Determination of Gentamicins C₁, C₁a, and C₂ in Plasma and Urine by HPLC

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Background: Gentamicin is an aminoglycoside antimicrobial agent produced by fermentation of Micromonospora purpurea or M. echinospora. It has a wide spectrum of antimicrobial activity and is still considered a cornerstone in the treatment of severe infections caused by gram-negative aerobic bacteria (1). However, gentamicin is not a single molecule but a complex of three major and several minor components. Gentamicins C₁, C₁a, and C₂ are the three major components of the drug complex (Fig. 1). The C₂ component consists of two stereoisomers (C₂ and C₂a). The structural difference between the gentamicin components is comparable to the difference between gentamicins and other aminoglycosides such as tobramycin and netilmicin. Despite the wide therapeutic use of gentamicin for decades and the importance of gentamicin in clinical practice, chromatographic methods able to determine the individual components in biological fluids are few and are limited in performance (2). Consequently, the accuracy of the methods generally used for measuring total gentamicin concentration, such as immunoassays and bioassays, has not been confirmed as a function of gentamicin component ratio.

Gentamicin use is associated with severe side effects, the most important of which are nephrotoxicity and ototoxicity (1). Animal data indicated that the different gentamicin components differed in their toxicities (3), but human data were insufficient to confirm this (4). Because of its toxicity, gentamicin use is subject to therapeutic drug monitoring, especially in patients with renal impairment. The research concerning disposition of the individual gentamicin components has been practically nonexistent, apparently because of the lack of suitable analytical methods and calibrators. Gentamicin C₁ was reported to have different pharmacokinetics than the gentamicin complex when gentamicin C₁ was given separately to patients (4). Subsequently, results from a study using an HPLC method able to separate gentamicin C₁ from the two other components contradicted these results (5). Decisive determination of the pharmacokinetics of the individual gentamicin components may have important therapeutic and toxicological implications.

Currently, the set limits for the component ratio, as

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Received in revised form February 16, 2000; accepted March 24, 2000.

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Clinical Chemistry 46:6
837–842 (2000)
defined in the US Pharmacopoeia (6), are 25–50% for C₁, 10–35% for C₁a, and 25–55% for the sum of C₂ and C₂a. The British Pharmacopoeia (7) limits are 25–50% for C₁, 15–40% for C₁a, and 20–50% for the sum of C₂ and C₂a. However, the official methods for analysis of the gentamicin component ratio in drug standards have been criticized for failing to indicate the actual composition of a gentamicin complex (8). Consequently, the component ratio in commercial gentamicin preparations varies remarkably and is generally unknown (8). The importance of the variability in the component ratio and its implications to gentamicin toxicity remain to be established (9).

The chromatographic methods that separate gentamicins C₁, C₁a, and C₂ lack sufficient sensitivity and accuracy identification and/or quantification to measure therapeutically relevant concentrations of the individual components in plasma and urine (2). Therefore, fluorescence polarization immunoassays and radioimmunoassays currently are the principal methods used in therapeutic drug monitoring and pharmacokinetic studies of gentamicin, but microbiological assays have also been used (9). These methods lack the ability to identify and measure separately the three components. It also is not clear whether these methods are able to measure with equal accuracy the concentrations of the individual components, hence producing a potential bias in the total gentamicin concentration. In a recent study of a homogeneous immunoassay for gentamicin, this issue was addressed (10). Notwithstanding that the authors claimed similar response and that absolute recovery was not determined, the results indicated that differences up to 32% between the reactivities of the antibodies to the gentamicin components may exist. Remarkable intersubject variability has been reported for immunoassays in general, and the accuracy of the results has been questioned, especially at low concentrations (11). Thus, development of a chromatographic method for reliable detection, identification, and quantification of the three major gentamicin components in body fluids in the therapeutic range (0.1–20 mg/L) is essential for further research concerning gentamicin pharmacokinetics and toxicity.

The present study describes the development of a liquid chromatographic method able to determine concentrations of gentamicins C₁, C₁a, and C₂ in plasma and urine at relevant concentrations for therapeutic drug monitoring and pharmacokinetic studies.

**Materials and Methods**

Acetonitrile and methanol (LiChrosolv; Merck) were of HPLC gradient grade. Tris (Trizma® base), ammonium acetate, gentamicin sulfate, and gentamicin solution were purchased from Sigma Chemical Co. The 1-fluoro-2,4-dinitrobenzene (FDNB)³ was from Fluka, and the Oasis™ (30 mg) solid-phase extraction (SPE) cartridges were from Waters.

**INSTRUMENTATION**

A Hewlett-Packard H-P 1100 low-pressure mixing gradient HPLC equipped with a diode array ultraviolet-visible detector, autosampler, and column oven was used. An Esquire (Brucker and Hewlett-Packard) ion trap mass spectrometer with an atmospheric pressure chemical ionization (APCI) interface was utilized for mass spectrometric determinations. A Varian VXR-300S nuclear magnetic resonance (NMR) spectrometer was used for measuring the proton NMR.

**HPLC PROCEDURE**

Gentamicins C₁, C₁a, and C₂ were determined as their 2,4-dinitrophenyl derivatives using a mobile phase of 680 mL/L acetonitrile-320 mL/L Tris buffer (pH 7.0; 8.3 mmol/L, titrated to pH 7.0 with HCl) at a flow rate 1.2 mL/min. A Symmetry™ C₁₈ reversed-phase column (100 × 4.6 mm; 3.5 μm bead size; Waters) connected to a C₁₈ precolumn was used for separation. The injection volume was 20 μL, the column temperature was 25 °C, and the chromatographic eluent was monitored at 365 nm.

**PREPARATIVE SEPARATION OF THE GENTAMICIN COMPONENTS**

The gentamicin C₁, C₁a, and C₂ components were separated from gentamicin sulfate (lot no. 42H0610; Sigma) in a silica column according to the method described by Claes et al (8). The column was prepared from a silica (100 g) slurry in isopropanol-chloroform-170 mL/L ammonium hydroxide (1:2:1, by volume). The column was eluted with isopropanol-chloroform-250 mL/L ammonium hydroxide (1:2:1, by volume), and 10-mL fractions were collected. The elution was monitored using the thin-layer chromatographic method described by Wilson.

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³ Nonstandard abbreviations: FDNB, 1-fluoro-2,4-dinitrobenzene; SPE, solid-phase extraction; APCI, atmospheric pressure chemical ionization; NMR, nuclear magnetic resonance spectroscopy; and OPA, o-phthalaldehyde.
et al. (12). The solvent system for the thin-layer chromatography consisted of a lower phase of methanol-chloroform-250 mL/L ammonium hydroxide (1:1:1, by volume), and the gentamicins were visualized with ninhydrin spray (0.1 g ninhydrin in 100 mL of 950 mL/L n-butanol-50 mL/L glacial acetic acid). The appropriate fractions were combined and evaporated to dryness. The residue was redissolved in methanol and evaporated under reduced pressure to constant weight. The purities of the component calibrators were assessed by HPLC, thin-layer chromatography, NMR, and melting point. The proton NMR was measured, and the chemical shifts are all reported relative to tetramethylsilane. The antimicrobial potency of the calibrators was evaluated using Bacillus subtilis as test organism in an agar diffusion disk assay.

**PREPARATION OF CALIBRATORS AND CALIBRATION CURVES**

We prepared the calibrators by diluting each of the gentamicin components in deionized water. The stock solutions were added to plasma and urine to provide concentrations of 0, 0.1, 0.2, 0.4, 1, 5, 10, 15, 20, and 50 mg/L of the individual gentamicin components. The calibrators in water, plasma, and urine were derivatized in the SPE cartridge as described below. The calibration curves were obtained by plotting the peak areas as a function of the respective concentrations for each analyte and calculating the linear regression. Recoveries from plasma and urine were determined by comparison of samples to which the components had been added to aqueous calibrators, all prepared using SPE. The relative recovery of the SPE and derivatization was determined by comparing responses of aqueous calibrators prepared by SPE to aqueous calibrators evaporated to dryness and derivatized in a vial with 0.3 mL of the derivatization solution (100 °C for 1 h).

**DERIVATIZATION REAGENT**

The derivatization reagent consisted of 0.5 mL of 0.17 mol/L Tris buffer (pH 12.0), 0.5 mL of water, 2 mL of acetonitrile, and 50 mg of FDNB in 0.2 mL of acetonitrile. The reagent, except for the FDNB component, was stored in 3°C. The FDNB fraction was added to the reagent before use.

**PREPARATION OF PLASMA AND URINE SAMPLES**

The SPE cartridge was conditioned with 1 mL of methanol followed by 1 mL of 0.17 mol/L Tris buffer at pH 10.0. Five milliliters of Tris buffer (0.17 mol/L Tris, titrated to pH 12.0 with NaOH) was added to 1.0 mL of plasma or urine, vortex-mixed, and charged on the cartridge. The flow through the cartridge was kept at <0.3 mL/min. The cartridge was washed with 2 mL of 0.17 mol/L Tris buffer (pH 10) and dried; 300 μL of the derivatization mixture was then applied on the SPE cartridge. The top of the cartridge was closed with a conical polypropylene pipette tip (5–200 μL) to reduce evaporation. The cartridge was then incubated for 1 h in a laboratory oven at 100 °C. The derivatized gentamicin was eluted with 5 mL of acetonitrile by gravity into the tube holding the cartridge. The eluate was evaporated to dryness, redissolved in 300 μL of acetonitrile, and transferred to an autosampler vial. The blood samples from gentamicin-treated dogs or humans were collected in heparin-containing test tubes, and plasma was separated with centrifugation. The plasma samples were then stored at −30 °C until analysis as described above.

**MASS SPECTROMETRY**

The preparatively separated gentamicin C₁, C₁₅ and C₂ components were identified using APCI and ion trap mass spectrometry. The analytes were dissolved in a solution of methanol-0.05 mol/L ammonium acetate (1:1, by volume) and injected directly (by syringe pump) into the liquid chromatography-mass spectrometry interface. Positive ions were detected. The APCI heater temperature was set at 400 °C. The capillary exit was maintained at 100.0 V, skimmer 1 maintained at 30 V, and the cutoff was at 50 m/z. Nitrogen was used as a sheath gas, and helium was the collision gas.

**RESULTS**

**IDENTIFICATION OF COMPONENTS**

Mass spectrometry of the component calibrators using APCI produced a molecular peak of each of the components (C₁₅, m/z 450 [M+H]; C₂, m/z 464 [M+H]; and C₁, m/z 478 [M+H]). The mass spectral data are summarized in Table 1. The mass spectrum of gentamicin C₁ is presented in Fig. 2.

Proton NMR gave the following results:

<table>
<thead>
<tr>
<th>Component</th>
<th>Mr</th>
<th>Ions (relative abundance)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C₁₅</td>
<td>449</td>
<td>450 (55), 322 (50), 321 (100)</td>
</tr>
<tr>
<td>C₂</td>
<td>463</td>
<td>464 (100), 322 (90), 160 (5)</td>
</tr>
<tr>
<td>C₁</td>
<td>477</td>
<td>478 (100), 322 (25)</td>
</tr>
</tbody>
</table>
where $\delta$ is the chemical shift in parts per million downfield from the tetramethylsilane standard, $s$ is a singlet, $d$ is a doublet, and $m$ is a multiplet.

The melting point of C1 was 92–97 °C, and that of C2 was 105–112 °C. The melting point of gentamicin C1a was not determined because there was no reference value.

The purified components did not differ in their antimicrobial activity, as reported previously (13). In addition, the antimicrobial activity of the individual components was equal to that of the reference gentamicin mixture.

CHROMATOGRAPHIC SEPARATION

Chromatograms of the separation of the mixture of gentamicins in dog plasma and a blank chromatogram are presented in Fig. 3. The chromatographic peaks were identified using single component calibrators obtained as described above. The elution order of the gentamicins was C1a, C2 isomers, and C1. Only one peak was obtained after derivatization of each gentamicin component calibrator, indicating that derivatization was complete. Separation was obtained only within a narrow range of mobile phase constituent concentrations. When the acetonitrile concentration was 50 mL/L higher, the separation was lost, whereas when it was 50 mL/L lower, the retention times increased and the peaks became unacceptably wide.

METHOD VALIDATION

The method was validated for human and dog plasma and urine. The precision, linearity, and recovery of the method were evaluated. For the inter assay CVs, 20 repetitive analyses were performed within 2 months on three different concentrations ($0.5, 5, 50$ mg/L). The calculated CVs and recoveries for each analyte are presented in Table 2. The limit of quantification of the method, defined as nine times the background noise, was $0.1$ mg/L for gentamicins C2 and C1a, and $0.07$ mg/L for gentamicin C1 from plasma and urine samples. The plasma and urine calibration curves were linear with the following equations: gentamicin C1, $y = 340x$ ($r^2 = 0.999$); gentamicin C2a, $y = 169x$ ($r^2 = 0.998$); and gentamicin C1a, $y = 183x$ ($r^2 = 0.996$) for plasma; and gentamicin C1, $y = 470x$ ($r^2 = 0.999$); gentamicin C2, $y = 235x$ ($r^2 = 0.998$); and gentamicin C1a, $y = 245x$ ($r^2 = 0.998$) for urine. Amoxicillin, ampicillin, neomycin, tobramycin, streptomycin, sulfadiazene, sulfa methoxazole, oxytetracycline, and doxycycline were added to plasma samples for evaluation of possible interference. No interference from these drugs or common plasma and urine components was detected in 200 samples analyzed.

Table 2. Method validation results from plasma and urine.

<table>
<thead>
<tr>
<th>Component</th>
<th>Recovery, $^a$ (%)</th>
<th>Intraassay CV, $^b$ (%)</th>
<th>Interassay CV, $^b$ (%)</th>
<th>Intraassay CV, $^b$ (%)</th>
<th>Interassay CV, $^b$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gentamicin C1a</td>
<td>72, 98</td>
<td>2.1, 7.7</td>
<td>4.8, 13</td>
<td>6.1, 10</td>
<td>8.7, 12</td>
</tr>
<tr>
<td>Gentamicin C2</td>
<td>72, 98</td>
<td>4.2, 10</td>
<td>6.3, 12</td>
<td>4.2, 3.1</td>
<td>16, 14</td>
</tr>
<tr>
<td>Gentamicin C1</td>
<td>72, 98</td>
<td>1.1, 11</td>
<td>2.0, 7.7</td>
<td>7.8, 8.1</td>
<td>16, 12</td>
</tr>
</tbody>
</table>

$^a$ Plasma and urine, respectively.

$^b$ n = 10. The percentages are from the highest (50 mg/L) and the lowest (0.1 mg/L) concentrations.
Gentamicin is a very polar, hydrophilic drug that is soluble only in water and is slightly soluble in methanol. The hydrophilic character of gentamicin makes its extraction from biological matrices by common organic solvents impossible. Acidic and basic phosphate buffer, H₂SO₄, trichloroacetic acid, basic Tris buffer, methanol, acetonitrile, and combinations of the above were investigated for the ability to extract gentamicin from plasma; except for Tris buffer, they all failed. SPE was necessary to eliminate interference in derivatization and chromatography and to concentrate the samples. Gentamicin was not retained in a C₁₈ SPE cartridge. Weak cation-exchange SPE required complicated pH adjustment of the sample and elution with an acidic aqueous solution. Additional titration was needed because the derivatization was performed under basic conditions. Weak cation-exchange SPE was therefore considered inappropriate for the present application. Reversed-phase polymer SPE allowed selective extraction of gentamicin from plasma with Tris buffer. However, we were unable to elute the retained gentamicin with organic solvents, and on-column derivatization was adopted.

Gentamicin does not possess a chromophore in its structure and must be derivatized to enable ultraviolet detection. After preliminary trials with various reagents, we chose FDNB derivatization for the gentamicin derivatization. The 2,4-dinitrophenyl derivatives are very soluble in organic solvents and thus easy to elute from the SPE cartridge. The specific stability of the Oasis cartridges over a wide pH range enabled the use of basic buffers, which was essential for gentamicin retention in the cartridge and for on-column derivatization. The SPE material was stable in the temperatures used and allowed optimization of the derivatization yields. The SPE and on-column derivatization allowed the use of much higher plasma volumes than in earlier studies (generally <500 μL) (2). Consequently, assay sensitivity could be enhanced. The present sample preparation and derivatization procedure is also fast and simple to perform and allows throughput of hundreds of samples as required in pharmacokinetic studies. The high stability of the dinitrophenyl derivatives, superior to most other derivatization reagents, allows preparation of large numbers of samples simultaneously. A single analysis could be performed in less than 3 h.

Our results show that FDNB-derivatized gentamicin components produce different detector responses. o-Phthaldialdehyde (OPA) with mercaptan has been widely used for the derivatization of gentamicin (2). In preliminary studies, we found that OPA postcolumn derivatization with mercaptoethanol did not allow separation of the three components and that precolumn OPA derivatization did not produce the necessary assay sensitivity, probably because of the instability of the derivatized component. The British Pharmacopoeia and US Pharmacopoeia claim that when precolumn OPA derivatization is used, the individual peak areas, as percentages of the total peak area, can be used to quantify the gentamicin component ratio. Both the British and the US Pharmacopoeia methods for determining the ratio of gentamicin components have
been shown to be erroneous (8). It seems likely that the inaccuracy results from variations in detector response generally believed to correlate with the number of derivatized groups. Consequently, gentamicin C₁ should have a smaller response than the two other components because it has one less free amine and OPA reacts only with primary amines. Therefore, the differences in detector responses obtained here for the three gentamicin components substantiate the prerequisite that the components be analyzed independently and that the use of individual component calibrators is necessary for adequate quantification of total gentamicin.

The chemical differences between the gentamicin components are very small, and sufficient separation of the components is difficult to achieve. Barends et al. (14) reported derivatization of gentamicins with FDNB, but only C₁ₐ was separated from the two other components. In the present study, the dinitrophenyl derivatives of gentamicins C₁ₐ, C₂, and C₁ were sufficiently separated to allow quantification of the individual compounds. This was apparently attributable to differences in the column specifications because we could also achieve the same separation with ammonium acetate buffer (1.0 mmol/L at pH 4.2). The gentamicin C₂ₐ enantiomer eluted together with gentamicin C₂ on a standard C₁₈ column.

It must be emphasized that the gentamicin content in commercial calibration materials is given as the antimicrobial potency of an unknown mixture of at least three components. This severely invalidates accurate quantification based on use of these calibrators. Method development also becomes questionable because calibration curves cannot be prepared without the use of calibrators for the individual components.

**Applicability of the Method**

The appropriateness of the present method was assessed in a study performed to determine the pharmacokinetics of the gentamicin components in dogs. To our knowledge, this is the only study describing the individual pharmacokinetics of the three gentamicin components. The results indicated that the pharmacokinetic characteristics of the components were significantly different. The results of the study will be reported elsewhere (15). Currently, the method is used in a pharmacokinetic study of gentamicin components in human patients. Representative plasma concentration-time curves of the three gentamicin components from a patient are presented in Fig. 4.

The present method can be used to evaluate the accuracy of commonly used nonchromatographic gentamicin assays and to determine the component ratio in unknown samples. In light of the results of this study, it appears increasingly important to establish the effect of variations in the ratios of the components on the accuracy of methods unable to separate the components.

To our knowledge, this is the first time that FDNB was used for derivatization of amines in a SPE cartridge. The present extraction and derivatization technique may facilitate sample preparation of a wider class of hydrophilic amines that are difficult or impossible to extract or concentrate from biological matrices as the parent compounds.

In conclusion, the present method allows fast analysis of gentamicins C₁, C₂, and C₁ in plasma or urine. It is the first method able to separate the three major gentamicin compounds that has been applied to pharmacokinetic studies. Sample preparation using SPE and on-column derivatization with FDNB allowed use of large sample volumes and produced stable, concentrated gentamicin dinitrophenyl derivatives. The need for quantification of the individual components to determine the total gentamicin concentration was emphasized. The method can be used as a reference method for performance characterization of nonchromatographic methods.

We thank Rica Benita for skillful technical assistance.

**References**