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Determination of gentamicin after trimethylsilylimidazole and trifluoroacetic anhydride derivatization using gas chromatography and negative ion chemical ionization ion trap mass spectrometry

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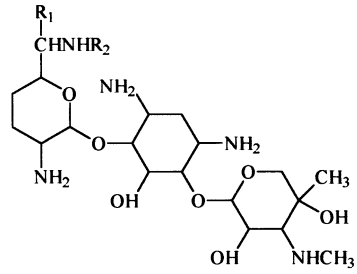
A gas chromatographic method for the determination of gentamicin, an aminoglycoside antibiotic, was developed. A two step derivatization method utilizing trimethylsilylimidazole for silylation of the hydroxyl groups and trifluoroacetic anhydride for acetylation of the amino groups was used. Chemical ionization and negative ion monitoring resulted in greatly improved sensitivity and more informative fragmentation of the gentamicin components compared with electron ionization and positive ion monitoring. Tandem mass spectrometry was used for the identification of the gentamicin components and for characterization of the derivatized groups. The optimized method also allowed the use of three ions for selected ion monitoring. The method significantly improved the determination of gentamicin compared with previously described gas chromatographic methods. Chemical ionization and negative ion monitoring with the use of an ion trap analyzer appeared superior to other detection modes.

1. Introduction

Gentamicin is an aminoglycoside antibiotic consisting of three major and several minor components. The structures of the major gentamicin components, gentamicin C_1, C_{1a} and C_2, are presented in Fig. 1. Gentamicin is a fermentation product of *Micromonospora purpurea* and *Micromonospora echinospora* and its composition with respect to the component ratio has been shown to vary greatly.[1] The commercially available gentamicin substance generally contains undefined amounts of each component and the quantification of the total gentamicin content is based on antimicrobial activity. Microbiological assays do not differentiate between the gentamicin components or possible additional antimicrobially active impurities. Consequently, there appears to be a definite need for confirmatory methods for the determination of gentamicin that are able to quantify the amounts of the components in analytical reference materials. Obtaining structural information on the separated components is often essential because the individual gentamicin components are not commercially available.



$R_1 = R_2 = CH_3$ Gentamicin C_1
$R_1 = CH_3$ $R_2 = H$ Gentamicin C_2
$R_1 = R_2 = H$ Gentamicin C_{1a}

Fig. 1 Structures of the major gentamicin components. The molecular weights of the components are C_1 477.6, C_2 463.6 and C_{1a} 449.5.

Gentamicin components are polar, non-volatile, water-soluble molecules with primary and secondary amine and hydroxyl groups in their structures. These compounds lack chromophores. According to a recent review concerning aminoglycoside analysis, most chromatographic methods for gentamicin determination include derivatization as part of the analytical procedure in order to improve solubility, detectability and/or volatility.[2] The presence of multiple functional groups makes derivatization of aminoglycosides for chromatographic analysis and, in particular, for gas chromatographic (GC) analysis exceptionally complicated. Unpredictable or partial derivatization reactions can be encountered.

Only a few GC methods for aminoglycosides have been reported.[2] A two step derivatisation, using trimethylsilyl imidazole (TMSI) for silylation of the hydroxyl groups and heptafluorobutyroimidazole (HFBI) for acetylation of amino groups, was reported for both packed[3] and capillary column[4] applications for gentamicin determination. The reason for this approach is the poorer stability of silylated amines compared with silylated hydroxyls, necessitating separate derivatisation of the amino groups. Whereas separation of the components in a packed column combined with electron capture detection (ECD) produced satisfactory results, capillary column separation and mass spectrometric (MS) detection resulted in a decrease in sensitivity. The lower sensitivity in GC-MS was believed to result from the high molecular weight of the derivatized analytes and the limited mass range of the benchtop MS instruments.

The determination of gentamicin using MS detection and liquid chromatographic separation is also complicated. The polarity of gentamicin results in poor retention in reversed phase columns and, in general, aminoglycosides are difficult to retain in these columns even with fully aqueous eluents.[5,6] Ion pair chromatography is, therefore, generally used. The most

commonly used ion pairing reagents are non-volatile sulfonic acid derivatives, which cannot be used in LC-MS applications. Fluorinated carboxylic acids have been used but numerous problems were encountered.[7] Multiple charging (in electrospray), thermal fragmentation and a low ion current (in atmospheric pressure chemical ionization) were observed. The optimum performance of electrospray and atmospheric pressure chemical ionization interphases is usually achieved at the cost of compromised chromatographic separation. The structural similarity of the gentamicin components and the lower separation capacity of LC compared with GC seem to advocate the use of GC for the separation of the gentamicin complex for MS analysis.

To the best of our knowledge, gas chromatography with chemical ionization and negative ion monitoring mass spectrometry of aminoglycosides has not been reported previously. The purpose of this work was to study the mass selective detection of gentamicin and the fragmentation patterns of the *N*-trifluoroacetylated and *O*-trimethylsilylated gentamicin components using an ion trap detector.

2. Experimental

2.1. Chemicals

Gentamicin sulfate, gentamicin cell culture solution, trimethylsilylimidazole and trifluoroacetic anhydride (TFAA) were purchased from Sigma (St. Louis, MO, USA). Pyridine was obtained from Merck (Darmstadt, Germany) and was of *pro analysi* grade. De-ionized, doubly distilled water was used throughout.

2.2. Apparatus

The GC-MS equipment consisted of a Finnigan MAT (Austin, TX, USA) GCQ gas chromatograph equipped with an external ion source and an ion trap tandem mass spectrometer. Manual injection utilizing the splitless mode was used and the injector temperature was set at 300 °C. The injection volume was 1 μl. An HP-5 Trace, capillary column (15 m × 0.25 mm id, 0.25 μm film thickness) (Hewlett-Packard, Palo Alto, CA, USA) was used to separate the derivatized gentamicin components. The temperature program was as follows: the initial temperature was set at 210 °C for 2 min, succeeded by a constant temperature gradient of 32 °C min^{-1} to the final temperature of 300 °C, which was held for 5 min. Helium (99.999%), filtered through R&D separators, was used as the carrier gas at a constant velocity of 40 cm s^{-1}.

The ion source and the transfer line temperatures were set at 200 and 300 °C, respectively. The spectral scanning speed was 1 s^{-1}. Automatic gain control was used throughout at the default value (50). The following mass spectrometric parameters were used: emission current 250 μA; trap offset, 10 V; multiplier voltage, 1400 V [negative chemical ionization (NCI)] and 1275 V [electron ionization (EI)]; and collision energy, 0.5 V for collision activated dissociation (CAD). Methane was used as the chemical ionization reagent gas.

2.3. Derivatisation procedure

Gentamicin solution was evaporated to dryness under a nitrogen stream in an autosampler vial and the dry residue was dissolved in 50 μl of anhydrous pyridine. To the pyridine solution, 100 μl of TMSI were added and the vial was closed and incubated for 15 min at 60 °C. Thereafter, 70 μl of TFAA were added and the vial was closed and incubated for 60 min at 60 °C.

2.4 Evaluation of repeatability, reproducibility and linearity

The repeatability of the derivatization procedure and the chromatographic analysis was determined by five replicate injections of the standards prepared as described above. The reproducibility of the method was determined by 10 replicate injections performed within 1 month. Linearity of the detector response was evaluated for total gentamicin by plotting the total peak area *versus* total concentration and performing linear least squares regression analysis.

3. Results and discussion

3.1. Optimization of the derivatization

Experiments were carried out for optimizing the derivatization conditions. Common silylating reagents alone failed to produce volatile and/or stable derivatives. Poor derivative stability has been reported for *N*-trimethylsilyldiethylamine (TMSDEA) derivatives of neomycin and kanamycin.[8] A two step derivatization was considered necessary. The previously reported HFBI derivatization of the amine groups and trimethylsilylation of the hydroxyl groups results in a molecular weight of close to 1600 Da of the gentamicin derivatives.[4] TFAA derivatization of the amines was, therefore, considered superior to HFBI because of the substantially smaller (100 Da for each derivatized group) increase in the molecular weight of the derivatives. The higher molecular weight complicates the mass spectral interpretation owing to potential loss of molecular ions and primary fragments resulting from the limited mass range of the analyzer.

Derivatization time, temperature and reagent volumes were varied and their effects on the derivatization yield and method sensitivity were evaluated (data not shown). The TFAA derivatization was highly time dependent whereas the TMSI derivatization was less sensitive. Partial derivatization was observed with non-optimal conditions. The derivatization temperature did not have a significant effect on the results within the range 45–65 °C.

3.2. Mass spectral analysis

Fig. 2 shows a scanned mass spectrum and specific fragments from the derivatized gentamicin C_1 obtained using EI and positive ion monitoring. The ions at *m/z* 310, 349 and 400 were selected for tandem mass spectrometric (MS-MS) experiments. The ions at *m/z* 310 and 400 were common for all gentamicin components whereas that at *m/z* 349 was specific to gentamicin C_1. Selected ion monitoring was attempted using the ions at *m/z* 310, 349 and 400 but the resulting chromatograms were inferior to those obtained by selected reaction monitoring.

A clear increase in ion abundance was observed (more than 100-fold) as a result of use of NCI instead of EI. The scanned mass spectra of the components and some specific fragments obtained with NCI are presented in Fig. 3. The negative ion mass spectra allowed the identification of the gentamicin components and MS-MS was carried out on the ions at *m/z* 742, 351, 337 and 366. Table 1 summarizes the main fragments after NCI-CAD. Cleavage of the glycosidic bonds was found to be the main fragmentation pattern. Three ions (*m/z* 351, 650 and 741, *m/z* 337, 636 and 727 and *m/z* 365, 665 and 755 for gentamicin C_2, C_{1a}, and C_1, respectively) were selected for the quantification of each of the gentamicin components.

It was reported previously that mass spectrometric analysis provided inferior sensitivity to ECD.[4] According to the present results, NCI provided better sensitivity than EI or ECD. The increased sensitivity was apparently related to at least two factors. The chemical ionization generally produces less

fragmentation, increasing the amount of high mass peaks in the mass spectrum. Consequently, the signal-to-noise ratio is improved. Moreover, the analytes apparently have high electron affinity. A significant amount of the total ion current is lost as negative ions when positive ions are monitored.

3.3. Quantification, repeatability and reproducibility

The elution order of the gentamicin components was gentamicin C_2 (5.8 min), C_{1a} (6.2 min) and C_1 (6.8 min). No interference of the electron capturing reagent (TFAA) was observed using negative ion monitoring. The limit of detection using EI was disappointing (in the low μg ml^{-1} region of total gentamicin). With the use of NCI, the limit of detection was below 10 ng ml^{-1} (20 fmol per injection) of total gentamicin. Quantification of the individual components was not attempted because analytical standards of the individual components were not available, nor was there any information about the component ratio of the reference material used in this study. The increase in total gentamicin peak area was linear within the investigated concentration range (20 ng ml^{-1}–10 μg ml^{-1}), resulting in a concentration *versus* response (peak height) curve $y = 189.4x - 16.4$. The intra- and inter-day relative standard deviations were 7.3 and 12% at 1 μg ml^{-1} (2 pmol per injection), respectively.

The sensitivity of an ion trap mass spectrometer over the entire mass range is good and permits the detection of relatively high molecular weights. In quadrupole instruments, the ion transmission through the mass filter decreases with increasing *m/z*. No attempts were made to evaluate possible internal standards. It is likely, however, that other aminoglycosides can be used as internal standards for the method.

The results of these novel derivatization and detection procedures for the gentamicin complex indicated that NCI greatly improved the sensitivity of gentamicin determination compared with both EI positive ion monitoring and ECD. The present method facilitates the use of various mass spectrometric quantification and detection methods in gentamicin determination. Total ion monitoring can be used in screening, selected ion monitoring for quantification and selected reaction monitoring when extreme selectivity is needed. Multiple options in selecting ions for detection are available depending on the analytical requirements. All of the components produced fragments specific to the individual component in addition to fragments common to all of the components. The sensitivity of the present method exceeds that of all other methods reported to date. In conclusion, this study demonstrated that GC-MS provides an alternative to LC-MS in the determination of non-volatile compounds when suitable derivatization is used.

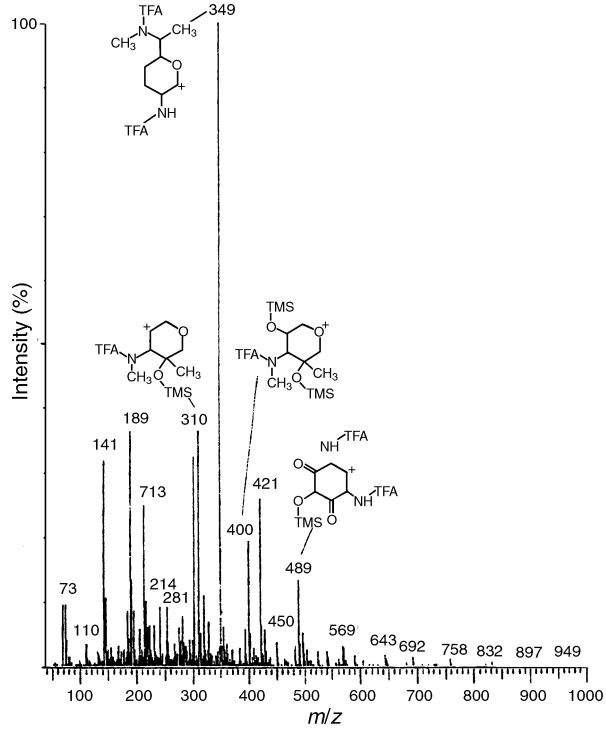


Fig. 2 Mass spectrum of gentamicin C_1 using EI and positive ion monitoring.

Table 1 MS-MS data obtained from specific selected ions from gentamicin components after NCI monitoring

Component	Parent ion (*m/z*)	Fragment ions (*m/z*) (abundance, %, in parentheses)
C_{1a}	337	337 (100), 267 (13), 319 (10), 299 (12), 279 (11)
C_1	365	365 (100), 347 (20), 295 (22), 251 (10), 181 (10)
C_2	351	351 (100), 333 (12), 313 (40), 293 (14), 281 (10), 197 (8)

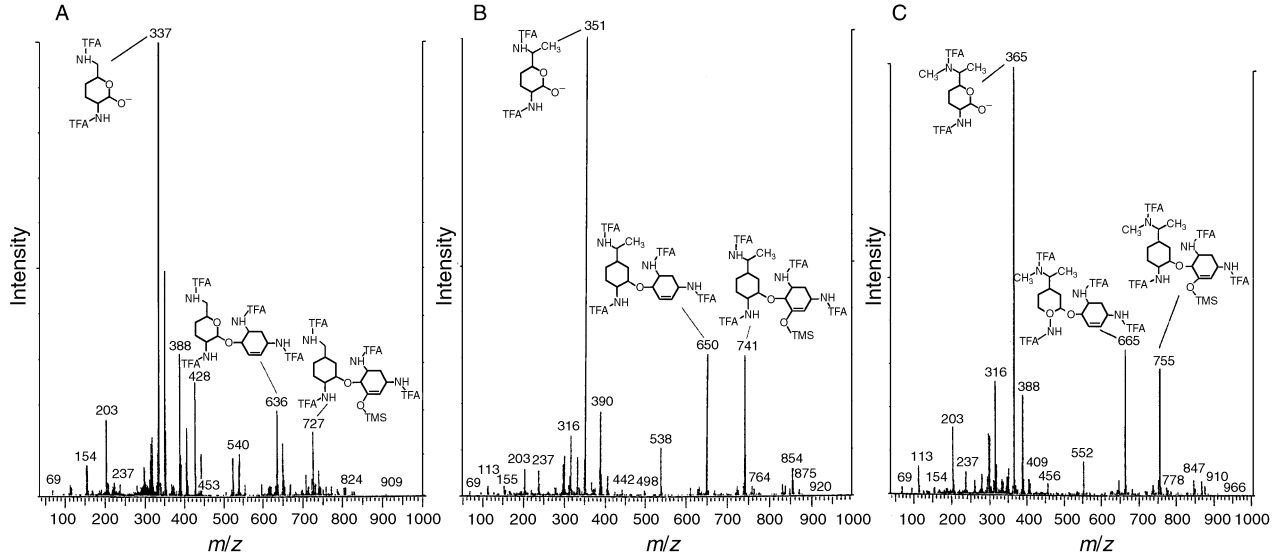


Fig. 3 Mass spectrum of gentamicin components, (A) gentamicin C_{1a}, (B) gentamicin C_2 and (C) gentamicin C_1, and some specific fragment ions obtained with chemical ionization and negative ion monitoring.

References

1 P. J. Claes, R. Busson and H. Vanderhaeghe, *J. Chromatogr.*, 1984, **298**, 445.
2 N. Isoherranen and S. Soback, *J. AOAC Int.*, 1999, **82**, 1017.
3 J. W. Mayhew and S. L. Gorbach, *J. Chromatogr.*, 1978, **151**, 133.
4 M. Preu, D. Guyot and M. Petz, *J. Chromatogr. A*, 1999, **818**, 95.
5 G. Inchauspe and D. Samain, *J. Chromatogr.*, 1984, **303**, 277.
6 L. Essers, *J. Chromatogr.*, 1984, **305**, 345.
7 L. G. McLaughlin, J. D. Henion and P. J. Kijack, *Biol. Mass. Spectrom.*, 1994, **23**, 417.
8 K. Tsuji and J. Robertson, *J. Anal. Chem.*, 1969, **41**, 1332.