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Liquid chromatographic analysis of streptomycin sulfate

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Abstract

The analysis of streptomycin sulfate using a column packed with base deactivated reversed phase silica gel and ultraviolet (UV) detection at 205 nm is described. The mobile phase consists of an aqueous solution containing 14 g/l of sodium sulfate, 1.5 g/l of sodium octanesulfonate, 50 ml/l of acetonitrile and 50 ml/l of a 0.2 M phosphate buffer at pH 3.0. The method allows separating streptidine, streptomycin B, streptomycin and dihydrostreptomycin, as well as several other components, which were not yet identified. The total time of analysis is 50 min. The effects of the different chromatographic parameters on the separation were investigated. A number of commercial samples were analyzed using this method. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Streptomycin; Liquid chromatography; Ultraviolet detection

1. Introduction

Streptomycin, produced by *Streptomyces griseus*, was the first aminoglycoside antibiotic described by Waksman et al. in 1944 [1]. It was shown to inhibit the growth of aerobic gram-positive and gram-negative bacteria as well as of tubercle bacilli [2]. Like other aminoglycosides, streptomycin is potentially oto- and nephrotoxic. Whall described a liquid chromatographic (LC) system for the analysis of streptomycin [3]. This method employs ion-pair reversed phase chromatography with ultraviolet (UV) detection at 195 nm. Although, it is mentioned that, it separates streptomycin from streptobiosamine, streptidine, dihydrostreptomycin and streptomycin B, also called mannosidostreptomycin (Fig. 1), a more selective method is desirable for drug purity control because some peaks in the neighborhood of the main peak cannot be well determined. A few years later, the method described by Whall was slightly adapted to determine streptomycin in serum [4]. Also, the determination of the main component streptomycin in pharmaceutical preparations using gas chromatography after silylation [5] and in animal tissue [6] and milk [7] using ion-pair LC combined with fluorescence detection after post-column derivatization with 1,2-naphtoquinone-4-sulfonic acid has been described. The European Pharmacopoeia (Ph. Eur.) [8] as well as the US Pharmacopeia (USP) [9]

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prescribe microbiology as assay technique, but only the Ph. Eur. limits the amount of streptomycin B to 3%, using thin-layer chromatography.

In this study an ion-pair LC method using a column packed with base deactivated reversed phase silica gel and UV detection at 205 nm is described. The chromatographic system used in this study is based on that described for the analysis of dihydrostreptomycin [10]. Besides the related substances mentioned above, also several other impurities are separated. The separation of another possible impurity, hydroxystreptomycin, was also investigated. This can be isolated from *Streptomyces griseocarneus* [11]. Pulsed electrochemical detection (PED) is used to see if no important peaks were overlooked when using UV detection. Also several types of stationary phases are examined. Finally, the chosen method is applied to analyze a number of commercial samples of streptomycin.

2. Experimental

2.1. Reagents and reference samples

Water was distilled twice from glass apparatus. Anhydrous sodium sulfate was obtained from Merck–Hitachi (Darmstadt, Germany); sodium 1-octanesulfonate monohydrate 98%, phosphoric acid 85% (m/m) and 2-methyl-2-propyl methyl ether from Acros Organics (Geel, Belgium); potassium dihydrogen phosphate and ethanol from BDH (Poole, UK) and acetonitrile grade S and tetrahydrofuran (THF) HPLC grade from Rathburn (Walkerburn, UK).

Dihydrostreptomycin was obtained from VMD (Arendonk, Belgium) and hydroxystreptomycin from Abbott Laboratories (North Chicago, IL, USA). Streptidine was prepared by acid hydrolysis of streptomycin [12]. A sample containing 10% of streptomycin B and a house standard of streptomycin sulfate were available in the laboratory. The streptomycin base content of this standard was 66.2% (m/m), expressed on the substance as is. The total base content of this standard was determined by an aqueous potentiometric titration with 0.1 M sodium hydroxide. The water content and the sulfate content were determined as described in the Ph. Eur. [8] and amounted to 12.0 and 20.1% (m/m), respectively. The total mass explained by titration, water and sulfate was 99.5% (m/m). The total content of impurities, determined by LC combined with UV detection and expressed as streptomycin base, was 5.2% (m/m).

Commercial samples of streptomycin sulfate were provided by VMD, Ludeco (Brussels, Belgium), Continental Pharma (Brussels, Belgium), Dopharma (Raamsdonksveer, The Netherlands), Office Chimique (Waterloo, Belgium) and Kela (Hoogstraten, Belgium).

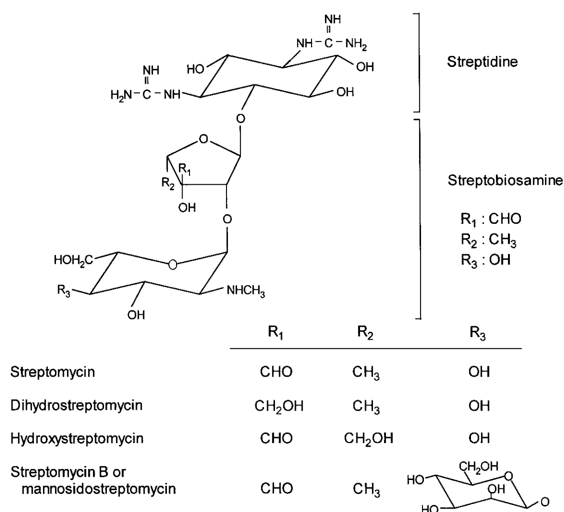

Fig. 1. Structure of some sreptomycin components.

2.2. Apparatus

The chromatographic analysis was carried out using a L-6200 Intelligent pump (Merck–Hitachi), a SpectraSERIES AS 100 autoinjector (Thermo Separation Products, Riviera Beach, FL, USA) with a fixed loop of 20 μl and an electronic integrator HP 3396 series II (Hewlett–Packard, Avondale, PA, USA). The Supelcosil LC-ABZ column (250 × 4.6 mm i.d.), packed with base deactivated reversed phase silica gel (100 Å, 5 μm), was obtained from Supelco (Bellefonte, PA, USA). The column was immersed in a water bath with a heating circulator (Julabo, Seelbach, Ger-

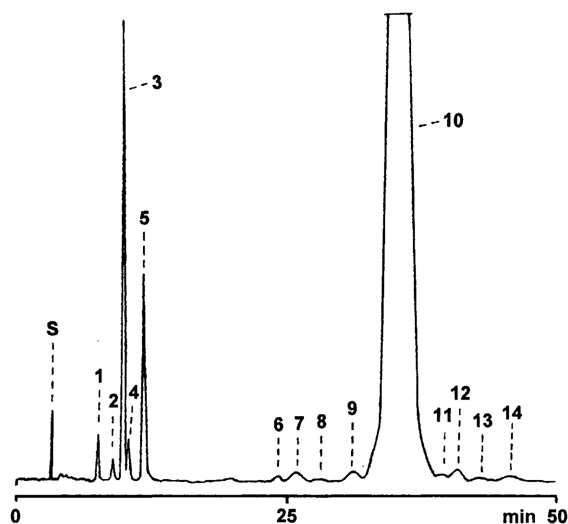

Fig. 2. Typical chromatogram obtained under the finally chosen chromatographic conditions. Stationary phase, Supelcosil LC-ABZ, 100 Å, 5 μm (250 × 4.6 mm). Column temperature, 45°C. Mobile phase, an aqueous solution containing 14 g/l of sodium sulfate, 1.5 g/l of sodium octanesulfonate, 50 ml/l of acetonitrile and 50 ml/l of a 0.2 M phosphate buffer (pH 3.0). Detection, UV at 205 nm; 1, unknown 1; 2, unknown 2; 3, streptidine; 4, unknown 3; 5, unknown 4; 6, unknown 5; 7, streptomycin B; 8, unknown 6; 9, unknown 7; 10, streptomycin; 11, unknown 8; 12, unknown 9; 13, dihydrostreptomycin; 14, unknown 10.

many). Other columns used were, Supelcosil ABZ Plus, 5 μm (250 × 4.6 mm i.d.) (Supelco); Inertsil ODS-2, 5 μm (250 × 4.6 mm i.d.) (Alltech, Deerfield, USA); Spherisorb S5 ODS B, 10 μm (250 × 4.6 mm i.d.) (PhaseSep, Queensferry, UK); Luna, 3 μm (100 × 4.6 mm i.d.) (Phenomenex, CA, USA); and Hypersil BDS C18, 5 μm (250 × 4.6 mm I.D.) (Hypersil, Runcorn, UK). The L-4200 UV–vis detector (Merck-Hitachi) was set at 205 nm. The PED-1 pulsed electrochemical detector (Dionex, Sunnyvale, CA, USA) was equipped with a gold working electrode, an Ag/AgCl reference electrode and a stainless-steel counter electrode.

2.3. Chromatography

The mobile phase consisted of an aqueous solution containing 14 g/l of sodium sulfate, 1.5 g/l of sodium 1-octanesulfonate, 50 ml/l of acetonitrile and 50 ml/l of 0.2 M phosphate buffer (pH 3.0). The latter was prepared by mixing a 0.2 M solution of phosphoric acid and a 0.2 M solution of potassium dihydrogen phosphate until a pH of 3.0 was achieved. The mobile phase was degassed by ultrasonication before use. The flow rate was 1.0 ml/min. The column temperature was maintained at 45°C. All substances to be analyzed were dissolved in water. The conditions used for PED were the same as described previously for the analysis of other aminoglycoside antibiotics [13–15]. Sodium hydroxide was added post-column through a tee and mixed with the mobile phase in a packed reaction coil, which was linked to the electrochemical cell. The time and voltage parameters of the PED detector were set as follows, E_1, E_2 and E_3 were, respectively, $+0.05$, $+0.75$ and -0.15 V with the assigned pulse durations t_1, 0.00–0.40 s; t_2, 0.41–0.60 s and t_3, 0.61–1.00 s. Integration of the signal occurred between 0.20 and 0.40 s.

3. Results and discussion

3.1. Chromatographic method

Based on the good results for dihydrostreptomycin [11], base deactivated reversed phase silica gel was used as the stationary phase. The mobile phase used for dihydrostreptomycin was adapted to improve the separation of the different streptomycin components, mainly those in the neighborhood of the main peak. This was mainly obtained by using a higher amount of sodium sulfate in the mobile phase. However, when the concentration of sodium sulfate is increased, the measured signal decreases, causing a diminished sensitivity. A concentration of 14 g/l was found to be a good compromise between selectivity and sensitivity. Although streptomycin in the finally chosen mobile phase has an absorption maximum at 200 nm, 205 nm was preferred because a better signal to noise ratio was obtained. A typical chromatogram of a commercial sample of streptomycin sulfate, obtained under the selected chromatographic conditions is shown in Fig. 2. Ten of the fourteen peaks correspond to compo-

nents of unknown identity. Hydroxystreptomycin coelutes with streptomycin B, but it is possible to separate these two components by using more octanesulfonate in the mobile phase. When the presence of hydroxystreptomycin in commercial samples was examined, no detectable amounts were found ($<0.08\%$). Since higher amount of octanesulfonate increases unnecessarily the total analysis time, the coelution of hydroxystreptomycin and streptomycin B was tolerated.

Other stationary phases, as mentioned in Section 2.2, were also examined. On most columns examined, the resolution in the neighborhood of the main peak was not sufficient. Only Spherisorb S5 ODS B gave an acceptable result, similar to Supelcosil LC-ABZ.

Since streptomycin impurities without guanidine groups cannot be detected by UV at 205 nm, the commercial samples were also examined by LC in combination with PED to see if more peaks were present. One additional peak, in some samples raising to 1% (expressed as streptomycin) could be detected in the front of the chromatogram. This very polar component, which nearly coelutes with ethanol, is probably a monosugar. Using PED, the resolution between the peaks was poorer because the peakwidth was broader (1.6 vs. 1.2 min for the main peak at half height). This can be attributed to the post-column addition of sodium hydroxide, necessary to allow PED. The quantitative repeatability was also poorer (R.S.D. = 2.4 vs. 0.9% on the area of the main peak ($n=4$)). This is mainly due to the presence of acetonitrile, which probably adsorbs to the electrode surface of the detector cell [16–18]. Attempts to replace acetonitrile by other organic modifiers, like THF or 2-methyl-2-propyl methyl ether, were not successful because they gave less good separations. Since only one peak was overlooked and for reasons of simplicity, UV detection was used in further experiments.

3.2. Factorial analysis

By means of a half-fraction five-factorial design, the importance of the individual chromatographic parameters and parameter interactions of this LC method was studied. The set-up of the applied factorial design was supported by a statistical graphics software system, Statgraphics version 6 (Manugistics, Rockville, MD, USA). The chromatographic parameters examined as variables were the concentration of sodium sulfate (Na_2SO_4), the sodium octanesulfonate (SOS) concentration, the pH of the mobile phase buffer, the amount of acetonitrile (CH_3CN) and the column temperature (temp). The values used in the design are shown in Table 1. In order to reduce the number of experiments, a half-fraction factorial design at two levels was chosen. This involves $2^5{:}2=16$ experimental measurements. The central level was repeated three times to estimate the experimental error. The measured response variables were the retention times of streptidine, streptomycin B, streptomycin and dihydrostreptomycin, because reference components of these substances were available.

A standardized Pareto chart, representing the estimated effects of the five chromatographic parameters and their interactions on the retention time of the main peak streptomycin, is shown in Fig. 3. A standardized Pareto chart consists of bars, the lengths of which are proportional to the absolute value of the estimated effects, divided by

Table 1
Factorial analysis, values corresponding to -1, 0 and $+1$

Chromatographic parameter	Low value (-1)	Central value (0)	High value ($+1$)
Sodium sulfate (g/l)	12	14	16
Sodium octanesulfonate (g/l)	1.3	1.5	1.7
pH of the mobile phase buffer	2	3	4
Acetonitrile (ml/l)	45	50	55
Column temperature (°C)	42	45	48

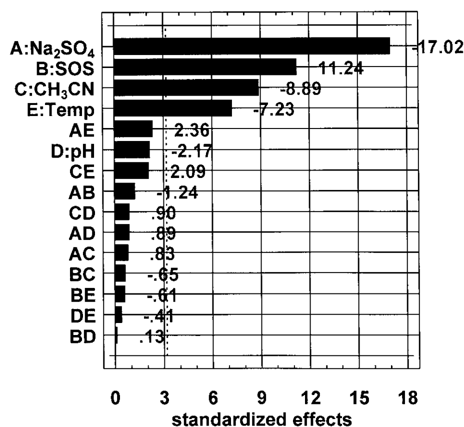

Fig. 3. Standardized Pareto chart, representing the estimated effects of the chromatographic parameters and parameter interactions on the retention time of streptomycin.

the S.E. The bars are displayed in order of the size of the effects, with the largest effect on top. The chart includes a vertical dashed line at the critical t-value for an alpha of 0.05. An effect smaller than the value, indicated by this line, is considered to be insignificant. Under the examined conditions, the LC system is principally influenced by the sodium sulfate concentration, which has a negative effect on the retention times. This means that the retention times of the streptomycin components examined will decrease with an increasing amount of sodium sulfate. The second most important factor, which has an effect on the retention times, is the sodium octanesulfonate concentration. This factor has a positive effect, so that the retention times will increase with an increasing amount of sodium octanesulfonate. The amount of acetonitrile and the column temperature were the next most important parameters. They both have a negative effect on the retention times of the streptomycin components examined. The pH of the buffer of the mobile phase has no significant effect on the retention times. No significant interactions between the different parameters were observed.

Using the same experimental results, also the selectivity between streptidine, streptomycin B, streptomycin and dihydrostreptomycin was examined. The selectivity factors for streptidine–streptomycin B ($\alpha_{\mathrm{Stid-StrB}}$), streptomycin B–streptomycin ($\alpha_{\mathrm{StrB-Str}}$) and streptomycin–dihydrostreptomycin ($\alpha_{\mathrm{Str-DHS}}$) were used as response variables. The standardized Pareto charts, representing the estimated effects of the five chromatographic parameters and their interactions on the selectivity factors are shown in Fig. 4. As can be seen all α are mainly influenced by the sodium sulfate concentration, which has a negative effect on $\alpha_{\mathrm{Stid-StrB}}$ and a positive on $\alpha_{\mathrm{StrB-Str}}$ and $\alpha_{\mathrm{Str-DHS}}$. This implement that higher amounts of sodium sulfate will improve the separations streptomycin B–streptomycin and streptomycin–dihydrostreptomycin. This confirms what was mentioned above, sodium sulfate is necessary in the mobile phase to obtain a good separation in the neighborhood of the main peak. An increase in the sodium sulfate concentration results in a less good separation between streptidine and streptomycin B, but this is not so important because these peaks are very well separated anyway. Acetonitrile has a negative effect on $\alpha_{\mathrm{Stid-StrB}}$, a positive on $\alpha_{\mathrm{StrB-Str}}$ and an insignificant on $\alpha_{\mathrm{Str-DHS}}$. The latter means that sodium sulfate cannot be replaced by acetonitrile. The sodium octanesulfonate concentration has a slightly positive effect on $\alpha_{\mathrm{Stid-StrB}}$ while it has an important negative influence on $\alpha_{\mathrm{StrB-Str}}$ and $\alpha_{\mathrm{Str-DHS}}$. The column temperature is only significant for $\alpha_{\mathrm{StrB-Str}}$ and $\alpha_{\mathrm{Str-DHS}}$. As expected, the pH of the buffer of the mobile phase has no significant influence on the separation and interactions between the parameters were also found to have no significant influence on the selectivity factors.

3.3. Quantitative aspects of the LC method

For the analysis of streptomycin an amount of 50 μg was used by injecting 20 μl of a 2.5 mg/ml solution. For this quantity the limit of detection (LOD, $s/n = 3$) and the limit of quantification (LOQ, R.S.D. $< 10\%$ for $n = 4$) for streptidine sulfate, streptomycin B sulfate and dihydrostreptomycin sulfate were determined. The results are shown in Table 2. The linearity of different streptomycin components was examined in the following concentration ranges, relative to the sample concentration (2.5 mg/ml), 20–120 and 0.5–10% for streptomycin, 0.04–10% for streptidine and 0.5–10% for streptomycin B and dihydrostrepto-

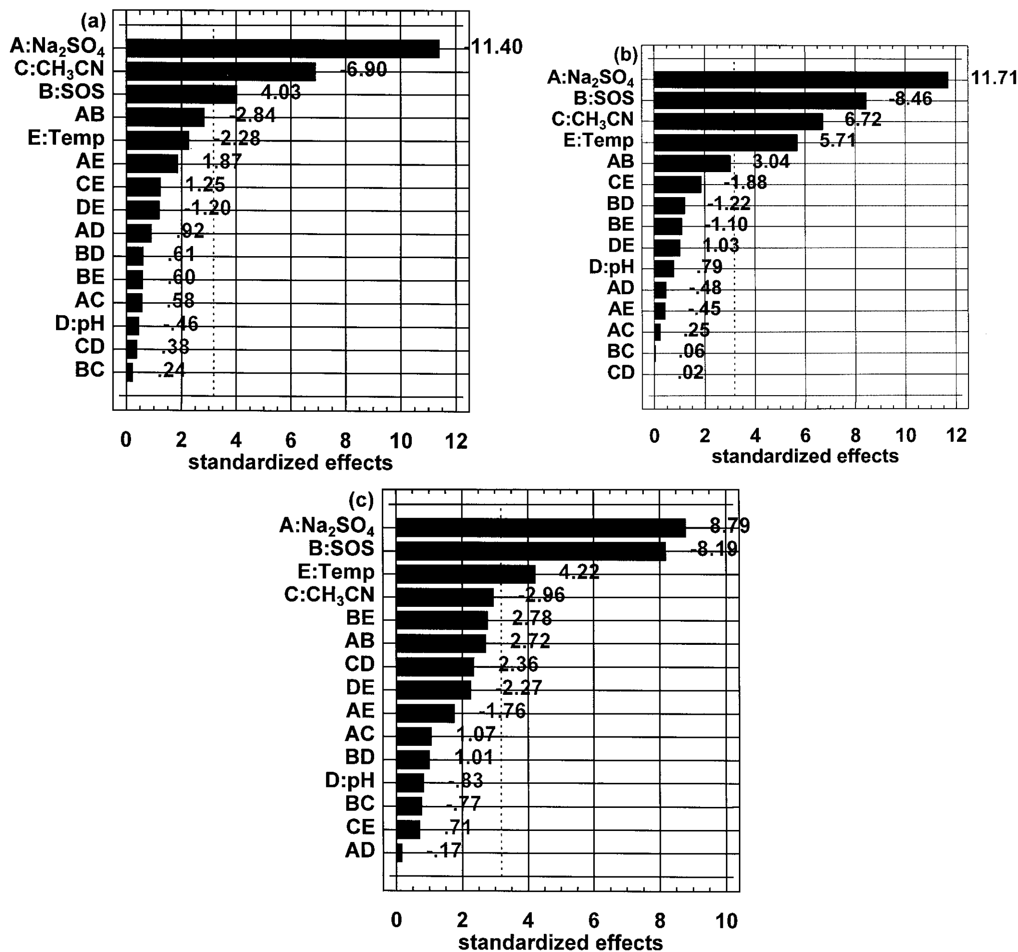

Fig. 4. Standardized Pareto chart, representing the estimated effects of the chromatographic parameters and parameter interactions on the selectivity factor for streptidine–streptomycin B (a), streptomycin B–streptomycin (b) and streptomycin–dihydrostreptomycin (c).

Table 2
Quantitative aspects of the system

	LOD (μg)	LOQ (μg)	Linearity			
			Range (μg)	y	r	$S_{y,x}$
Streptomycin			10–60	$1189x+1015$	0.9999	252
			0.25–5	$1249x+138$	0.9998	78
Streptidine	0.010	0.020	0.02–5	$5113x+312$	0.9996	333
Streptomycin B	0.100	0.250	0.25–5	$1064x+17$	0.9999	18
Dihydrostreptomycin	0.125	0.250	0.25–5	$1271x+43$	0.9995	90

Table 3
Composition of commercial streptomycin samples (%, m/m), expressed as streptomycin base on the substance as is[a]

Sample	Unk. 1	Unk. 2	Streptid.	Unk. 4	Unk. 5	Str. B	Unk. 6	Unk. 7	Str.	Unk. 8	Unk. 9	DHS	Unk. 10
1	±0.11	0.20	1.45	0.12	ND	ND	ND	1.13	66.0	ND	0.39	±0.18	±0.19
2	0.56	0.32	1.08	1.03	0.42	0.93	ND	0.52	65.1	ND	0.61	ND	±0.30
3	±0.09	±0.08	0.42	ND	±0.14	±0.26	±0.13	0.56	70.2	ND	±0.22	ND	±0.40
4	0.59	0.29	1.08	1.60	±0.29	0.63	ND	0.55	65.9	±0.19	0.70	ND	±0.21
5	0.37	±0.09	0.84	0.27	±0.22	0.39	ND	0.36	68.6	ND	±0.30	ND	±0.19
6	0.48	0.29	1.06	2.05	±0.29	0.67	±0.16	0.64	71.6	±0.17	0.56	±0.17	±0.40
7	±0.11	±0.06	0.37	±0.07	±0.25	0.40	±0.14	±0.27	69.7	ND	±0.17	ND	±0.20
8	0.16	0.36	0.61	±0.08	±0.18	±0.19	ND	0.43	68.8	ND	±0.20	ND	±0.28
9	0.49	0.32	1.04	1.51	ND	±0.18	ND	0.38	71.0	±0.17	0.51	ND	±0.20
10	0.75	0.20	1.29	0.84	±0.29	0.63	ND	0.70	65.8	±0.17	0.55	±0.19	±0.23

[a] Unk., unknown; streptid., streptidine; Str. B, streptomycin B; Str., streptomycin; DHS, dihydrostreptomycin; ND, not detected (below LOD), ± is used for values below LOQ.

mycin. The results are also shown in Table 2, where y = peak area/1000, x = amount of sample injected (µg), r = coefficient of correlation and $S_{y,x}$ = S.E. of estimate. The repeatability was checked by analyzing a 2.5 mg/ml solution of streptomycin four times. The R.S.D. on the area of the main peak was 0.9%.

3.4. Analysis of commercial samples

Several samples of streptomycin sulfate were analyzed using the described method. The composition of the commercial samples is shown in Table 3. All substances are expressed as streptomycin base on the substance as is, calculated with reference to the streptomycin house standard (66.2%, m/m as is). The content of the minor components was calculated using reference chromatograms obtained with a 5% (v/v) dilution (0.125 mg/ml) of the streptomycin house standard. As can be seen, the purity of the examined samples is quite variable and a lot of impurities are presented in concentrations around the LOD, making quantitation difficult. For most samples, streptidine is the most important impurity, followed by the peaks corresponding to unknown 4 and unknown 7.

4. Conclusion

The described method allows the separation of 14 components of streptomycin. The total time of analysis is 50 min. It is the first time that quantitative results are reported for so many streptomycin components. It is not only possible to separate the known potential impurities, but also several impurities of unknown identity. The method shows good repeatability, linearity and sensitivity without derivatization.

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