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Analysis of kanamycin sulfate by liquid chromatography with pulsed electrochemical detection

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Abstract

The analysis of kanamycin sulfate by liquid chromatography using a column packed with poly(styrene–divinylbenzene) and pulsed electrochemical detection on a gold electrode is described. A two-step gradient was necessary to obtain a good separation together with a reasonable analysis time of maximum 45 min. The mobile phases consisted of an aqueous solution of 20 g/l or 60 g/l sodium sulfate, 1.3 g/l sodium octanesulfonate and 50 ml/l 0.2 *M* phosphate buffer pH 3.0. Sodium hydroxide was added post-column. The influence of the different chromatographic parameters on the separation was investigated. A number of commercial samples were analyzed using this method. Besides the previously reported impurities, such as kanamycins B and C, two other impurities were separated, one of which is called kanamycin D. In total, eight components were separated.

Keywords: Kanamycin sulfate; Antibiotics; Pharmaceutical analysis

1. Introduction

Kanamycin, which is mainly used as the sulfate, is a water soluble broad spectrum aminoglycoside antibiotic produced during fermentation of *Streptomyces kanamyceticus* [1]. It has a narrow therapeutic range and is potentially oto- and nephrotoxic like other aminoglycosides.

The main component is kanamycin A (Fig. 1). Small amounts of the structurally related components kanamycin B and kanamycin C were reported to be present [2]. The presence of other minor constituents, paromamine and 6-O-(3-amino-3-deoxy-α-D-glucopyranosyl)deoxystreptamine (6-O-(3-AG)-DS), was reported by Claes et al. [3]. The structure and antimicrobial activity of 4-O-(6-amino-6-deoxy-α-D-glucopyranosyl)deoxystreptamine (4-O-(6-AG)-DS) obtained by hydrolysis of kanamycin A, were reported previously [4]. Its presence in commercial samples is reported in this paper. The isolation from a commercial sample of the substance called kanamycin D was carried out in this laboratory. After analysis it appeared to be identical to compound NK-1001, which was isolated from culture filtrates and described in 1970 by Murase et al. [5]. The latter two products, 4-O-(6-AG)-DS and kanamycin D, were not yet reported to be present in commercial kanamycin samples.

Chromatographic separation of the different kanamycin components is quite difficult. Paper chromatography [2], ion-exclusion chromatography [2,3,6], gas-liquid chromatography after silylation [7] and

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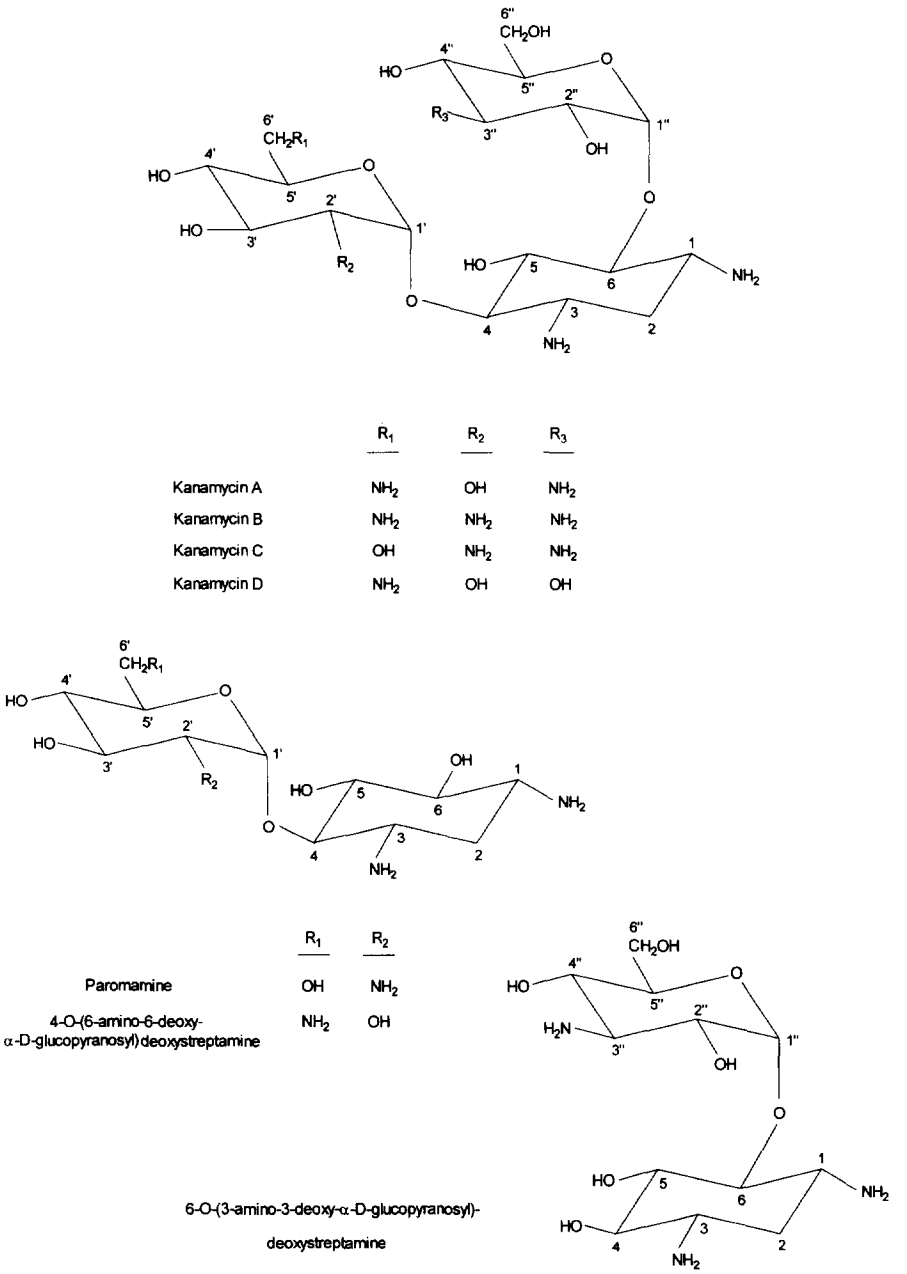

Fig. 1. Structures of the different kanamycin components.

reversed-phase liquid chromatography (LC) [8] have been reported for the analysis of kanamycin. Detection is also problematic because kanamycin has no strong UV absorbing chromophore. Detection systems based on conductivity [3], colorimetry after reaction with ninhydrin [6] and pre-column derivatization with 2,4,6-trinitrobenzenesulphonic acid have been used [8].

In this work an ion-pair LC method is described using a column packed with poly(styrene–divinylbenzene) and pulsed electrochemical detection. The composition of the mobile phase was based on

that previously used for the analysis of neomycin sulfate, another aminoglycoside antibiotic [9]. The method has been used to analyze a number of commercial samples.

2. Experimental

2.1. Reagents

Water was distilled twice from glass apparatus. The buffer solution was made 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. These solutions were prepared with phosphoric acid 85% m/m and potassium dihydrogen phosphate (Acros Chimica, Geel, Belgium). Anhydrous sodium sulfate was obtained from Merck (Darmstadt, Germany); sodium 1-octanesulfonate, monohydrate 98% from Acros Chimica and helium from Air Liquide (Machelen, Belgium). The 0.5 *M* sodium hydroxide solution was made using sodium hydroxide 50% (m/m), aqueous solution (Baker, Deventer, Netherlands).

2.2. Reference substances

Kanamycin B was obtained from Bristol Laboratories (Syracuse, NY, USA) and kanamycin C from Merck (Rahway, NJ, USA). Deoxystreptamine was prepared in the laboratory from neamine [10] and paromamine was isolated from commercial neomycin samples [11]. Kanamycin D was isolated from a commercial kanamycin sulfate sample using open column chromatography (250×45 mm) on Amberlite CG 50 type I ion-exchange resin, 100–200 mesh (Serva, Heidelberg, Germany), in the NH_4^+ form. After applying a solution of 20 g of commercial kanamycin in water, the column was washed with 1000 ml of water. The elution was performed by using 0.2 *M*, 0.25 *M* and 1.0 *M* NH_4OH. Fractions of 50 ml were collected. To monitor the fractions, thin-layer chromatography (TLC) on silica gel (150A K5, Whatman, Maidstone, UK) was carried out, using methanol–20% (m/v) sodium chloride solution (50:50) as the mobile phase. Spots were visualized by spraying with ninhydrin solution (1.0 g in 50 ml alcohol and 10 ml of glacial acetic acid) and heating at 100–105°C for 10 min. The first 20 fractions were eluted with 0.2 *M* NH_4OH. They contained no kanamycin components. Fractions 21–47 were eluted with 0.25 *M* NH_4OH. Kanamycin D was present in fractions 31–37. These fractions were evaporated to dryness under vacuum and the structure was determined by MS and ^{13}C-NMR. It corresponds to that of NK-1001 [5].

Kanamycin derivatives 6-O-(3-AG)-DS and 4-O-(6-AG)-DS were obtained by hydrolysis of kanamycin A, based on the method described by Umezawa and Tsuchiya [4]. For the preparation of 6-O-(3-AG)-DS, 2 g of kanamycin sulfate were refluxed in 20 ml of 2 *M* hydrochloric acid for 10 min. The solution was evaporated to dryness under reduced pressure after it was neutralized with ammonia to avoid further hydrolysis. To separate the different components of the residue, the same procedure as described above was followed. The first 20 fractions, which were eluted with 0.2 *M* NH_4OH, contained no kanamycin components. Fractions 21–40 were eluted with 0.25 *M* NH_4OH and 6-O-(3-AG)-DS was present in fractions 21–29. These fractions were evaporated to dryness under vacuum and the structure was confirmed by MS and ^{13}C-NMR. Fractions 30–40 contained a mixture of several components. Kanamycin A was eluted with 1.0 *M* NH_4OH.

Since 4-O-(6-AG)-DS is less stable than 6-O-(3-AG)-DS, 2 g of kanamycin sulfate were refluxed in 20 ml of 2 *M* hydrochloric acid for only 6 min. A shorter reflux time gave more kanamycin A and a longer one more degradation products like deoxystreptamine. The isolation and identification procedures were similar as above, but Amberlite CG 50 II, 200–400 mesh (Fluka, Buchs, Switzerland) was used.

Commercial kanamycin sulfate and acid sulfate samples were obtained from VMD (Arendonk, Belgium), Kela (Hoogstraten, Belgium), Continental Pharma (Machelen, Belgium), Dopharma (Raamsdonksveer, Netherlands) and Fluka (Buchs, Switzerland).

A house standard of kanamycin A base was prepared in the laboratory starting from commercial kanamycin sulfate, using the above-mentioned chromatographic procedure with Amberlite CG 50 I and NH_4OH. The base content (95.81%) of the house

standard was determined by non-aqueous potentiometric titration with 0.1 *M* perchloric acid using glacial acetic acid as the solvent. The water content was determined by Karl Fischer titration to be 3.90%. The total mass explained by titration and water determination was 99.71%. The percentage of impurities, determined by LC and expressed as kanamycin A, was 0.95%. So the purity of this standard was 94.86% (m/m), expressed on the substance as is.

2.3. Apparatus

The chromatographic procedure was carried out using a L-6200 Intelligent Pump (Merck-Hitachi, Darmstadt, Germany), a Marathon autosampler (Spark Holland, Emmen, Netherlands) with a fixed loop of 20 μl, a laboratory-made pneumatic device, allowing pulse-free post-column addition of sodium hydroxide solution and an electronic integrator HP 3393 A (Hewlett-Packard, Avondale, PA, USA). The column (250 mm×4.6 mm I.D.) was packed with poly(styrene–divinylbenzene) PLRP-S 1000 Å, 8 μm (Polymer Laboratories, Shropshire, UK). The temperature of the column was maintained at 45°C using a water bath with a circulator (Julabo, Seelbach, Germany). The PED-1 pulsed electrochemical detector from Dionex (Sunnyvale, CA, USA) was equipped with a gold working electrode with a diameter of 3 mm, a Ag/AgCl reference electrode and a stainless steel counter electrode. The detector was put in a laboratory-made hot-air oven to keep the temperature at 35°C.

2.4. Chromatography

All substances to be analyzed were dissolved in water. A two-step gradient was necessary to obtain a good separation between the first eluted compounds and to elute the others within a reasonable analysis time. Mobile phase A consisted of an aqueous solution containing 20 g/l sodium sulfate, 1.3 g/l octanesulfonate and 50 ml/l 0.2 *M* phosphate buffer pH 3.0. Mobile phase B consisted of an aqueous solution containing 60 g/l sodium sulfate, 1.3 g/l octanesulfonate and 50 ml/l 0.2 *M* phosphate buffer pH 3.0. The mobile phases were sonicated before use. The gradient was chosen as follows: 0–11.0 min: 100% A; 11.1–23.0 min: 60% A–40% B; 23.1–32.0 min: 10% A–90% B; 32.1 –45.0 min: 100% A. The flow-rate was 1 ml/min. Through a mixing tee 0.5 *M* NaOH was added post-column from a helium-pressurized reservoir (1.6 bar) and mixed in a packed reaction coil (1.2 m, 500 μl) from Dionex which was linked to the electrochemical cell. The post-column addition of the base must be pulse-free and is necessary to raise the pH of the mobile phase to approximately 13 to improve the sensitivity of the detection [12]. The base was added at a flow-rate of 0.3 ml/min. Although the flow-rate is not critical, it should be constant. The NaOH solution was made starting from a 50% m/m aqueous solution which was pipetted into helium degassed water because it is very important to avoid carbonates that foul the electrodes. For this reason it is advisable to pipette the NaOH solution from the center of the bottle and to use only two thirds of the bottle [13].

The time and voltage parameters for the detector were the same as previously used for neomycin [9] and were set as follows: E_1, E_2 and E_3 were respectively +0.05 V, +0.75 V and −0.15 V with the assigned pulse durations t_1: 0–0.40 s, t_2: 0.41–0.60 s, t_3: 0.61–1.00 s. Integration of the signal occurred between 0.2 and 0.4 s. Although the sequence of the potentials theoretically cleans the electrode surface, it is necessary to polish the gold working electrode after about 40 analyses to obtain a good repeatability. After the electrode is cleaned with fine polishing compound it is sonicated in water for 10 min. It takes about one hour to obtain a stable baseline with a freshly polished electrode.

3. Results and discussion

3.1. Chromatographic method

A typical chromatogram of a commercial sample of kanamycin sulfate, obtained under the selected chromatographic conditions, is shown in Fig. 2 (Scheme 1 shows chromatographic conditions). It is clear that the total analysis time can be shortened by shortening each step of the gradient. Here, the longer analysis time was applied to make sure that no impurity was overlooked.

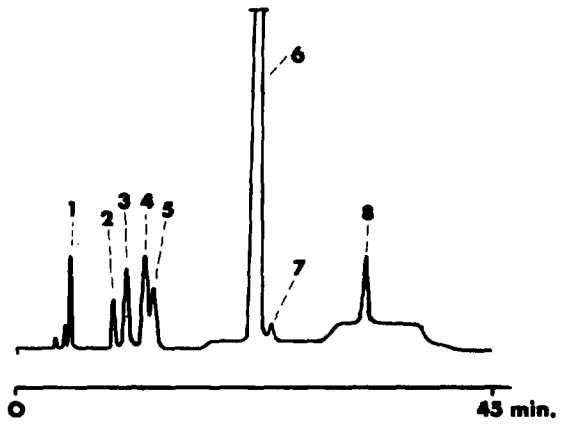

Fig. 2. Typical chromatogram of a commercial kanamycin sulfate sample. See Scheme 1 for chromatographic conditions. 1=deoxystreptamine, 2=kanamycin D, 3=4-O-(6-AG)-DS, 4=6-O-(3-AG)-DS, 5=paromamine, 6=kanamycin A, 7=kanamycin C, 8=kanamycin B.

Poly(styrene–divinylbenzene) was chosen as the stationary phase because of its remarkable stability and between batch reproducibility. In order to examine the robustness of the method, the influence of the different chromatographic parameters on the separation of the kanamycin compounds was evaluated using the capacity factors (k'). Only one parameter was changed while the others were kept constant. Methanol was used to determine t_0. For the calculation of k', the average retention time of two analyses was used.

The influence of the pH of the mobile phase on the k' values of the kanamycin components is illustrated in Fig. 3. As can be seen, there are little changes between pH 2.0 and pH 4.0. By further increase of the pH, the retention times decrease since less amino groups are protonated and the interaction with octanesulfonate decreases. The influence of the column temperature was examined at 40, 45 and 50°C. As expected, the k' values of the components decrease when the column temperature is increased. Sodium octanesulfonate acts as an ion-pairing agent. It is added to retain the kanamycin molecules, which are positively charged at pH 3.0. The sodium octanesulfonate concentration of the mobile phase was

Stationary phase : PLRP-S 1000 Å, 8 μm, 250 mm × 4.6 mm I.D., Polymer Laboratories, Shropshire, UK.

Mobile phase :	A	B
sodium sulfate	20 g/l	60 g/l
sodium 1-octanesulfonate	1.3 g/l	1.3 g/l
phosphate buffer pH 3, 0.2 M	50 ml/l	50 ml/l
water	up to 1 l	up to 4 l

Two-step gradient :		
	0 - 11.0 min	100 % A
	11.1 - 23.0 min	60 % A - 40 % B
	23.1 - 32.0 min	10 % A - 90 % B
	32.1 - 45.0 min	100 % A

Flow rate : 1 ml/min
Injection volume : 20 μl
Column temperature : 45 °C

Post-column addition of 0.5 M NaOH : 0.3 ml/min

Pulsed electrochemical detector :
Working electrode : gold
Reference electrode : Ag/AgCl
Counter electrode : stainless steel

Detector settings :	t (s)	E (volt)
	0 - 0.40	0.05
	0.41 - 0.60	0.75
	0.61 - 1.00	-0.15

Integration period : 0.20 - 0.40 s
Sensitivity : 1 μC
The detector was kept at 35 °C.

Scheme 1. LC conditions

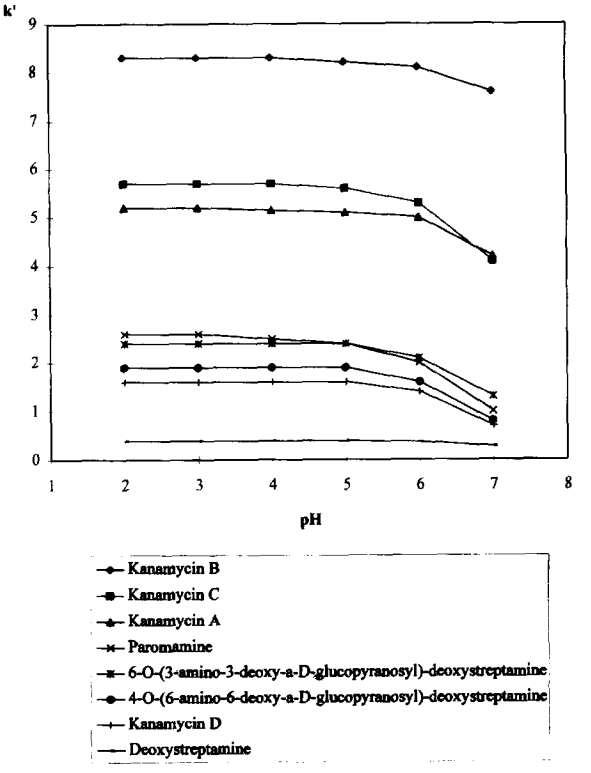

Fig. 3. Influence of the pH of the mobile phase on the k' values.

varied in the range from 1.1 to 1.5 g/l. As expected, the capacity factors decrease slightly by decreasing the sodium octanesulfonate concentration. Below 1.2 g/l the separation between 4-O-(6-AG)-DS and kanamycin D is insufficient (resolution <1.25). Sodium sulfate has been used in ion-pairing mobile phases to shorten retention times as the sulfate anions are more hydrophilic than the anions of the ion-pairing agent [14].

The chromatography was also performed using PLRP-S materials having smaller pore sizes of 100 Å and 300 Å. On the narrow pore materials the peak symmetry was poor and the retention times were higher. Similar differences were observed in the analysis of neomycin [9], as well as in the analysis of macrolide antibiotics like erythromycin [15], tylosin [16] and josamycin [17].

3.2. Quantitative aspects of the LC method

For the determination of the impurities in kanamycin a 10 μg sample was used by injecting 20 μl of a 0.5 mg/ml solution. For this quantity the limit of detection for kanamycin D and paromamine was 0.03% (m/m) (3 ng) and for kanamycin B 0.05% (m/m) (5 ng), determined at a signal-to-noise ratio of 3. The limit of quantitation was 0.1% (m/m) for kanamycin D (R.S.D.=8.2%; $n=4$) and paromamine (R.S.D.=9.8%; $n=4$) and 0.15% (m/m) for kanamycin B (R.S.D.=9.2%; $n=4$). The linearity of kanamycin D, paromamine and kanamycin B was examined in the concentration range corresponding to 0.3% to 10% of the sample concentration (0.5 mg/ml). The following results were found: for kanamycin D: $y=897\,813x+917$; $r=0.9997$ and $S_{y,x}=460$; for paromamine: $y=1\,372\,512x+1863$; $r=0.9994$ and $S_{y,x}=1006$ and for kanamycin B: $y=646\,217x+549$; $r=0.9991$ and $S_{y,x}=565$, where $y=$ peak area/1000; $x=$ concentration in mg/ml; $r=$ coefficient of correlation and $S_{y,x}=$standard error of estimate. The kanamycin A content was determined using a sample concentration of 0.05 mg/ml (1 μg injected) since there is no good linearity in the range from 0.06 mg/ml to 0.5 mg/ml, probably due to overloading of the electrodes. The linearity of kanamycin A was examined in the concentration range corresponding to 20% to 120% of the sample concentration (0.05 mg/ml). The following results were found: $y=631\,964x+223$; $r=0.9992$ and $S_{y,x}=588$. The repeatability was checked by analyzing 6 times a 0.05 mg/ml solution of kanamycin sulfate. The R.S.D. on the area of kanamycin A was 1.4%.

3.3. Analysis of commercial samples

Several samples of kanamycin sulfate and acid sulfate were analyzed using the described method. The composition of the commercial samples is shown in Table 1. All substances are expressed as kanamycin A base, calculated with reference to the kanamycin house standard (94.86%, m/m, as is). The content of the minor components was calculated using reference chromatograms obtained with a 10% (m/V) dilution (0.05 mg/ml) of kanamycin A base. The following contents were found in the 16 samples examined: less than 1.51% of deoxystreptamine, 0.40–2.85% of kanamycin D, 0.12–2.96% of 4-O-(6-AG)-DS, 0.04–4.04% of 6-O-(3-AG)-DS, less than 2.77% of paromamine, 54.7–80.5% of kanamycin A, less than 0.42% of kanamycin C and 0.11–1.83% of kanamycin B.

4. Conclusion

The described method, using poly(styrene–divinylbenzene) as the stationary phase, allows to separate 8 components of kanamycin. The maximum analysis time is 45 min. This study reports for the first time the presence of kanamycin D and 4-O-(6-AG)-DS in commercial samples. These compounds, present in significant amounts, are found to be the more important impurities. The use of pulsed electrochemical detection needs some experience to obtain reproducible results. However, compared to the few chromatographic methods previously published, this method allows sensitive detection of kanamycins without derivatization.

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Table 1
Composition of kanamycin sulfate and acid sulfate (*) samples (% (m/m)), expressed as kanamycin A base

	Deoxy-streptamine	Kanamycin D	4-O-(6-AG)-DS[a]	6-O-(3-AG)-DS[b]	Paromamine	Kanamycin A	Kanamycin C	Kanamycin B
1	ND	0.96	0.12	0.10	ND	76.4	0.05	1.24
2	1.51	1.03	2.96	4.04	2.77	54.7	0.35	1.83
3	ND	2.85	0.85	0.23	ND	61.4	ND	0.17
4	ND	1.43	0.43	0.21	ND	76.6	±0.02	0.24
5	ND	0.40	0.45	0.04	ND	80.5	ND	0.11
6	ND	0.66	0.49	0.30	ND	76.4	ND	1.23
7	ND	0.94	0.61	0.41	ND	70.8	±0.02	0.93
8	0.16	1.49	1.03	0.42	ND	75.6	±0.02	0.55
9	ND	0.99	0.59	0.38	ND	77.9	±0.02	1.32
10	ND	1.84	0.59	0.18	ND	73.9	ND	0.39
11	ND	1.20	0.44	0.14	ND	78.2	0.05	0.40
12	0.06	1.75	0.55	0.34	ND	75.9	0.07	0.36
13	ND	1.49	0.85	0.29	ND	75.8	0.07	0.78
14	ND	1.53	0.43	0.35	ND	75.2	ND	0.30
15*	0.59	2.06	1.42	1.08	2.17	57.8	0.42	3.25
16*	ND	1.76	0.75	0.16	ND	61.4	ND	0.12

[a] 4-O-(6-amino-6-deoxy-α-D-glucopyranosyl)deoxystreptamine.
[b] 6-O-(3-amino-3-deoxy-α-D-glucopyranosyl)deoxystreptamine.
ND=not detectable.

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