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Analysis of gentamicin sulfate by high-performance liquid chromatography combined with thermospray mass spectrometry

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ABSTRACT

The quantitative analysis of gentamicin sulfate by high-performance liquid chromatography (HPLC) with mass spectrometry was performed on-line utilizing thermospray mass spectrometry (TSP-MS). Chromatographic reversed-phase separation utilizing trifluoroacetic acid as an ion pair reagent resulted in the observation by TSP-MS of the major components (C_{1a}, C_2 and C_1) of gentamicin sulfate as well as an additional minor component. This minor component had the identical $[M+H]^+$ ion and fragmentation pattern of the major fraction C_2 which indicated that the minor component may be the C_{2a} component. Method development and optimization of the mobile phase for HPLC–TSP-MS were accomplished with a variable simplex algorithm. HPLC with electrochemical detection was utilized in conjunction with the simplex algorithm to establish a mobile phase suitable for the HPLC–TSP-MS analysis of gentamicin sulfate. Bulk preparations of gentamicin sulfate were assayed by HPLC–TSP-MS for the major components by comparison with an external standard, and by a comparison of peak areas obtained for the individual components *vs.* the totaled peak areas.

INTRODUCTION

The aminoglycoside antibiotic gentamicin is produced from the fermentation of *Micromonospora purpurea*. The analysis of gentamicin sulfate is a difficult and challenging task for two particular reasons. Firstly, gentamicin is a multicomponent mixture primarily made of three major constituents, the C_{1a}, C_2, and C_1 fractions as shown in Fig. 1. The small substituent difference on the aminomethyl saccharide (F_3, Fig. 1) makes separation of these major constituents non-trivial. Furthermore, there exist minor constituents (C_{2a} and C_{2b}) which may represent a substantial percentage of the gentamicin antibiotic in newer formulations [1]. The second reason that makes the analysis of gentamicin difficult is the lack of chromophores in the gentamicin

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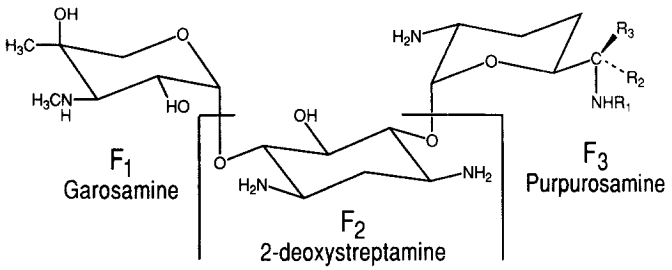

GENTAMICIN	R_1	R_2	R_3
C_1	Me	H	Me
C_2	H	H	Me
C_{1a}	H	H	H
C_{2a}	H	Me	H
C_{2b}	Me	H	H

Fig. 1. Structures of the components of the gentamicin complex.

moieties (Fig. 1). This lack of a chromophore does not readily allow the detection of gentamicin by conventional ultraviolet (UV) spectroscopic methods.

Gentamicin was first isolated by Weinstein *et al.* [2] in 1963. Numerous analytical methods have been applied to assay gentamicin. These methods initially employed paper chromatography to determine the C_1 and C_2 composition of gentamicin [3], and later the C_{1a} component was separated [4]. The paper chromatographic method followed by microbiological assay of the separated components was adopted as the official Food and Drug Administration (FDA) protocol [5]. Subsequently, additional investigations by paper and thin-layer chromatography established that several other minor components existed in gentamicin [6–8]. Detection with a conductivity bridge after ion-exchange chromatography separated and detected the major components of gentamicin [9]. Thomas and Tappin [10] employed ion-exchange column chromatography with optical rotation detection for gentamicin analysis. The first utilization of an ion pair reagent with a reversed-phase column for gentamicin analysis was reported by Anhalt [11]. Detection of gentamicin sulfate was accomplished with fluorescence after post-column derivatization with *o*-phthalaldehyde (OPA) [11,12]. Similar high-performance liquid chromatography (HPLC) with pre-column derivatization by OPA [13] and dansyl chloride [14] followed by fluorescence was performed for gentamicin analyses. Other workers examined gentamicin by HPLC followed by derivatization in plasma and urine samples analyzed in the range of 0.25 to 25 $\mu g\ ml^{-1}$ [15]. Freeman *et al.* [1] employed pre-column derivatization with an OPA–thioglycollic acid reagent followed by UV detection at 330 nm. They reported that the C_{2a} component represented a significant proportion of the gentamicin antibiotic. Later studies [16,17] confirmed the importance of monitoring the C_{2a} component. Claes *et al.* [18] utilized ion pair HPLC and pre-column derivatization with UV detection at 350 nm for analysis of the C_{2a} component. Other gentamicin analyses were accomplished with pre-column derivatization by 2,4,6-trinitrobenzenesulphonic acid [19]. Recently, the effect of inorganic cations on the separation of OPA derivatives of

gentamicin by HPLC was examined [20]. The concentration of these inorganic cations affected the separation order of the gentamicin components. Seidl and Nerad [21] used isocratic ion-exchange chromatography with post-column OPA reaction followed by fluorescence to detect the C_1, C_{1a}, C_2, C_{2a}, and C_{2b} components. The separation order for this ion-exchange chromatography [21] was C_{1a}, C_2, and C_1 which supported the observed effect of potassium iodide concentration on the elution order of OPA derivatives by reversed-phase HPLC [20]. Inchauspé and Samain [22] were able to separate several aminoglycoside antibiotics by utilizing perfluorinated carboxylic acids as ion pair reagents in reversed-phase HPLC with refractive index (RI) detection. The separation of gentamicin sulfate was in the elution order of C_{1a}, C_2, and C_1 with an unidentified gentamicin peak labeled "X".

All the ion pair reversed-phase HPLC analyses cited using UV or fluorescence detection required derivatization prior to assay. RI detection was used after the perfluorinated carboxylic acid separations without derivatization [22,23]. Previous studies have shown that HPLC with electrochemical detection (ED) is useful for detecting gentamicin sulfate without the need for derivatization [24].

Mass spectrometry (MS) of gentamicin sulfate has been reported by chemical ionization (CI), and the type of ions formed evaluated [25]. Parfitt *et al.* [26] discussed the electron impact (EI), CI by isobutane, and field desorption (FD) mass spectrometry of gentamicins. The EI-MS of gentamicin was reported to have no diagnostic value for evaluating commercial mixtures, and FD-MS was used to obtain the $[M + H]^+$ ions with little glycosidic cleavage for the various gentamicin components at optimum conditions. FD-MS with emitter CI was also discussed by Takeda *et al.* [27]. Inchauspé *et al.* [28] examined gentamicin sulfate by FD-MS after off-line separation by HPLC using perfluorinated carboxylic acids as ion pair reagents. Plasma desorption MS was applied for analysis of several aminoglucoside antibiotics, including gentamicin sulfate [29]. Atmospheric pressure ionization MS with corona discharge has been employed successfully for a wide variety of aminoglycoside antibiotics [30]. The applicability of thermospray mass spectrometry (TSP-MS) utilizing trifluoroacetic acid (TFA) as an ion pair reagent for on-line reversed-phase HPLC for separating and detecting the components of gentamicin sulfate has been discussed [31]. Fast atom bombardment MS of aminoglycoside antibiotics has also been accomplished [32].

This study reports the details for the on-line HPLC–TSP-MS determination of gentamicin sulfate. Before performing on-line HPLC–TSP-MS, the mobile phase was optimized using a simplex algorithm and LC–ED. Several bulk preparations of gentamicin sulfate were examined for C_{1a}, C_2, and C_1 content and for possible characterization of previously unidentified components by HPLC–TSP-MS.

EXPERIMENTAL

For the HPLC–TSP-MS analysis, a Hewlett-Packard (HP) 5988A mass spectrometer with HP Chemstation data handling system (version 3) was utilized. A Vestec (Houston, TX, USA) Thermospray source and controller were used. The HPLC pump was an SSI, Model GS400 (College Station, PA, USA). The conditions for TSP-MS were control, 110°C and block, 308°C with filament and discharge off. Scan range was approximately m/z 100 to 500. Selected ion currents (SIC) were

plotted after a full scan measurement. For the HPLC with ED analysis, a Brinkmann/Metrohm EA-1096 cell (Westbury, NY, USA) with Ag/AgCl reference electrode and glassy carbon working and auxiliary electrodes was employed. A BAS (West Lafayette, IN, USA) CV-1B was used as the potentiostat in conjunction with this cell. The HPLC pump for HPLC–ED was a Spectra-Physics (San Jose, CA, USA) SP-8700 with ternary solvent capability. The potential of the electrochemical cell was set to approximatgely +1.2 V.

In both cases, HPLC–TSP-MS and HPLC–ED, the flow-rate was 1.0 ml min^{-1} and a 3-μm ODS-II reversed-phase column (100 × 4.6 mm I.D.; LC Custom, Houston, TX, USA) was employed. A Rheodyne (Cocati, CA, USA) 7125 injector with 20-μl loop was utilized.

Prior to the HPLC–TSP-MS analysis of gentamicin, the mobile phase was optimized employing a variable simplex algorithm software program compatible with a PC based computer (Statistical Products, Houston, TX, USA) and HPLC–ED. Only mobile phase composition was considered in the optimization and three factors were selected. These factors consisted of solvent A which contained 0.22 *M* trifluoroacetic acid (TFA) raised to a pH of 3.6 with ammonium hydroxide in deionized water, solvent B was deionized water only, and solvent C was methanol. The simplex program normalized the percent composition of each solvent (factor) to 100%. These percentages were then selected for each solvent on the SP-8700 HPLC pump which was capable of handling a three-solvent system for the mobile phase. This set-up allowed for rapid change of the mobile phase based on the response of the simplex calculation for every new experimental point designated. The final mobile phase utilized for HPLC–TSP-MS consisted of 0.11 *M* aqueous TFA–methanol (94:6) where the aqueous TFA was adjusted to pH 3.6 with ammonium hydroxide.

Gentamicin sulfate samples were bulk preparations. The standard was a USP standard, Lot H, rated at a potency of 663 μg/mg. The percent components as stated for Lot H were C_{1a} = 31.5%; C_2 = 31.6%; C_1 = 36.9%. Analysis by paper chromatography followed by microbiological assay [5] gave C_{1a} = 31.33%; C_2 = 30.31%; C_1 = 38.37% for Lot H (see Table III). The HPLC–TSP-MS percentages are compared directly with the microbiological assay after taking into account the activities of the three major gentamicin components [5]. Samples and standards for HPLC–TSP-MS were diluted in deionized water at a nomial concentration of 1 mg ml^{-1}.

RESULTS AND DISCUSSION

The TSP-MS of the gentamicin sulfate components in the positive ion mode produced low ion intensity for the $[M + H]^+$ ions, but high ion intensity for the fragments resulting from the cleaving of the glycosidic bonds [31,32]. The resultant reduction in ion intensity for the $[M + H]^+$ ions is probably due to the thermal lability of the compound via TSP-MS. A distinct fragment ion corresponding to each of the major components of gentamicin sulfate (Fig. 1) was observed. These distinct ions arose from the differences in the number of methyl groups bonded to a terminal aminomethyl substituent on the F_3 fragment. The distinctive F_3 fragment and $[M + H]^+$ ions are listed in Table I for each gentamicin sulfate component. Two fragments (F_1 and F_2) were identical in all components (Fig. 1) and their corresponding ions were also identical (Table 1). The ion indicative of the F_2 fragment resulted from the

TABLE I

DISTINCTIVE FRAGMENT AND $[M + H]^+$ IONS FOR EACH COMPONENT OF GENTAMICIN SULFATE BY TSP-MS

Component	*m/z*			
	$[F_1]^{+a}$	$[F_2 + 3H]^{+b}$	$[F_3]^{+c}$	$[M + H]^+$
C_{1a}	160	163	129	450
C_2	160	163	143	464
C_1	160	163	157	478
C_{2a}	160	163	143	464
C_{2b}	160	163	143	464

[a] Terminal saccharide without aminomethyl substituent (Fig. 1).
[b] Central fragment ion resulting from addition of three hydrogens.
[c] Distinctive terminal saccharide with aminomethyl substituent (Fig. 1).

retention of the two glycosidic oxygens to this fragment after cleavage followed by addition of three hydrogens to this fragment. This generated the $[F_2 + 3H]^+$ ion at *m/z* 163 as listed in Table I. The nature of the $[F_1]^+$ and $[F_3]^+$ ions has not been clearly defined. The ion indicative of fragment F_1 gave a *m/z* of 160, as indicated in Table I. For fragment F_1, the removal of the glycosidic oxygen leaves a total mass of 160 a.m.u. One possibility that may produce an ion at *m/z* 160 for F_1 is a proton abstraction to form a double bond, which makes the F_1 fragment 159 a.m.u., followed by addition of a proton to generate the observed ion fragment at *m/z* 160. This ion at *m/z* 160 may also be due to the formation of a cation on the ethereal oxygen to produce a $[F_1]^+$ ion. The same possibilities exist for the nature of the ion indicative of the distinctive F_3 fragment. Fragmentation of gentamicin sulfate observed in FD-MS [27] resulted in ions corresponding to the F_1 and F_3 fragments identical to those indicated in Table I. These fragment ions were considered to have a positive charge on the ethereal oxygen [27]. A similar ion was suggested for the F_1 fragment by EI-MS, and identical ions appeared in the isobutane CI mass spectra [26]. At this time, it is not clear what the true nature of the formation of the $[F_1]^+$ and $[F_3]^+$ ions in TSP-MS is; however, it is definite that the ions indicated in Table I are correlated to the particular fragments from studies dealing with analogues of gentamicin sulfate [32].

As stated earlier, the separation and detection of the components of gentamicin sulfate is not a trivial task. The reversed-phase separation by HPLC usually required some ion-pair reagent to exaggerate the small differences in the gentamicin components. Anhalt and co-workers [11,12] used a mobile phase containing sodium pentanesulfonate (SPS) and sodium sulfate to achieve separation of the C_{1a}, C_2, and C_1 components followed by post-column derivatization with OPA and fluorescence detection. Initial attempts to use a similar mobile phase containing SPS for HPLC–TSP-MS were unsuccessful due to the non-volatility of the salts used. Ion exchange of protons for sodium in SPS and substitution of ammonium sulfate for sodium sulfate in the mobile phase were also unproductive due to the incompatibility of this mobile phase with the thermospray (TSP) source. A volatile ion pair reagent was needed to accomplish the separation of the gentamicin sulfate components and be compatible

with the TSP source. Inschauspé and Samain [22] utilized trifluoracetic acid (TFA) as an ion-pair reagent to separate the gentamicin sulfate components. Using a TFA mobile phase with reversed-phase HPLC, the on-line separation and detection of gentamicin sulfate by TSP-MS was successful [31].

In order to improve the TFA mobile phase for gentamicin sulfate analysis by HPLC–TSP-MS, the separation of the various components was optimized with respect to the mobile phase composition. A variable simplex algorithm with three factors was used. These three factors represented three solvents which were incorporated into the mobile phase. The response factor (R) involved two goals: (1) to minimize the analysis time, and (2) to achieve good separation of the various components by comparing the capacity factors (k') of each neighboring chromatographic peak to some ideal predetermined difference in capacity factors of neighboring chromatographic peaks. Based on these goals, an equation for R was generated. As R approached zero, this was considered to be the best separation.

$$R = \frac{(k'_1 - k'_0) - I_0}{2} + \ldots + \frac{(k'_{n+1} - k'_n) - I_n}{2} \quad (1)$$

where k'_0 is the capacity factor of the solvent front ($k'_0 = 0$); k'_1 is the capacity factor of the first chromatographic peak, in this case, component C_{1a}; I_0 is the ideal predetermined difference in $k'_1 - k'_0$, in the first instance, $I_0 = 1$. For $n + 1$ number of chromatographic peaks, a summation is carried out for all of the peaks. One change is that I_n is greater than I_0 for late eluting peaks. The increase in I for late eluting peaks is due to band broadening over chromatographic time. This aids in maintaining good separation between late eluting components. The value for I should be chosen with respect to the particular HPLC column size, column type and flow-rate. Of the two goals stated above, good separation was considered a higher priority than length of analysis time. For this reason, when $[k_{n+1} - k_n) - I_n] < 0$, there was no division by two for that particular difference in eqn. 1. In cases where no separation occurred between neighboring peaks, $(k_{n+1} - k_n) = 0$, a value of ten was arbitrarily given to this separation.

Using the simplex algorithm and HPLC–ED, a suitable mobile phase was achieved after 13 vertices, three of which were beyond value limits. This final mobile phase is described in the Experimental section. A chromatogram of gentamicin sulfate by HPLC–ED is shown in Fig. 2. For analytical purposes, the chromatographic

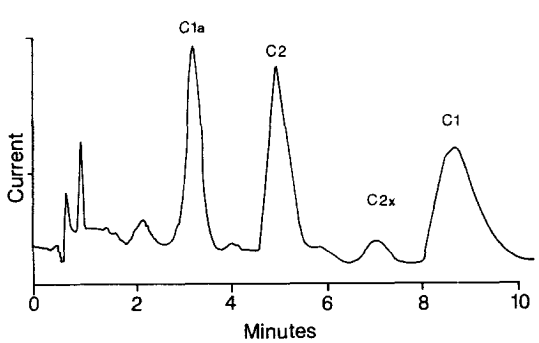

Fig. 2. HPLC–ED of gentamicin sulfate using the trifluoroacetic acid ion pair mobile phase. Peak C_{2x} is considered as component C_{2a} as described in text. Approximately 15 μg injected on-column.

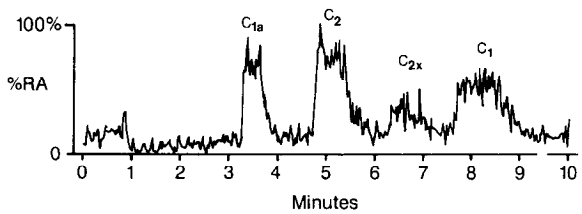

Fig. 3. HPLC–TSP-MS of gentamicin sulfate as a function of percent relative abundance (RA) *vs.* chromatographic time. Peak C_{2x} is considered as component C_{2a}, and the mobile phase is identical to the one used for HPLC–ED (Fig. 2). Approximately 15 μg injected on-column.

peak C_{2x} represents the C_{2a} and C_{2b} components of gentamicin sulfate. The occurrence of C_{2x} in Fig. 2 between the C_2 and C_1 chromatographic peaks has the identical location to peak "X" employing a TFA containing mobile phase [22].

The HPLC–TSP-MS of gentamicin sulfate using the same mobile phase employed for HPLC–ED is seen in Fig. 3. The total ion current (TIC) trace is identical to the HPLC–ED. In Fig. 4, the selected ion current (SIC) is plotted for the $[F_3]^+$ ions indicated in Table I. As shown in Fig. 4, these fragment ions are distinctive for each component of gentamicin sulfate. It is also seen that the peak labeled C_{2x} is related to C_2 in that the $[F_3]^+$ ion is identical.

The mass spectrum for each chromatographic peak in Fig. 3 was examined after background subtraction. The ions listed in Table 1 were observed in the mass spectra of the various components (Fig. 5a–d). In comparing Fig. 5b for the C_2 component with Fig. 5d, it is clearly indicated that C_{2x} is related to the C_2 component. Because the amount of component C_{2b}, also known as sagamicin, is expected to be very low [21], the C_{2x} will be considered to be the C_{2a} component of gentamicin sulfate. For analytical purposes, the peak area contributed by C_{2a} (C_{2x}) will be totaled with the C_2

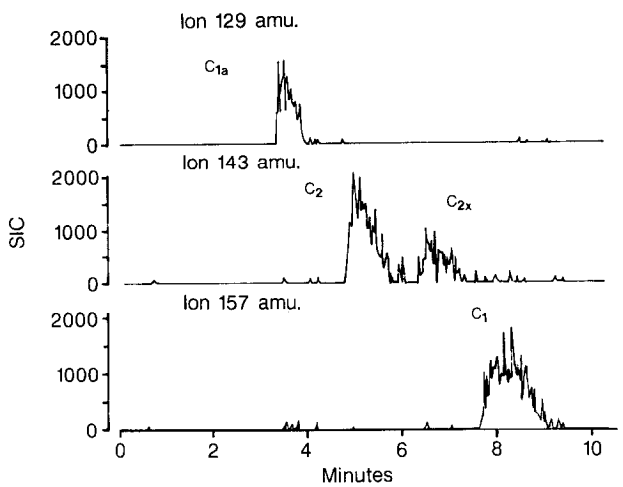

Fig. 4. Selected ion current (SIC) for the individual distinctive ions representing the F_3 part of the gentamicin sulfate complex as described in Table I.

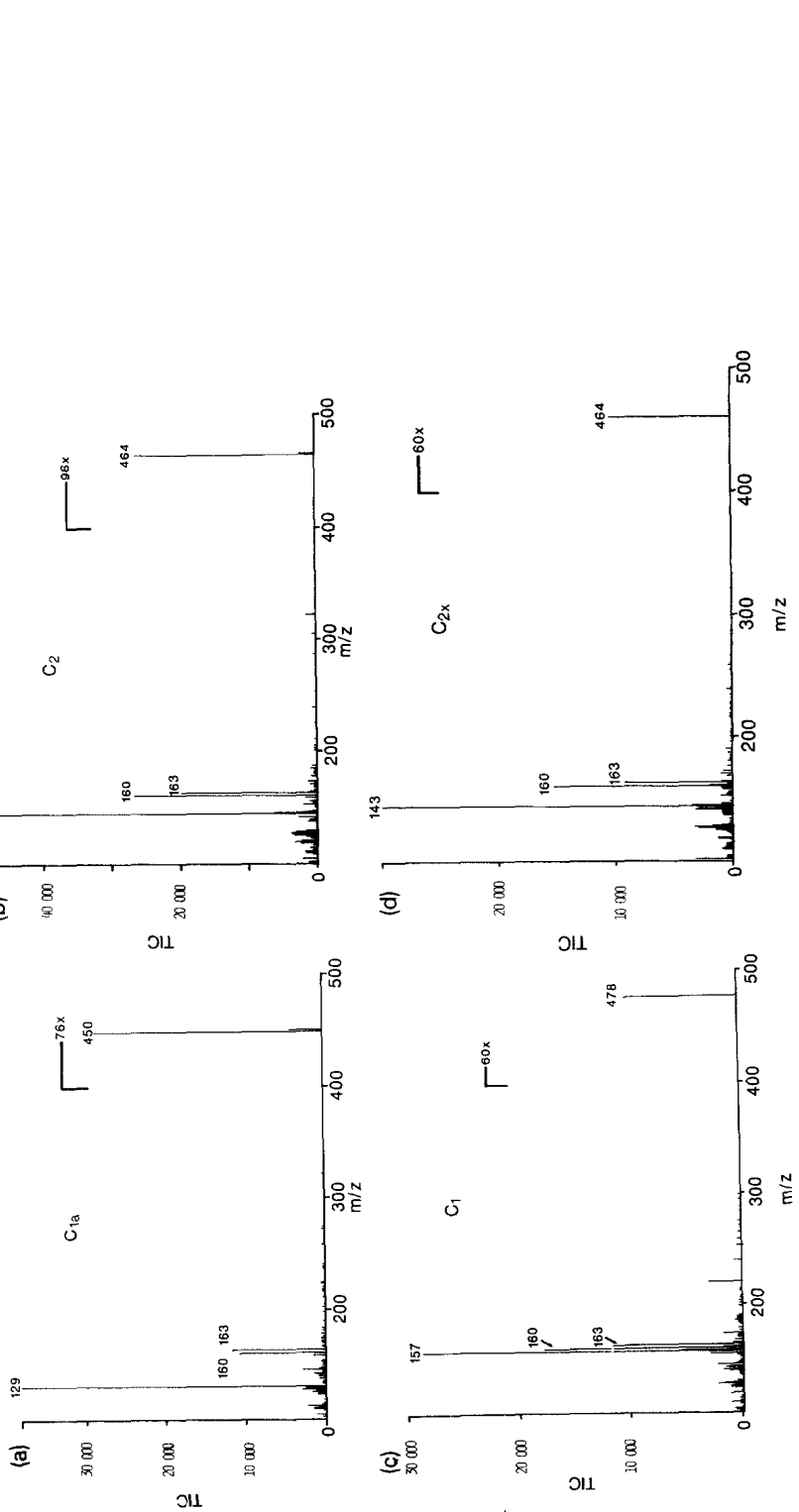

Fig. 5. (a) Thermospray mass spectrum of the gentamicin sulfate C_{1a} component. (b) Thermospray mass spectrum of the gentamicin sulfate C_2 component. (c) Thermospray mass spectrum of the gentamicin sulfate C_1 component. (d) Thermospray mass spectrum of the C_{2x} fraction considered to be the gentamicin sulfate C_{2a} component.

peak area. Finally, two additional points should be made clear with respect to the HPLC–TSP-MS of gentamicin sulfate. Firstly, the ion intensity for the $[M + H]^+$ ions is close to zero for low concentrations of the gentamicin sulfate injected on-column, thus the need to monitor the distinct ion produced from the F_3 fragment (Fig. 1). Secondly, an ion is sometimes observed at *m/z* 322 for all components of gentamicin sulfate. It was concluded that this ion was representative of the combined $F_1 + F_2$ (Fig. 1) fragments producing a $[F_1 + F_2 + 2H]^+$ ion. The corresponding $[F_2 + F_3 + 2H]^+$ ions were not observed suggesting that the first glycosidic bond breakage was between the F_2 and F_3 segments. This behavior was noted for analogues of gentamicin sulfate [32]. The FD-MS of gentamicin [27] did detect the $[F_2 + F_3 + 2H]^+$ ion.

The linearity of the signal *vs.* solute injected on-column by HPLC–TSP-MS for gentamicin was evaluated. The correlation coefficient (*r*) for each of the components is shown in Table II. The sample used for this linearity study was a bulk preparation. The USP standard for gentamicin sulfate was observed to have a very low amount of C_{2a}, thus the utilization of a bulk preparation so that a signal *vs.* concentration curve could be generated for C_{2a}. The *r* for all components was 0.98 or better. The amount injected on-column is the total amount of the gentamicin complex injected. The actual amount for each component is unknown, but the approximated amount is indicated in Table II. Because the exact amount are unknown for this bulk preparation, a detection limit could only be approximated to be 0.4 μg injected for the total gentamicin sulfate complex. In order to assay the bulk preparation, it was necessary to compare the composition of the gentamicin sulfate USP standard to the peak area obtained by HPLC–TSP-MS. The stated composition of the USP standard does not take into account any C_{2a} component. For assay purposes, the peak area obtained for C_{2a} was added to the C_2 peak area. Three standards were weighed out and each standard was analyzed in triplicate. A signal *vs.* μg injected on-column response was calculated using the percent composition of the gentamicin sulfate USP standard. It is important to note the μg injected represents microbiological activity and takes in account the relative activity for each component [5]. The results of HPLC–TSP-MS analysis of the standard are shown in Table III.

Three bulk preparations of gentamicin sulfate were assayed. One noticeable difference was that the peak area associated with the C_{2a} component in the bulk

TABLE II

LINEARITY OF GENTAMICIN SULFATE COMPONENTS AS DETERMINED BY THE CHROMATOGRAPHIC PEAK AREA *VS.* THE AMOUNT OF BULK GENTAMICIN SULFATE INJECTED ON-COLUMN

Component	Range of μg injected[a]	Correlation coefficient (*r*)
C_{1a}	0.3–6.0	0.985
C_2	0.3–6.0	0.994
C_{2a}[b]	0.1–2.0	0.9996
C_1	0.3–6.0	0.996

[a] This range was based on 30% of the total amount of the gentamicin sulfate complex injected for the three major components, and 10% for C_{2a}. It represents only approximate amounts.

[b] The component C_{2x} was considered to be C_{2a} with no major contribution from C_{2b}.

TABLE III

RESPONSES OF GENTAMICIN SULFATE STANDARD BY HPLC–TSP-MS COMPARED TO QUANTITIES INJECTED

μg Injected on-column[a]				Signal[c]/μg Injected		
Total[b]	C_{1a}	C_2	C_1	C_{1a}	C_2	C_1
7.20	2.26	2.18	2.76	2.10	2.99	2.80
9.92	3.10	3.00	3.80	1.97	3.11	2.84
8.00	2.50	2.42	3.06	1.98	3.26	2.76
Mean				2.02	3.12	2.80
R.S.D.[d]				3.6%	4.3%	1.4%

[a] The μg injected for each component was based on percentage obtained from microbiological assay as noted in Experimental and represents a relative amount related to the activity of the individual components.
[b] Amount of C_{2a} was observed to be negligible in standard.
[c] (Signal from integrated peak area) · 10^{-5}.
[d] R.S.D. = Relative standard deviation, $n = 3$.

preparations was substantially higher than the USP standard. Table IV shows the results of the HPLC–TSP-MS assay using an external standard compared to the official paper chromatographic method. As can be seen from Table IV, the values obtained by paper chromatography *vs.* HPLC–TSP-MS do not agree exactly. Several points should be considered when comparing these two methods. The paper chromatography method does not take into account the C_{2a} component. For HPLC–TSP-MS, the peak area from the C_{2a} was summed with the C_2 peak area. This may not represent the situation in paper chromatography. The C_{2a} component may be summed with either the C_1 or C_{1a} components, thus affecting the percentages obtained by paper chromatography. Weigand and Coombes [33] considered the problems with the analysis of the C_{2a} component by HPLC compared to the paper chromatographic assay. They reported that for the two methods to agree, C_{2a} must coelute with C_2. From HPLC–TSP-MS, the peak area corresponding to C_{2a} repre-

TABLE IV

ASSAY OF GENTAMICIN SULFATE BULK PREPARATIONS BY HPLC–TSP-MS COMPARED TO PAPER CHROMATOGRAPHIC METHOD AS CALCULATED BY EXTERNAL STANDARD METHOD

Sample	Paper chromatography (%)			HPLC–TSP-MS[a] (%)		
	C_{1a}	C_2	C_1	C_{1a}	C_2[b]	C_1
A	28.10	32.96	38.94	29.3	41.8	28.9
B	20.60	36.78	42.65	20.2	45.8	34.0
C	17.59	35.18	47.22	22.5	40.9	36.5

[a] These percentages compare directly with paper chromatography values after accounting for the microbiological activities for each component as indicated in ref. 5.
[b] Component C_{2a} combined with C_2.

TABLE V

PERCENT COMPOSITION OF THE MAJOR COMPONENTS FOR GENTAMICIN AS CALCULATED BY USP PROCEDURE (REF. 35) VIA TOTALING PEAK AREAS *VS.* COMPONENT PEAK AREA

Sample	C_{1a}[a] (%)	C_2 (%)	C_{2a} (%)	C_1[b] (%)	$C_2 + C_{2a}$[c] (%)
A	21.9	35.6	12.6	29.9	48.2
B	14.6	31.8	19.4	34.1	51.2
C	16.5	34.8	11.6	37.1	46.4

[a] USP limits 10–35%.
[b] USP limits 25–50%.
[c] USP limits 25–55%.

sented 12 to 19% of the total peak area, as seen in Table V, for the bulk preparations. Another point to consider is that the paper chromatography percentages were based on microbiological methods and the potency of the various components. This percent potency does not correlate directly with percent composition since the microbiological activity of the various components differ.

Although the percentages do not agree completely in Table IV for the two methods, trends are noted in the percentages. Comparing sample A with sample B, it is seen that C_{1a} is less in sample B (28.10% to 20.60%) as measured by the paper chromatography method. A similar decrease is observed for C_{1a} in sample B in the HPLC–TSP-MS method (29.3% to 20.2%). Likewise, an approximate 4% increase in both methods is noted for the C_2 component going from A to sample B, and an approximate 3 to 5% increase for the C_1 component. The trend is not as clear with sample C; however, this may be due to the effect of the C_{2a} composition.

The difficulties in comparing analyses by HPLC to microbiological assay for gentamicin sulfate [33] and other antibiotics have been discussed by Thomas [34]. Because of the question of C_{2a} composition and other difficulties, USP has established guidelines for approving bulk preparations of gentamicin sulfate by HPLC assay [35] without an external standard. The HPLC assay established by USP is similar to the method of Freeman *et al.* [1] and employs UV detection at 330 nm with precolumn derivatization with OPA. The procedure consists of totaling the chromatographic peak areas for C_{1a}, C_2, C_{2a}, and C_1 and calculating the percent peak areas contributed by the fractions. The C_{2a} peak area is incorporated with the C_2 peak area [35]. Using this procedure for HPLC–TSP-MS, the percentages for the major components for the gentamicin sulfate bulk preparations are given in Table V. As seen from Table V, the gentamicin sulfate components are within USP limits for all samples.

Finally, the elution order of the major components of gentamicin should be considered. The elution order for the USP method [1,35] is C_1, C_{1a}, and then C_2 for the OPA derivatized components. Modifying the mobile phase may affect the elution order [12]. With the HPLC–TSP-MS method reported in this study, any change in elution order will be detected immediately by monitoring the distinctive ions for each component. Furthermore, there is no need to derivatize the gentamicin components, although it may be a point of interest to observe what effects derivatization may have on detection limits.

CONCLUSIONS

The analysis of the major components of gentamicin sulfate by on-line HPLC–TSP-MS was achieved using volatile TFA as an ion pair reagent with reversed-phase HPLC, and required no derivatization. The utilization of simplex optimization with HPLC–ED permitted a rapid and simple means by which a compatible mobile phase for TSP-MS was selected. The technique of HPLC–ED detected the major components of gentamicin sulfate without derivatization. An advantage of using an alternate detection method, such as HPLC–ED, for optimization before performing HPLC–TSP-MS is that valuable and expensive instrumental time is not consumed on a mass spectrometer. Adjustment of thermospray vaporizer and block temperatures are ususally necessary after altering the mobile phase composition. With the mobile phase optimized and selected by HPLC–ED, an advantage is that only one set of temperature conditions for HPLC–TSP-MS required optimizing instead of adjusting the temperature conditions for each new mobile phase.

The quantitative analysis of gentamicin sulfate was accomplished by an external standard method, and by the USP procedure of comparing individual peak areas to the totaled peak area [35]. Few studies have discussed quantification by HPLC–TSP-MS. The degree of reproducibility for this HPLC–TSP-MS analysis was indicated by a 1 to 4% relative standard deviation for triplicate assays of the standard. For the assay of gentamicin sulfate, the advantage of TSP-MS is demonstrated by the ability to characterize the C_{2a} component. Other detection schemes, such as HPLC–ED, could not definitively ascertain that a particular chromatographic peak was due to a fraction without a comparison of retention times with an authentic standard of the individual components. The distinctive fragmentation pattern and observation of the $[M + H]^+$ ion for C_2 and C_{2a} allowed for a confident summation of these two chromatographic peak areas for analytical purposes.

One drawback of TSP-MS is the incompatibility of non-volatile salts in the ion source. The incorporation of TFA as an ion pair reagent in the mobile phase for on-line HPLC–TSP-MS, as exemplified by this analysis of gentamicin sulfate, may be applied to other aminoglycoside antibiotics and similar compounds that are difficult to separate by reversed-phase HPLC.

The detection limits for gentamicin sulfate via HPLC–TSP-MS was approximately 400 ng injected on-column compared to 16 μg for HPLC–ED [24]. The UV detection method with derivatization was estimated to be 10 μg on-column [1]. Fluorescence detection after derivatization is very sensitive giving an estimated detection limit of 10 ng [15]. For routine assay procedures, the UV and fluorescence methods would be adequate provided that standards are available to assure the identity of the components by retention times. The HPLC–TSP-MS technique has the advantage for characterizing the components without standards, and changes in retention times due to column degradation or contamination would not affect the HPLC–TSP-MS identification. It should be noted that the mass spectra were full scan measurements; selective ion monitoring (SIM) modes may give detection limits similar to the fluorescence methods.

The nature of the ions observed for the F_1 and F_3 fragments (Fig. 1) has not been defined; however, this unique fragmentation as exemplified for gentamicin sulfate at the glycosidic bond will make characterization of similar unknown aminogly-

coside antibiotics possible. Further studies will be needed to confidently identify the type of ions indicative of the F_1 and F_3 fragments produced by thermospray ionization.

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