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Note

Analysis of aminoglycoside antibiotics as benzoyl derivatives by high-performance liquid chromatography and its application to the quantitation of neomycin in the perilymph

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It is well known that application of ototoxic drugs directly to the middle ear often causes severe sensorineural deafness [1]. Severe inner ear pathology or endolymphatic hydrops is sometimes observed in association with otitis media [2, 3]. It is most probable in such instances that the drug or inflammation in the middle ear spreads to the inner ear by way of the round window membrane, and recently the permeable character of the round window membrane has drawn the attention of many investigators.

We also have undertaken investigations on the subject, and have already shown that the round window membrane of the guinea pig is permeable to neomycin, by observing damage to the organ of Corti after topical application of neomycin on the round window membrane [4]. To clarify how much of the neomycin permeates from the middle ear into the inner ear through the round window membrane, it is necessary to determine the concentration of

neomycin in the perilymph, which is collected from the inner ear only in an extremely small quantity.

At present, the level of aminoglycoside antibiotics in the perilymph is determined by microbiological assay [5], radioimmunoassay [6] and radioenzymatic assay [7]. However, these methods include rather laborious steps or the use of biohazardous radioactive materials. To overcome these shortcomings, we established a method using high-performance liquid chromatography (HPLC). As is well known, HPLC is very useful for the ultra-sensitive and high-speed determination of various biological materials. As for the determination of aminoglycoside antibiotics, derivatization of amino groups in the molecule with o-phthalaldehyde [8, 9] or 1-fluoro-2,4-dinitro-benzene [10] has been reported. Instead of these derivatives, we chose benzoylation of both hydroxyl and amino groups in order to increase the sensitivity of detection. This paper describes a convenient method for the analysis of aminoglycoside antibiotics and its application to the determination of neomycin in the perilymph.

EXPERIMENTAL

Materials

Benzoyl chloride, pyridine, n-hexane, tetrahydrofuran and sodium carbonate were purchased from Wako, Osaka, Japan. Neomycin, kanamycin, streptomycin and dihydrostreptomycin were obtained from Nippon Kayaku, Tokyo, Takeda, Osaka, Kaken, Tokyo, and Meiji Seika Kaisha, Tokyo, Japan, respectively. Chloroform and methanol were distilled once before use. The structure of neomycin is shown in Fig. 1.
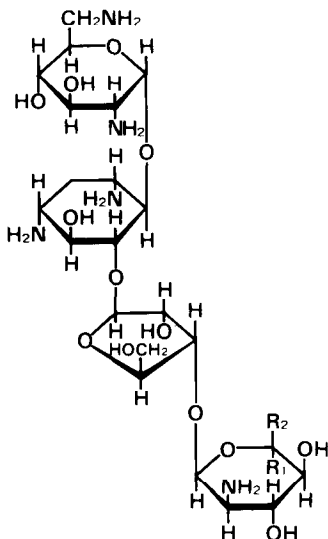
neomycin B : $R_1 = H$ $R_2 = CH_2 \cdot NH_2$
neomycin C : $R_1 = CH_2NH_2$ $R_2 = H$

Fig. 1. Structure of neomycin. Neomycin is a mixture of two stereoisomers, neomycin B and C.

Benzoylation with benzoyl chloride

Benzoylation of aminoglycoside antibiotics was performed according to the method reported previously [11]. Briefly, about 100 μg of neomycin and other aminoglycoside antibiotics (kanamycin, streptomycin and dihydrostreptomycin) were each dissolved in 90 μl of pyridine, and 10 μl of benzoyl chloride were added. The reaction was allowed to proceed at 80°C for 30 min. After the reaction, pyridine was evaporated under a flow of nitrogen. Then, 1 ml of methanol was added, and the mixture was again heated at 80°C for 10 min to convert excess reagent to volatile methyl benzoate. To the solution were added 50 mg of sodium carbonate powder and 1 ml of methanol which was previously saturated with sodium carbonate. The solution was washed three times with 2 ml of *n*-hexane to remove methyl benzoate, and the hexane layer produced by adding 1 ml of water was further removed. The derivative was then extracted with 3 ml of chloroform. The lower chloroform layer was washed three times with 1 ml of methanol—water (1:1, v/v), and the solution was evaporated to dryness.

High-performance liquid chromatography

The derivative was dissolved in 50 μl of chloroform and 2 μl of the solution were injected into a stainless-steel column (25 cm × 4.6 mm I.D.) packed with surface porosity silica gel (Zorbax SIL, 5—6 μm average particle diameter; DuPont, Wilmington, DE, U.S.A.). For the analysis of less than 10 μg of neomycin, the derivative was dissolved in 15 μl of chloroform, and 5 μl of the solution were injected into the column. The mobile phase was a mixture of *n*-hexane and tetrahydrofuran (1:1, v/v), and a flow-rate of 2 ml/min was maintained with a HPLC system (LC-3A; Shimadzu, Kyoto, Japan). The column effluent was monitored at 230 nm using a variable-wavelength ultraviolet (UV) monitor (SPD-2A; Shimadzu). The peak areas were measured with a computer (C-RIA; Shimadzu) of which the attenuation was set to either 2^7 or 2^4.

Collection of perilymph samples

Three guinea pigs (body weight 250—400 g) were anaesthetized with Nembutal® (Abbot, North Chicago, IL, U.S.A.) and a post-auricular incision was made. The middle ear bulla was opened retroauricularly to visualize the round window membrane. Neomycin (5 mg) was absorbed by a small piece of Gelfoam® (Upjohn, Kalamazoo, MI, U.S.A.) soaked in water, and this piece was placed directly onto the round window membrane of each of the guinea pigs. After the intervals of 15 min, 60 min and 120 min, respectively, the Gelfoam was removed from the round window membrane, and immediately a small hole was drilled on the otic capsule into the scala tympani of the basal turn with an electric motor drill. A sample of 6 μl of the perilymph was collected from each animal with a glass micro-pipette (Drummond Scientific, Broomall, PA, U.S.A.). The perilymph samples were lyophilized, and were directly benzoylated by the method described above.

RESULTS

Benzoylation of aminoglycoside antibiotics

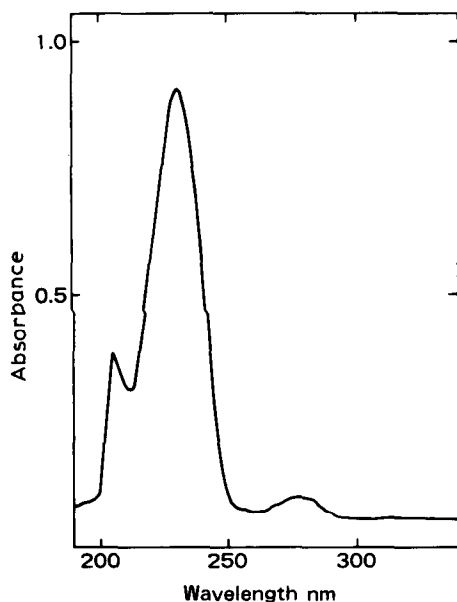Fig. 2 shows the UV spectrum of benzoylated neomycin. The maximum

Fig. 2. UV spectrum of benzoylated neomycin. The concentration of derivative was 6.32 μM in ethanol; a cell with a 1-cm light path was used for the analysis.

absorption of the derivative was at about 230 nm, and the molar extinction coefficient at 230 nm was 142,000, indicating that eleven to thirteen benzoyl chromophores are introduced into the molecule. In order to select a suitable mixture of solvents for HPLC analysis, the benzoylated derivatives of several aminoglycoside antibiotics were analysed by thin-layer chromatography (TLC) on silica gel GF plates (0.25 mm thick; E. Merck, Darmstadt, F.R.G.). They were developed with several mixtures of organic solvents, such as n-hexane—diethyl ether (7:3, v/v), n-hexane—tetrahydrofuran (1:1, v/v), n-hexane—dioxane (1:1, v/v) and n-hexane—acetonitrile (1:1, v/v). Since detection after HPLC was carried out at 230 nm, the solvents giving a wavelength cut-off shorter than 220 nm were chosen as the developing solvents for TLC. Under UV light, the derivatives were observed as black quenched spots on a fluorescent background. The sharpest bands with significantly high resolution were obtained by development with n-hexane—tetrahydrofuran (1:1, v/v), giving the following R_F values: 0.07 for neomycin, 0.11 for kanamycin, 0.19 for streptomycin, and 0.21 for dihydrostreptomycin.

Analysis of benzoylated aminoglycoside antibiotics by HPLC

On the basis of the above results obtained by TLC, n-hexane—tetrahydrofuran (1:1, v/v) was selected as the best solvent for HPLC elution and the peaks were monitored at 230 nm, the maximum absorption of the benzoyl derivative. Fig. 3 shows HPLC chromatograms of benzoylated derivatives of neomycin, kanamycin, streptomycin and dihydrostreptomycin. Kanamycin, streptomycin and dihydrostreptomycin were eluted close to each other, whereas neomycin was clearly separated from the other antibiotics. Three peaks of neomycin having the retention times of 8.11, 9.28 and 10.08 min were thought to be
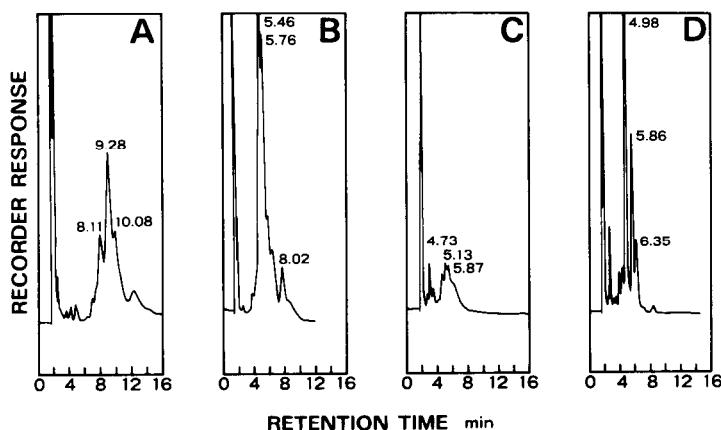
Fig. 3. HPLC chromatograms of various aminoglycoside antibiotics. The elution solvent was *n*-hexane—tetrahydrofuran (1:1, v/v), and the detection was carried out at 230 nm. A = neomycin, B = kanamycin, C = streptomycin, D = dihydrostreptomycin.

structural isomers of neomycin and the relative ratio of the peak areas was constant in different amounts of neomycin.

Quantitation of neomycin by HPLC

Judging from the absorption of benzoylated neomycin eluted from the HPLC column along with the molar extinction coefficient, the recovery of neomycin through derivatization and HPLC analysis was almost quantitative and reproducible (100.0 ± 1.5%). Accordingly, by reacting known amounts of neomycin and injecting the same volume into the HPLC column, a standard curve was made for the quantitation of neomycin. The curve was linear up to 100 μg of neomycin injected, and the lower limit of detection was about 10 ng of neomycin.

Determination of neomycin level in the perilymph

Table I shows the concentration of neomycin in the perilymph at various time intervals after the application of neomycin on the round window membrane. Usually, 6 μl of the perilymph could be collected and the samples were directly benzoylated after lyophilization. The derivatives were dissolved in

TABLE I

CONCENTRATION OF NEOMYCIN IN THE PERILYMPH OF GUINEA PIGS AS MEASURED BY HPLC

After topical application of neomycin on the round window membrane, perilymph samples were collected at the various time intervals indicated, and directly benzoylated as described in the text.

Time (min)	Concentration of neomycin (μg/ml)
15	140
60	350
120	750

15 μl of chloroform and the determination was performed by injecting 5 μl of each solution. The chromatograms thus obtained were essentially the same as that in Fig. 3A, indicating that any material after prewashing did not interfere with the elution of benzoylated neomycin. The results indicate that neomycin can pass through the round window membrane in a short time.

DISCUSSION

Aminoglycoside antibiotics in biological fluids have been determined by a variety of methods [12]. These include microbiological assay which has long been used for measuring antibiotic concentrations in body fluids and tissues, radioimmunoassay which is extremely sensitive, radioenzymatic assay, gas—liquid chromatography, HPLC and others. With the development of flow cells, well controlled surface-porosity column packings and high-pressure pumping systems, HPLC has become a rapid, precise and sensitive method for quantitating a variety of compounds including antimicrobial agents [13]. Determination of aminoglycoside antibiotics by HPLC has been reported by many investigators, for example by Tsuji et al. [10] for neomycin, by Mays et al. [9] for kanamycin, and by Maitra et al. [14] for gentamicin. HPLC has also been applied to the determination of antibiotics other than aminoglycosides [15]. In these methods, antibiotics have been converted to UV-absorbing or fluorescent derivatives by introducing chromophores into amino groups of the molecule.

Since aminoglycoside antibiotics contain multiple hydroxyl and amino groups, we have selected the reaction with benzoyl chloride in pyridine, which introduces the UV-absorbing benzoyl chromophores into hydroxyl and amino groups in a form of ester and acidamide linkages, respectively. As a result, eleven to thirteen chromophores could be introduced into neomycin, and this allowed us to determine a small amount of neomycin in the perilymph. Without any pretreatment of the perilymph, such as deproteinization, the derivatization of neomycin could be successfully performed, and any contamination could be removed in the washing steps of the derivatization procedure. Further protection of the column from contamination was achieved by placing a precolumn (5 cm $\times$ 4.6 mm I.D.) packed with reversed-phase packing (C_{18}; DuPont) between the injector and the analytical column. Direct derivatization is quite useful for samples only available in a small quantity, such as perilymph.

The results of the present study clearly showed the permeable property of the round window membrane to neomycin. The level of aminoglycoside antibiotics in the perilymph as a function of time after their systemic administration has already been investigated [6, 16], but analysis of neomycin in the perilymph after its topical application on the round window membrane has not yet been fully undertaken. The application of the procedure to further analysis of the dynamics of neomycin in the perilymph is now in progress in our laboratory. Furthermore, improvement in the accuracy and precision of the procedure is also being investigated by using an internal standard. Kanamycin is one of the candidates for internal standard as shown in Fig. 3.

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