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Identification and control of impurities in streptomycin sulfate by high-performance liquid chromatography coupled with mass detection and corona charged-aerosol detection

Ulrike Holzgrabe[a,*], Cees-Jan Nap[b], Nathalie Kunz[b], Stefan Almeling[b]

[a] University of Wuerzburg, Institute of Pharmacy and Food Chemistry, Am Hubland, 97074 Wuerzburg, Germany
[b] European Directorate for the Quality of Medicines and Health Care, Strasbourg, France

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ABSTRACT

For the control of impurities in streptomycin sulfate a reversed phase ion-pair high performance liquid chromatography (HPLC) method using charged aerosol detection (CAD) was developed. With this method, 21 impurities could be separated and tentatively identified using a combination of exact mass measurement by TOF-MS and MS/MS experiments with a triple quadrupole MS. For three impurities the suggested structures could be confirmed by in situ formation. The CAD detector response was found to be linear over 2 orders of magnitude allowing a straightforward quantification of all impurities. A limit of quantification of 0.09% for streptomycin sulfate and of 0.008% for streptidine sulfate (referred to the concentration of the 5 mg/ml test solution) could be achieved. The HPLC method was applied to the purity testing of 12 samples of commercially available streptomycin sulfate from different manufacturers. Impurity levels between 4.6% and 16.0% were found. The current European Pharmacopoeia monograph for streptomycin sulfate only limits streptomycin B by a TLC test to 3.0%. Therefore, the results of this study underline the importance of introducing a state-of-the-art test for the control of impurities in the monograph. The new HPLC-CAD method is considered suitable for this purpose.

1. Introduction

In 1943, streptomycin (SM) was first isolated from *Streptomyces griseus* by Albert Schatz, a graduate student in the laboratory of Selman Abraham Waksman at the Rutgers University, New Jersey, USA [1]. It is an inhibitor of the protein biosynthesis which irreversible binds to the S12 protein of the 30S subunit of the bacterial ribosome [2] and consequently leads to the death of microbial cells. Streptomycin is bacteriocidal to both Gram-positive and Gram-negative bacteria.

It belongs to the group of aminoglycoside antibiotics. Like other members of this family (e.g. gentamicin, tobramycin, amikacin, netilmicin, kanamycin and neomycin) it is normally applied parenteral. Whilst for human use streptomycin is nowadays mainly employed as a second line therapeutic against tuberculosis, it is still a first line antibiotic in veterinary medicine for the treatment of Gram-negative bacteria in large animals, e.g. horses, cattle and sheep. A major disadvantage of all aminoglycoside antibiotics is their pronounced ototoxic side effect [3].

Streptomycin, also known as streptomycin A (cf. Fig. 1), is an oligosaccharide with basic properties. Like other representatives of the aminoglycoside family it consists of a monosaccharide, an amino sugar and a guanidino substituted cyclohexane with at least 3 hydroxyl functions. The three units are linked via α-glycosidic bonds. In the case of streptomycin these three units are streptidine (a diguanidino derivative of inositol), L-streptose (a relatively rare 3-methylpentose), and N-methyl-L-glucosamine. The streptose carbonyl function is mainly present in its hydrated form [4].

In the current European Pharmacopoeia (Ph.Eur.) monograph of streptomycin sulfate [5], no test for related substances is described and only the pharmacologically less active streptomycin B is limited to 3.0% using a TLC test after hydrolysis of the molecule employing mannose as a reference substance. This is particularly striking since streptidine was described to be a possible source for the ototoxicity of streptomycin [6].

Several papers have been published proposing HPLC methods for the identification and control of the impurities in streptomycin sulfate using UV detection [7,8] mass spectrometric [9] and pulsed amperometric detection (PAD) [10]. Moreover, a capillary zone electrophoresis (CZE) method with UV detection was reported [11]. However, each of the proposed methods suffers from several drawbacks. Due to the lack of a suitable chromophore in streptomycin and most of its impurities, the analysis by UV-spectrophotometry

* Corresponding author. Tel.: +49 931 3185460.
E-mail address: u.holzgrabe@pharmazie.uni-wuerzburg.de (U. Holzgrabe).

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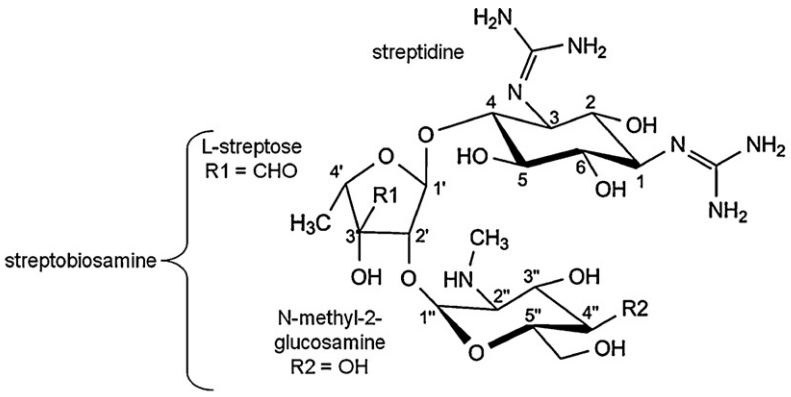


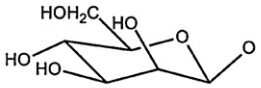
	R1	R2	m/z-ratio $[M+H]^+$
Streptomycin A	CHO	OH	582.2735
Streptomycin B	CHO		744.3258
Dihydrostreptomycin	CH_2OH	OH	584.2886

Fig. 1. Structural formulae of streptomycin components.

requires high concentrations and a low detection wavelength (about 200 nm). Moreover, impurities lacking the UV-absorbing guanidino-groups of streptidine will not be detected. Mass spectrometry is a useful tool for the identification of impurities, but without having them available as individual external standards, an appropriate quantification is difficult to achieve. PAD is technically difficult to apply and requires a skilled operator. Furthermore, non-oxidizable compounds are not detected. Additionally, the selectivity of most of the proposed methods in terms of resolution of streptomycin from its impurities is not satisfactory.

Detection by CAD was considered to be a promising alternative to overcome the aforementioned limitations. Thus, the present paper reports the development of a highly selective and sensitive HPLC method using CAD for the purity testing of streptomycin sulfate. 12 samples of streptomycin sulfate from different manufacturers were analysed. 21 impurities could be separated and tentatively identified by HPLC–MS.

2. Experimental

2.1. Reagents and chemicals

Water was delivered by an ELGA PureLab Ultra system (Elga Antony, France). Perfluoropropionic acid (PFPA), sodium hydrogensulfite ACS reagent, N-acetylglucosamine and acetone p.a. were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Streptidine sulfate as well as the samples of streptomycin sulfate originating from VMD, Ludeco (Brussels, Belgium), Continental Pharma (Brussels, Belgium), Dopharma (Raamsdonksveer, The Netherlands), Office Chimique (Waterloo, Belgium), Kela (Hoogstraten, Belgium), were kindly provided by Prof. Hoogmartens (Faculty of Pharmacy, University of Leuven, Belgium). Additionally, a sample of streptomycin sulfate European Pharmacopoeia (Ph.Eur.) CRS 3 was made available by the European Directorate for the Quality of Medicines and HealthCare (EDQM, Strasbourg, France). All samples and reagents were used without further purification. Nitrogen + 99% was delivered by a Peak Systems NM18LA or NM30LA nitrogen generator (Lab Gaz Systems, Massy, France). Nitrogen N 50 used as MS/MS collision-gas came from Air Liquide (Illkirch, France).

2.2. Apparatus

A Waters Alliance Separation Module 2695 including thermostated autosampler, quarternary pump and column oven (St. Quentin-en-Yvelines, France) equipped with Waters Empower Pro data processing software was used for liquid chromatography. Detection by CAD was performed by means of a Corona CAD (ESA Bioscience INC. – Vendor: Eurosep Instruments, Cergy Pontoise, France). For HPLC/MS-MS analysis an Agilent 1200 Series Rapid Resolution HPLC system coupled with an Agilent 6400 Series Triple Quadrupole HPLC/MSD (Waldbronn, Germany) was employed. Exact mass measurement was conducted with a Bruker μ-TOF-MS (Wissembourg, France). The YMC-Pack Pro column was purchased from Interchim (Montlucon, France).

2.3. Method

2.3.1. HPLC-UV-method

For the search for the streptomycin sulfate sample with the most impurities an HPLC method using a Supelcosil ABZ alkylamide column (250 mm × 4.6 mm, 5 μm) at a temperature of 45 °C was utilized. Detection was performed by UV at 205 nm. 20 μl of a 2.5 mg/ml aqueous test solution of streptomycin sulfate was injected at a flow-rate of 1.0 ml/min. The method run-time was 50 min. For the preparation of the mobile phase 14 g sodium sulfate and 1.5 g disodium 1-octanesulfonate were dissolved in 400 ml of water. After addition of 50 ml of 0.2 molar phosphate buffer (pH 3.0) and 50 ml of acetonitrile, the solution was diluted to 1 l with water. The phosphate buffer solution was prepared by dissolving 2.72 g of potassium dihydrogen phosphate in 80 ml of water. After

adjustment to pH 3.0 with phosphoric acid 85%, the buffer solution was further diluted to 100.0 ml with water.

The limit of quantification (based on a signal-to-noise ratio of 10) of streptomycin sulfate was extrapolated from the detector response of a solution containing 12.5 μg/ml of streptomycin sulfate. The same solution was used as external standard for the quantification of the impurities.

For the calculation of the streptidine content a correction factor of 0.51 was applied. This factor was determined by HPLC measuring the detector response of solution containing 250 μg/ml of streptidine sulfate and 250 μg/ml of streptomycin sulfate in water.

2.3.2. HPLC method conditions used for CAD and MS analysis

The HPLC-CAD and MS analysis was performed using a YMC-Pack Pro column (250 mm × 4.6 mm; particle size 3 μm) at a temperature of 40 °C. A mobile phase containing 20 mmol/L of PFPA in water/acetone (99/1, v/v) was used. The method run-time was 70 min at a flow-rate of 0.8 ml/min. 10 μl of a 5 mg/ml solution of streptomycin sulfate in water was injected as a test solution.

2.3.3. Detector settings

2.3.3.1. CAD-detection. The detection was performed using a gas pressure of 35 psi in the 100 pA detection range.

2.3.3.2. TOF-MS. A 1/10 T-split of the mobile phase eluting from the HPLC column was applied for the HPLC-TOF-MS coupling. The TOF-MS parameters were as follows: source type and polarity, ESI-positive; nebulizer gas pressure, 2.0 bar; drying gas, 8.0 l/min; dry heater, 250 °C; capillary, 4500 V; end plate, 500 V; scan range, 50–3000 m/z; capillary exit, 100 V; hexapole RF, 100 V; skimmer 1, 50 V; hexapole 1, 25 V; pulsar pull, 0 V, pulsar push, 825 V; reflector, 1709 V, flight tube, 8589 V, detector, 1880 V.

2.3.3.3. MS/MS. The MS/MS parameters were as follows: source type and polarity, ESI-positive; nebulizer gas-pressure 35 psi; dry gas, 12 l/min; dry heater 350 °C; capillary, 4000 V; scan range, 50–1300 m/z; fragmentor, 135 V; collision energy 0–60 eV.

2.3.4. Calibration and CAD-response factor

The calibration curve used for the quantification was recorded using 0.1% (5 μg/ml), 0.2% (10 μg/ml), 0.5% (25 μg/ml), 1.0% (50 μg/ml), 2% (100 μg/ml) and 3% (150 μg/ml) dilutions of a 5 mg/ml solution of streptomycin sulfate sample 11. All dilutions were prepared from a 5 mg/ml stock solution using water as a solvent. For the verification of the linearity of the range, a 5% (250 μg/ml) dilution of the streptomycin sulfate test solution was injected instead of a 3% dilution.

The determination of the CAD correction factor of streptidine sulfate was carried out measuring the detector response of streptidine sulfate and streptomycin sulfate in solutions containing 25 μg/ml and 50 μg/ml of both substances, respectively. The CAD limits of quantification (based on a signal-to-noise ratio of 10) were extrapolated from the detector response of a solution containing 5 μg/ml of streptomycin sulfate and 5 μg/ml of streptidine sulfate.

2.3.5. In-situ formation of impurities

In order to confirm that hydrogensulfite adducts occur as streptomycin related impurities, they were generated in situ by adding 20 μl of a 100 mg/ml solution of sodium hydrogensulfite to a solution of 5 mg of streptomycin sulfate in 1 ml of water. The solution was subsequently analysed by HPLC–MS.

For the generation of streptomycin acid, 20 μl of a 0.1 N potassium permanganate solution was added to a 5 mg/ml aqueous solution of streptomycin sulfate. 20 μl of each solution was analysed by HPLC using the aforementioned method with MS detection.

For the verification of the identity of the impurity 1 with an *m/z*-ratio $[M+H]^+$ of 222 (cf. Table 1), a 5 mg/ml test solution of streptomycin sulfate sample 10 spiked with about 0.5% of N-acetylglucosamine was analysed using the HPLC method described under Section 2.3.2 and detection by means of CAD.

3. Results and discussion

3.1. HPLC method development

The aim of this study was to develop a selective and sensitive HPLC method for the control of impurities in streptomycin sulfate. Since apart from streptidine no individual impurities were available, 12 commercially available samples of streptomycin sulfate from different manufacturers were analysed using a HPLC-UV method described in literature [7]. This method was selected because the published batch results showed that considerable amounts of impurities could be separated from streptomycin sulfate. Therefore the method is suitable to give a good indication for the selection of the most appropriate sample for a further method development. The analysis performed in this study showed 13 impurities being separated. The total amount of impurities in the analysed samples ranged between 2.2% and 15.6%. A limit of quantification for streptomycin sulfate of about 0.5% (referred to the concentration of the test solution of 2.5 mg/ml), calculated on the basis of a signal-to-noise ratio of 10, was determined. Since a correction factor of 0.51 (compared to the detector response of streptomycin sulfate) was found for streptidine sulfate, the peak area of the peak due to streptidine was corrected accordingly. However, it must be borne in mind that even this correction does not necessarily deliver a precise estimate of the amount of impurities, since under the conditions of this method streptidine probably co-elutes with other impurities which may not have the same correction factor.

Samples 10 (cf. Fig. 2) and 4 were found to contain the most impurities and were therefore selected for the method development.

For the development of the HPLC-CAD method the suitability of trifluoroacetic acid (TFA), pentafluoropropionic acid (PFPA), heptafluorobutyric acid (HFBA) and perfluoroheptanoic acid (PFHA) as volatile ion-pair reagents was tested. PFPA was found to deliver the best results in terms of resolution and retention time. For the selection of an appropriate organic modifier methanol, tetrahydrofuran, acetonitrile and acetone were examined. Acetonitrile and acetone were found most suitable. Due to better results in terms of resolution and because of its higher volatility, acetone was selected. The best chromatographic profile was obtained with a mobile phase containing 20 mmol/L of PFPA in a mixture of water/acetone 99/1 (v/v) at a flow rate of 0.8 ml/min. A YMC-Pack Pro column with a particle size of 3 μm, at a temperature of 40 °C was found most appropriate. However, almost identical results were obtained when using an Inertsil ODS 3 column (250 mm × 3.0 mm, particle size: of 3 μm).

Under these conditions, all streptomycin sulfate samples were screened. Up to 21 impurities could be separated from streptomycin sulfate (cf. Fig. 3).

3.2. Identification of impurities by HPLC-TOF-MS and HPLC-MS/MS

The HPLC-CAD method described in this paper was directly compatible to mass detection. Therefore, the exact mass of all observed impurities was determined by HPLC coupled with time-of-flight mass spectrometry (TOF-MS). Moreover, MS/MS experiments were performed using a triple–quadrupole mass spectrometer. An

Table 1
Results of the exact mass measurement by TOF-MS and the neutral loss by MS/MS for the impurities found in streptomycin sulfate.

Peak no.[a]	*m/z*-ratio $[M+H]^+$ observed by TOF-MS	*m/z*-ratio theoretical $[M+H]^+$	Rel. error (ppm)	Supposed formula	MS/MS neutral loss (product m/z)	Suggested name
1	**222.0975**	**222.0972**	**−1.47**	**$C_8H_{15}NO_6$**	**N.A.**	**N-acetyl-inositolamine**
2	222.1084	222.1084	0.2	$C_7H_{15}N_3O_5$	N.A.	N-amidino-scyllo-inosamine [9]
3	**194.1020**	**194.1023**	**1.71**	**$C_7H_{15}NO_5$**	**18 (176)**	**N-methylglucosamine**
4/5	**356.1542**	**356.1551**	**2.58**	**$C_{13}H_{25}NO_{10}$**	**18 (338); 180 (176)**	**Mannosido-N-methylglucosamine**
4/5	**370.1709**	**370.1708**	**−0.26**	**$C_{14}H_{27}NO_{10}$**	**18 (352); 180 (190)**	**Mannosido-N,N-dimethylglucosamine**
6	221.1240	221.1244	1.96	$C_7H_{16}N_4O_4$	N.A.	3-deguanidino-3-aminostreptidine [8]/N-amidinostreptamine [9]
7	263.1462	263.1462	0.13	$C_8H_{18}N_6O_4$	N.A.	Streptidine [8,9]
8	**664.2448**	**664.2454**	**0.93**	**$C_{21}H_{41}N_7O_{15}S$**	**82 (582)**	**Streptomycin sulfite-adduct**
9	**664.2450**	**664.2454**	**0.66**	**$C_{21}H_{41}N_7O_{15}S$**	**82 (582)**	**Streptomycin sulfite-adduct**
10	571.2571	571.2570	−0.26	$C_{20}H_{38}N_6O_{13}$	162 (409)	2′-O-de-N-methylglucosamino-2′-O-mannosido DHS [8]
11	409.2025	409.2041	4.09	$C_{14}H_{28}N_6O_8$	146 (263)	2′-O-de-N-methylglucosamine DHS [8]/O-L-dihydrostreptose-(1′->4)-streptidine [9]
12	367.1807	367.1823	4.42	$C_{13}H_{26}N_4O_8$	146 (221)	O-L-dihydrostreptose-(1->4)-N-amidinostreptamine [9]
13	**598.2665**	**598.2679**	**2.31**	**$C_{21}H_{39}N_7O_{13}$**	**175 (423); 422 (176)**	**Streptomycin acid**
14	**612.2823**	**612.2835**	**1.96**	**$C_{22}H_{41}N_7O_{13}$**	**189 (423); 422 (190)**	**2″-N-dimethylstreptomycin acid**
15	744.3353	744.3258	0.28	$C_{27}H_{49}N_7O_{17}$	162 (582); 406 (338); 568 (176)	SM B [8]/mannosido-streptomycin [9]
16	568.2559	568.2573	2.45	$C_{20}H_{37}N_7O_{12}$	161 (407); 406 (162)	2″-N-dimethyl SM[8]/N-dimethylstreptomycin [9]
17	**N.A.**	**N.A.**	**N.A.**	**N.A.**	**N.A.**	**Unknown structure**
18	596.2875	596.2886	1.86	$C_{22}H_{41}N_7O_{12}$	189 (407); 406 (190)	N,N-dimethyl-L-glucosamine-(1″->2′)-L-streptose-(1′->4′)-streptidine [9]
19	570.2727	570.2729	0.38	$C_{20}H_{39}N_7O_{12}$	161 (409); 408 (162)	N-dimethyl DHS [9]
20	584.2886	584.2886	0.05	$C_{21}H_{41}N_7O_{12}$	175 (409); 408 (176)	DHS [8,9]
21	625.2790	625.2788	−0.35	$C_{22}H_{40}N_8O_{13}$	43 (582)	6-dehydro-6-carbamate streptomycin [8]

Bold face indicates impurities not yet reported in literature.
N.A., not available; SM, streptomycin; DHS, dihydrostreptomycin.
[a] Peak-no. as referred to in Fig. 3.

overview of the *m/z* values of all observed impurities, measured by TOF-MS, is given in Table 1. Moreover, Table 1 contains the neutral loss (NL) determined by HPLC–MS/MS where appropriate as well as the suggested name of the impurity. The supposed structural formulae of the impurities are presented in Fig. 4.

For those impurities where the results of the exact mass measurement and the neutral loss obtained by MS/MS, where available, were in agreement with the data already reported in the literature [8,9,12], the names of the compounds together with a link to the corresponding literature were indicated. For the impurities not yet reported in literature, the results are discussed below. This includes also impurities 13, 14 and 17 where the analytical data obtained in this study were not in agreement with the structures suggested in the literature.

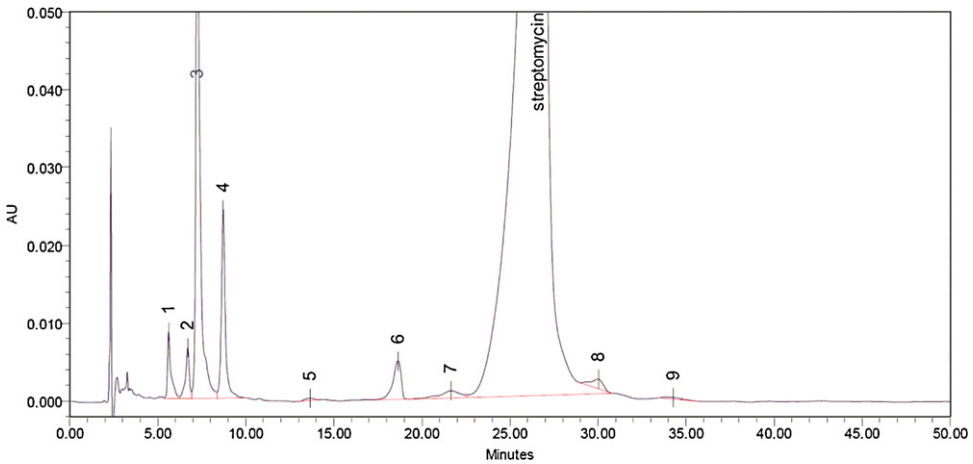


Fig. 2. HPLC chromatogram of streptomycin sulfate sample 10 with UV detection at 205 nm; column: Supelcosil ABZ alkylamide (250 mm × 4.6 mm, particle size 5 μm); column temperature: 45 °C; mobile phase: an aqueous solution containing 14 g/l sodium sulfate, 1.5 g/l disodium 1-octanesulfonate, 50 ml/l acetonitrile and 50 ml/l of a 0.2 molar phosphate buffer (pH 3.0).

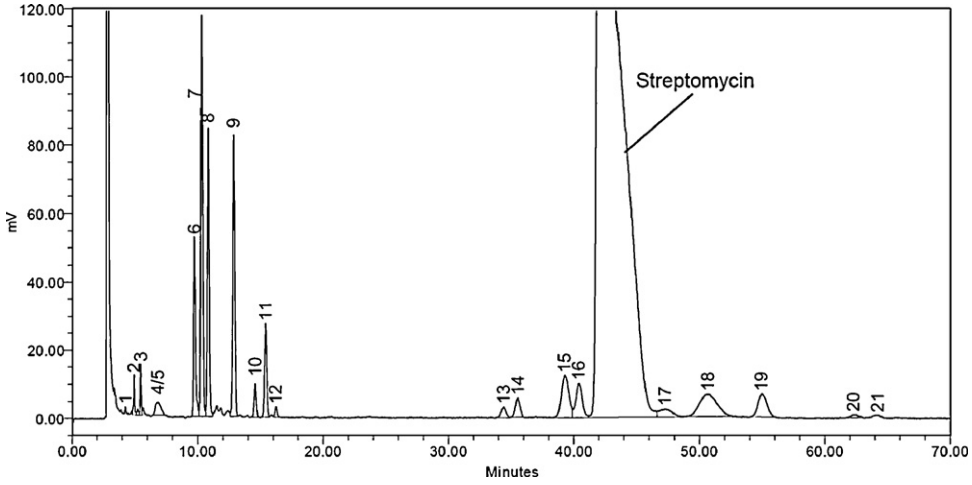


Fig. 3. HPLC chromatogram of streptomycin sulfate sample 4 with detection by CAD; column: YMC-Pack Pro column (250 mm × 4.6 mm; particle size 3 μm); column temperature: 40 °C; mobile phase: 20 mmol/l of PFPA in a mixture of water/acetone 99/1 (v/v); flow rate: 0.8 ml/min; injection of 10 μl of a 5 mg/ml solution of streptomycin sulfate in water.

In the following, more detailed explanations concerning the identification of the 8 newly detected impurities (cf. Table 1 bold names) are given.

Peak 1 [M + H]⁺ m/z 222: Based on the results of the exact mass measurement by TOF-MS the supposed sum formula of this compound corresponded to $C_8H_{15}NO_6$. The signal was not found to be very abundant and no neutral loss could be detected in the MS/MS spectrum. The peak exhibited a mass 42 U higher than glucosamine, which corresponds to an acetyl group. Since uridine diphosphate N-acetylglucosamine was shown to occur in *S. griseus* [13], it was primarily hypothesized that this peak is due to N-acetylglucosamine. However, this assumption could not be confirmed by a HPLC spiking experiment with N-acetylglucosamine. Since inositolamine exhibits the same sum formula as glucosamine and it is also known to occur as an intermediate in the biosynthesis of

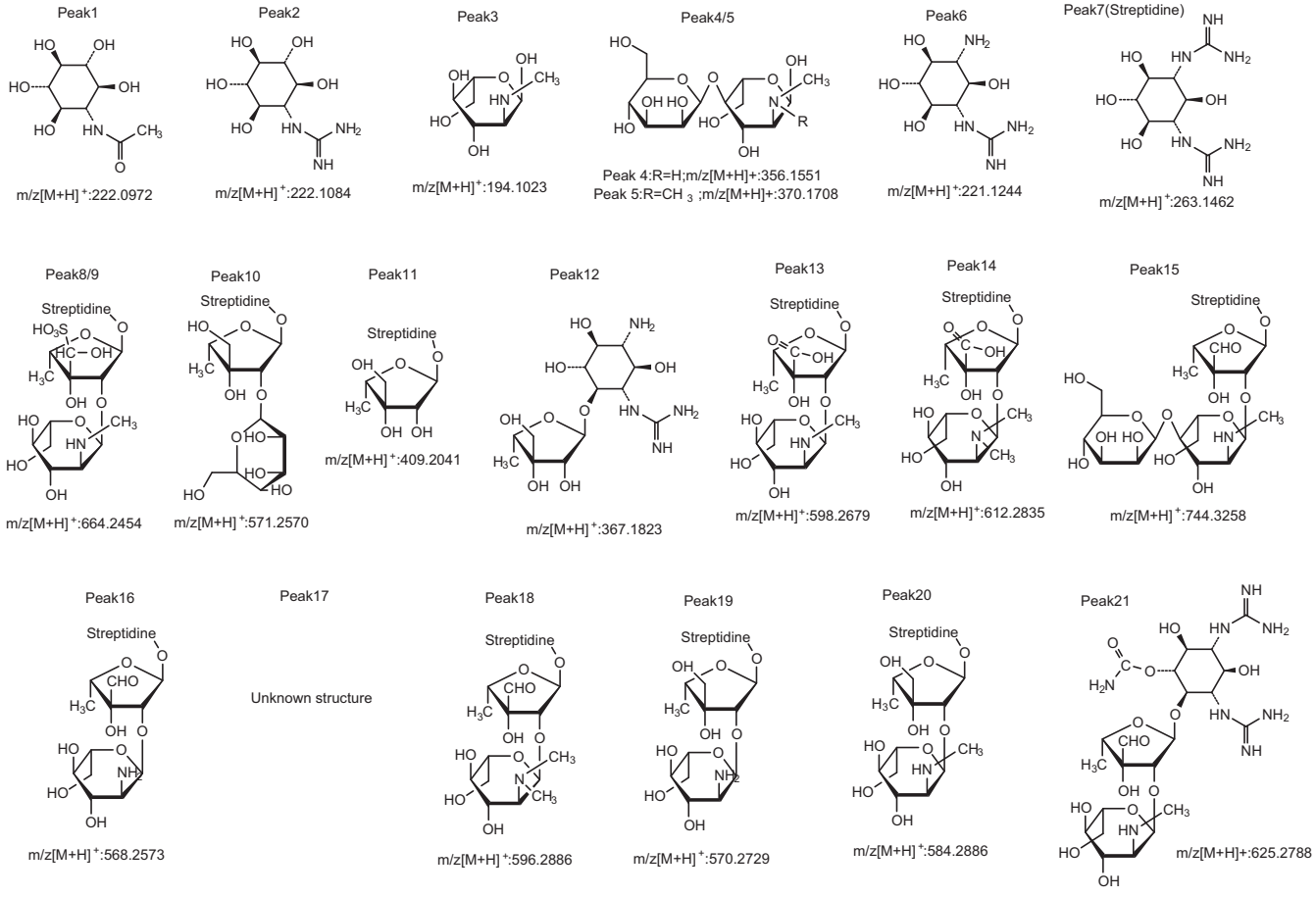


Fig. 4. Proposed structural formulae of the impurities found in streptomycin sulfate.

streptomycin [14], peak 1 was tentatively assigned as N-acetyl-inositolamine.

Peak 3 [M+H]⁺ m/z 194: Apart from a neutral loss of 18 U in the MS/MS spectrum corresponding to the loss of water, no further fragmentation could be observed. The *m/z* ratio of 194.1020 measured by TOF-MS was in very close agreement with the one of N-methylglucosamine (*m/z* M+H⁺: 194.1023). N-methylglucosamine can occur from hydrolysis of streptomycin or can be present as a by-product produced by *S. griseus* [15,16]. Moreover, the neutral loss of 18 was also observed for other impurities containing N-methylglucosamine (cf. Table 1 – peak 4 and 5). Therefore it was concluded that peak 3 is due to N-methylglucosamine.

Peak 4/5 [M+H]⁺ m/z 356: This peak was found to contain two co-eluting impurities. The first impurity exhibited an exact mass corresponding to the mannosido-N-methylglucosamine moiety of streptomycin B. This was confirmed by a neutral loss of 180 U in the MS/MS experiment which is in agreement with the loss of the mannose sugar.

Peak 4/5 [M+H]⁺ m/z 370: The exact mass of the second impurity of this peak complied with the sum formula of mannosido-N,N-dimethylglucosamine. Similar to peak 4/5*m/z* 356, a neutral loss of 180 U, corresponding to the mannose sugar, was found.

Peak 8 and 9 [M+H]⁺ m/z 664: Whilst all compounds possessing a carbonyl group as R1 (cf. Fig. 1) showed an abundant signal with a *m/z* of the hydrated form of the corresponding carbonyl group (geminal diol) ([M+H]⁺+18) [4], this was not the case for peaks 8 and 9. Therefore, a modification of R1 was likely. The exact mass of these peaks was found to be in very good agreement with the mass of the hydrogen sulfite adducts of the streptomycin carbonyl group. This assumption was supported by a neutral loss of 82 U in MS/MS, corresponding to H_2SO_3. Moreover, both peaks exhibited approximately the same peak surface indicating the formation of a chiral C-atom upon addition of hydrogensulfite to the streptomycin carbonyl function. Since hydrogensulfite is an antioxidant known to be added to streptomycin sulfate solutions [17], a sample devoid of peaks 8 and 9 was spiked with sodium hydrogensulfite. Analysis of the spiked solution by HPLC-MS showed the increase of both peaks indicating the in situ formation of streptomycin hydrogen sulfite-adducts

Peak 13 [M+H]⁺ m/z 598. In the MS/MS, a neutral loss of 175 U, corresponding to N-methyl-glucosamine, was found. As for peaks 8 and 9 no signal due to the streptomycin geminal diol was detected in the mass spectrum. Moreover, peak 13 did not decrease upon addition of hydrogensulfite, whilst this was the case for the other peaks of compounds possessing a carbonyl group in position R1 (cf. Fig. 1). Therefore it was concluded that peak 13 was not an isomer of hydroxy-streptomycin as suggested by [12] but a streptomycin analogue with a modification of R1. The *m/z*-ratio [M+H]⁺of 598.2665 observed in the ESI-positive mode was in good agreement with the sum formula of streptomycin acid which has been reported in the literature as an oxidation product of streptomycin [18]. After addition of potassium permanganate to a streptomycin test solution, peak 13 increased. Moreover, a sample enriched with this reaction product was isolated and was measured by direct infusion TOF-MS in the ESI-negative mode. A signal with a *m/z* [M−H]⁻ of 596.2591, corresponding to streptomycin acid (theoretical *m/z* (M−H)⁻ 596.2533) was found indicating that the peak 13 can be assigned to streptomycin acid.

Peak 14 [M+H]⁺ m/z 612. In the MS/MS, a neutral loss of 189 U, corresponding to N,N-dimethyl-glucosamine, was found. As for peak 13, the signal of the geminal diol of the carbonyl group at R1 was missing and no decrease of the peak size after addition of sodium hydrogensulfite was found. In analogy to peak 13, peak 14 was assigned to 2″-N,N-dimethyl-streptomycin acid.

Peak 17. An impurity with an integer mass of 728 is described in the literature [8] to be 4′-(O-dihydrostreptose)-streptomycin. In a screening performed by single ion monitoring (SIM), a *m/z* ratio of 728 could be measured for peak 17. However, the determination of the exact mass by TOF-MS revealed a mass error of 98 ppm when compared with the structure of 4′-(O-dihydrostreptose)-streptomycin. Moreover, the signal of *m/z* 728 was found to be very faint and it was not possible to decide whether the peak really corresponds to this *m/z*-ratio. For this reason peak 17 was listed as an impurity with unknown structure.

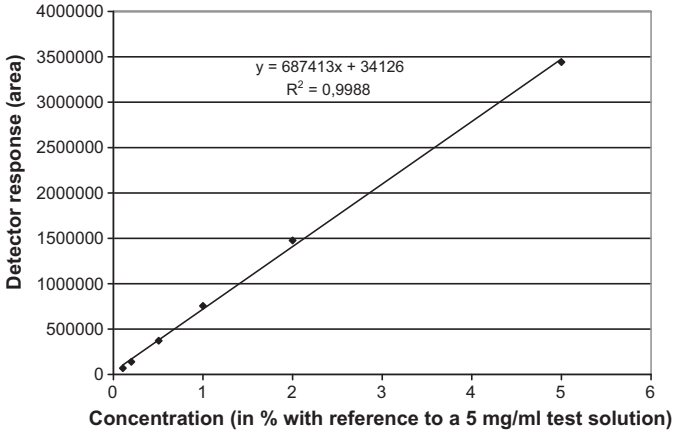


Fig. 5. HPLC-CAD calibration curve of streptomycin sulfate from 5 μg/ml to 250 μg/ml (6 concentration levels). Conditions as described in Fig. 3.

3.3. Quantification of impurities by HPLC-CAD

3.3.1. Linearity and range

The response of CAD is not linear over a broad concentration range [19], but it has been demonstrated in several studies that a quasi-linear detector response can be obtained over a limited range of about two orders of magnitude [20–22].

In this study, a verification of the linearity was performed using 6 solutions containing streptomycin at 5 μg/ml, 10 μg/ml, 25 μg/ml, 50 μg/ml, 100 μg/ml and 250 μg/ml, corresponding to a concentration range from 0.1% to 5% referred to a 5 mg/ml test solution. A linear correlation between the concentration and the detector response was found (cf. Fig. 5):

3.3.2. Limit of quantification (LOQ) and relative response factor (RRF)

The limits of quantification of the CAD – calculated on the basis of a signal-to-noise ratio of 10 – were extrapolated from the detector response of 5 μg/ml solutions of streptomycin sulfate and streptidine sulfate. The limits of quantification were determined to be 4.5 μg/ml for streptomycin sulfate and 0.4 μg/ml for streptidine sulfate (0.09% and 0.008%, respectively – referred to the concentration of a 5 mg/ml test solution). The significantly lower LOQ of streptidine compared to streptomycin can be explained by the fact that streptidine elutes much earlier and as a very narrow and high peak.

The CAD-response for different compounds is reported to be very similar in many cases [19,23,24]. However, sometimes differences in the detector response were observed [20]. Therefore, the response factor of streptidine sulfate relative to streptomycin sulfate was determined using a 25 μg/ml and a 50 μg/ml solution containing streptomycin sulfate and streptidine sulfate. The RRF of streptidine sulfate was determined to be 1.0.

3.3.3. Purity testing of streptomycin sulfate samples

The purity of 12 commercially available samples of streptomycin sulfate from different manufacturers including also the

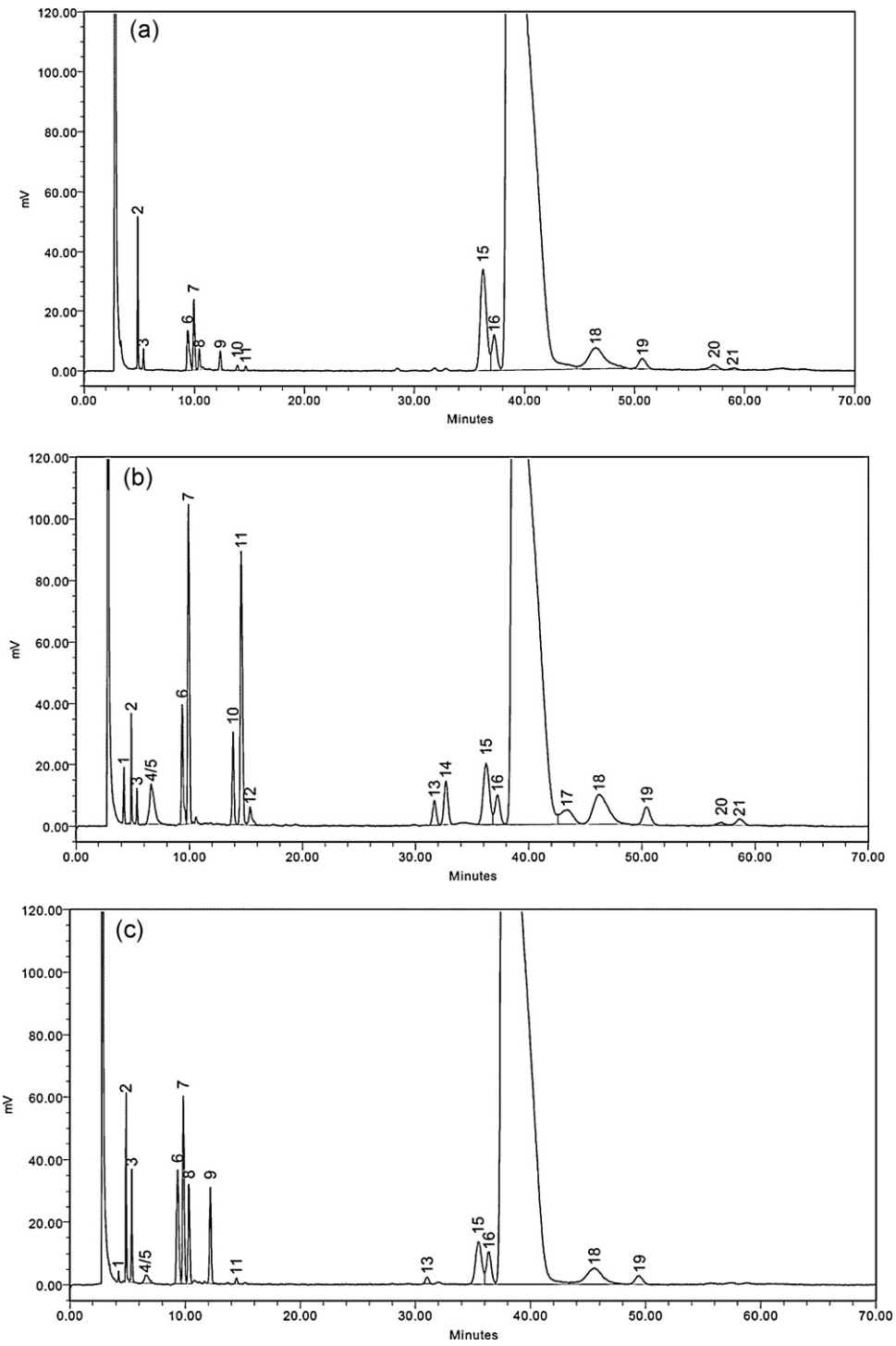


Fig. 6. HPLC chromatograms of the test solutions of (a) streptomycin sulfate Ph.Eur. CRS 3, (b) samples 10, and (c) sample 7. HPLC conditions cf. Fig. 3.

current Ph.Eur. reference standard was determined using the above described HPLC-CAD method (cf. 2.3.2). The quantification of the impurities was performed using a linear calibration curve of streptomycin sulfate from 0.1% to 3% (referred to a 5 mg/ml test solution). The total amount of impurities was found between 4.6% and 16.0%. Representative chromatograms of samples 7, 10 and Ph.Eur. CRS 3 are presented in Fig. 6. An overview of the results of the batch analysis is given in Table 2.

The amount of streptomycin B, currently limited to 3% by the Ph.Eur. using a TLC test, was found to be between 0.68% and 2.9%. Streptidine was present at levels between 0.50% and 2.9%. Moreover high amounts of impurities 6, 8, 9, 11 and 18 of up to 2.6% (impurity 11 in sample 10) were found. Impurities 2, 4/5, 10, 14, 16, 17 and 19 appeared at levels between 0.5% and 1.0%. Amounts below 0.5% were observed for impurities 1, 3, 12, 13, 20 (DHS) and 21 in all samples.

Compared with the results of the HPLC-UV method (cf. 3.1) the total amount of impurities determined by the newly developed HPLC-CAD method was found to be between 0.4% and 3.3% higher. On the one hand this can be explained by the higher method selectivity, i.e. separation of more impurities from the major peak of streptomycin, and on the other hand by the higher method

Table 2
Amounts of impurities found in commercial samples of streptomycin sulfate (%, m/m), expressed as streptomycin sulfate on the substance as is.

Sample	Peak No. (cf. Fig. 3 and Table 1 for peak identity)																				
	1	2	3	4/5	6	7 SD	8	9	10	11	12	13	14	15 SMB	16	17	18	19	20 DHS	21	Total
1	ND	0.05	0.21	0.31	0.80	0.95	ND	ND	ND	ND	ND	0.06	±0.04	0.68	0.65	ND	1.2	±0.09	ND	ND	5.1
2	0.07	0.19	0.19	0.36	0.88	2.4	1.1	1.1	0.38	1.5	0.21	0.30	0.72	1.2	0.77	0.70	2.4	0.70	0.11	0.11	15.4
3	0.04	0.24	0.39	0.09	1.3	1.8	0.69	0.67	0.19	0.49	0.11	0.23	0.39	0.69	0.68	0.24	1.4	0.93	0.11	±0.06	10.7
4	0.03	0.14	0.19	0.26	1.3	2.9	2.1	2.2	0.25	0.74	0.08	0.18	0.32	1.2	0.82	0.37	1.5	0.74	±0.09	±0.08	15.5
5	0.02	0.38	0.15	0.92	0.83	1.6	0.67	0.61	0.04	0.05	0.04	0.07	ND	0.72	0.62	ND	0.99	0.15	ND	ND	7.9
6	0.06	0.16	0.19	0.33	0.91	2.6	1.1	1.0	0.32	1.3	0.24	0.30	0.65	0.22	0.76	0.72	2.3	0.70	0.11	± 0.07	14.0
7	0.04	0.67	0.46	0.17	1.1	1.4	0.71	0.71	ND	0.05	ND	0.11	ND	1.1	0.70	ND	1.2	0.28	ND	ND	8.7
8	0.04	0.21	0.28	0.11	1.2	2.6	1.2	1.1	0.54	1.4	0.05	0.27	0.78	1.7	0.96	0.33	1.8	0.98	0.11	0.12	15.8
9	ND	0.23	0.22	0.48	1.2	1.3	0.31	0.26	ND	ND	ND	± 0.05	ND	0.79	0.74	ND	1.2	0.10	ND	ND	6.9
10	0.26	0.39	0.15	0.90	0.96	2.5	ND	ND	0.77	2.6	0.25	0.39	0.77	1.7	0.68	0.81	2.0	0.59	±0.08	0.21	16.0
11	ND	0.03	0.14	ND	0.84	0.56	ND	ND	ND	± 0.02	ND	0.06	± 0.05	0.88	0.57	ND	1.4	± 0.09	ND	ND	4.6
Ph.Eur. CRS 3	ND	0.52	0.08	ND	0.46	0.50	0.15	0.15	0.04	0.03	ND	ND	ND	2.9	0.81	ND	1.8	0.36	0.17	± 0.06	8.0

ND, not detected (below LOD), ± is used for values below LOQ, these results were taken into account for the calculation of the total; SD, streptidine; SMB, streptomycin B; DHS, dihydrostreptomycin.

sensitivity of the HPLC-CAD method (LOQ streptomycin sulfate: 0.09%) compared to the HPLC-UV method (LOQ streptomycin sulfate: 0.4%).

4. Conclusion

A HPLC-CAD method for the separation and quantification of impurities in streptomycin sulfate using PFPA as a volatile ion-pair reagent was developed. With this method 21 impurities could be separated from streptomycin sulfate.

A direct coupling of the HPLC with a TOF-MS detector enabled the determination of the exact mass of all impurities: Moreover, MS-fragmentation data were obtained by HPLC–MS/MS coupling. For 12 impurities structures could be suggested based on comparison with literature data. For 8 further impurities tentative structures were proposed based on the work carried out in this study. For one impurity, it was not possible to suggest a structural formula.

The HPLC-CAD method allowed the quantification of the impurities with a limit of quantification of at least 0.1%, referred to the concentration of the streptomycin sulfate test solution. The analysis of 12 samples of streptomycin sulfate from different manufacturers resulted in a total amount of impurities between 4.6% and 16.0%.

Based on the results of the analysed samples, the following tentative specification for the impurities in streptomycin sulfate could be considered: streptomycin B, max. 3.0%; streptidine, max. 3.0%; impurity 18, max. 2.0%; impurities 6, 8, 9, 11, max. 1.5%, impurities 2, 4/5, 10, 14, 16, 17, and 19, max. 1.0%; impurities 1, 3, 12, 13, 20 (DHS), and 21, max. 0.5%; unspecified impurities, max. 0.2%; sum of impurities, max. 16.0%.

To the best of our knowledge the method presented in this paper offers a combination of selectivity and sensitivity that has not been reported so far and could be used to improve the quality control of streptomycin sulfate in the corresponding Ph.Eur. monograph.

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