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Journal of Chromatography A, 674 (1994) 255–261

JOURNAL OF CHROMATOGRAPHY A

Forensic investigation of gentamicin sulfates by anion-exchange ion chromatography with pulsed electrochemical detection

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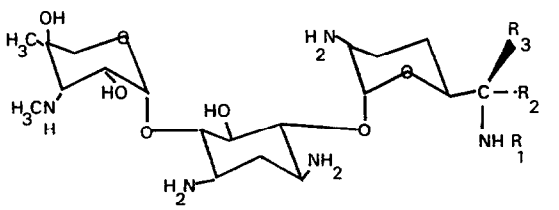
Abstract

Many methods exist for the separation of gentamicin C complex components, C_1, C_{1a}, C_2 and C_{2a}. In an investigation of possible counterfeit suppliers of gentamicin sulfate, a new method utilized high-pH anion-exchange separation on a carbohydrate column, with pulsed electrochemical detection on a gold electrode. Component ratios and the presence and/or absence of additional peaks were used to link or dissociate forensic samples.

1. Introduction

The aminoglycoside antibiotic gentamicin is produced by the growth of *Micromonospora purpurea*, and consists of four major components C_1, C_{1a}, C_2 and C_{2a} [1]. As many as four minor components have also been identified [2,3]. Numerous methods for the analysis of gentamicin components have been published including: thin-layer paper chromatography [4], high-performance liquid chromatography (HPLC) [1,5–7], and HPLC–thermospray mass spectrometry [8]. The official Food and Drug Administration method for the determination of percent composition is a paper chromatographic separation followed by microbiological assay [9]. Current United States Pharmacopeia (USP) protocol specifies an HPLC separation of pre-column derivatized components for the determination of percent composition and a microbial assay for potency [1]. The lack of strong UV chromophores in gentamicin (refer to Fig. 1) makes direct detection difficult. The hydroxyl moieties, however, present the possibility of electrochemical detection as used for carbohydrate analysis. Aminoglycosides are anionic at high pH and therefore are retained on an anion-exchange column. The aminoglycosides are then oxidized at high pH on a gold electrode [10]. High-performance anion-exchange chromato-



Gentamicin	R_1	R_2	R_3	M_r
C_1	CH_3	H	CH_3	477
C_{1a}	H	H	H	449
C_2	H	H	CH_3	463
C_{2a}	H	CH_3	H	463
C_{2b}	CH_3	H	H	463

Fig. 1. Structures of major gentamicin C complex components.

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SSDI 0021-9673(94)00013-Y

graphic (HPAEC) separation was used with pulsed amperometric detection on a gold electrode (HPAEC–PAD) by Statler for the determination of tobramycin [11], an aminoglycoside antibiotic similar to gentamicin.

Forensic investigations of bulk drugs and injectable preparations at the National Forensic Chemistry Center (NFCC) attempted to link or dissociate various sources of gentamicin. Although studies by other researchers have shown the considerable variation in the percent composition of commercial gentamicin, most gentamicin tended to be within the USP allowable range [6]. USP allowable ranges are: $C_1 = 25–50\%$; $C_{1a} = 10–35\%$; $C_2 + C_{2a} = 25–55\%$, where percent of each component is calculated as the peak responses of each peak divided by the sum of peak responses for all four peaks [1]. A method was sought which could determine C_1, C_{1a}, C_2 and C_{2a}, and which might also be sensitive enough to detect trace components in order to compare samples. Analysis of contaminants in bulk drugs may be used as a "chemical fingerprint" to track drug sources since various manufacturing processes may contribute characteristic residual chemicals to the fingerprint. This study reports the use of HPAEC–PAD for the comparison of gentamicin from various sources.

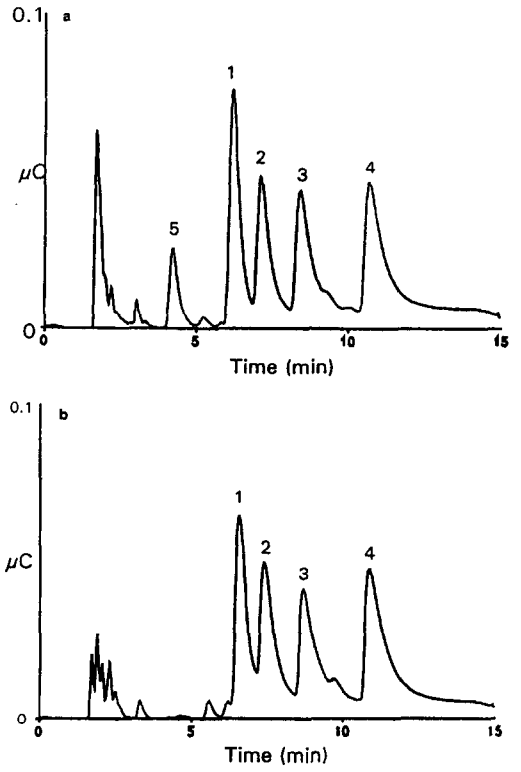

Fig. 2. Comparison of bulk gentamicin sulfate sources. (a) Manufacturer A; (b) manufacturer B. Peaks: 1 = C_{1a}; 2 = C_2; 3 = C_{2a}; 4 = C_1; 5 = fifth peak. Column: Carbopac PA-1; flow-rate: 1.0 ml/min; injection volume: 20 μl. Post column reagent: 0.5 *M* NaOH at 0.5 ml/min. Gradient program: eluent 1: 18 MΩ water; eluent 2: 10 m*M* NaOH.

Time(min)	Eluent 1 (%)	Eluent 2 (%)	Comments
0.0	70	30	Re-equilibration
5.0	70	30	
5.1	70	30	Injection occurs
20.0	50	50	

2. Experimental

2.1. Reagents and standards

Gentamicin sulfate USP reference standard lot I-1 was obtained from United States Pharmacopeial Convention (Rockville, MD, USA) with a labeled potency of 682 μg gentamicin base/mg standard. Eluent and post column reagent were prepared using 50% (w/w) sodium hydroxide (Fisher Scientific, Fair Lawn, NJ, USA). Care was taken to minimize carbonate contamination of the eluent since carbonate would alter the eluent strength. Deionized distilled water (18 MΩ), purified using a Milli-Q water system (Millipore, Milford, MA, USA), was sparged with helium to eliminate CO_2, prior to the addition of hydroxide. Sisomicin sulfate, kanamycin A, tobramycin sulfate, clindamycin hydrochloride, cloxacillin sodium salt, cefazoline sodium salt, penicillin G potassium salt, and neomycin sulfate standards were obtained from Sigma (St. Louis, MO, USA).

2.2. Apparatus and chromatography

The instrumentation used was a Dionex 4500 ion chromatograph (Dionex, Sunnyvale, CA, USA) consisting of a gradient pump (GPM-1); a

Rheodyne 9126 injector equipped with a 20-μl sample loop; a pulsed electrochemical detector (PED-1) with gold working electrode operated in the integrated amperometry mode; an automated sampler (ASM-1); a reagent-delivery module (RDM) consisting of a pressurized reservoir, mixing tee, and reaction coil; and an AI-450 software program for instrument control and data collection.

An anion-exchange column, Dionex Carbopac PA-1 analytical column (250 × 4 mm), and PA-1 guard (50 × 4 mm) were used. Gradient conditions are listed with Fig. 2.

Eluent flowed through the column to the mixing tee, where 0.5 *M* NaOH was added from the pressurized reservoir, and mixed in the reaction coil prior to the electrochemical cell. The post-column addition of base was necessary to raise the pH of the mobile phase to approximately 13 to improve sensitivity and stabilize the gradient baseline [12]. The detector settings were potential $E_1 = 0.10$ V, time $t_1 = 300$ ms; $E_2 = 0.60$ V, $t_2 = 120$ ms; $E_3 = -0.80$ V, $t_3 = 300$ ms. The pulse sequence cleans the electrode surface on a continuous basis, resulting in improved reproducibility and lessened electrode fouling.

Percent composition was determined by the following formula: % composition = area of component peak/sum of areas of four peaks $(C_{1a} + C_2 + C_{2a} + C_1) \times 100$. Potency was calculated based on total area of the four peaks compared to USP standard of known potency. Samples of gentamicin were diluted in water to a concentration of approximately 100 to 200 μg gentamicin sulfate/ml.

3. Results and discussion

The separation of gentamicin components C_{1a}, C_2, C_{2a} and C_1 by gradient HPAEC–PAD is shown in Fig. 2. Peak identifications were made based upon comparison of the percent composition calculated for the USP standard compared to the reported values based upon HPLC. In assigning C_2 as the peak prior to C_{2a}, since the USP did not report values for C_2 and C_{2a} individually, it was assumed that positional isomers would elute near one another. The first peak was assigned as C_2 since the area of that peak was larger and C_2 is generally present in higher proportions than C_{2a}. These peak assignments make sense chromatographically concerning size and steric hindrance but do not take into account effects on pK_a. Retention time and area response reproducibility of ten replicate injections on the same day, as well as linear range data are presented in Table 1. The minimum detection limit for gentamicin sulfate is 20 ng on column, defined as three times the standard deviation of a blank divided by peak response factor. The detection limit calculated based on the smallest peak in the USP standard, C_{2a}, was used as the detection limit of gentamicin sulfate. The detection limits reported for other methods include: 10 ng on column for HPLC with fluorescence of derivitized components [13]; approximately 10 μg on column for HPLC–UV with derivatization [14]; 16 μg on column for HPLC–electrochemical detection [15]; and 400 ng on column for HPLC–thermospray MS [8]. Several antibiotics which are used

Table 1
Short-term reproducibility of area response and retention time for 190 μg/ml gentamicin sulfate

Peak	t_R (min)	R.S.D. (%)[a]	R.S.D. (%)	Linearity 5–200 μg/ml	
		t_R	Area	Slope	Correlation coefficient
C_{1a}	5.91	0.8	1.1	3 113 258	0.9998
C_2	6.66	0.7	1.5	5 304 894	0.9999
C_{2a}	8.04	0.7	4.0	2 918 240	0.9999
C_1	10.05	0.7	4.0	4 565 322	0.9999

[a] $n = 10$.

Table 2
Comparison of gentamicin component values determined by ion chromatography with manufacturer's certified values

Manufacturer	Lot	Ion chromatography values[a]					Manufacturer's values[b]		
		C_{1a} (%)	C_1 (%)	$C_2 + C_{2a}$ (%)	C_2 (%)	C_{2a} (%)	C_{1a} (%)	C_1 (%)	$C_2 + C_{2a}$ (%)
A	Feb 90	30.1 (0.5)	22.8 (0.7)	41.1 (0.2)	27.7 (0.4)	13.4 (1.2)	NA[c]	NA	NA
	May 91	22.8 (0.4)	29.0 (1.0)	48.3 (0.5)	32.7 (0.4)	15.5 (0.6)	22	31	48
	Aug 91	32.4 (1.5)	26.3 (1.8)	41.4 (0.2)	22.7 (1.8)	18.6 (2.1)	30	31	46
	Dec 91	25.6 (0.8)	29.9 (1.7)	44.5 (0.7)	24.0 (1.7)	20.6 (0.4)	23	34	46
	Feb 92	24.3 (2.0)	32.5 (0.6)	43.2 (0.7)	22.3 (0.4)	20.8 (1.4)	26	32	42
B	Sept 91	29.4 (0.3)	30.0 (0.7)	40.6 (0.5)	24.5 (0.4)	16.1 (1.0)	30.2	37.5	32.3[d]
	Feb 92	21.7 (0.5)	30.8 (1.0)	47.5 (0.4)	33.4 (0.6)	14.0 (0.7)	21.9	30	48.1[e]
USP	I-1	20.5 (1.0)	28.3 (1.2)	51.2 (0.9)	33.8 (1.0)	17.5 (1.0)	21	29	50

[a] Averages of four analyses. Sum of four components taken as 100%. Numbers in parentheses are of component ratios. R.S.D. (%).
[b] HPLC analysis, qualified to USP XXII.
[c] NA = Not available.
[d] $C_2 = 18.5$; $C_{2a} = 13.9$.
[e] $C_2 = 33.5$; $C_{2a} = 14.6$.

concomitantly with gentamicin or are structurally similar (kanamycin, tobramycin, clindamycin, cloxacillin, cefazoline, penicillin G, and neomycin) were injected onto the ion chromatograph and did not interfere with the determination of gentamicin.

Table 3
Comparison of percent C_2 to C_{2a} ratios and presence of fifth peak in bulk drugs, as determined by ion chromatography

Manufacturer	Lot	$C_2 + C_{2a}$ (%)	C_2 (%)	C_{2a} (%)	C_2 (%)/C_{2a} (%)	Area 5th peak[a]
A	Feb 90	41.1	27.7	13.4	2.07	ND[b]
	May 91	48.3	32.7	15.5	2.11	ND
	Aug 91	41.4	22.7	18.6	1.22	1.92E9
	Dec 91	44.5	24.0	20.6	1.16	2.10E9
	Feb 92	43.2	22.3	20.8	1.07	1.39E9
B	Sept 91	47.5	33.4	14.0	2.38	6.3E4
	July 92	40.6	24.5	16.1	1.52	ND
USP	I-1	51.2	33.8	17.5	1.93	ND

[a] Retention time = 4.3 min.
[b] ND = Not detected.

3.1. Bulk product

Several samples of gentamicin sulfate from two manufacturers were analyzed by HPAEC–PAD. The percent composition of the components as determined by ion chromatography was compared to the manufacturers' certified values obtained by HPLC according to USP protocol (values were obtained from manufacturers' certificates of analysis). Reproducibility of percent composition by ion chromatography ranged from 0.2 to 2.0% relative standard deviation for replicate injections. The values determined by ion chromatography generally agreed well with the manufacturer's values (refer to Table 2). No distinct pattern to distinguish between manufacturers was detected using the percent composition as reported by USP protocol: C_1, C_{1a}, and $C_2 + C_{2a}$. The ratio between C_2 and C_{2a} was then calculated (refer to Table 3). Differences between lots were then noticed. For manufacturer A, whenever the ratio C_2/C_{2a} was approximately 1, an additional peak at 4.3 min was detected (labeled as fifth peak). For C_2/C_{2a} ratio approximately 2, no fifth peak was detected. In manufacturer B samples, an opposite trend was noted. Fig. 2a and 2b show representative chromatograms for manufacturer A (with fifth peak) and manufacturer B, respectively. Distinct differences between manufacturer A and B were seen in the early part of the chromatograms. The patterns of the early peaks were the same for all lots of the same manufacturer. An additional peak can be seen in Fig. 2b after C_{2a} which may be the minor component C_{2b}. Although these peaks have not yet been identified, they still form useful patterns with which manufacturers can be distinguished.

3.2. Injectables

Four injectable solutions of gentamicin sulfate were also analyzed by HPAEC–PAD. The samples consisted of a control (with labeled potency of 100 mg gentamicin base/ml), and three unknowns. The four solutions had already been analyzed using capillary electrophoresis for potency and percent C_1, C_{1a} and $C_2 + C_{2a}$ and compared to microbial assay [16]. Analysis was required to determine if unknowns 1, 2 and 3 were all from the same source. Results of the ion chromatographic analysis is presented in Table 4. There was very little variation in the percent composition of C_1, C_{1a}, and $C_2 + C_{2a}$. Once again, as with the bulk samples, the ratio C_2/C_{2a} was calculated. The ratios for unknown 1 and 2

Table 4
Analysis of gentamicin in injectable solutions

Sample[a]	C_{1a} (%)	C_1 (%)	C_2 (%)	C_{2a} (%)	$C_2 + C_{2a}$ (%)	Total peak area[b]	Gentamicin base mg/ml
USP[c]	20.5 (1.0)	28.3 (1.2)	33.8 (1.0)	17.5 (1.0)	51.2 (0.9)	2260599 (4.9)	0.0658
Control[d,e]	20.5 (1.2)	26.6 (1.8)	36.6 (0.9)	16.3 (0.5)	52.9 (0.5)	3275903 (1.1)	95.4 ± 1.1
Unknown 1[e]	20.2 (0.7)	27.2 (0.6)	36.2 (0.2)	16.3 (0.7)	52.5 (0.1)	3689941 (0.5)	107.4 ± 0.6
Unknown 2[e]	20.3 (0.2)	27.0 (0.5)	36.3 (0.3)	16.3 (0.9)	52.6 (0.3)	3493696 (0.9)	101.7 ± 0.9
Unknown 3[e]	22.8 (0.5)	25.8 (0.2)	28.4 (0.2)	23.0 (0.5)	51.4 (0.1)	3322589 (0.5)	96.7 ± 0.5

[a] $n = 3$.
[b] Number in parentheses = % relative standard deviation.
[c] 0.0965 mg gentamicin sulfate USP standard/ml × 0.682 mg gentamicin base/mg USP standard.
[d] Labeled as 100 mg gentamicin base/ml.
[e] Dilution = 1000.

were the same (2.22) and different from unknown 3 (1.23). Also, as was found for the bulk product, there were differences in the early part of the chromatograms prior to C_{1a} which distinguished unknowns 1 and 2 as from the same source, and different from unknown 3 (refer to Fig. 3a–c).

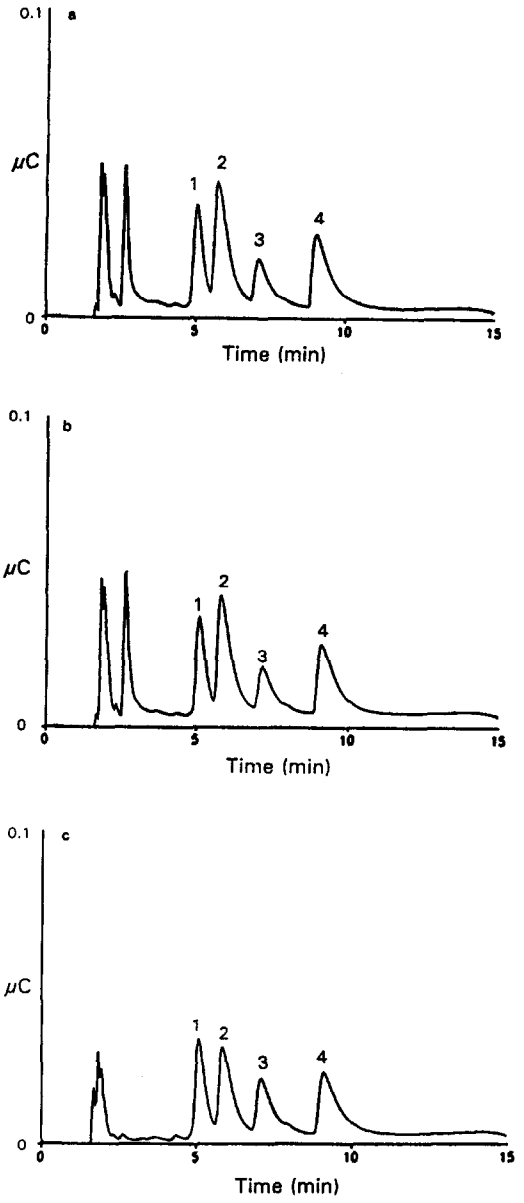

Fig. 3. Comparison of three gentamicin injectable solutions. (a) unknown 1, (b) unknown 2, and (c) unknown 3. Peaks: $1 = C_{1a}$; $2 = C_2$; $3 = C_{2a}$; $4 = C_1$.

The potency of the injectables was calculated using the total peak area of C_1, C_{1a}, C_2 and C_{2a}. The control was found to contain 95.4 ± 1.1 mg gentamicin base/ml compared to the labeled value of 100. The potencies of the unknowns were compared to results of microbial assay: unknown 1, 107.4 ± 0.6 by ion chromatography compared to 116 by microbial assay; unknown 2, 101.7 ± 0.9 by ion chromatography compared to 111; and unknown 3, 96.7 ± 0.5 by ion chromatography to 90.2. Based on the limited analysis of the injectables, ion chromatography appears to be a viable technique for the determination of potency for gentamicin.

4. Conclusions

The quantitative analysis of the major components of gentamicin sulfate has been accomplished using high-pH anion-exchange ion chromatography with pulsed electrochemical detection. Although a gradient run is required, the total analysis time is only 20 mins. The components were detected without derivatization with a detection limit of 20 ng gentamicin sulfate on column. The qualitative analysis of other unidentified components or impurities in the early portion of the chromatograms was useful in investigative comparisons of both bulk product and injectable solutions.

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