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Liquid chromatographic analysis of dihydrostreptomycin sulfate

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Abstract

The analysis of dihydrostreptomycin sulfate using a column packed with base deactivated reversed phase silica gel and ultraviolet detection at 205 nm is described. The mobile phase consists of an aqueous solution containing 4 g/l of sodium sulfate, 1.5 g/l of sodium octanesulfonate, 100 ml/l of acetonitrile and 50 ml/l of a 0.2-M phosphate buffer at pH 3.0. The method allows separation of streptidine, dihydrostreptomycin B, streptomycin, dihydrostreptomycin and deoxydihydrostreptomycin, as well as some other components which were not identified. The total time of analysis is 55 min. The effects of the different chromatographic parameters on the separation were also investigated. A number of commercial samples were analyzed using this method.

Keywords: Dihydrostreptomycin; Liquid chromatography

1. Introduction

Dihydrostreptomycin sulfate (DHS) is a water soluble, broad spectrum aminoglycoside antibiotic. Although most dihydrostreptomycin is produced by chemical reduction of streptomycin [1–3], it can also be produced electrolytically [4] or by direct fermentation processes [5,6]. Like other aminoglycoside antibiotics, DHS is potentially toxic, especially causing damage in vestibular and auditory function. The only liquid chromatographic (LC) method that can be considered for the analysis of DHS was described by Whall in 1981. This method employed ion-pair reversed phase chromatography with ultraviolet (UV) detection at 195 nm. Although it is mentioned that DHS is separated from related substances like dihydrostreptobiosamine, streptidine, streptomycin and mannosidodihydrostreptomycin, also called DHS B (Fig. 1), a more selective method is necessary for drug purity control. Also quantitative results for commercial samples are only given for streptidine and streptomycin [7]. More recently, some other papers were published to determine DHS in animal tissues [8,9] and milk [10,11] using ion-pair LC combined with fluorescence detection after post-column deriva-

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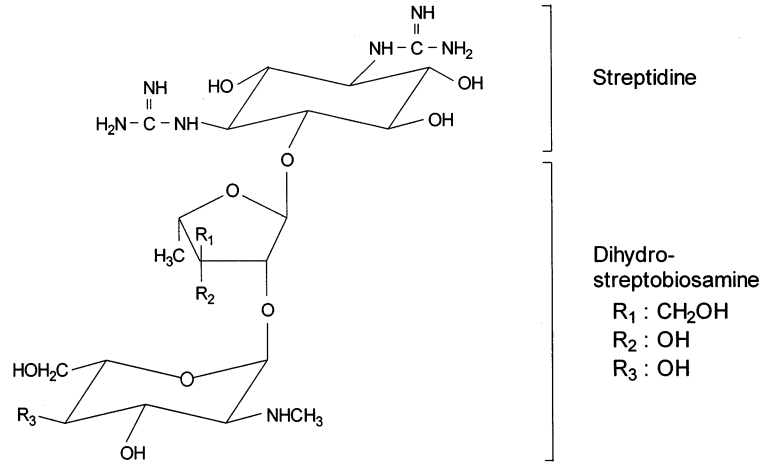
tization with 1,2-naphthoquinone-4-sulfonic acid [8–10] or ninhydrin [11]. The European Pharmacopoeia (Ph. Eur.) and the United States Pharmacopeia (USP) prescribe a spectrophotometric method to determine the streptomycin content, which is limited in the Ph. Eur. to 1% and in the USP to 3%, except when it is labeled as being crystalline (< 1%) or if it is labeled for oral use solely (< 5%). As assay technique in these monographs, microbiology is prescribed [12,13].

In this work an ion-pair LC method using a column packed with base deactivated reversed phase silica gel and ultraviolet detection at 205 nm is described. Besides the related substances mentioned above, the separation of dihydrodeoxystreptomycin (Fig. 1), first described by Ikeda et al. [14,15], and of several other impurities was also investigated. The composition of the mobile phase used in this study is based on that described by Whall [7] and on that previously used for other aminoglycosides like neomycin, kanamycin and amikacin [16–18]. Since pre- and post-column derivatization are time consuming and give some problems with quantitation, two other detection techniques were investigated: direct UV detection at low wavelengths and pulsed electrochemical detection. Although some experience is required for the latter method, it was included because of the good results already obtained for other aminoglycoside antibiotics [16–18]. Several types of stationary phases were also examined. Finally, the chosen method has been applied to analyze a number of commercial samples of DHS.



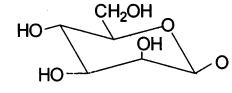
	R_1	R_2	R_3
Dihydrostreptomycin (DHS)	CH_2OH	OH	OH
Streptomycin	CHO	OH	OH
Dihydrodeoxystreptomycin	CH_2OH	H	OH
Mannosido-dihydrostreptomycin (DHS B)	CH_2OH	OH	

Fig. 1. Structure of some dihydrostreptomycin components.

2. Experimental

2.1. Reagents and reference samples

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

Streptomycin sulfate was obtained from VMD (Arendonk, Belgium). Dihydrodeoxystreptomycin was prepared according to Ikeda et al. [14], mannosidodihydrostreptomycin by the sodium borohydride reduction of streptomycin B [3,7], and streptidine by acid hydrolysis of DHS [19].

A house standard of DHS sulfate was available in the laboratory. The DHS base content of this standard was 70.8% (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. [12] and amounted to 7.0 and 19.5%, respectively. The total mass explained by titration, water and sulfate was 100.1%. The total content of impurities, determined by LC combined with UV detection and expressed as DHS base, was 5.6%.

Commercial samples of DHS sulfate were provided by VMD, Kela (Hoogstraten, Belgium), Dopharma (Raamsdonksveer, the Netherlands), Ostermann (Osnabrück, Germany) and Novo Industri (Bagsværd, Denmark).

2.2. Apparatus

The chromatographic analysis was carried out using an L-6200 Intelligent pump (Merck-Hitachi, Darmstadt, Germany), 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 temperature of the column was maintained at 45°C by immersion in a water bath with a circulator (Julabo, Seelbach, Germany). Other columns (250 × 4.6 mm i.d.) used were: Bakerbond C18, 5 μm (Baker, Phillipsburg, NJ, USA), Rsil C18 HL, 5 μm (Biorad, Eke, Belgium), Spherisorb S5 ODS B and ODS 1, 10 μm (PhaseSep, Queensferry, UK) and PLRP-S, 1000 Å, 8 μm (Polymer Laboratories, Shropshire, 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 4 g/l of sodium sulfate, 1.5 g/l of sodium 1-octanesulfonate, 100 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. All substances to be analyzed were dissolved in the mobile phase. The conditions used for pulsed electrochemical detection were the same as described previously for the analysis of other aminoglycoside antibiotics [16–18].

3. Results and discussion

3.1. Development of the chromatographic method

Based on the good results obtained for other aminoglycosides [16–18], LC on poly(styrene-divinylbenzene) (PLRP-S 1000 Å, 8 μm) combined with pulsed electrochemical detection was first investigated. The polymer stationary phase was chosen because of its remarkable stability and batch reproducibility. A disadvantage is the lower efficiency. Using this type of column and a mobile

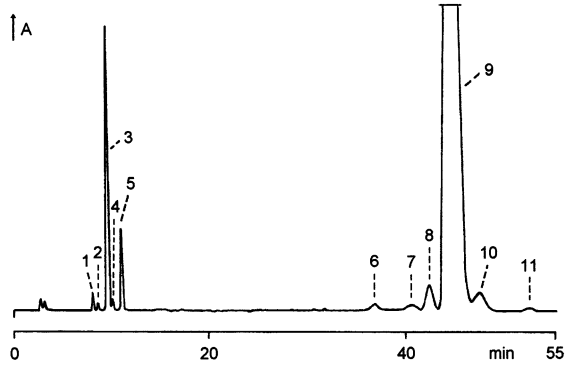


Fig. 2. Typical chromatogram obtained under the finally chosen chromatographic conditions. Stationary phase: Supelcosil LC-ABZ, 100 Å, 5 μm (250 × 4.6 mm). Column temperature: 45°C. Mobile phase: an aqueous solution containing 4 g/l of sodium sulfate, 1.5 g/l of sodium octanesulfonate, 100 ml/l of acetonitrile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

phase containing sodium sulfate, sodium octanesulfonate, a phosphate buffer of pH 3.0 and water, the different DHS components, mainly those in the neighbourhood of the main peak, could not be well separated. Also the peak shape of the principal peak was poor. Neither the use of a gradient, nor the addition of organic modifiers, like THF, tert-butyl methyl ether or acetonitrile to the mobile phase, could really improve the separation. Nevertheless, slightly better results were obtained with acetonitrile, giving a better separation of unknown five from the main peak. This organic modifier, also used by Whall [7], was further incorporated into the mobile phase. However, acetonitrile gives poor quantitative repeatability in combination with pulsed electrochemical detection, probably due to adsorption of the organic solvent to the electrode surface of the detector [20–22]. For this reason UV detection at low wavelengths was examined. Since it gave similar results, it was used for further method development.

Despite the advantages of polymer stationary phases, they were not satisfactory for the analysis of DHS. Classical C18 columns of different manufacturers, like Bakerbond C18, 5 μm, Rsil C18 HL, 5 μm and Sperisorb ODS 1, 10 μm, also were not able to separate well the different DHS components. Only the so-called base deactivated silica columns, like Supelcosil LC-ABZ and Spherisorb S5 ODS B gave an acceptable separation. The mobile phase was further optimized experimentally using the Supelcosil LC-ABZ column in combination with UV detection. The optimum found was confirmed by the computer-simulation software program DryLab (LC Resources, Berlin, Germany). The theoretical parameters obtained by the program were in good agreement with the experimental parameters. The mobile phase finally chosen is that described in Section 2.3. Sodium sulfate is necessary to obtain a good separation between streptomycin and DHS. However, when the sodium sulfate concentration is increased, the measured signal decreases, causing a diminished sensitivity. A concentration of 4 g/l was found to be a good compromise between selectivity and sensitivity. Although DHS in the finally chosen mobile phase has an absorption maximum at 200 nm, 205 nm was preferred because the sensitivity at this wavelength is sufficient and a more stable baseline is obtained. A typical chromatogram of a commercial sample, obtained under the selected chomatographic conditions, is shown in Fig. 2. Of

Table 1
Factorial analysis: nominal values corresponding to −1, 0 and +1

Chromatographic parameter	Low value (−1)	Central value (0)	High value (+1)
Sodium sulfate (g/l)	3.5	4	4.5
Sodium octanesulfonate (g/l)	1.3	1.5	1.7
pH of the mobile phase buffer	2	3	4
Acetonitrile (ml/l)	95	100	105
Column temperature (°C)	40	45	50

Table 2
Quantitative aspects of the system

	LOD (μg)	LOQ (μg)	Linearity			
			Range (μg)	y	r	$S_{y,x}$
DHS			10–60	$1985x+669$	0.9998	901
Streptidine	0.005	0.01	0.01–5	$4004x+135$	0.9998	151
Streptomycin	0.040	0.10	0.1–5	$1969x+144$	0.9999	64
Dihydrodeoxystreptomycin	0.050	0.10	0.1–5	$2135x+114$	0.9998	82

the eleven peaks, six correspond to components of unknown identity. Since DHS impurities without guanidine groups cannot be detected by UV at 205 nm, the commercial samples were also examined by LC in combination with pulsed electrochemical detection to see if more peaks were present. Only two small additional peaks ($<0.2\%$), probably corresponding to hydrolysis products like dihydrostreptobiosamine, could be detected in the front of the chromatogram. The resolution between the peaks however was somewhat poorer because the peakwidth was broader (1.3 vs. 1.1 for the main peak at half height). This can be attributed to the post-column addition of sodium hydroxide, necessary to allow pulsed electrochemical detection. Since UV detection gave similar results and also because of its simplicity, it was preferred above pulsed electrochemical detection.

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 were 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 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 at least $2^5 : 2 = 16$ experimental measurements. The central level was repeated three times and was also included in the design. The measured response variables were the retention times of the different DHS components. Under the examined conditions, the LC system is principally influenced by the sodium octanesulfonate concentration which has a positive effect on the retention times. This means that the retention time of the different DHS components will increase with an increasing amount of sodium octanesulfonate. The column temperature, amount of acetonitrile and sodium sulfate concentration were the next most impor-

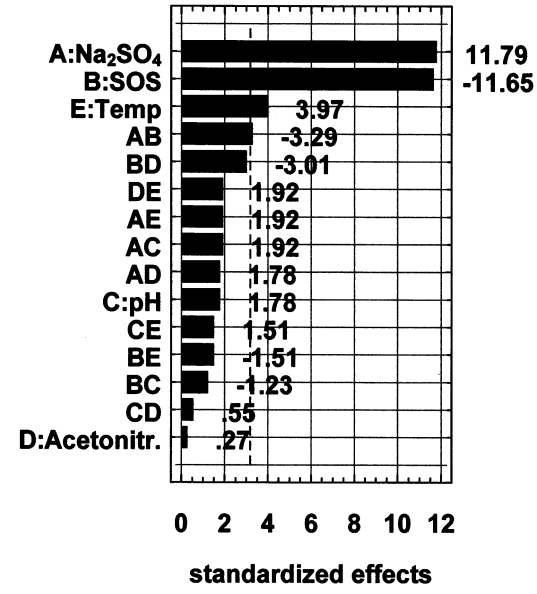


Fig. 3. Standardized pareto chart, representing the estimated effects of the chromatographic parameters and their interactions on the selectivity factor for streptomycin and DHS.

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

Sample	Unk. 1	Unk. 2	Streptid.	Unk. 3	Unk. 4	DHS B	Streptom.	Unk. 5	DHS	Unk. 6	DHdeoxyS
1	0.52	0.07	2.56	0.12	1.29	0.51	0.62	1.41	66.8	0.34	±0.07
2	0.12	0.22	0.55	0.40	0.74	0.16	0.37	0.85	68.1	0.55	±0.15
3	0.11	0.30	0.41	0.13	0.34	0.45	±0.06	0.68	61.6	8.70	0.23
4	0.07	0.06	0.43	0.03	0.05	ND	±0.09	1.38	75.4	±0.06	ND
5	0.08	0.07	0.53	0.20	0.15	0.32	±0.14	1.30	70.8	±0.09	ND
6	0.11	0.15	0.54	0.18	0.21	0.38	0.32	0.91	70.0	±0.09	±0.08
7	0.09	0.06	0.35	ND	0.20	0.36	0.20	0.73	70.7	±0.10	±0.09
8	0.10	0.22	0.75	0.25	0.20	0.22	±0.11	0.62	71.7	±0.12	ND
9	0.19	0.10	1.44	0.20	0.95	0.24	0.43	1.08	67.8	0.25	±0.08
10	0.22	0.15	1.20	0.18	0.30	0.20	±0.08	0.94	70.3	0.24	ND
11	0.08	0.21	0.51	ND	0.06	0.32	ND	0.35	71.7	±0.13	±0.09
12	0.08	0.08	0.50	ND	0.04	0.58	ND	0.44	72.2	±0.10	±0.09

[a] DHdeoxyS, dihydrodeoxystreptomycin; ND, not detected; streptid., streptidine, streptom., streptomycin; Unk., unknown; ± is used for values below LOQ.

tant parameters, having about the same significance. They all have a negative effect on the retention times of the different DHS components, so that the retention times will decrease when one of these parameters is increased. The pH of the buffer of the mobile phase has only a small negative effect on the retention times. No important interactions between the different parameters were observed.

Using the same experimental results, also the separation between streptomycin and DHS was examined. The separation between these two compounds was chosen because reference components of both substances were available. The selectivity factor for streptomycin and DHS (α_{SD}) was used as response variable. The standardized pareto chart, representing the estimated effects of the five chromatographic parameters and their interactions on α_{SD} is shown in Fig. 3. As can be seen, α_{SD} is mainly influenced by the sodium sulfate and sodium octanesulfonate concentration. The sodium sulfate concentration however has a positive effect on the separation whereas the sodium octanesulfonate concentration has a negative effect. This implements that higher amounts of sodium sulfate will improve the separation between streptomycin and DHS. As mentioned above, this is also the reason why sodium sulfate is necessary in the mobile phase and cannot be replaced by acetonitrile because the latter has no significant effect on α_{SD}. Also neither the column temperature, nor the pH of the mobile phase buffer, nor possible interactions were found to have an influence on α_{SD}.

3.3. Quantitative aspects of the LC method

For the analysis of DHS 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) for streptidine sulfate, streptomycin sulfate and dihydrodeoxystreptomycin sulfate were determined. The results are shown in Table 2. The linearity of different DHS components was examined in the following concentration ranges, relative to the sample concentration (2.5 mg/ml): 20–120% for DHS, 0.02–10% for streptidine and 0.2–10% for streptomycin and dihydrodeoxystreptomycin. The results are also shown in Table 2, where y = peak area/1000, x = amount of sample injected (μg), r = coefficient of correlation and $S_{y,x}$ = standard error of estimate. The repeatability was checked by analyzing a 2.5-mg/ml solution of DHS six times. The RSD on the area of the main peak was 0.9%.

3.4. Analysis of commercial samples

Several samples of DHS sulfate were analyzed using the described method. The composition of the commercial samples is shown in Table 3. All substances are expressed as DHS base on the substance as is, calculated with reference to the DHS house standard (70.8%, 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 DHS house standard. As can be seen, the purity of the examined samples is quite variable. It is also noteworthy that they contained nearly no streptomycin.

4. Conclusion

The described method, using a base deactivated column as the stationary phase, allows the separation of 11 components of DHS. The total time of analysis is 55 min. It is the first time that quantitative results are reported for so many DHS components. This method is highly selective since all known potential impurities and also several impurities of unknown identity are separated from each other. The method shows good repeatability, linearity and sensitivity without derivatization.

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