration decreased the elution strength of the mobile phase, thus leading to increasing retention and dramatically decreasing column efficiency (Fig. 2A). The partitioning model for HILIC assumed the presence of water-rich liquid layer on the stationary phase surface [31]. Mobile phases containing high amounts of organic solvent make the salt prefer to be in the water-rich liquid layer. Higher salt concentration would drive more solvated salt ions into the water-rich liquid layer resulting to an increase in volume or hydrophilicity of the liquid layer [30]. This might lead to stronger retention of the solutes.

3.2. The effect of buffer pH and column temperature on retention

Similar to reversed-phase separation, HILIC separation commonly employs mixture of water and acetonitrile as the mobile phase, but requires much higher organic content to ensure significant hydrophilic interaction [31]. The content of organic solvent in the mobile phase is the factor that has the largest influence on retention.

All HILIC columns used in this study were silica based. In HILIC, ionization of different groups attached to the silica surface and silanol on the silica surface can be an important contributor to the analyte retention. Mobile phase pH has very significant influence on retention and selectivity in HILIC by affecting solute ionization. In this study, the effect of buffer pH and buffer content on retention of Lys was investigated. The buffer volume fraction (φ) in the mobile phase was adjusted along with appropriate changing the pH of buffer (potassium citrate/phosphate) while keeping buffer concentration constant at 5 mM. Temperature was held constant at 35 °C. The low salt concentration was selected due to the low solubility of salt in the mobile phase with high acetonitrile content.

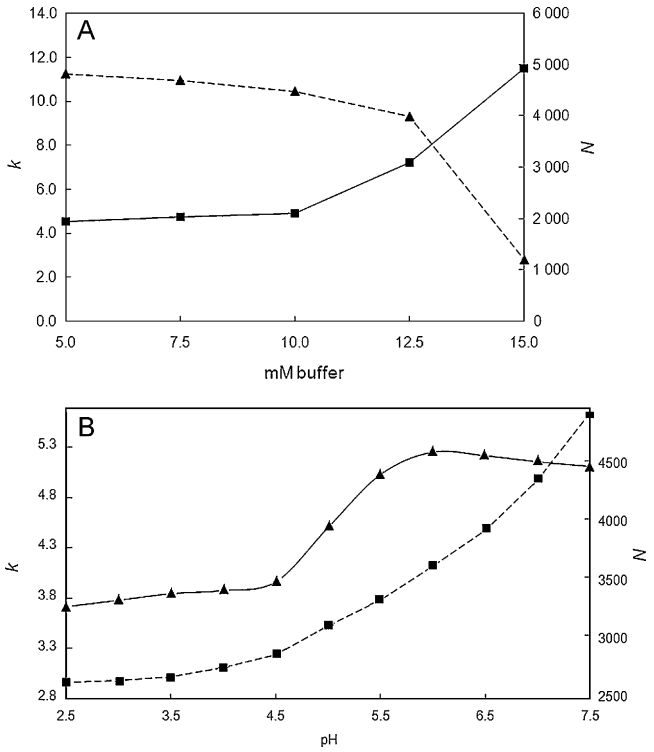

Fig. 2. (A) Influence of buffer concentration on capacity factor *k* (■) and column efficiency *N* (▲) of Lys. (B) The effect of buffer pH on the capacity factor *k* (■) and column efficiency and column efficiency *N* (▲) of Lys.

At first, the capacity factor and column efficiency (expressed as *N*) of Lys for different buffer pH in pH range from 2.5 to 7.5 under constant buffer volume fraction (φ = 0.25) were measured. Fig. 2B illustrates the effects of buffer pH on the retention and column efficiency. In general, increasing of the buffer pH caused an increased in the retention and efficiency of Lys. The observations can be explained by the electrostatic interaction between Lys and the stationary phase surface. In this case, the ion exchange as secondary retention mechanism might play important role due to ionization of Lys, which is largely positively charged under the experimental conditions. The retention and efficiency were sensitive to changes of pH especially in the region of pH from 4.0 to 5.8. Therefore, this region was selected for farther study.

In RP-HPLC, Eq. (1a) can be used to evaluate the influence of organic solvent fraction φ in mobile phase on retention:

$$\log k = \log k_a - m\varphi \quad (1a)$$

where k_a is the (extrapolated) value of *k* for φ = 0 and *m* is a constant for particular solute [32,33]. This approach was applied also in HILIC, where φ is buffer volume fraction and constant k_a is the

Table 2
The calculated coefficients of Eq. (1a).

	Mobile phase pH													
	4.0		4.3		4.6		4.9		5.2		5.5		5.8	
	Arg	Lys	Arg	Lys	Arg	Lys	Arg	Lys	Arg	Lys	Arg	Lys	Arg	Lys
m	−6.42	−6.98	−6.45	−7.01	−6.59	−7.15	−6.89	−7.71	−7.16	−7.85	−7.76	−8.54	−8.65	−9.49
k_a	107.55	169.22	118.07	186.31	132.51	209.44	171.82	319.68	219.71	377.02	336.79	609.84	607.11	1132.05
r	−0.9951	−0.9948	−0.9949	−0.9949	−0.9940	−0.9942	−0.9979	−0.9959	−0.9934	−0.9934	−0.9922	−0.9923	−0.9892	−0.9885

extrapolated value for $\varphi = 0$ (acetonitrile as mobile phase only). The calculated coefficients of Eq. (1a) for buffer volume fraction $\varphi = 0.200–0.275$ for different pH buffer (in range 4.0–5.8) are shown in Table 2.

The effect of buffer volume fraction in mobile phase on separation selectivity (α) is following:

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

where the coefficients are the same as in Eq. (1a). Due to the difference in values of constants m in Eq. (1b) the volume fraction of buffer has significant influence on selectivity of separation. The buffer pH has no significant influence on selectivity of separation (see Fig. 3A). The above mentioned equations allow prediction of retention and selectivity of Arg and Lys in studied chromatographic system (for mentioned ranges of buffer volume fraction). It was observed very good agreement with theory and calculated difference between theoretical and experimental data was not more than 0.9%. Fig. 3B illustrates the significant influence of buffer volume fraction on resolution between Arg and Lys due to changes in separation selectivity.

The effect of temperature on the retention in RP-HPLC has been previously examined, e.g. by Melander et al. [34]. The expected temperature dependence of retention can be expressed using van't Hoff's equation [35]:

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

where ΔH°, ΔS° is standard enthalpy and standard entropy, respectively, 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. If the retention of polar compounds in HILIC is through partitioning between the mostly organic mobile phase and a water-rich liquid layer on the packing surface as proposed by Alpert [31], the van't Hoff's equation should apply to HILIC. In this study linear van't Hoff plot was obtained over narrow temperature range (20–45 °C) by varying the buffer volume fraction in the mobile phase (0.200–0.275) while keeping mobile phase pH constant at pH 4.6 under constant salt concentration at 5 mM. The calculated coefficients of Eq. (2a) for temperature range 20–45 °C are mentioned in Table 3. As expected, retention of Arg and Lys decreased with increasing temperature. The influence of temperature on separation selectivity (α) can be expressed using following equation:

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

where the coefficients are the same as in Eq. (2a). The temperature has no significant influence on separation selectivity of Lys and Arg due to slight difference of constants B in Eq. (2b). The influence of temperature on resolution between Arg and Lys is negligible due to the small change selectivity with temperature and the main contribution to resolution is volume fraction of acetonitrile (Fig. 3C). Nevertheless, the above mentioned equation allows prediction of Lys and Arg retention in studied chromatographic system (for investigated ranges of temperature). With these, very good agreement between theory and experimental data was found. The calculated difference between theoretical and experimental data was not more than 1.0%.

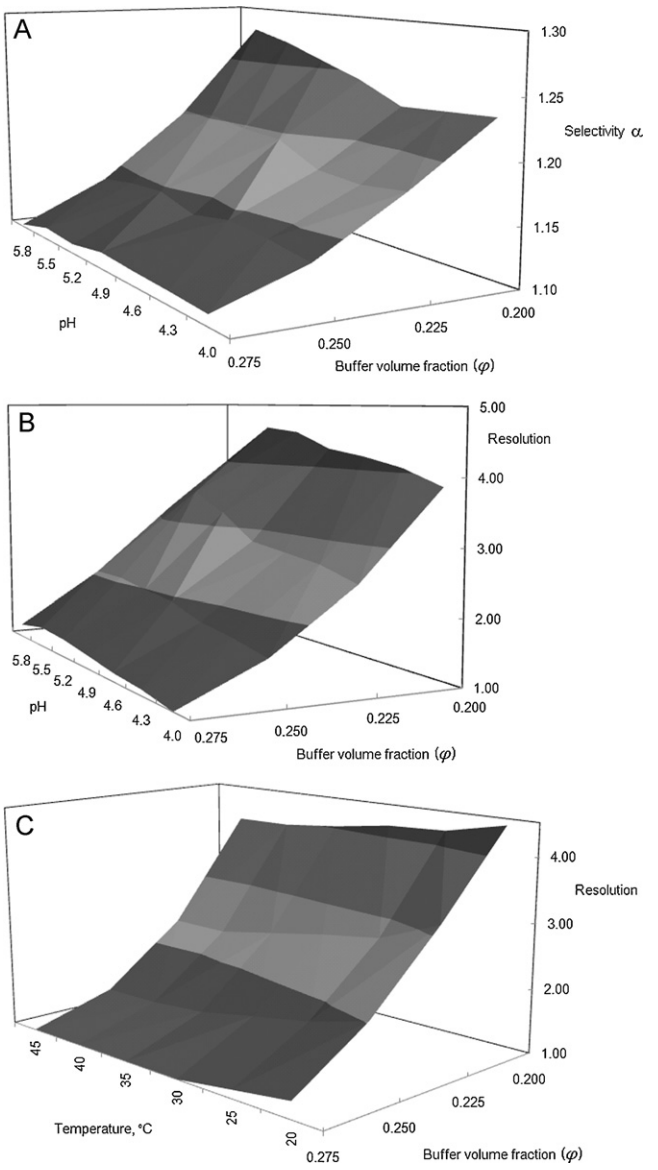

Fig. 3. (A) Influence of buffer pH and buffer volume fraction (φ) on selectivity. (B) Influence of buffer pH and buffer volume fraction (φ) on resolution between Arg and Lys. (C) Influence of temperature and buffer volume fraction (φ) on resolution between Arg and Lys.

The optimal mobile phase contains 225 volumes of 5 mM potassium citrate buffer adjusted to pH 4.6 with phosphoric acid and 775 volumes of acetonitrile.

Table 3
The calculated constants of van't Hoff equation (2a) for temperature range 20–45 °C.

Buffer volume fraction (φ)	Arg			Lys		
	A	B	Correlation coefficient r	A	B	Correlation coefficient r
0.200	−2.330	1271.6	0.9765	−2.293	1323.6	0.9682
0.225	−2.747	1247.9	0.9860	−2.862	1331.9	0.9824
0.250	−2.940	1190.3	0.9896	−3.128	1286.3	0.9868
0.275	−2.849	1072.5	0.9880	−3.111	1182.6	0.9872

Table 4
The effect of flow rate on retention data of Arg and Lys.

Flow rate	Analysis time (min)	*N*	Resolution	Selectivity
0.1	115.1	5963	2.75	1.19
0.2	57.9	6275	2.71	1.18
0.3	38.9	6367	2.63	1.18
0.4	29.3	6359	2.54	1.18
0.5	23.4	6350	2.51	1.17
0.6	19.1	6300	2.40	1.17
0.7	16.2	6250	2.46	1.17
0.8	14.1	6200	2.43	1.16
0.9	12.5	6100	2.37	1.16
1.0	11.2	6150	2.30	1.16
1.1	10.0	6100	2.30	1.16
1.2	8.9	6050	2.29	1.16

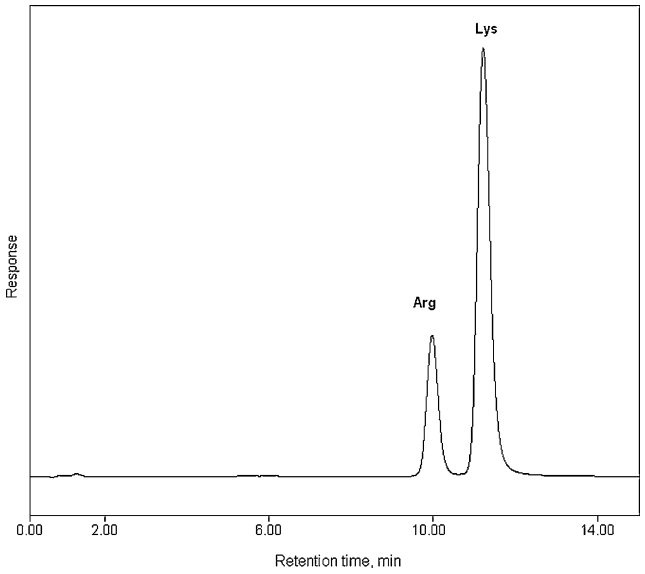

Fig. 4. The HILIC separation of Arg and Lys under method conditions on column Luna 5μ HILIC (mobile phase composition: 5 mM potassium citrate buffer adjusted to pH 4.6 – acetonitrile, 225 + 775 (v/v), 1 ml min^{-1}).

3.3. The effect of flow rate on retention data

The influence of flow rate on selected retention data (retention time, column efficiency, selectivity and resolution) was investigated. The optimal composition of mobile phase described above was chosen. The flow rate of mobile phase was varied from 0.1 to 1.2 ml min^{-1}. The flow rate has no significant influence on retention data and flow rate at 1.0 ml min^{-1} was preferred to achieve acceptable analysis time (Table 4). The separation Arg and Lys under optimal conditions is shown in Fig. 4.

3.4. Selectivity of polar stationary phases

The all experiments described above were carried out on diol stationary phase (Luna 5μ HILIC). Lys and Arg contains carboxyl and amino groups which may interact with the diol phase trough mechanism such as hydrogen bond formation. The retention behavior of Lys and Arg on different stationary phases (Atlantis HILIC – silica phase; TSKgel Amide-80 – amide phase; Kromasil 60-5DIOL – diol phase; Nucleosil 100-5 N(CH3)2 – dimethylaminopropyl phase; ZIC HILIC – sulfobetaine phase) was investigated. The surface chemistry of the stationary phase in these columns is very different hence the different retention mechanism (ion exchange) can be asserted. In this study, the mobile phases were prepared by mixing appropriate volumes of acetonitrile and 5 mM potassium citrate buffer adjusted to pH 4.6. The buffer content in mobile phase was adjusted so that the capacity factor of Lys was comparable in all investigated chromatographic systems. The retention data are illustrated in Table 5. The ZIC-HILIC and Silica columns showed larger retention for Lys with comparable column efficiency; however, the selectivity of ZIC-HILIC was poor in comparison with silica stationary phase. The best selectivity exhibits dimethylaminopropyl phase (Nucleosil 100-5 N(CH3)2) whereas the column efficiency is remained unchanged. The HILIC TSK gel amide was not suitable for separation of Arg and Lys under these conditions. The Luna 5μ HILIC, Nucleosil 100-5 N(CH3)2 and Atlantis HILIC Silica columns are suitable for separation of Lys and Arg with regard to obtained retention data (especially selectivity).

3.5. 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. The post-column derivatization was performed using capillary coil reactors, because this type of the reactor is the most suitable for fast reaction.

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 derivatization reagent flow rate. The linear dependence of residence time (s) versus relative detector response (%) was calculated using linear regression analysis. The dependence showed slope 2.07, *y*-intercept 37.8 and correlation coefficient of 0.9994. The optimal flow rate was of 0.2 ml min^{-1}.

The repeatability (expressed as relative standard deviation) of the derivatization reaction and of the retention time was evaluated by relative standard deviation of eight repetitive injections of working stock solution. The retention time repeatability of Lys was 0.16% and area repeatability was 0.81%.

3.6. Validation parameters

The method was validated according to ICH Q2(R1) guideline [36].

Table 5
Selectivity of different polar stationary phases – retention data.

Column (stationary phase)	Volume fraction of buffer in mobile phase (φ)	Capacity factor *k*	Column efficiency *N*	Resolution R_S	Selectivity (α)	Symmetry factor A_S
HILIC Diol Luna	0.225	4.58	8728	2.62	1.17	1.16
HILIC TSK gel amide	0.300	4.55	6432	1.97	1.10	1.74
Diol Kromasil	0.260	4.50	11 542	2.86	1.14	1.18
Atlantis HILIC Silica	0.325	4.56	8447	3.06	1.19	1.19
Nucleosil 100-5 N(CH3)2	0.310	4.54	9307	4.22	1.25	1.01
ZIC-HILIC	0.360	4.57	9463	1.99	1.10	0.97

Table 6
Assay of Lys in three different commercial samples.

Sample	Declared amount of lysine (%)	Found amount (%)	Recovery (%)	Confidence interval (± %)
Clonixin lysinate	35.75	35.56	99.47	0.18
DL-Lysine acetylsalicylate	37.84	37.88	100.11	0.11
Cephalexin lysinate	29.61	29.42	99.36	0.15

3.6.1. System suitability

The system suitability test is performed to assure that the analytical method can be executed within the existing HPLC system. The system suitability test of the chromatographic system was performed before each validation run. The area and retention time RSD and resolution between Arg and Lys, for the five injections of a system suitability/calibration standard (at concentration of 5 mg l^{-1}) were determined. For all samples analyses, the resolution more than 2.5 was achieved, RSD of peak area was ≤1.2% and RSD of retention time was ≤0.7%.

3.6.2. Linearity and range

A set of six standard solutions at the concentration range of 2.0, 3.0, 4.0, 5.0, 6.0 and 8.0 mg L^{-1} was prepared. The linearity range corresponds to the range of analytical method. Each sample was analysed 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 6 074 337, *y*-intercept 1 383 404 and correlation coefficient of 0.9991 that indicates an excellent linearity. The linearity of calibration curve was investigated using the other statistical tests like the quality coefficient QC [37]. If the quality coefficient QC fulfils the criterion QC < 5%, the linearity of calibration model is demonstrated. The calculated QC was 0.96%.

3.6.3. Precision, accuracy and determination in commercial preparation

The precision of the assay was evaluated by determining real samples of tablets (Ibalgin Fast 400 mg, Zentiva, Czech Republic), in six repetitive analyses. The samples were prepared under the same conditions as described above (2.5 sample preparation). The precision was expressed as RSD and it was 0.61%.

Model samples consisting of placebo and active substance (API) with different additions of Lys (80, 100 and 120% of specification limits) were prepared to test the accuracy of the developed method. For each level, three analyses were performed. The average overall recovery of Lys was 99.5% with a RSD of 1.1%. Determined contents (c_d) were compared with expected ones (c_e) using linear regression. The regression equation (significance level $P = 0.95$) was $c_d = (3.26 \pm 37.90) + (0.973 \pm 0.239)c_e$ and $R^2 = 0.9998$. The first and second constants were not statistically different from zero and one, respectively. It can be concluded that analytical method gives accurate results for pharmaceutical preparations.

The developed method was applied to the determination of Lys in commercially available samples – clonixin lysinate, L-Lys acetylsalicylate, cephalexin lysinate (Chemos, Czech Republic). All samples were measured in triplicate. Table 6 shows a comparison of assay values with declared contents in commercial samples and the results are in good agreement with theory.

3.6.4. Selectivity

The specificity of the method was tested by analysing Lys in the presence of the interfering ingredients (ibuprofene and excipients such as lactose, starch, silica, cellulose, hydroxypropyl cellulose and sodium stearyl fumarate) showed good separation of the target compounds. The selectivity was evaluated by determining real samples of tablets (Ibalgin Fast 400 mg, Zentiva, Czech Republic) as well as (Fig. 5) and the results are mentioned above (3.6.3 Precision, accuracy and determination in commercial preparation). No interference due to presence of active pharmaceutical ingredients and excipients was detected. This demonstrates the suitability of the method for routine analysis and quality control of the Lys in pharmaceutical formulations.

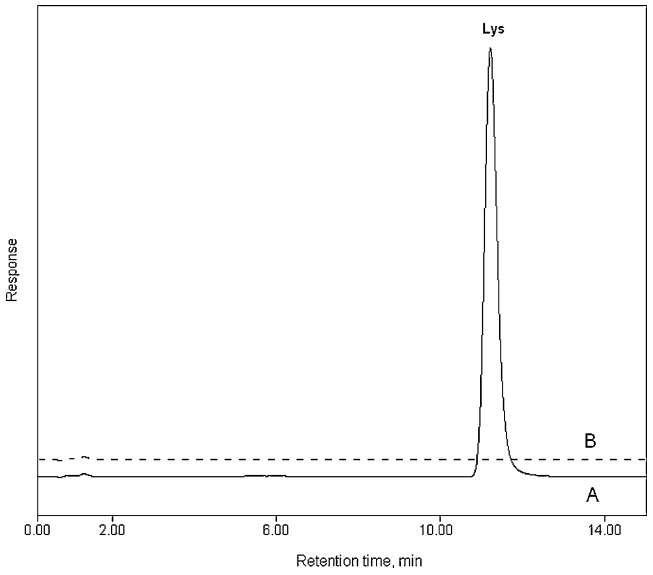

Fig. 5. The HILIC separation of Lys in real sample (content 283 mg Lys per tablet); (A) extract of real sample, dilution = 500; (B) blank. HPLC conditions are described in text.

4. Conclusion

The developed HILIC procedure allows short analysis (below 14 min) with satisfactory fluorescence detection and it is convenient for determination of Lys in pharmaceuticals preparation. In comparison with previously described RP-HPLC methods for determination of Lys, developed HILIC method is very simple, rapid and sensitive enough for determination. The precolumn derivatization step is not required to achieve retention and separation of Lys and Arg. Elimination of interfering compounds, without loss of target analyte, is achieved using selective fluorescence detection. Evaluation of method demonstrates satisfactory statistical parameters for its application to Lys determination in studied matrices. Preparation of samples in series and short chromatographic run also offers the application of developed method in routine laboratory assays. The Arg can be used as an internal standard for determination of Lys and for system suitability testing.

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