High-Pressure Liquid-Chromatographic Method for Determination of Gentamicin in Plasma

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A rapid, specific method for measuring gentamicin in plasma by high-pressure liquid chromatography was developed. After deproteinization, gentamicin in the supernate was dansylated and extracted into ethyl acetate. The organic extract was chromatographed on a microparticulate reversed-phase column, which was eluted with aqueous acetonitrile. Use of the dansyl derivative enables fluorometry, for more sensitive quantitation. Various factors that could affect the assay sensitivity were investigated. With 0.2-ml plasma samples, the method can accurately measure as little as 1 mg of gentamicin per liter. We encountered no interferences from plasma supplemented with various drugs or plasma of patients who were on therapy with other drugs. This method can also separate gentamicin C1 from C1a and C2, all of which are present in various ratios in commercial dosage forms. This method is also applicable to gentamicin determination in urine.

Additional Keyphrases: antibiotic assay • urine • monitoring therapy • gentamicin components • aminoglycoside assay

Gentamicin is a widely used, potent, broad-spectrum aminoglycoside antibiotic. It has a narrow therapeutic range in plasma (1–3). At peak concentrations of less than 2 mg/liter, this chemotherapy not only is ineffective, but the incidence of bacterial resistance increases (4). Concentrations exceeding 12 mg/liter can cause serious ototoxicity or nephrotoxicity, or both (2, 5). For these reasons, many methods of various kinds have been developed for monitoring concentrations of gentamicin in plasma or serum (6–18).

Unfortunately, gentamicin concentrations are often greatly influenced by physiological and pathological factors such as inter- and intra-subject variations in plasma gentamicin concentrations after a given dosage (19, 20), the patient’s age (2, 21), fever (22), burns (23), or renal-function impairment (4, 24–26). Because gentamicin is principally excreted in the urine (26, 27), its accumulation, and consequent serious toxicity, can result in patients with renal-function impairment. All these factors mandate fast, accurate monitoring if this antibiotic is to be safely used, particularly in patients whose renal function is changing (28).

The reported microbiological assays are inexpensive and simple, but they share several disadvantages (29). For example, they are subject to the variations caused by the sensitivity of microorganism strains being tested and have limited specificity, owing to cross reaction with other antimicrobial agents that may be present in the same sample (16, 17). Furthermore, such assays usually require more than 6 h.

The enzymatic assays are more specific and accurate, but require substantially purified enzyme (13, 14). The specificity of the enzyme preparations also affects this type of assay, and in some cases normal constituents of plasma may interfere with the assay (30).

The radioimmunoassay methods can be quite specific and sensitive, but they also depend on the specificity of the antibodies, and cross reactions have been reported (15–17).

We report here a simple, sensitive, specific, and rapid high-pressure liquid chromatographic method for determining gentamicin in plasma.

Materials and Methods

Reagents

Gentamicin sulfate, donated by Schering Corp., Bloomfield, N.J. 07003, was used as received. The labeled potencies are 620, 641, 788, and 578 μg of the base per milligram of the components C1, C1a, C2, and the mixture (to be referred to as gentamicin), respectively. 5-Dimethylamino-1-naphthalene sulfonyl chloride (dansyl chloride) and dansylamide were from Pierce Chemical Co., Rockford, Ill. 61105. The organic solvents—acetonitrile, methylene chloride, and ethyl acetate—were glass-distilled (Burdick & Jackson Lab, Muskegon, Mich. 49442). All other chemicals were of reagent grade.
Preparation of Standard Curves

In 13 × 100 mm disposable culture tubes (Fisher Scientific Co., Fairlawn, N. J. 07410), 0.2-ml aliquots of plasma were supplemented with various amounts of gentamicin sulfate from concentrated aqueous stock solutions, to give gentamicin concentrations from 1.2 to 23 mg/liter (calculated as free base). After adding alkalinized phosphate buffer (0.8 ml, pH 7.4, 66 mmol/liter, plus 30 μl of sodium hydroxide solution, 1.25 mol/liter) and 2.5 ml of acetonitrile, the samples were thoroughly vortex-mixed for 10 s and centrifuged at about 900 × g for one min. The clear supernatant solution was then poured directly into another culture tube containing 2 ml of methylene chloride. After vortex-mixing for 10 s and centrifuging for 1 min, 0.5 ml of the separated aqueous (upper) layer was pipetted into another culture tube and mixed with 0.3 ml of acetonitrile containing 2.5 mg of dansyl chloride. The culture tube was screw-capped, incubated in darkness for 5 min in a water bath set at 75 °C, and then cooled in icewater. Next, 0.5 ml of ethyl acetate and 6 ml of carbonate buffer (0.5 mol/liter sodium bicarbonate and 0.5 mol/liter sodium carbonate, pH 9.5) were added to the cooled reaction mixture, which was vortex-mixed for 10–15 s and centrifuged. The organic phase, 5 μl, was chromatographed. Peak-height measurements were used to construct the standard curves. The standard curve for component C1 was also prepared in the same way, with the use of a stock solution of pure C1 sulfate.

At least one plasma blank study was carried out concurrently with each standard curve preparation. Before being used, the disposable culture tubes were pre-rinsed with distilled water and dried. They were not re-used for the assay because a higher blank reading resulted, even after thorough cleaning.

Plasma and serum samples from a rabbit (in our preliminary animal study) and from patients were deproteinized, dansylated, and chromatographed in the same manner. Some urine samples, after proper dilution with water or pH 7.4 buffer, were also analyzed by the same method.

Some Analytical Variables

Effect of plasma dilution: From the concentrated stock solution, 3 μg of gentamicin sulfate was added to a 1-ml mixture of the pH 7.4 buffer and plasma in different proportions ranging from 100% plasma to 100% buffer. These samples were alkalinized with sodium hydroxide, “deproteinized” with 2.5 ml of acetonitrile, extracted with 2 ml of methylene chloride, dansylated, and chromatographed as described above.

Effect of amount of dansyl chloride used: One milliliter of plasma containing 15 μg of gentamicin sulfate was diluted with 4 ml of the alkalinized phosphate buffer, treated with 12.5 ml of acetonitrile, and extracted with 10 ml of methylene chloride. Aliquots of 0.5 ml of the aqueous solution were then reacted with various amounts of dansyl chloride, ranging from 0.5 to 6 mg. The reaction mixtures were treated with ethyl acetate and carbonate buffer and chromatographed as described.

Effect of sodium hydroxide: Plasma samples, 0.2 ml containing 0.3 μg of gentamicin sulfate, were diluted with phosphate buffer, alkalinized with various volumes (0–100 μl) of the sodium hydroxide solution, deproteinized, dansylated, and chromatographed as described. Similar studies were also conducted using 1 mg of dansyl chloride for the dansylation at 100 °C for 15 min.

Effect of temperature and time: Deproteinized samples were dansylated with 2.5 mg of dansyl chloride at 45, 75, and 100 °C for different intervals.

Intra- and inter-assay reproducibility: Pooled human plasma samples, supplemented with known concentrations of gentamicin, were used for these studies, the latter of which extended over one month.

Drug interference studies: Pooled human plasma samples were supplemented (10 mg/liter) with stock drug solutions of kanamycin, netilmicin, chloramphenicol, sulfisoxazole, ampicillin, phencetin, aspirin, and acetaminophen. We also examined a 40 mg/liter solution of cephalothin and a 50 mg/liter solution of salicylic acid, in plasma, for possible interference, as well as plasma samples from patients on simultaneous gentamicin and carbenicillin therapy.

Preliminary Studies in a Rabbit and in Humans

Gentamicin sulfate, 200 mg, in a 5 ml sodium chloride solution (9 g/liter) was administered intravenously to a 4-kg albino rabbit via the medial vein of one ear. Blood samples, 0.5 ml, collected at various times from the marginal vein of the other ear, were analyzed for gentamicin as described under Preparation of standard curves. We also analyzed 25 plasma samples collected...
Results

Figure 1 shows typical chromatograms for gentamicin from human and rabbit plasma. Although gentamicin has three components, only two peaks, with retention times of 5.1 and 5.9 min, are seen that could be attributed to gentamicin. The chromatograms for gentamicin in human and rabbit plasma are essentially the same. The human plasma blanks usually showed no peak or negligible peaks that would interfere. The human plasma blanks that we investigated included pooled human plasma, plasma and (or) serum from healthy subjects, and plasma from patients with or without known renal impairment. The chromatograms of gentamicin components C1, C1a, and C2, used individually for supplementation, are shown in Figure 2. Components C1a and C2 had the same retention time, 5.1 min; C1 had a retention time of 5.9 min. Accordingly, the peak in the chromatograms of gentamicin with a retention time of 5.1 min was assigned to the mixture of C1a and C2, and the other peak, for which the retention time was 5.9 min, to C1.

Dansylation of gentamicin could conceivably result in the formation of multiple products, because it has three major components, each of which has five amino groups that could react with dansyl chloride. However, this complication was not observed. Attempts to resolve components C1a and C2 were not successful. Many of the peaks that eluted earlier in the chromatograms could not be identified because of detector overloading and therefore we are not sure that only a single product was formed in the dansylation of each component. The nature of the dansylation products of the two peaks attributed to gentamicin was not investigated in this study, but they evidently were nonpolar; they could be extracted into organic solvents and were eluted relatively slowly from the column.

Figure 3 shows standard curves for gentamicin and component C1. Good linear relationships were observed for both gentamicin and C1 in all the concentrations investigated (up to 40 mg/liter). From these standard curves, we calculated that 29% of the gentamicin sulfate we used was component C1. The slopes of the standard curves were fairly reproducible when the detector was properly calibrated with dansylamide.

Sodium hydroxide alkalinization of the samples before deproteinization is essential. No gentamicin could
be detected when sodium hydroxide was added after deproteinization; however, gentamicin added to the above supernate of deproteinized plasma could be quantitatively accounted for.

Dilution of the plasma samples with phosphate buffer improved the results, probably owing to dilution of some normal plasma constituents that otherwise would interfere with the dansylation. The effect on the gentamicin peak heights of the quantity of phosphate buffer used to dilute plasma samples is shown in Figure 4. Up to a point, peak height increased with increased proportions of the buffer. The amount of sodium hydroxide used to alkalinize the plasma samples also affected the peak heights of gentamicin (Figure 5). The pH's of the deproteinized plasma samples are also shown in Figure 5. With either 2.5 mg or 1.0 mg of dansyl chloride, use of 30 µl of the sodium hydroxide solution, which gave a final pH of about 10, appeared to be optimal. Thus both pH and the volume of buffer of the reaction media influenced the dansylation.

The effect of the amount of dansyl chloride on the dansylation reaction is shown in Figure 6. The gentamicin peak heights increased sharply with an increase in the quantity of dansyl chloride up to 1 mg, but further increase in dansyl chloride resulted in proportionately smaller increases in peak height and peak heights appeared to decrease when more than 4 mg of dansyl chloride was used. Results seemed to be optimal with 2.5 mg of dansyl chloride, when other factors such as the interference and the overloading of the detector caused by the large excess of this reagent were considered. With respect to the amount of acetonitrile present during the dansylation of gentamicin in phosphate buffer, a 6/4 mixture (by vol) of buffer to acetonitrile gave the best results.

Temperature and duration of dansylation also affected the results (Figure 7). At 45 °C, the dansylation appeared to be continuing, even after a 20-min reaction. At 100 °C, the gentamicin peaks became maximal in only about 3 min but declined sharply thereafter, probably because of decomposition of the reaction.
products. At 75 °C, peak heights that were near the plateau value were attained in about 3 min, and the products appeared to be stable at this temperature because the peak heights changed little for as long as 20 min later. Therefore, we chose a reaction time of 5 min at 75 °C for the routine analysis. The products of gentamicin dansylation were stable in ethyl acetate at ambient temperature for at least 6 h, during which time there were no significant changes in peak heights.

Table 1 shows the results of the within-run study conducted at two different concentrations, and Table 2 the results of the between-run study. In both cases the reproducibility was good, the largest coefficient of variation being only about 8%. We attempted to find a suitable internal standard to minimize assay variation, but such efforts were unsuccessful. We strongly recommend that a standard curve based on two or three points or more be determined daily. A linear relationship between concentrations and peak heights was always obtained in over 50 separate standard curves prepared during several months.

Of all the drugs we evaluated for possible interference with gentamicin, only netilmicin, a new aminoglycoside antibiotic, interfered—in this case with the measurement of the second gentamicin peak. Gentamicin concentrations can be determined from either one of the two peaks alone when the ratio of the peak heights is similar to that of drug standard, so the presence of netilmicin will not seriously interfere with the present assay. Kanamycin, another aminoglycoside that occasionally is administered together with gentamicin, caused no interference. No interfering peaks were seen in chromatograms for plasma from 10 patients who were receiving gentamicin and other drug therapy.

Figure 8 shows the change in gentamicin concentrations with time in the plasma of the rabbit treated with gentamicin sulfate. Both C₁ and the mixture of C₁₅ and C₂ showed a biexponential decay pattern with a short (about 25 min) distribution phase and a parallel elimination phase with a biological half-life of 60 min.

The average ratio of the two gentamicin peak heights (about 0.54, second vs. first) obtained from all the rabbit plasma samples was identical to that found for aqueous gentamicin. Similarly constant ratios were also found in all the patients’ plasma samples. These results indicate that measurement of either one of the two peaks is sufficient for the gentamicin quantitation in patients.

Discussion

The lack of ultraviolet chromophore and fluorophore in the gentamicin molecule presents some difficulty in monitoring it, especially when the concentrations are as low as those encountered in patients clinically receiving the drug. In some similar situations, chemical derivatizations have been used to enhance ultraviolet absorbance or to produce fluorescent compounds (33–35) in liquid-chromatographic analysis. One example of this is the fluorescent labeling of amino acids, peptides, and amines with dansyl chloride (34, 35). This was applied to our assay of gentamicin.

Gentamicin is very soluble in water and insoluble or very poorly soluble in nonpolar organic solvents. Thus it is difficult and impractical to extract these compounds directly from plasma with an organic solvent. If whole plasma was used for direct dansylation, gentamicin could not be detected, and detection of gentamicin in phosphate buffer was greatly hindered by the presence of human serum albumin. Therefore, deproteinization is essential, but trichloroacetic acid or tungstic acid evidently precipitate gentamicin with the protein, because no gentamicin could be detected in the supernate of plasma samples so treated. Attempts to use
improvement over the microbiological, enzymatic, and radioimmunooassay methods.

The major advantage of the present method for gentamicin assay over the microbiological, enzymatic, or radioimmunoassays is that any interfering substance can be easily detected by its effect on the shape and ratio of the peak height of the two gentamicin peaks on the chromatogram. Our method is also useful for the determination of gentamicin in urine.

The present method, or a modification of it, may also be used to quantify other aminoglycosides in biological fluids. Results of these studies will be published later.

References