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Note

Column liquid chromatographic determination of isepamicin in nasal cavity using gauze

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Isepamicin (ISP), a new semisynthetic aminoglycoside antibiotic obtained by modification of gentamicin-B, is widely used. It shows a broad spectrum of activity against both gram-positive and gram-negative bacteria, including strains resistant to other aminoglycoside antibiotics (AGs). The AGs have been often used for paranasal sinusitis symptoms [1–3], but their quantitations have been hampered by concerns of assay methodology. Monitoring of the drug distribution in the nasal cavity is necessary to estimate its effectiveness in paranasal sinusitis and post-operation treatments. It has been evaluated by measuring its surface concentration in nasal mucosa after aerosol therapy [4].

However, it is difficult to collect biological specimens from patients owing to the low concentration, the operating technique, the assay method and a reluctance to provide samples. In addition, the distribution of ISP is often greatly influenced by physiological and aerosol therapy conditions, such as inter- and intra-patient variations in specimen concentrations after a given dosage, the dosage concentration and the aerosol-generating instrument.

This paper describes the development of a simple, rapid and reproducible procedure for the extraction and measurement of ISP in whole blood instead of pituita. ISP in gauze was placed in the nasal cavity of patients and was analysed by high-performance liquid chromatography (HPLC) following de-

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rivatization with *o*-phthalaldehyde (OPA) and β-mercaptopropionic acid (β-MP) [5].

EXPERIMENTAL

Chemicals

Isepamicin sulphate and isepamicin injection (200 mg per 2 ml) were obtained from Toyo Jozo (Shizuoka, Japan) and Schering-Plough Kabushiki Kaisha (subsidiary of Schering-Plough U.S.A., Osaka, Japan), respectively. 1-Heptanesulphonic acid (PIC B-7®) was purchased from Nihon Waters (Osaka, Japan). OPA and β-MP were purchased from Nakarai Tesque (Kyoto, Japan). Antibiotic medium No. 5 was purchased from Difco (U.S.A.). Distilled water was used for the preparation of the mobile phase, derivatizing reagent and media. All other chemicals were of analytical-reagent grade.

Chromatographic system

The chromatographic system consisted of a 510 pump (Waters) for the mobile phase and a 420-AC fluorescence detector (Waters). The derivatizing reagent was delivered by a KHU-16 mini-micro pump (Kyowa Seimitsu, Tokyo, Japan) with a mixing tee. The reaction coil, made of PTFE (5 m$\times$0.25 mm I.D.) was placed between the column and the detector and maintained at ambient temperature (25 ± 2°C) to promote the reaction.

Separation was accomplished with a μBondapak C_{18} column (300 mm$\times$3.9 mm I.D., 5-μm particles, Waters) and a mobile phase of 0.16 *M* sodium sulphate–0.005 *M* 1-heptanesulphonic acid (PIC B-7). The derivatizing reagent was prepared by dissolving OPA (0.3 g) in 5 ml of methanol, followed by the addition of 1.25 ml of β-MP and 500 ml of 0.4 *M* potassium borate buffer (pH 10.4). The solution was filtered (HA 0.45-μm filter, Millipore) under reduced pressure.

The retention time of the ISP was adjusted to 12 min by controlling the flow-rate of the mobile phase (ca. 0.6 ml/min). The flow-rate of derivatizing reagent was 0.3 ml/min, the sample size was 50 μl, and the column effluent was monitored at λ_{ex} 360 nm (band-pass filter, Waters) and λ_{em} 440 nm (long-pass filter, Waters), respectively. Chromatograms were recorded using a Hitachi 833 integrated recorder, and quantitation was based on peak areas.

Microbiological assay

The microbiological assay was carried out by a standard agar-well diffusion technique using antibiotic medium No. 5 [6]. ISP standards were prepared in 0.1 *M* phosphate buffer (pH 8.0). The test organism was *Bacillus subtilis* (ATCC 6633). The seeded agar was incubated at 35°C, and the diameter of the zone of growth inhibition was measured 16–18 h later. Zone size was plotted on semilogarithmic paper vs. known concentration of ISP.

Preparation of standard samples

For calibration, an aqueous solution containing ISP was added to gauze (10 mm × 10 mm) to provide concentrations in the range 0.1–2500 μg per gauze.

For evaluation of subject studies, 100 μl of whole blood (anticoagulant: EDTA 3K) and aliquots of ISP stock solution to provide concentrations at 25 μg per gauze were spotted on to a gauze (10 mm × 10 mm). The gauzes were allowed to dry at ambient temperature and stored in an air-tight plastic sampling tube before use.

Subjects studied

Twenty patients presenting to the Osaka Municipal Hospital took part in the study. The gauze (10 mm × 10 mm) was placed in five points of patient's nasal cavity (both right and left side of nasal vestibule, middle nasal meatus and pharynx) before and after paranasal sinusitis operation, then 25 mg/ml ISP saline solution (0.25 ml of ISP injection plus 0.75 ml of saline) was administered by jet-nebulizer for 3 min. Each sample was stored in an air-tight plastic sampling tube at −20 °C until measured.

Elution of ISP from the gauze

The gauzes containing ISP were dipped in air-tight plastic sampling tubes containing 500 μl of 0.5 *M* Na_2HPO_4 solution [7,8] and placed for various times (15, 30, 45 and 60 min) in a water-bath (35 °C) or kept at ambient temperature. An appropriate portion of this solution was injected into the chromatographic column. For the bioassay, 100 μl of the eluate were dispensed into an air-tight plastic sampling tube followed by the addition of 900 μl of 0.1 *M* phosphate buffer (pH 8.0).

RESULTS AND DISCUSSION

Extraction procedure

The optimal temperature and time conditions for the extraction of ISP from the gauze were investigated: the extraction was judged to be complete by 15 min at 35 °C, and by 60 min at ambient temperature. We selected an extraction time of 30 min at 35 °C to keep the time for the total assay within acceptable limits.

Limit of quantitation and recovery

The detection limit was 0.1 μg/ml under the present conditions. The recovery and the precision for ISP in the range 0.1–2500 μg per gauze were quite acceptable. The recovery was more than 90%, and the intra-assay coefficient of variation (C.V.) was less than 3.8%. The analytical recovery of ISP added to gauze was calculated by comparing the peak area from blood samples con-

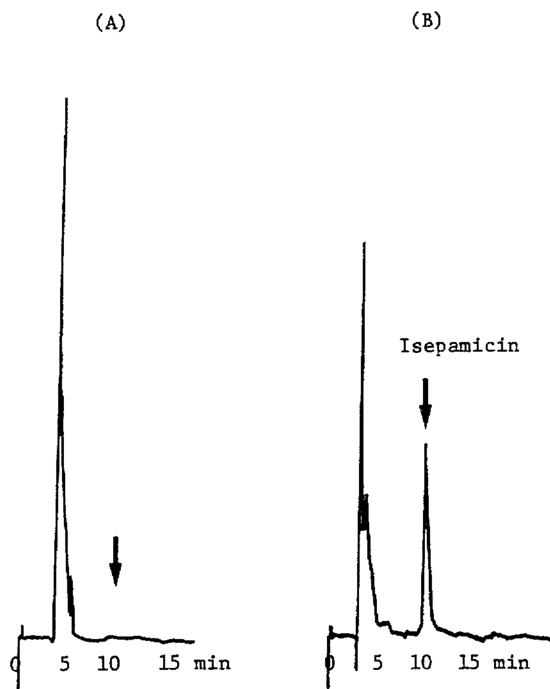

Fig. 1. Chromatograms obtained from (A) a gauze spiked with 100 μl of whole blood and (B) a gauze placed in the nasal cavity of a patient. Conditions as described in Experimental.

taining 50 μg/ml (25 μg per gauze) ISP with that from the same concentration of aqueous solution. The mean recovery of ISP was 93.5% (C.V. 4.0%, $n=5$).

Fig. 1 shows chromatograms obtained from gauze spiked with 100 μl of whole blood and from the patient. The ISP peak is well resolved, and apparently free from interferences in blood.

Correlation with HPLC assay and bioassay

A total of 25 samples from six patients receiving ISP as an aerosol were assayed by the present HPLC assay and by bioassay. The correlation between the two assays is shown in Fig. 2; the equation of the line is $y=1.05x-4.15$ ($r=0.997$).

Clinical results

The distribution data of ISP for twenty patients who underwent surgical operation are shown in Fig. 3. Although many factors are known to influence the distribution of AGs, their relative effects on morphology and on the nebulizing method are difficult to quantify. Our data indicate that a simple

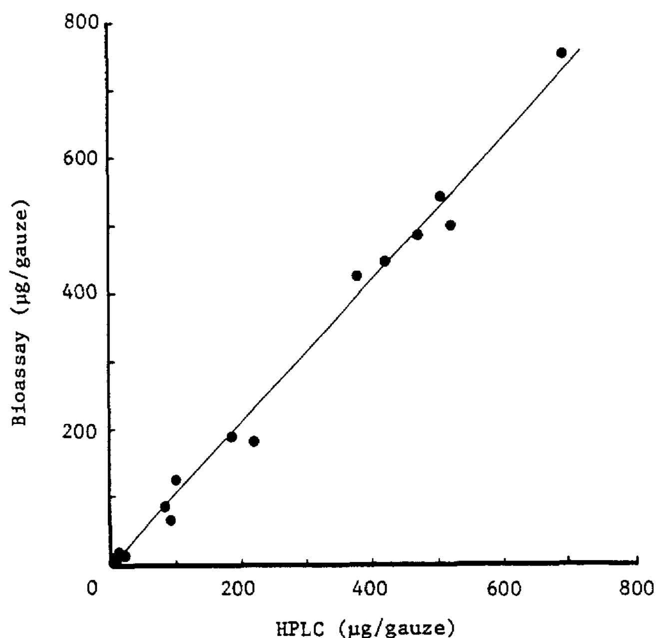

Fig. 2. Correlation between bioassay and HPLC assay values of isepamicin concentrations in gauzes placed in patients' nasal cavities. $y=1.05x-4.15$ ($r=0.997$).

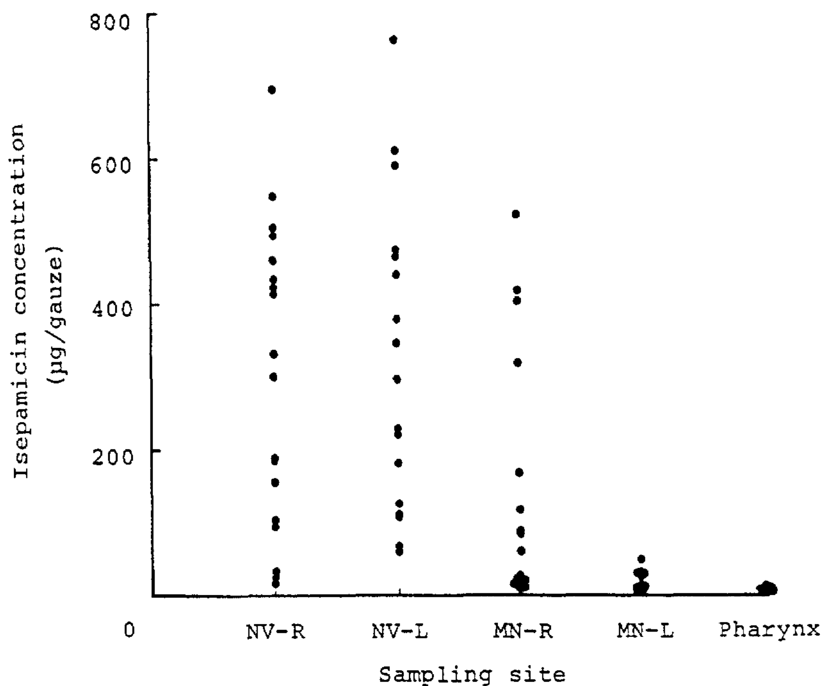

Fig. 3. Distribution of isepamicin at five sites in the nasal cavity: NV-R, right side of nasal vestibule; NV-L, left side of nasal vestibule; NM-R, right side of nasal meatus; MN-L, left side of nasal meatus; pharynx.

method, using gauze, appears to be acceptable for adjustment of AG dosage in a clinical setting.

Our results confirmed that ISP can be determined even when the gauze is contaminated with whole blood or other biological fluid.

As the low-molecular-mass primary amines in biological fluid interfered with the determination of ISP by HPLC with OPA and β-MP, deproteinization procedures, such as organic solvent extraction [9], ultrafiltration [7] or ion-exchange with CM-Sephadex (C_{25}) [10] were required to remove these amines as well as proteins. Because the present method requires only the extraction of ISP, it avoids any trouble and errors in sample preparation. Hence this method is suitable for the determination of the nasal cavity distribution of ISP in investigating the efficacy of aerosol therapy.

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