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Application of hydrophilic interaction liquid chromatography for the quantification of succinylcholine in Active Pharmaceutical Ingredient and medicinal product. Identification of new impurities of succinylcholine chloride

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Abstract

A new method, using hydrophilic interaction chromatography (HILIC), for quantification of succinylcholine and impurities in Active Pharmaceutical Ingredient (API), as well as in the medicinal product, was developed. Additionally, the new impurities in API were discovered using ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UHPLC-QTOF) technique. The substances were quantified with the application of a UV detector ($\lambda = 214$ nm). Chromatographic separation was performed isocratically with the application of 30% phosphate buffer (pH 4,0; 0.05 M) in

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ACN as a mobile phase. A major feature of the developed method is a very high resolution (Rs $>$ 3), between succinylcholine and its main impurity - succinylmonocholine, whereas the width of peak bands does not exceed 0.7 min. A low value of limits of detection (LOD) were obtained for succinic acid, succinylmonocholine and for succinylcholine, which amounted to 2.4, 6.0 and 11.5 μg ml^{-1}, respectively. Another feature of the developed method is linearity in a very wide range of concentrations: 7.3 μg ml^{-1} − 670 μg ml^{-1} amounting to R^2 = 0.999. The recovery provided by this method at three different fortification levels for all analytes remained within the following range: 95.7 − 98.9 %. Intra-day and inter-day precision remained within the following range: 1.0 − 5.9 % coefficient of variation (CV), whereas accuracy within the range: −1.3 − 6.3. The developed method of hydrophilic interactions made it possible to quantify two new impurities, probably originating from the synthesis of an API: [2-(trimethylaminium)ethyl]-[2′-(trimethylaminium)vinyl] succinate and [2-(dimethylamino)etyl]-[2′-(trimethylaminium)etyl] succinate, which were identified and described for the first time. In addition, a physicochemical form of peak doublet described in the USP as impurities was studied and it was demonstrated that these peaks are the result of the specific physicochemical interactions in ion pair chromatography.

Keywords: Pharmaceutical science, Analytical chemistry

1. Introduction

Succinylcholine chloride (SCh) is a medication, which still finds a wide application in medicine, emergency service, and anesthetic, in order to bring about muscle flaccidity. It is applied, among others, in surgery, in the course of procedures in which it is required to bring about a rapid and short-time muscle paralysis, including endotracheal intubation, endoscopic examinations and electroshocks or convulsive therapy [1]. The mechanism of action of SCh consists in acetylcholine's competing for nicotinic acetylcholine receptor (type 2) of the end myoneural junction and after connecting with this receptor, it induces depolarization inside a muscle cell. In spite of significant side effects and complications connected with the application of this neuromuscular blocker, SCh still remains, among many available on the market, useful in clinical terms as the only neuromuscular blocker which is extremely rapid, and whose action lasts for a very short time [2].

From the point of view of the maximum biological effect of SCh with minimal side effects connected with the application of this neuromuscular blocker, chemical purity plays a very significant role [2, 3, 4]. Research into the chemical quality of succinylcholine chloride is described in British and in American monograph [5, 6]. The United States Pharmacopeia (USP) describes the HPLC method of

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quantifying chemical impurities in Active Pharmaceutical Ingredient (API) and in a pharmaceutical formulation, whereas BPh describes the method of quantifying SCh with the application of conductometric titration. SCh is a chemically unstable compound, particularly in aqueous solutions, in which it undergoes a gradual hydrolysis [7]. The most important products of the degradation of SCh are succinic acid (SA), succinylmonocholine (SmCh) and choline (Chol). For that very reason, the analysis of the quality of API or a medicinal product in terms of these principal impurities is very significant, because it conclusively indicates the safety of those products. In accordance with pharmacopeial requirements set forth in the USP, the contents of SA have to be below the level of 0.1%, of SmCh <0.4%, and that of any different individual impurity may not exceed 0.2% [6]. The presence of known and unknown impurities significantly limits the application of SCh, by intensifying side effects or even reducing the therapeutic effectiveness [1, 8, 9].

The chemical analysis of SCh, including the contents of impurities, is not straightforward, due to its high polarity caused by the double quaternary amine and because of that, the standard chromatographic analysis with the application of reversed phases requires different approaches in terms of separation and detection. The most frequent method of examining the quality of SCh is the ion pair chromatography described in the USP with the application of 1-PenSO_3Na as an ion pair reagent [6]. The disadvantages of this method are very high values of the mass transfer resistance between an active substance and a stationary phase and applying a very high concentration of the examined substance into a chromatographic column. In such a case, a SCh peak is overabundant and very wide (even a several-minute one), which brings about the fact that possible impurities descending in the regions of an active substance in the USP method will be invisible. Taking the above into consideration, the USP method is only suitable for content determination of principal impurities - SA and SmCh. For that reason, a number of analytical methods were developed, for impurities and active substance assay in API or in pharmaceutical formulations. For the separation and quantification impurities and SCh, the following have been applied: ion pair chromatography applying a different a counter couple and electrochemical or conductometric detection [9, 10, 11, 12], liquid chromatography in the system of reversed phases with fluorescence detection [13], UV [14], capillary electrophoresis [15], liquid chromatography combined with mass spectrometry [1, 16, 17] and gas chromatography [8]. The majority of the methods described in the available literature are based on reversed phase liquid chromatography with or without the application of ion pair. Such analytical approach very frequently makes it impossible to separate SCh and products of its degradation.

The analysis of impurities in medicinal products is very important particularly if there exist numerous different methods of the synthesis of API. The process of

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the synthesis of SCh may be conducted in different ways upon the basis of the reactions of: succinic anhydride with choline [18], methylene chloride with bis(2-dimethylaminoethyl)succinate at an elevated temperature [19, 20, 21], 2-dimethylaminoethanol with dimethylsuccinate in the presence of LiNH in a reduced pressure and at an elevated temperature [20], various esters of SA with Chol [22], or succinic chloride with Chol. It means that the probability of the occurrence of substrates or the intermediates of synthesis SCh in API or in a medicinal product is relatively high, which results from the applied process of synthesis and purification. There exists the necessity of having at disposal selective and high-resolution analytical methods in order to conduct more precise qualitative research, so important from the therapeutic point of view in terms of SCh.

The objective of this elaboration was to apply hydrophilic interaction chromatography (HILIC) for quantifying impurities in API and in a medicinal product referred to as SCh, with a classic UV detection. In addition, the application of the same method for quantifying the contents of an active substance (SCh) was investigated. The developed method was fully validated and applied for research into the quality of different APIs and the most common medicinal product available on the Polish market.

2. Materials and methods

2.1. Materials

The research material was constituted by six different API, purchased on the following website: Pharma Compass Grow Your Pharma Business Digitally; for each API, $n = 10$. One product (Suxamethonium Chloride 50 mg ml^{-1} solution for injection) available on the Polish market was researched additionally; the product's $n = 10$.

2.2. Chemicals

Sodium phosphate dibasic 99.95% trace metals basis, phosphoric acid crystalline, $\geq$ 99.999% trace metals basis, acetonitrile (ACN) for UHPLC for mass spectrometry, sodium 1-pentanesulfonate (1-PenSO_3Na) monohydrate for ion pair chromatography $\geq$99.0%, sodium chloride 99.99 suprapur®, sulfuric acid puriss p.a., 95−97%, succinylmonocholine chloride United States Pharmacopeia (USP) reference standard, succinic acid United States Pharmacopeia (USP) reference standard, succinylcholine chloride United States Pharmacopeia (USP) reference standard, sodium formate analytical standard liquid chromatography mass spectrometry grade (1 mM internal standard for high resolution of QTOF) were bought in Sigma−Aldrich (Merck) branch in Poznan, Poland.

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2.3. Methods

2.3.1. Sample preparations for HILIC method

100 mg ± 5 mg of a sample (API or a product) was weighed up into 10 ml measuring flasks and dissolved in a chromatographic phase (in case of the optimized phase composition, the samples were dissolved in a 30% phosphate buffer, pH: 4.0; 0.05 M in ACN). 25μl of a sample was injected onto a chromatographic column.

2.3.2. Sample preparation according to USP method

The samples were prepared in accordance with method specified in the USP [6]. To determine the SCh content, the sample was diluted 25 times to the final concentration approx. 400 μg ml^{-1}.

2.3.3. Impurities and SCh analysis according to HILIC interactions

Chromatographic separation was performed with the application of a liquid chromatography (Thermo Fininigan, model Surveyor Plus^{TM} HPLC system). The HPLC was provided with a two-piston pump, four-component low-side blender, an integrated degasser, an autosampler with integrated thermostat for chromatographic columns and UV/Vis detector (PDA Plus Detection with Patented LightPipe Technology). Chromatographic separation was performed on a HILIC column (250 × 4.6 mm, particle size 5 μm) of the COSMOSIL company. Separation was performed isocratically in the conditions of constant flow rate of 1 ml min^{-1}. The temperature of the chromatographic column was 30 °C. Different composition of mobile phases was applied in order to choose the best conditions of chromatographic separation. A mobile phase was constituted by the following mixture: a 30% phosphate buffer (concentration within the following range: 0.01−0.05 M and the values of pH within the following range: 2,0−5,0) in ACN. The analyzed compounds were quantified with an analytical wavelength of λ = 214 nm ± 0.5 nm [6]. Upon the basis of retention times and peaks width at the basis, the chromatographic system selectiveness (α) and the resolving power (R_s) of the chromatographic system was calculated.

2.3.4. Impurities analysis according to USP method

Analysis of the contents of impurities in the researched APIs and the medicinal product was performed in accordance with the methodology described in the USP. The separation was performed on ACE 5 C18 column (250 × 4.6 mm, 5 μm particle size). The work was performed in an isocratic system in the

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conditions of constant flow rate of 1 ml min^{-1}. Phase composition: 5 % ACN in a buffer composed of 3.85 g of L^{-1} of 1-PenSO_3Na, 2.9 g of L^{-1} of sodium chloride, and 1% of v/v 1N sulfuric acid. 50μl of the analysed solution was injected into the chromatographic column. The separation was conducted at the temperature of 30 °C, whereas the detection was performed with an analytical wavelength of $\lambda = 214$ nm $\pm$ 0.5 nm. In case of the experiment described in the discussion section, chromatographic separation was conducted, as well, in isocratic conditions, but by means of mixing earlier: a buffer (phase A), ACN (phase B) and water (phase C), which was presented in Table 1. The purpose of such a chromatographic approach was the ability to change, rapidly and fluently, the concentration of 1-PenSO_3Na – an ionic pair.

2.3.5. Calculating the contents of impurities

The contents of particular impurities for both of the methods was calculated in accordance with pharmacopeial requirements and with the application of the following equation: $I = 10C\left(\frac{r_i}{r_s}\right)F$ [6]. Response coefficient for SA was 0.63 and for the remaining impurities, F = 1.

Table 1. Composition of phases applied in case of API 5 separation. The per cent share of particular phases for a particular chromatogram was not changed. In order to eliminate the necessity of preparing numerous phases, appropriate mutual phase mixing (low-side) was applied, which is presented in the Table below.

Chromatogram	Phase A [%] 3.85 g L^{-1} 1-pentanesulfonic acid sodium salt, 2.9 g L^{-1} sodium chloride and 1% v/v 1N sulfuric acid	Phase B [%] acetonitrile	Phase C [%] water
1 (USP method)	95	5	0
2	90	5	5
3	80	5	15
4	70	5	25
5	60	5	35
6	55	5	40
7	50	5	45
8	40	5	55
9	30	5	65
10	20	5	75
11	10	5	85

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2.3.6. Preparing the samples of unknown impurities for UHPLC-QTOF analysis

Preparing the samples of unknown impurities consisted in collecting the fractions of unknown peaks identified in some of API samples with the application of the HILIC or the USP method, in accordance with the above-described methodology, into round-bottom flasks having the volume of 25 ml. The collected quantities were 20 or 40 of the fraction of each impurity into flasks, and afterward the solvent was evaporated in a vacuum evaporator at the temperature of 35 °C, condensing the sample to approximately 1 ml. The samples prepared this way were analysed with the application of UHPLC-QTOF injecting 25 μl it into a chromatographic column.

2.3.7. Identification of unknown impurities

The identification of unknown impurities in collected fractions was performed with the application of UPLHC-QTOF (Ultimate 3000 Dionex system consisting of: a pump, a degasser, an autosampler and a column heater, QTOF maXis 4G, Bruker Daltonic). The collected fractions were analysed by the HILIC method described above (2.2.4) using the following modification of the mobile phase composition: 30% ammonium acetate buffer (pH 4.0; 0.025 M) in ACN. The QTOF settings were: electrospray ionization (ESI) in the positive ion mode, dry gas (nitrogen) flow rate 8.0 L min^{-1}, the dry heater 180 °C, nebulizer 1.6 bar, the capillary voltage 4500 V and end plate offset −500 V. MS data were recorded in full scan mode (from 50 to 3000 m/z) and Auto MS/MS mode at the same time. Parameters for Auto MS/MS mode: precursor ions max 5 for threshold ≥1000 cts, fragmentation automatically was adjusted based upon isolation and fragmentation list: for 200 m/z with isolation width 5.0 m/z the collision energy was 20eV, next for 400 m/z with isolation width 5.0 m/z the collision energy was 25eV and for 800 m/z with isolation width 5.0 m/z the collision energy was 35eV. The collision gas was nitrogen with the flow rate 1 ml min [1]. The mass spectrometer was working in the high resolution mode (R = 60000), and the internal calibrant (1 mM aqueous solution of sodium formate) was applied in order to make a precise measurement of mass. Data Processing was carried out with Chromeleon 6.8 and Chromeleon Validation ICH software (Dionex) and microTOF control together with Compass Data Analysis from Bruker Daltonic.

2.4. Method validation

Quantitative analysis was performed by means of the standard-addition method. In this way, besides estimating the unknown amount of the analytes occurring in the different API, it was possible to evaluate sensitivity and linear dynamic range. Recoveries, matrix effect, linearity, accuracy (repeatability and reproducibility), limits

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of detection (LOD) and limits of quantitation (LOQ) were calculated after having determined the levels of SA, SmCh and SCh in the specific API and product.

2.4.1. Preparation of stock and calibration solutions, LOD and LOQ calculation

Primary stock solution of SA (1 mg ml^{-1}), SmCh (1.0 mg ml^{-1}) and SCh (1 mg ml^{-1}) was prepared in 30% phosphate buffer (pH 4,0; 0.05 M) in ACN. Calibration curves were prepared with the mixed intermediate solutions of the analytes and all these solutions were stored at 0 °C ± 1 °C, while protecting it against the light. Six calibration curve points for SA: range from 5.0 to 170 μg ml^{-1}, six calibration curve points for SmCh: range from 8.0 to 160 μg ml^{-1} and six calibration curve points for SCh: range from 20.0 to 670 μg ml^{-1} were prepared. LOD and LOQ were calculated based on standard deviation of the response and the slope of the analytical curve: $LOD = \frac{3.3\sigma}{S}$ and $LOQ = \frac{10\sigma}{S}$ where σ − the standard deviation of the response, and the S − the slope of the calibration curve. Mean LOD and LOQ were calculated from n = 10. Additionally the LOD and the LOQ were calculated for USP method using the same procedure as above, but the analytes were dissolved in mobile phase described in USP [6].

2.4.2. Recovery

The recovery of the analytes was studied with the application of the standard addition method. The recovery was studied on three different levels of enrichment for SA and SmCh: 20 μg ml^{-1}, 40 μg ml^{-1} and 80 μg ml^{-1}, but for SCh: 100 μg ml^{-1}, 150 μg ml^{-1} and 200 μg ml^{-1} on a final solution. The recovery was studied independently for each studied samples. The global recovery was calculated for each level of fortification (the average of all the API and one product with n = 10 was demonstrated).

2.4.3. Matrix effect (MF)

MF was calculated by 100% − ([peak area of studied component in presence of matrix/mean peak area of studied component in absence of matrix] × 100%). Was calculated mean MF, n = 10.

2.4.4. Precision and accuracy

Precision and accuracy were evaluated by intra-day and inter-day parameters. Intra-day precision was calculated as % coefficient of variation (CV) from mean amount of quality control sample (SA, SmCh, SCh USP Reference Standards, n = 10 and the concentration for each component was always 100 μg ml^{-1}) by a single person on the same equipment, under the same conditions, and in a short period of time (one

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day). Inter day precision was calculated as % CV from mean amount of quality control sample (SA, SmCh, SCh USP Reference Standards and the concentration for each component was always 100 μg ml^{-1}) by a three independent people on the same equipment, under the same conditions and in a long period of time (3 days). The accuracy of this analytic method was assessed as the percentage relative error (100 × [found − added]/added]. The analytical standard was added always after the whole procedure of sample preparation and was added always 10 μg ml^{-1} for SmCh, SA and 50 μg ml^{-1} for SCh. The accuracy was measured for one randomly selected API, n = 10. Precision and accuracy was performed according data published previously [23, 24].

3. Results

In Table 2, Figs. 1 and 2, the impact of pH was presented, together with the concentration of a phosphate buffer, on the possibility of separating SA, SmCh and SCh, with the application of a chromatographic column with HILIC stationary phase. Due to the fact that SA and SmCh are substances with similar retention time in developed method, selectiveness (α) and chromatographic resolution (R_s), were calculated in relations to these compounds. The optimum pH, from the point of view of the selectiveness of a chromatographic system, is pH 3.0, whereas that of the resolution of the system pH 3.0 and pH 4.0, in the conditions of a constant ionic strength of a buffer (0.02 M). As well as that, the impact of the varied ionic strength of a buffer in the conditions of pH 3.0 and 4.0, on selectiveness and the resolving power of a chromatographic system, were researched. Increase in the ionic strength of a buffer was accompanied by an increase in the resolving power of the system, whilst maintaining a virtually constant value of α. The greatest chromatographic resolution was obtained when the ionic strength amounted to 0.05 M pH value of 4,0. Increasing the ionic strength of buffer above 0.07 M causes the precipitation of a buffer in ACN and in connection with that it was concluded that the optimum composition of a mobile phase in terms of separating SA, SmCh and SCh is the mixture of 30% of phosphate buffer (pH 4,0; 0.05 M) in ACN. For such a mobile phase composition, the following were obtained: $\alpha = 1.8$, $R_s > 3$, whereas the width of peaks at the basis amounted to ≤0.7 min. In Fig. 3, the comparison of the developed

Table 2. Impact of pH and ionic strength of a buffer on a chromatographic separation of succinic acid and succinylmonocholine chloride in the system of a 30% phosphate buffer in acetonitrile, n = 10.

	pH 2,0 0.02 M	**pH 3,0 0.02 M**	**pH 4,0 0.02 M**	**pH 5,0 0.02 M**	**pH 3,0 0.01 M**	**pH 3,0 0.02 M**	**pH 3,0 0.05 M**	**pH 4,0 0.05 M**
α	2.0^{b}	2.4^{c}	1.5^{a}	1.4^{a}	2.8^{d}	1.9^{b}	1.9^{b}	1.8^{b}
R_s	0.81^{a}	2.19^{c}	2.03^{c}	1.30^{b}	0.87^{a}	2.19^{d}	2.42^{e}	3.39^{f}

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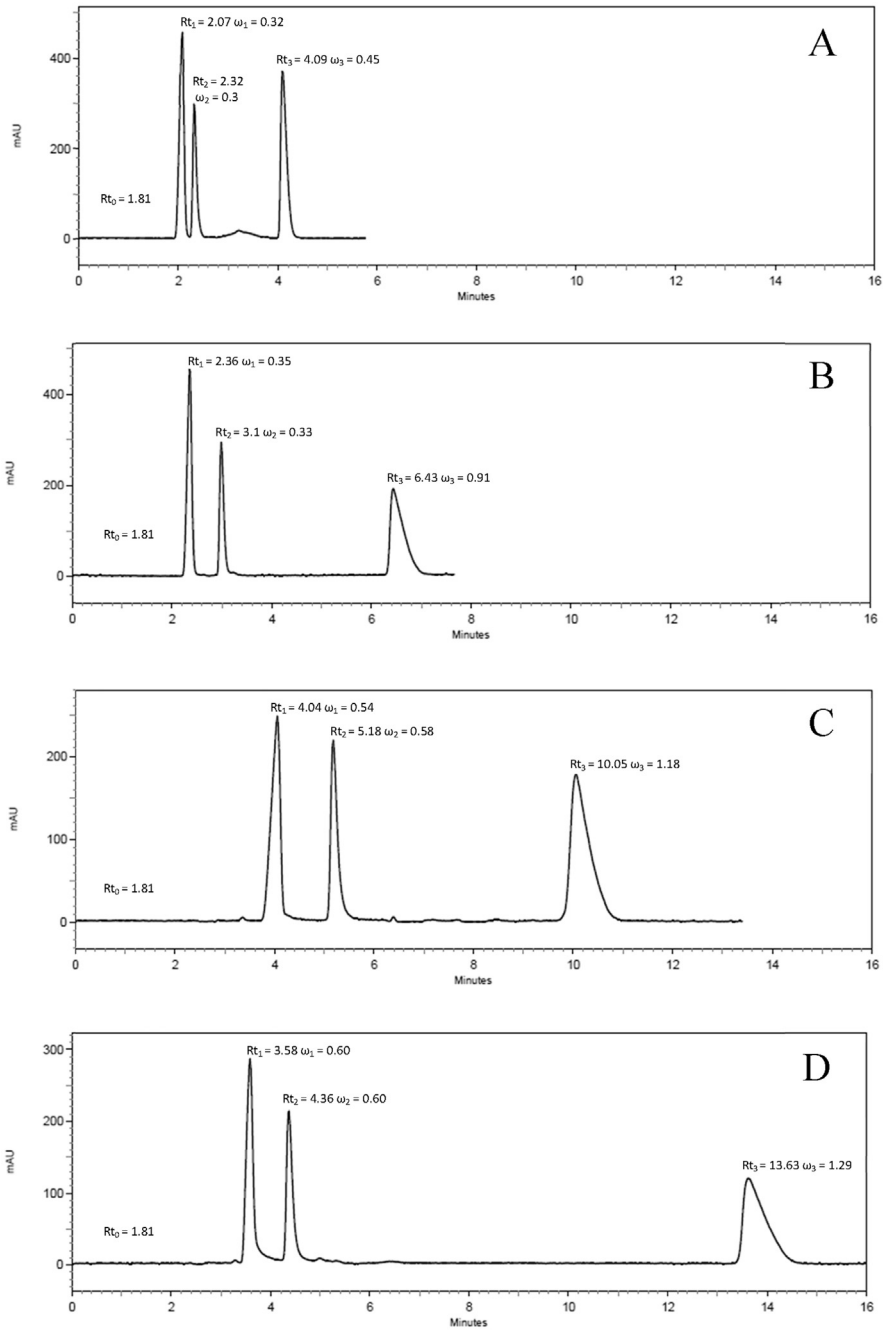

Fig. 1. Mixture of standard settings' chromatographic separation on the following column: COSMOSIL HILIC 250 × 4,6 mm, particle size: 5μm, isocratic separation, mobile phase composition: a 30% phosphate buffer pH = 2,0; 0.02 M (Figure A), pH = 3,0; 0.02 M (Figure B), pH = 4; 0.02 M (Figure C), and pH = 5,0; 0.02 M (Figure D) in acetonitrile. R_{t1} − succinic acid, R_{t2} − succinylmonocholine and R_{t3} −succinylcholine.

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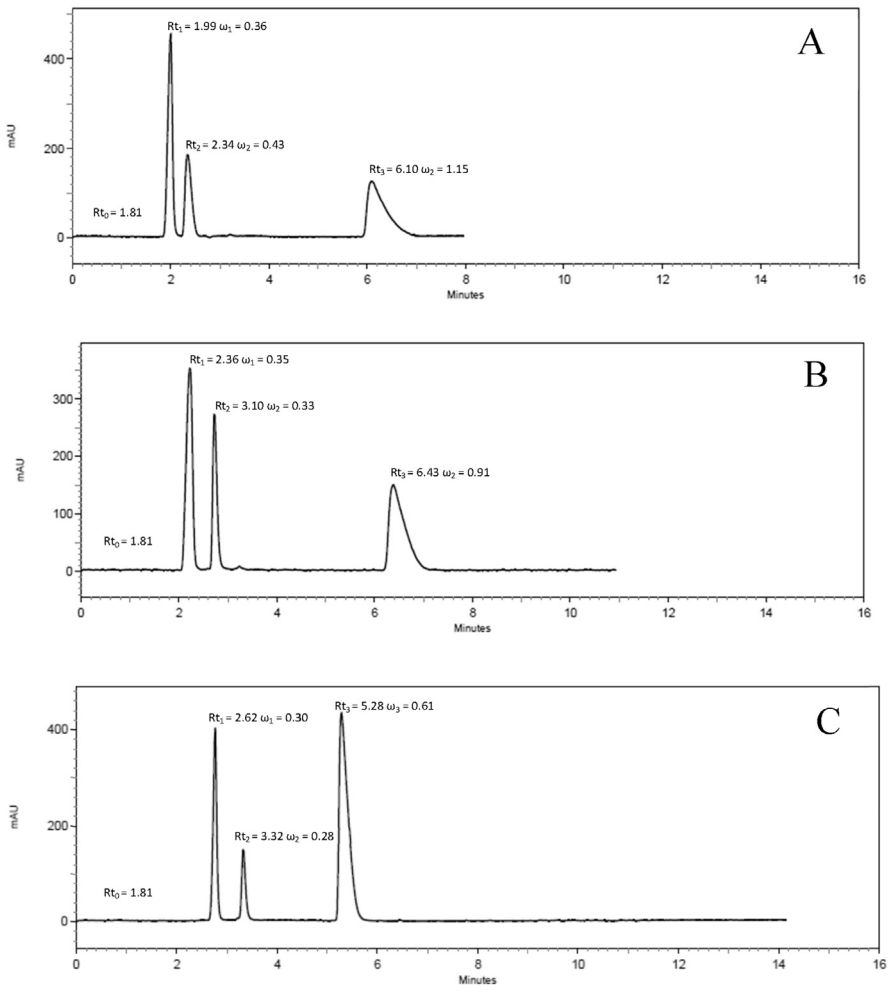

Fig. 2. Mixture of standard settings' chromatographic separation on the following column: COSMOSIL HILIC 250 × 4,6 mm, particle size: 5μm, isocratic separation, mobile phase composition: a 30% phosphate buffer pH = 3,0: 0.01 M (Figure A), 0.02 M (Figure B) and 0.05 M (Figure C) in acetonitrile. R_{t1} − succinic acid, R_{t2} − succinylmonocholine and R_{t3} −succinylcholine.

chromatographic separation method for SA, SmCh and SCh on HILIC column (Fig. 3A) were presented, with the USP method of separation and quantifying impurities in API and a medicinal product (Fig. 3B). In addition, in case of the USP method, a citric acid was added to a mixture of standard solution, because the resolution of a chromatographic system in the USP method is calculated in relations to the peak of SA and to that of the citric one. Chromatographic resolution with the application of a chromatographic column with HILIC phase amounts to ~3.3, whereas of the USP method based upon separation with the application of a C18 phase and ion couples ~1.6. In addition, the HILIC method allows to obtain a narrow and Gaussian peak of SCh contrary to the USP method. Additionally, the application of HILIC phase makes it possible to separate impurities better and it is possible as well to determine the contents of an active substance in API or in a

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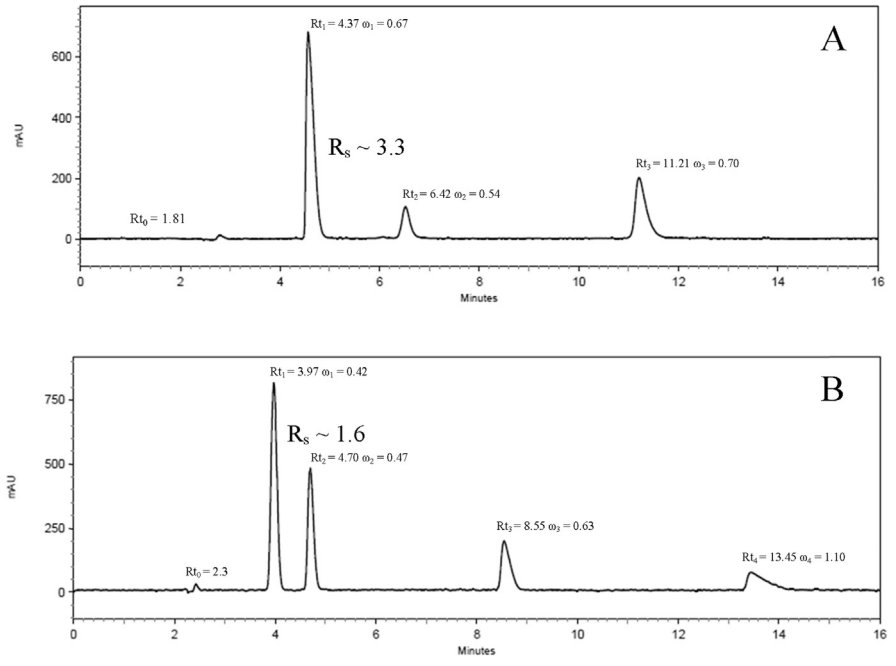

Fig. 3. Mixture of standards in different chromatographic conditions: A: column: COSMOSIL HILIC 250 × 4,6 mm, particle size: 5μm, isocratic separation with composition of mobile phase: 30% phosphate buffer pH = 4,0; 0.05 M in acetonitrile, where R_{t1} − succinic acid (SA), R_{t2} −succinylmonocholine (SmCh) and R_{t3} − succinylcholine (SCh); B: column: ACE 5 C18 250 × 4,6 mm, particle size: 5μm. Isocratic separation, phase composition: 5 % acetonitrile in a buffer composed of 3.85 g L^{-1} 1-pentanesulfonic acid sodium salt, 2.9 g L^{-1} of sodium chloride and 1% of v/v 1N sulfuric acid in accordance with the method in the United States Pharmacopeia (USP), with the application of ion pair chromatography, where R_{t1} − citric acid, R_{t2} − succinic acid (SA), R_{t3} − succinylmonocholine (SmCh) and R_{t4} − succinylcholine (SCh).

medicinal product. The developed method of chromatographic separation was applied for researching the contents of impurities and active substance, in API and in a medicinal product. In Table 3, the validation parameters of the developed method were collated. The validation of the method was conducted for randomly selected API and for a medicinal product, always repeating it 10 times. In Table 3, the mean values obtained in a validation experiment were presented. A major feature of the developed method is sensitivity, which is approximately 3 times higher in relation to the researched compounds in comparison with the USP method. A higher sensitivity of the method makes it possible to reduce the volume of the sample injected into a chromatographic column. The satisfying results were obtained for the injection volume of 25 μl achieving lower LOD and LOQ in comparison with the USP method, in which 50 μl was used. Another feature of the developed method is a very high recovery provided by this method for SA, SmCh, and SCh at three different fortification levels, always amounting to >95%. Matrix effect is virtually not noticeable and is at the level below 1%. Additionally, developed method has a very good linearity in a wide range of concentrations: 6.0 μg ml^{-1} to 670 μg ml^{-1}.

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Table 3. Validation parameters for HILIC method and additionally LOD and LOQ for the United States Pharmacopeia (USP) method, n = 10.

	HILIC method (25μl on column)		
	Succinic acid	**Succinylcholine**	**Succinylmonocholine**
LOD [μg ml^{-1}]	2.4	11.5	6.0
LOQ [μg ml^{-1}]	7.3	34.9	18.3
Additionally LOD and LOQ for USP method (50μl on column)			
LOD [μg ml^{-1}]	**3.6**	**25.6**	**12.8**
LOQ [μg ml^{-1}]	**8.2**	**51.2**	**25.6**
Recovery [%]:			
Spiked level 1	95.1	98.5	96.2
SD	1.2	3.5	1.3
Spiked level 2	98.6	97.3	97.6
SD	1.1	5.3	2.1
Spiked level 3	98.9	95.7	97.1
SD	1.8	6.1	1.9
Matrix effect (ME) [%]	<1	<1	<1
SD	-	-	-
Linear (range in μg ml^{-1} and R^2)	y = 3501x + 5695 7.3−170.0 R^2 = 0.999	y = 1196.3x + 84936 34.9−670 R^2 = 0.995	y = 1792.5x + 16933 18.3−160.0 R^2 = 0.998
Precision and accuracy:			
Intra-day precision in % CV	1.9	3.1	1.0
Intra-day accuracy	1.8	6.3	1.1
Inter-day precision in % CV	1.8	6.9	5.9
Inter-day accuracy %	-0.7	-1.1	-1.3

The precision and accuracy of the method, both in a short and long period of time (intra-day, inter-day precision and accuracy), are very satisfying, and they do not exceed 10 % CV in case of the precision of the method, whereas the measurement error is within the limits: -1.3−6.3. In Tables 4 and 5, the results for the contents of impurities and for an active substance, for the developed method and for the method based upon separation with the application of ion pair compatible with the methodology described in the USP for SCh, were presented [6]. With the application of both methods, the percentage stated contents of SA and SmCh, are similar. However, in case of the USP method, the contents of SA are below the limit of quantification. In the chromatographic method, based upon hydrophilic interactions in API 2 and API 4, the significantly larger contents of other unknown impurities (respectively 0.77% and 3.28%) in comparison with the USP based method were ascertained (for each API and for each medicinal product, the contents of unknown impurities were below 0.2%). In Fig. 4 A−C, the example of a chromatogram for API

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Table 4. Application of HILIC method for determination of succinic acid (SA), succinylmonocholine (SmCh) as impurities [mg g^{-1} and %], other impurities [%] and succinylcholine (SCh) [mg g^{-1}].

	Succinic acid	% as impurities	Succinylmonocholine	% as impurities	% Other impurities	Succinylcholine
API 1	0.3	0.09	2.6	0.32	0.08	978.1
SD	*0.04*	*0.01*	*0.03*	*0.01*	*0.01*	*28.7*
API 2	< LOD (0.10)	0.08	3.1	0.40	0.77	982.8
SD	*0.01*	*0.01*	*0.02*	*0.05*	*0.08*	*15.5*
API 3	0.3	0.09	3.0	0.36	0.12	980.9
SD	*0.03*	*0.01*	*0.1*	*0.02*	*0.01*	*16.2*
API 4	0.3	0.09	2.4	0.30	3.28	988.3
SD	*0.02*	*0.02*	*0.2*	*0.04*	*0.7*	*22.1*
API 5	0.3	0.09	3.0	0.37	0.20	979.4
SD	-	-	*0.1*	*0.07*	*0.04*	*19.3*
API 6	0.2	0.08	2.3	0.29	0.09	972.3
SD	*0.9*	*0.06*	*0.1*	*0.03*	*0.01*	*18.8*
Product 1	0.3	0.09	1.8	0.23	0.10	985.6
SD	*1.1*	*0.02*	*0.07*	*0.01*	*0.01*	*12.4*

Table 5. The United States Pharmacopeia (USP) method for determination of succinic acid (SA), succinylmonocholine (SmCh) as impurities [mg g^{-1} and %] and other impurities [%].

	Succinic acid	% according to USP	Succinylmonocholine	% according to USP	% Other impurities according to USP
API 1	< LOD	0.03	2.8	0.29	0.18
SD	-	*0.01*	*0.03*	*0,01*	*0,01*
API 2	< LOD	0.03	2.3	0.23	0.16
SD	-	*0.01*	*0.03*	*0.02*	*0.01*
API 3	< LOD	0.03	3.2	0.34	0.20
SD	-	*0.02*	*0.02*	*0.01*	*0.02*
API 4	< LOD	0.04	2.3	0.25	0.20
SD	-	*0.01*	*0.02*	*0.03*	*0.03*
API 5	< LOD	0.04	2.4	0.25	0.20
SD	-	*0.02*	*0.01*	*0.02*	*0.01*
API 6	< LOD	0.05	2.1	0.22	0.13
SD	-	*0.01*	*0.1*	*0.03*	*0.01*
Product 1	< LOD	0.04	1.2	0.13	0.13
SD	-	*0.02*	*0.2*	*0.01*	*0.02*

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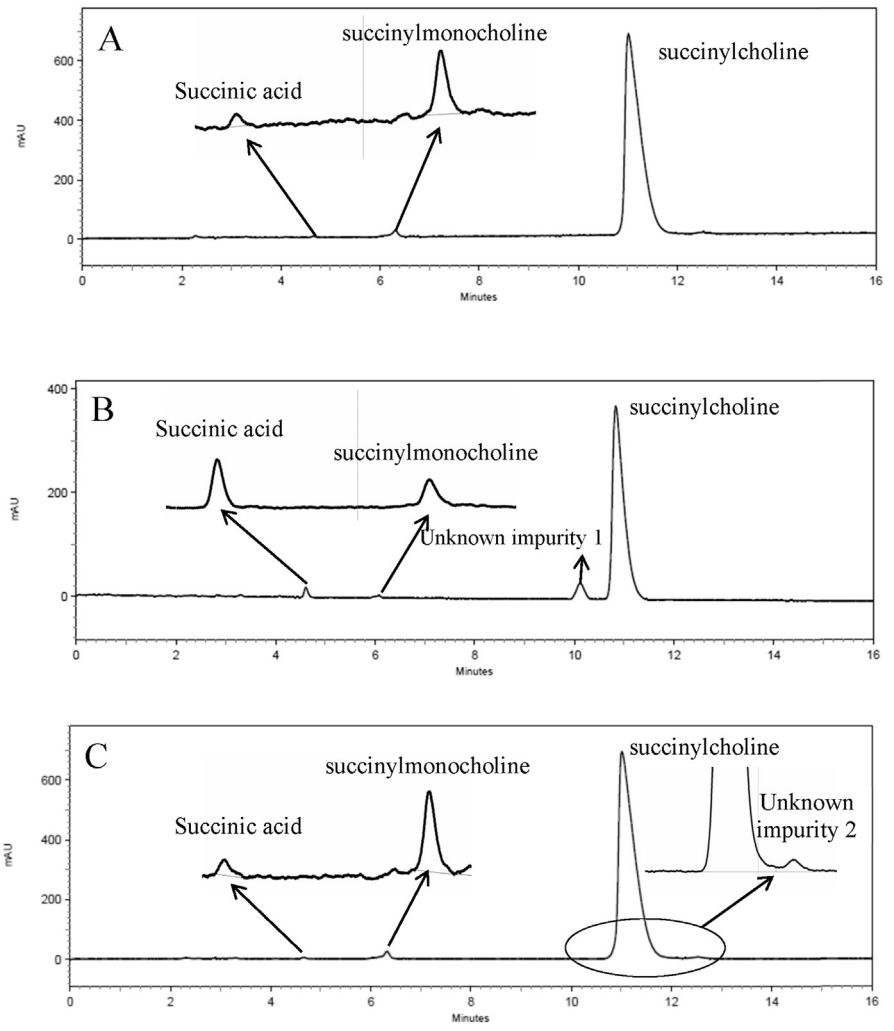

Fig. 4. Examples of a chromatographic separation of various API with quantifying classical chemical impurities for succinylcholine chloride (SCh). Figure A: API 5, and also new revealed impurities, Figure B: API 4 and C: API 2. Column: COSMOSIL HILIC 250 × 4,6 mm, particle size: 5μm, isocratic separation, mobile phase composition: 30% phosphate buffer pH = 4,0; 0.05M in acetonitrile.

was presented; in it, the contents of all impurities are within the acceptable limits in accordance with pharmacopeial requirements (Fig. 4A), and also chromatograms for API 2 and 4, in which the large contents of unknown impurities with the application of the developed chromatographic method, were ascertained. In addition, in Fig. 5, chromatograms for these same API obtained with the application of ion pair chromatography according to USP for SCh were presented. The analysis of impurities in accordance with the method set forth in the USP does not reveal additional peaks in the regions of an active substance, but solely typical doublets of peaks in the region 5−6 min after SA. These doublets may be interpreted as impurities, and match impurities identified with the application of HILIC method. The identified doublets will be discussed in depth in the section: discussion of the results. The developed

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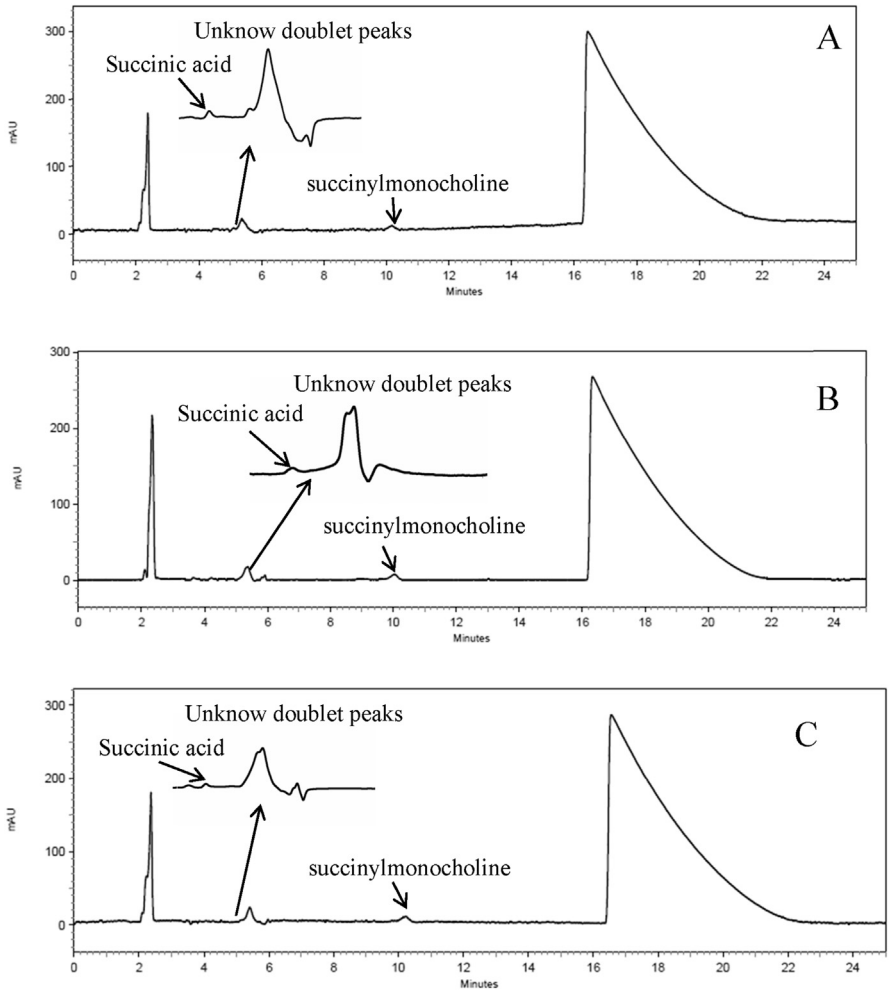

Fig. 5. Example of a chromatographic separation of different API in accordance with the United States pharmacopeia method. Column ACE C18, 250 × 4,6 mm, particle size: 5μm. Isocratic separation. Phase composition: 5 % acetonitrile in a buffer composed of 3.85 g L^{-1} 1-pentanesulfonic acid, 2.9 g L^{-1} of sodium chloride and 1% of v/v 1N sulfuric acid in accordance with the USP with the application of ion pair chromatography. Figure A: API 5, B: API 4) and C: API 2.

method, based upon HILIC separation, made it possible to separate the unknown impurities precisely (Fig. 4B and C), and afterwards, the identification of them with the application of UHPLC-QTOF was done. In Fig. 6, the fragmentation spectra of the masses of the analysed unknown fractions (Fig. 6A and C), with addition of a spectrum for SCh, were presented. The analysis of obtained fragmentation spectra made it possible to ascertain that the first compound at m/z = 144.1021 is a double-charged chemical compound (M^{++}) with two constant electric charges on the two nitrogen atoms of quaternary amine residues having the following molecular formula: $C_{14}H_{28}N_2O_4$, and the actual molecular mass of 288.2042 g mol^{-1}. For the identified compound, the mass measurement error does not exceed 2.0 ppm of the absolute value, whereas the mSigma isotope profile 13.1, thanks to which matching

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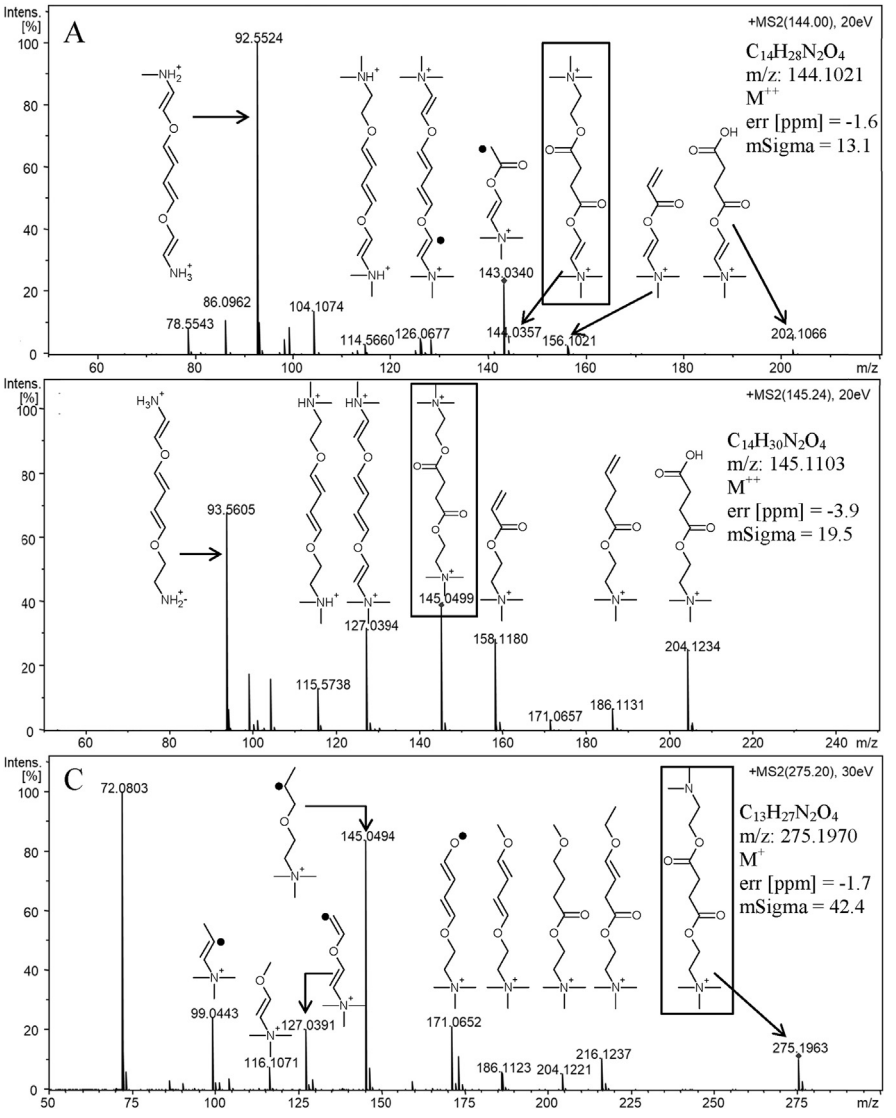

Fig. 6. Spectra QTOF HRMS of new identified impurities: Figure A: unknown impurity I, Figure B: succinylcholine and Figure C: unknown impurity II.

the molecular formula amounts to 99.9999 %. The analysis of the fragmentation spectrum (Fig. 6A) made it possible to build a molecule similar in structural terms to SCh with a single difference consisting in the fact that one of the choline residues contains a double bond between 2nd and 3rd carbon atom in choline carbon chain. The proposed name of the impurity is: [2-(trimethylaminium)ethyl]-[2′-(trimethylaminium)vinyl] succinate. Moreover, the identified molecule undergoes fragmentation very similarly to SCh, which constitutes the additional confirmation of the proposed chemical structure. Impurity 2, whose fragmentation spectrum is visible in Fig. 6C, is a chemical compound having m/z = 275.1970, and being a single-charged molecule (M^+) in the conditions of constant electric charge located on the nitrogen atom of quaternary amine having the following molecular formula

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$C_{13}H_{27}N_2O_4$, and the actual molecular mass of 275.1970 g mol^{-1}. For the identified compound, the mass measurement error does not exceed 2.0 ppm of the absolute value, whereas the mSigma isotope profile 42.1, thanks to which matching the molecular formula amounts to 99.9990% [24]. The analysis of the fragmentation spectrum (Fig. 6C) makes it possible to ascertain that impurity 2 is also similar in structural terms to SCh. However, instead of one of choline residues, one of the carboxyl residues of SA, namely: n-ethane-2-hydroxy-N,N-dimethylamine, is substituted. The proposed name of the impurity 2 is: [2-(dimethylamino)etyl]-[2′-(trimethylaminium)etyl] succinate.

4. Discussion

Developing new methods of a chromatographic separation of chemical substances always requires conducting a number of experiments, the result of which has to be satisfying the initial premises of the method. The premise of the new method of quantifying impurities in API and in a medicinal product referred to as SCh, was obtaining the chromatographic resolution $R_s > 3$, obtaining the narrow symmetrical bands of peaks ($\omega \leq 0.75$), particularly in case of an active substance itself, which would made it possible to, simultaneously, quantify it and the accompanying impurities. Increasing the resolving power of the new method in relation to the one described in the USP would make it possible to control the quality of API itself, as well as that of medicinal products, better from the point of view of the contents of known and unknown impurities in SCh. The application of a chromatographic column based upon HILIC makes it possible to separate very polar, including, as well, strongly ionized, chemical substances in the ACN: water system, which is impossible in the classic approach with the application of reversed phases [25]. Chromatography upon the basis of HILIC interactions is a specific kind of the chromatography of normal phases, in which the largest retention (maximizing hydrophilic interactions: an analyte − a stationary phase) is obtained on such aprotone solvents as acetone, ACN and tetrahydrofuran. Increasing the share of proton solvents such as, water, methanol or ethanol, causes disturbances in hydrophilic interactions: an analyte − a stationary phase, and, *ipso facto*, reduces the retention and elution of analytes from a column [25]. In HILIC chromatography and in other types of chromatography, a mobile phase plays, in combination with a stationary phase, a crucial role in obtaining the satisfying chromatographic effect. However, the application of any desired mobile phase composition in HILIC chromatography is not possible, e.g., from the point of view of the application of a UV detector for quantifying impurities and an active substance. In case of low detection wavelength ($\lambda =$ 214 nm), the possibilities of the application of different mobile phases are very limited due to the absorption of UVs radiation in this range [14, 26]. Essentially, in case of ($\lambda = 214$ nm), chromatographic separation on a HILIC column may be

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performed in ACN, and in water. Separated chemical substances on a HILIC phase are strongly ionic compounds, which requires buffering a mobile phase [25]. The application of phosphate buffer, for which the UV cut off is lower than 190 nm, in the range of 0.01−0.05 M and in range of pH 2,0−5,0 was chosen to obtain the assumed resolving power of the system. Chromatographic separation of ionic substances is strongly dependent upon a pH and ionic strength of phase [27], which was confirmed by conducted experiments, choosing the optimum phase composition: 30% of phosphate buffer (pH 4,0; 0.05 M) in ACN. A major feature of the developed method is a larger sensitivity in comparison with the USP method based upon separation with the application of ion-pair reagent. That is caused by a smaller molar load with dissolved substances in a mobile phase in the HILIC method. Phosphate buffer shows a significantly smaller absorption of UV radiation than 1-PenSO_3Na, whose concentration in the phase of the USP method amounts to approximately 0.02 M. In HILIC method, the molar concentration of phosphates amounts to approximately 0.15 M because the share of phosphate buffer in a phase amount to only 30%. In addition, a major feature of water is a larger UV radiation absorption coefficient (cut off: 190 nm) in comparison with ACN (cut off: 185 nm) [26]. In the HILIC method, the share of water amounts to 30%, whereas in the USP method it is as high as 95%. A high sensitivity of HILIC method makes it possible to reduce the volume injected into a chromatographic column, which is significantly reflected in reducing the width of peaks and their symmetry.

The results obtained with the application of developed HILIC method were set against the results obtained with the application of the USP method based upon separation with the application of ion pair. Both of the methods gave similar results concerning the contents of SA, and of SmCh. However, developed HILIC method made it possible to separate and identify new unknown impurities (Fig. 5A and B), which are not conclusively revealed with the application of the USP method. Identified impurities have never before been revealed in API or in the medicinal product referred to as SCh, even with the application of mass spectrometry for identifying an active substance [1, 8, 16, 17]. It needs to be pointed out that a fragmentary spectrum of SCh is completely compatible with data in the literature, which means that the observed mass is a double-charged ion, and the results of its fragmentation are the formation of both single- and double-charged fragments [16, 17]. Upon the basis of, among others, the mechanism of ionization of the fragmentation of SCh, the identification of unknown compounds visible in Fig. 4 and in Fig. 6, was performed.

The USP monograph describes two unknown peak doublets as specific impurities of SCh, which are visible in Fig. 5, and in Fig. 7 chromatogram 1. An attempt was made to identify this doublet by means of UHPLC-QTOF analysis after their fractioning, precisely the way it was performed for two peaks visible in Fig. 4B and C. Mass spectrometry analysis did not reveal any chemical substances in the range of scanned mass (50−3000 mass-to-charge ratio). The peak doublet described in the USP is not

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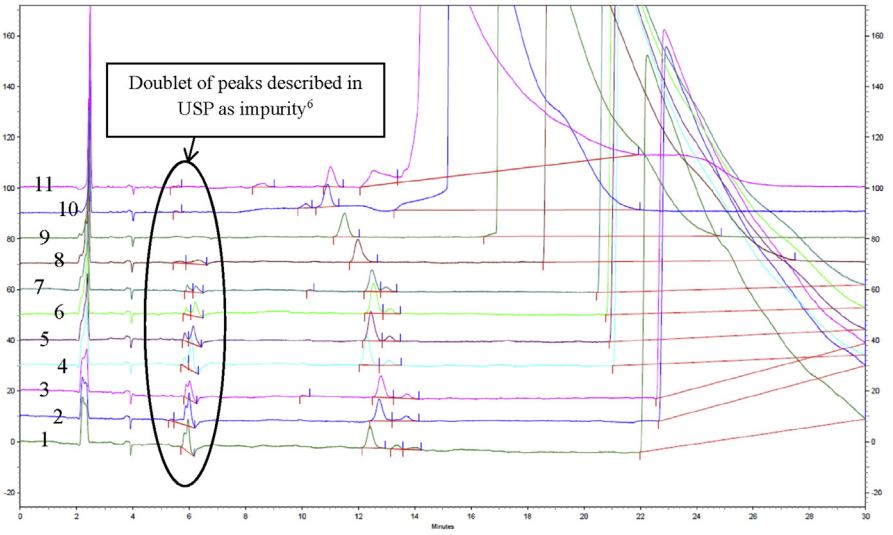

Fig. 7. API 5 concentration of 10 mg ml^{-1} (injection on a column: 50µl) depending on mobile phase composition (different concentration of sodium 1-pentanesulfonate). Column ACE 5 C18 250 × 4,6 mm, particle size: 5µm. Isocratic separation – phase composition: in accordance with Table 1.

made of new compounds identified with the application of the developed HILIC method. The doublets described in the USP for impurities in SCh are most likely to be system peaks connected with specific interactions of ion couples [28]. An experiment confirming this hypothesis was conducted. The experiment consisted in the analysis of an example chromatographic solution, API 5, having the concentration of 10 mg ml^{-1} (in accordance with the requirements set forth in the USP). Chromatographic separation was performed in an isocratic system by means of, respectively, mixing low-side three phase compositions (A, B and C) – Table 1 with the application of the method USP, and described in the section: Materials and methods of research [6]. The results of the experiment were presented in Fig. 7. The conducted research gives rise to the conclusion that reducing the concentration of ion pair reagent (in this case, of 1-PenSO_3Na) significantly impacts reducing the intensity of analysed peak doublets, which suggests that identified peak doublets are formed as a result of the specific interaction of a sample with a mobile and a stationary phase in a chromatographic column, and are not chemical impurities *sensu stricto* as it is described in the USP monograph. Forming peak doublets described in the monograph, and also identified by means of the analysis of API in accordance with the procedure described in the USP, is connected with the mechanism of ion pair itself [6]. In accordance with one of the models of the formation of ion pair, the ions of the dissolved substance A (analyte) interact with L ions (constituting a component of eluent) forming LAL complex (Fig. 8). This complex may be reversibly bound with a non-polar surface of a stationary phase S, which is constituted by so-called 'reversed phase ODS – octadecylsilane phase C18, i.e. a phase having the polarity lower than eluent, resulting in LAL complex of either type: S

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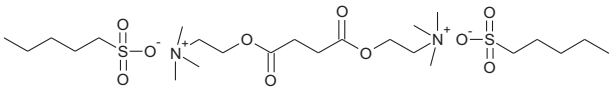

Fig. 8. Ionic pair of a pentosulfonate anion with succinylcholine.

or SLALS. Separated sample ions (in the form of LAL complex, or other ALs, like in case of SmCh) differ from one another in terms of the time of interaction with a stationary phase, which is connected with their different affinity to a non-polar surface of a stationary phase [28]. This phenomenon regarded in general constitutes the basis of chromatographic separation with the application of ion pair. The other theory assumes the existence of another mechanism consisting in the fact that the lipophilous an ion pair residue of an ion pair located in eluent (pentane residues) are adsorbed on the surface of a stationary phase forming LS complex. On the surface of a non-polar stationary phase, a dynamic ionic exchanger, with which the ions of the dissolved substance (A) interact, is formed. In the course of chromatographic separation, an analyte penetrates the inside of a layer formed by LS complexes, interacting with it and forming reversible complexes: ALS, or even SLALS. The time for which the complexes remain in a stationary phase (C18) is determined by adsorption. It should be pointed out that the formation of ion pair may be performed as a result of the following types of physicochemical interactions, whilst omitting interaction with a stationary phase [28]: ion-ion, ion-constant dipole, ion-induced dipole, constant dipole-constant dipole, induced dipole-constant dipole, induced dipole-induced dipole. Depending upon the kind of an interaction mechanism, separation with the application of ion pair will undeniably be different [28]. In case of quantifying SCh, the interactions of an analyte with an ion pair are undeniably ionic. Dissolving a relatively high concentration (approximately 0.3 mol L^{-1}) of SCh in a phase and feeding a relatively large volume of it (50μl) into a chromatographic column causes the formation of typical physical phenomena connected with chromatography of ion pairs, and also the van der Waals interactions typical for the classic reversed phases. In case of the premise behind the first theory of ion pairs, a large volume of free pentanesulfonate residues, and also LAL complexes, is fed, to immediately interact with a stationary phase forming the complexes SLAL and/or SLALA, which, in turn, causes 'throwing out' of pentanesulfonate residues with from active sites of a stationary phase, which are occupied by fed analyte. The excessive quantity of pentanesulfonate (L) may not react with active sites, i.e. ODS chains in a stationary phase, because their applied concentration in a mobile phase is larger than the adsoprtion ability of a stationary phase. Free pentanesulfonate residues interact with one another in the lateral pentane chains undergoing associations. Simultaneously, anionic residues of pentanesulfonate (SO_3^-) exert influence in accordance with the ion-dipole principle induced with silane residues (Si-OH) of a stationary phase. The excessive quantity of associated pentanesulfonates is visible in the form of a system peak visible between 5 a 6 min (Fig. 5, and also Fig. 7). The confirmation of this

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phenomena are the results of the experiments presented in Fig. 7. Gradual reducing of the concentration of an ionic pair causes, to a smaller degree, their interaction with a stationary phase S forming a smaller quantity of LS complexes. A smaller quantity of LS complexes results in increasing the number of active centres on a stationary phase C18, which are able to interact, in accordance with the principle of the van der Waals interaction, with the lipophilous chains of the pentane associates of an ion pair. The result of this phenomena is reducing the intensity of peaks between 5 a 6 min and simultaneous reducing the share of an A phase, i.e. *de facto* 1-PenSO_3Na (ionic pair). Upon the basis of the above considerations, it is not possible to treat the peaks observed in 5−6 min of the USP method as chemical impurities, and solely as system signals being the result of wrongly chosen chromatographic conditions for quantifying impurities in API and in a medicinal product referred to as SCh. The lack of additional impurities ascertained with the application of the USP method in comparison with HILIC method is probably caused by the width of a SCh peak, which amounts to several minutes (Figs. 5 and 7). Such a width results from the fact of overloading a column with an analyte in order to identify impurities, which have a lower concentration. The negative aspect of the USP method applied to identify impurities is probably covering impurities similar, in terms of structure, to an active substance under a wide band. Such a negative phenomenon does not occur in HILIC method, thanks to which it was possible to reveal, and afterwards to identify, new and hitherto unknown impurities in an API. New identified impurities probably originate from the process of the synthesis of SCh, which may take a different course upon the basis of the reaction between succinic anhydride with choline [18], methylene chloride with bis(2-dimethylaminoethyl)succinate at an elevated temperature [19, 20], 2-dimethylaminoethanol with dimethylsuccinate in the presence LiNH under a reduced pressure and at an elevated temperature [20], different esters of succinic acid with choline [22], or succinic chloride with choline. Most likely, the new identified impurities are formed at the stage of synthesis, and *ipso facto,* penetrate API. In accordance with the records of Drug Master File for API 2, the process of synthesis progressed as a result of a reaction of methylene chloride with bis(2-dimethylaminoethyl)succinate, which would make forming impurity II clear. API 4 was synthesised as a result of the reaction of succinic chloride with Chol in the medium of triethylamine and other solvents. Probably as a result of this reaction, there occurs the elimination (in the carbon chain) of Chol, the outcome of which is formation of one double bond in one of the Chol residues and the formation of impurity I.

5. Conclusion

A high-resolution and sensitive method, designed upon the basis of hydrophilic interactions and UV detection, for quantifying impurities and SCh was developed. The

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HILIC method made it possible to separate and identify new unknown chemical impurities ascertained in some API, which formed, most likely, in the process of the synthesis of SCh. The developed method makes it possible to obtain similar results concerning the contents of SA and SmCh, as observed in the USP method. Comparing the results obtained with the application of both of the methods, peak doublets described as impurities in the USP were identified. The described peak doublets were found to be system peaks being the result of physicochemical phenomena occurring in ion pair chromatography. In the period of the dynamic development of the pharmaceutical and chemical industry, there exists the necessity of developing new and better, having a better resolution and more sensitive, separation and detection methods in order to achieve better control and, *ipso facto*, to increase the safety of the API applied in manufacturing medicinal products, which will be reflected in the benefits for patients.

Declarations

Author contribution statement

Arkadiusz Szterk: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Adam Zmysłowski: Performed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Shela Gorinstein: Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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