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High-Performance Liquid Chromatographic Determination of the Stability of Sisomicin in Hydrophilic Petrolatum Ointment

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For the purpose of investigating of its suitability for topical application, the stability of sisomicin incorporated in hydrophilic petrolatum was investigated under various storage conditions by high-performance liquid chromatography. Sisomicin was extracted from the oleaginous vehicle, which was kept in a liquid state by adding liquid paraffin, using pH 6.5 phosphate buffer. An appropriate retention behavior of the antibiotic is obtained on a μBondapak-C_{18} column with a mobile phase of 2.0% (w/v) sodium sulfate–0.005 M sodium 1-pentanesulfate (detection at 214 nm). The degradation of sisomicin follows first-order kinetics with regard to the remaining antibiotic in aqueous solution: the apparent first-order rate constants are $2.63 \times 10^{-3}\,d^{-1}$ at 5 °C and $1.70 \times 10^{-2}\,d^{-1}$ at 25 °C. When incorporated in hydrophilic petrolatum, the antibiotic is 5 to 7 times more stable than in an aqueous medium. This is due to the reduction of its surface oxidation. Accordingly, the ointment is therapeutically effective for at least 3 months when preserved under shielding from light at 5 °C.

Keywords——sisomicin; hydrophilic petrolatum; sisomicin extraction; oleaginous phase; HPLC; stability sisomicin ointment; sisomicin stability

Sisomicin, produced by *Micromonospora inyoensis* is an aminoglycoside antibiotic having a broad antibacterial activity against both gram-positive and gram-negative bacteria such as *Staphylococcus aureus* and *Pseudomonas aeruginosa*.[1] The gross structure of sisomicin has been established; it contains the branched chain amino–sugar garosamine, 2-deoxystreptamine and an unsaturated amino–sugar moiety, sisosamine.[2]

Wagman *et al.* reported that sisomicin was stable in citric acid/phosphate buffers in the range of pH 2—8 and in borate buffers up to pH 10 at 100 °C (boiling water bath) for at least 30 min.[3] However, it was also stated that the color produced during the early stages of heating was yellow as opposed to light gray in the case of gentamicin C_{1a}. Although the mechanism of sisomicin degradation is unknown, 2-deoxystreptamine and garamine have been detected as hydrolyzed products under strongly acidic conditions.[2a,3]

No change was observed for the sisomicin injection under a nitrogen atmosphere in a sealed transparent ampoule, but the aqueous solution turned orange brown and finally dark when exposed to light and surface oxidation for longer period. Therefore, as an essential preliminary to the preparation of sisomicin ointment, intended to be used impetigo, pyoderma and burn patients, it was necessary to investigate the stability in such an environment.

Antibiotics have been generally assayed by the microbiological method, which usually requires technical skill in handling bacterial organisms and time-consuming operations. In the present study, threfore, we followed the stability of sisomicin by high-performance liquid chromatography (HPLC) after extraction of the antibiotic from the oleaginous vehicle. The sisomicin ointment was stored under various conditions for up to 3 months.

Experimental

Materials——Sisomicin sulfate and sisomicin injection (SISEPTIN, 75 mg as potency/ampoule) were supplied by Essex Japan (Osaka) and Yamanouchi Pharmaceutical Co. (Tokyo), respectively. Hydrophilic petrolatum, J.P, was used (Maruishi Pharmaceutical Co., Osaka). DL-α-Tocopherol, sodium sulfate (anhyd.) and liquid paraffin were obtained from Wako Pure Chemical Ind. (Osaka). Sodium 1-pentanesulfonate (LOW-UV PIC B5) was obtained from Waters Assoc. (Milford, Mass). HPLC quality distilled water was used (Wako Pure Chemical Ind., Osaka).

Preparation of Ointments——Ointments were prepared by the incorporation method: hydrophilic petrolatum (100 g) was first taken into a mortar, to which the sisomicin injection (300 mg, as potency in 6 ml) was gradually added and mixed with a pestle. During this process, the tocopherol alcohol solution was added so as to give a final concentration of 0.05% (w/w), when necessary. The resulting ointment (0.3% (w/w) sisomicin) was packaged in porcelain–white plastic jars (3 cm in diameter and 2 cm in depth).

Storage Conditions of Ointments——The sisomicin ointment packaged in jars with ordinary caps was maintained under the following conditions: (1) shielded from light at 5 °C, (2) shielded from light at room temperature (*ca.* 25 °C), (3) exposed to light at room temperature, *i.e.* placed in front of a north-facing window where the illumination was approximately 2000 lux in the daytime, (4) exposed to light as above at 40 °C.

Procedure for Assay——A 0.5 g portion of the sample was weighed accurately and transferred into a 50 ml centrifugal tube. Approximately 2 ml of liquid paraffin was added and the tube was mildly heated to melt the contents. Next, 40 ml of pH 6.5, 0.1 M phosphate buffer warmed at 70 °C was added, and the content was subjected to vigorous mixing on a vortex mixer for 5 min. The extraction of the agent was afterward continued for 12 h with constant shaking and with shielding from light.

The resulting emulsion was centrifuged at 3000 rpm for 5 min, the upper emulsified phase was aspirated out, the aqueous phase was passed through a Sep-Pak C_{18} cartridge (Waters Assoc.), and a 50 μl of the sample was injected into a port of the chromatograph system.

Chromatographic Conditions——An HPLC apparatus (Model 520, Gasukuro Kogyo, Tokyo) equipped with ultraviolet (UV) detector (UVIDEC 100-II, JASCO, Tokyo) and a reversed phase column (μBondapak-C_{18}, 10 μm, 30 cm × 3.9 mm i.d., Waters Assoc.) was used. The mobile phase was a mixture of 2.0% (w/v) sodium sulfate (anhyd.) and 0.005 M sodium 1-pentanesulfonate. The flow rate was maintained at 1.0 ml/min with an operating pressure of 120 kg/cm^2. The column eluate was continuously monitored at 214 nm with a sensitivity of 0.04 a.u.f.s. and a chart speed of 5 mm/min. Peak areas were obtained with a Shimadzu C-RIA CHROMATOPAC data analyzer (Kyoto). All analyses were performed at room temperature.

Thin-Layer Chromatography (TLC)——The TLC separation of decomposed products of sisomicin injection was carried out by spotting the equivalent of 50 μg of sisomicin on a silica gel plate (Tokyo Kasei Kogyo Co., Tokyo) and developing with the system chloroform–butanol–ethanol–ammonia water (2 : 4 : 5 : 5). Ninhydrin spraying revealed five spots, including sisomicin; the *Rf* values were 0.58 (unknown product, trace), 0.4 (sisomicin), 0.28 (unknown, main product), 0.14 (unknown, trace) and 0 (brown color left at the orgin). Two additional spots which were detected by fluorescence on illumination at 253.6 nm were methylparaben (*Rf* = 0.63) and propylparaben (0.70).

Calibrations——The concentration of unchanged sisomicin was determined by referring to a regression line that was constructed using known concentrations of the agent ranging from 12.5 μg/ml to 75 μg/ml. The buffer solution used here was fully exposed to the ointment base at 70 °C prior to use. The relationship between the concentration (ordinate) and the peak area (abscissa) was linear, giving intercept 1.35, slope 0.011 and correlation coefficient 0.998. The amount of the unchanged agent in the ointment was calculated as follows: A (μg/g of ointment) = $(0.011X + 1.35) \times 40/w$ (weight of ointment sampled).

Stability Studies——The remaining amount of sisomicin in the ointment was determined by HPLC, after storage of the ointment for 30, 60 and 90 d under various conditions as mentioned above. Samples were taken from both the surface (1—1.5 mm in depth) and the interior of the packed ointment.

The sisomicin injection and the bulk solution (pH 6.5, 0.1 M phosphate buffer) were also examined in the same manner. The solutions were stored in contact with air at 5 °C (shielded from light) and 25 °C (exposed to light). The initial drug concentration was 1.12×10^{-1} M, which was equivalent to that of the sisomicin injection.

Results and Discussion

HPLC Determination of Sisomicin

The HPLC method has been presented as an alternative to microbiological methods for the determination of antibacterial agents, since the HPLC procedures involved are generally rapid and assure specific separations of samples with high precision.[4] The method, however, has not been applied for the determination of sisomicin.

Appropriate HPLC conditions were developed using a reversed–phase column. Although

it was reported that sisomicin exhibited no absorption peaks in the UV range (200—400 nm),[3] a peak was found at 204 nm (log $\varepsilon = 4.06$ in pH 6.5 phosphate buffer). This may be because sisomicin is a dehydro derivative of gentamicin C_{1a} (or an isomer thereof) and contains a vinylic ether group in the molecule. In any case, this made the use of a UV detector possible after extraction of the antibiotics from the vehicle.

To find a suitable mobile phase, phosphate buffer, acetate buffer and sodium 1-pentanesulfonate were examined: sisomicin was not retained at all with the phosphate and acetate buffers and was eluted at V_0. Sodium 1-pentanesulfonate provided retention with a slight tailing of the peak, which was improved by adding sodium sulfate. Finally, the most suitable phase was found to be 2.0% (w/v) sodium sulfate and 0.005 M sodium 1-pentanesulfonate. The UV detector was operated at 214 nm since this gave a better calibration curve with little deviation from the origin.

To examine whether unidentified products interfere with the HPLC determination of sisomicin, TLC studies were also carried out on sisomicin solution which had turned orange-brown. Five spots were detected on the ninhydrin-sprayed chromatogram. The materials which were not equivalent to sisomicin were recovered by extracting the silica gel and injected into the HPLC system.

Figure 1 shows the chromatograms of sisomicin extracted from the ointment, the blank extraction solution and the products detected on the TLC plate.

The retention time of sisomicin was 4.8 min, while no peaks of products were observed at all. The products seem to have no UV absorption, rather than having longer retention times. Accordingly, the stability of sisomicin could be followed by determination of the remaining drug.

Extraction of Sisomicin from Ointments

The extraction of sisomicin, which is very soluble in water, from hydrophilic petrolatum was performed using pH 6.5 phosphate buffer. The advantage of the addition of liquid paraffin is that it keeps the oleaginous phase liquid during the extraction at room temperature.

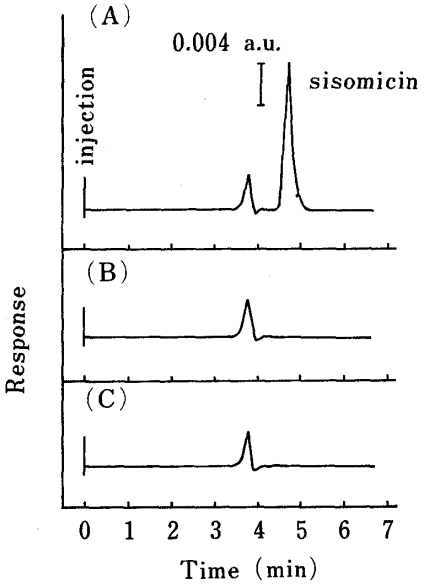

Fig. 1. Chromatogram of Sisomicin Extracted from Hydrophilic Petrolatum

(A) sisomicin (50 μg/ml of injected sample), (B) degradation products from sisomicin and (C) control (extraction fluid, phosphate buffer). a.u.f.s. = 0.04 at 214 nm.

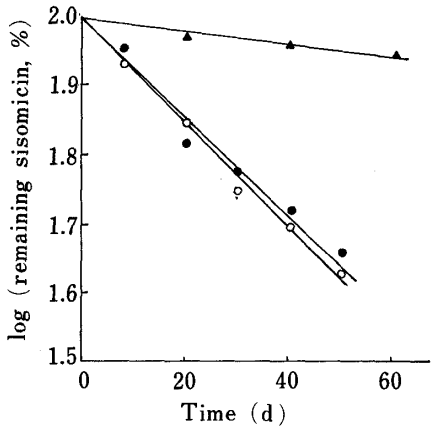

Fig. 2. First-Order Kinetics of Sisomicin Degradation in Aqueous Solutions

Open circles indicate sisomicin bulk solution (pH 6.5 phosphate buffer) and closed symbols show sisomicin injection (intact) at 5 (▲) and 25°C (●). Initial concentration: 1.12×10^{-1} M.

TABLE I. Recovery of Sisomicin from Sisomicin Ointment

Sisomicin content (%)	Recovery (%)[a] mean ± S.D.
0.1	100.0 ± 2.1
0.2	97.8 ± 1.7
0.4	98.3 ± 1.4
0.8	99.4 ± 2.3

a) Each value is the mean of five determinations.

TABLE II. Stability of Sisomicin Incorporated in Hydrophilic Petrolatum

Storage conditions			Remaining sisomicin (%, mean ± S.D.)[a]					
			30 d		60 d		90 d	
Temp. (°C)	AO[b]	ILM[c]	Sur[d]	Int[e]	Sur	Int	Sur	Int
5	+	−	97.2 ± 2.1	101.1 ± 1.5	96.4 ± 2.6	98.5 ± 1.9	93.1 ± 1.7	98.9 ± 2.0
5	−	−	100.2 ± 3.3	100.5 ± 1.9	96.2 ± 1.4	99.4 ± 1.1	94.2 ± 1.4	97.8 ± 2.2
Rm[f]	+	+	87.5 ± 1.4	95.8 ± 2.0	80.4 ± 3.1	85.2 ± 2.6	71.5 ± 1.8	82.4 ± 1.1
Rm	−	+	88.2 ± 1.5	97.1 ± 1.3	81.4 ± 2.9	89.6 ± 1.7	73.6 ± 1.3	77.4 ± 1.5
Rm	+	−	92.4 ± 2.2	97.4 ± 1.6	86.3 ± 3.1	90.1 ± 1.1	78.2 ± 2.6	82.9 ± 2.1
Rm	−	−	93.5 ± 1.6	96.2 ± 3.0	88.1 ± 1.5	91.2 ± 1.8	77.4 ± 1.4	85.3 ± 1.2
40	+	+	63.2 ± 1.9	70.5 ± 1.8	55.1 ± 1.5	60.4 ± 3.1	49.3 ± 2.0	54.1 ± 2.3
40	−	+	60.5 ± 1.2	65.5 ± 2.4	54.4 ± 1.8	58.8 ± 1.5	53.5 ± 1.3	57.8 ± 1.6

a) Each value is the mean of six determinations. b) Antioxidant (α-tocopherol).
c) Exposed to light. d) Ointment surface. e) Ointment interior. f) Room temperature.

The initial temperature of the aqueous phase added seemed to be important for efficient extraction. When it was 70 °C, the recovery was more than 97%, but it was only about 80% at room temperatures. The effect of such a relatively high temperature on the stability of the antibacterial agent was negligible during the extraction process because it was limited to an initial short period. The extraction solution was then passed through a Sep-Pak C_{18} cartridge to remove interfering peaks of other components in hydrophilic petrolatum which might have been transferred jointly to the aqueous phase.

Table I shows the recovery of sisomicin from the ointment phase; quantitative extraction (>97%) was generally obtained at various contents of the drug incorporated.

Stability of Sisomicin in Aqueous Solution

Firstly, the drug stability of sisomicin bulk solution and a commercially available injection were examined for comparison with that in hydrophilic petrolatum under conditions (1) and (3) where the temperature was fixed at 25 °C. The transparent solution became light yellow in the earlier stages and finally dark brown, but there was no response to visible spectrophotometric scanning. The color product, therefore, has an extremely low molar extinction coefficient and seems to be a very polar substance, since it was not developed at all on the TLC plate.

Figure 2 shows that the degradation process followed first-order kinetics with regard to the remaining sisomicin, and little difference between the bulk solution and the injection was found at 25 °C. The apparent first-order rate constants were $2.63 \times 10^{-3} d^{-1}$ and approximately $1.7 \times 10^{-2} d^{-1}$ under conditions (1) and (3), respectively.

Stability of Sisomicin in Hydrophilic Petrolatum

The stability of sisomicin incorporated in hydrophilic petrolatum was investigated under various storage conditions for up to 90 d: the effects of temperature, antioxidant (α-tocopherol) and exposure to light are included. Ointment samples were collected from both the surface and interior, and the former must be more exposed to the atmosphere. The results are shown in Table II. α-Tocopherol had hardly any effect under all the storage conditions. This is perhaps due to the facts that the sisomicin injection incorporated into the ointment contains an antioxidant (sodium pyrosulfate, 0.3% (w/v)), and α-tocopherol is water-insoluble and exists in the oleaginous phase of the ointment.

At 5 °C (condition (1)), the antibiotic remained essentially intact for at least 90 d in the ointment interior but about 5% degradation was observed at the surface portion.

At room temperature (conditions (2) and (3)), there was a slight difference between the exposed and nonexposed (to light) surface samples, while little difference was observed for the interior. The magnitude of the degradation generally ranged from 15 to 30% in 90 d. The apparent first-order rate constants approximated from three points were $2.4 \times 10^{-3} d^{-1}$ for the nonexposed surface sample and $3.7 \times 10^{-3} d^{-1}$ for the exposed surface sample, and about $2.2 \times 10^{-3} d^{-1}$ for the interior portions. The magnitude of the surface degradation, however, should depend on the superficial thickness taken as a sample. There is a possibility of obtaining rather conservative values of the rate constant since the top portion of the ointment was sampled to a depth of 1—1.5 mm, where air penetration was probably limited. This may also be the case under condition (1).

Compared with the decomposition rate in aqueous solution, the incorporated sisomicin was about 5 to 7 times more stable, presumably due to the diminished opportunity for surface oxidation.

Under condition (4), which may be rarely found in practice, the magnitude of the degradation reached about 40% for the surface sample and 30% for the interior in the first 30 d, followed by a slower decomposition. The physical appearance of the ointment deteriorated with time as a result of phase separation and moisture evaporation. As such changes progressed, the dispersed aqueous phase containing sisomicin seems to be condensed to a viscous phase, resulting in a slower decomposition rate.

Sisomicin is much more susceptible to surface oxidation than to light, and the storage temperature could be the most important factor affecting the stability of the antibiotic when prepared as a topical dosage form. Accordingly, the therapeutically effective content in the ointment is well maintained for at least 3 months when the ointment is stored at 5 °C, and for about one month at room temperature with the proviso that the extreme superficial portion may be less effective.

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