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PRE-COLUMN DERIVATIZATION OF SISOMICIN WITH *o*-PHTHALALDEHYDE–*β*-MERCAPTOPROPIONIC ACID AND ITS APPLICATION TO SENSITIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION WITH FLUORIMETRIC DETECTION

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SUMMARY

The stability of the *o*-phthalaldehyde (OPA) derivatives of sisomicin obtained using *β*-mercaptopropionic acid was investigated by reversed-phase high-performance liquid chromatography. One of the fluorescent derivatives of sisomicin was stable at least for 6 h in 50% methanol under the optimal conditions used (OPA concentration, pH and temperature). When plasma samples spiked with sisomicin were analysed, the response was linear in the calibration range 136–900 pg of sisomicin per injected volume (40 *μ*l). As little as 0.06 *μ*g of sisomicin per 1 ml of plasma could be detected with signal-to-noise ratio ≥ 2. For plasma samples spiked with 0.2 *μ*g/ml sisomicin, the recovery was $97.1 \pm 6.6\%$ (mean $\pm$ S.D., $n = 5$) with a within-run coefficient of variation of 6.8% and a day-to-day coefficient of variation of 7.2%. The method was also applied to plasma samples from rabbit after a subcutaneous injection of 1 mg/kg sisomicin.

INTRODUCTION

Aminoglycoside antibiotics (Ags) are widely used against aerobic Gram-negative bacillary infections, but have the potential for nephrotoxicity and ototoxicity if not carefully monitored. To assure therapeutic serum concentrations and

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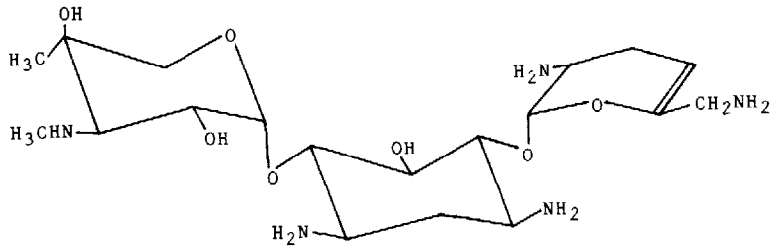

Fig. 1. Structure of sisomicin.

to minimize these toxicities, frequent and careful monitoring of serum Ags levels is essential for a considerable time after administration.

Various high-performance liquid chromatographic (HPLC) procedures have been reported for the determination of Ags as the most sensitive approach, although most require either pre- or post-column derivatization [1]. Although post-column derivatization is adequate, it requires special reaction chambers as part of the chromatographic system and so is dedicated to one type of analysis. Pre-column derivatization offers greater efficiency and higher sensitivity than the post-column technique, but little has been published. In many investigations using pre-column derivatization the Ags have been labelled with 1-fluoro-2,4-dinitrobenzene (FDNB) [2–4] or related nitrophenylation reagents [5–7] for ultraviolet (UV) detection. The drawback of using FDNB or related reagents is their toxicity and difficulties in establishing the optimal derivatization conditions, although HPLC with UV detection is well suited for routine analysis in clinical laboratories for therapeutic drug monitoring.

The derivatization reaction of Ags with *o*-phthalaldehyde (OPA)–*β*-mercaptoethanol (*β*-ME), which yields intensely fluorescent 1,2-disubstituted isoindole derivatives [8], is more sensitive, but it is impossible to use it for pre-column derivatization because of the instability of the derivatives [9], so it is most frequently used for post-column fluorescent derivatization [10–12]. Recently, it was suggested that increasing the steric bulk of the thiol side-chain should enhance the stability of the OPA derivatives of various primary amines, and Stobaugh et al. [13] recommended the use of 3-mercapto-1-propanol in the derivatization of amino acids, but this thiol cannot be obtained commercially. Kucera and Umagat [14] found that the OPA–*β*-mercaptopropionic acid (*β*-MP) adduct of alanine gave good stability and fluorescence response. More recently, this reagent system has been developed for the sensitive HPLC of individual amino acids in biological fluids [15].

Here we describe an investigation of the stability of the derivatives of sisomicin (Fig. 1), a dehydro analogue of gentamicin C_{1a}, by reversed-phase HPLC (RP-HPLC) using OPA–*β*-MP and its application as a pre-column derivatization reagent for the micro-determination of sisomicin in plasma.

EXPERIMENTAL

Chemicals

All chemicals were of analytical-reagent grade. Distilled water was filtered through a Milli-Q II water purification system (Nippon Millipore, Tokyo, Ja-

pan). Methanol of HPLC grade, OPA and β-MP were obtained from Nakarai Chemicals (Kyoto, Japan). Sisomicin sulphate in the injectable form (labelled potency 75 mg per 1.5 ml) was obtained from Yamanouchi Pharmaceutical (Tokyo, Japan). A 50 mmol/l buffer of pH 7.0–9.0 was prepared using sodium borate and potassium dihydrogenphosphate (KH_2PO_4) and a buffer of pH 10.0–12.0 using sodium borate and sodium hydroxide.

High-performance liquid chromatography

Reaction mixtures were monitored using a Model ERC-8710 liquid chromatograph pump (Erma Optical Works, Tokyo, Japan) equipped with a Model RF-500LC fluorescence spectromonitor (Shimadzu, Kyoto, Japan) and a 200 mm × 4 mm I.D. column packed with 5-μm Nucleosil C_{18} (Macherey & Nagel, Düren, F.R.G.). The reaction mixture was injected into a Model 7105 valve (Rheodyne, Berkeley, CA, U.S.A.). The mobile phase was a solution of 600 ml of methanol in 400 ml of K_3EDTA (2 g/l). The flow-rate was maintained at 0.6 ml/min at ambient temperature and the column effluent was monitored at λ_{ex} 340 nm and λ_{em} 450 nm.

Sample preparation for kinetic study

The degradation of sisomicin derivatives was monitored by direct injection of sample aliquots of 20 μl into the HPLC system at regular time intervals. A working solution was prepared from 800 μl of sisomicin solution (50 μl/l), 500 μl of methanolic OPA solution of various concentrations, 100 μl of methanolic β-MP solution (0.1 mol/l) and a definite volume of organic solvent. The resulting mixture was diluted to 10 ml with 50 mmol/l buffer and allowed to react at 20, 30 or 60 °C.

Plasma sample preparation for analysis

Sisomicin in plasma was purified according to Anhalt's method [16]. A column was prepared from CM-Sephadex (C_{25}) (Pharmacia, Uppsala, Sweden) with a bed volume of 1.0 ml. The column was washed with 2 ml of 0.2 mol/l sodium sulphate solution (rinse buffer). A 200-μl volume of plasma was applied to the column, followed by 1 ml and 4 ml of the rinse buffer, and then 600 μl of an alkaline buffer containing 10 mmol/l sodium hydroxide in 0.2 mol/l sodium sulphate solution (elution buffer). After the column had drained completely, 800 μl of the elution buffer were added and the eluate was collected. Standard plasma samples supplemented with various concentrations were prepared from 800 μl of the eluate, 500 μl of methanolic OPA solution (2 mg/ml), 100 μl of methanolic β-MP solution (0.1 mol/l), 1.7 ml of 50 mmol/l KH_2PO_4–borate buffer (pH 9.0) and 1.9 ml of methanol. The resulting mixture was allowed to react at 20 °C for 1 h and 40 μl of the final solution were injected on to the HPLC column. The resulting mixture was also placed in a water-bath at 30 °C for 40 min, then 1 ml of the mixture was transferred into a centrifuge tube and 5 ml of dichloromethane were added. After shaking for 5 min and centrifugation at 500 *g* for 5 min, 40 μl of the supernatant were injected into the HPLC system.

Rabbit study

A healthy rabbit received 1 mg of sisomicin per kg body weight by intramuscular injection. Blood samples were collected after 15 min, 1, 2, 5 and 10 h by venepuncture and plasma was separated from the collected blood by centrifugation (1100 *g*, 10 min) and stored at −20 °C prior to analysis. The concentration of sisomicin in the plasma as a function of time was determined by the above method after dilution with 50 mmol/l KH_2PO_4–borate buffer.

RESULTS AND DISCUSSION

Derivatization of sisomicin with OPA-β-MP

The three substances giving rise to peaks A, B and C (Fig. 2) were separated for the derivatization of sisomicin with OPA–β-MP. The retention times were 10, 16 and 23 min with the reversed-phase system used (Fig. 2), but their structures could be elucidated with certainty in this study. A blank plasma extract showed no peaks.

The optimal conditions for fluorescent derivatization and the stability of the

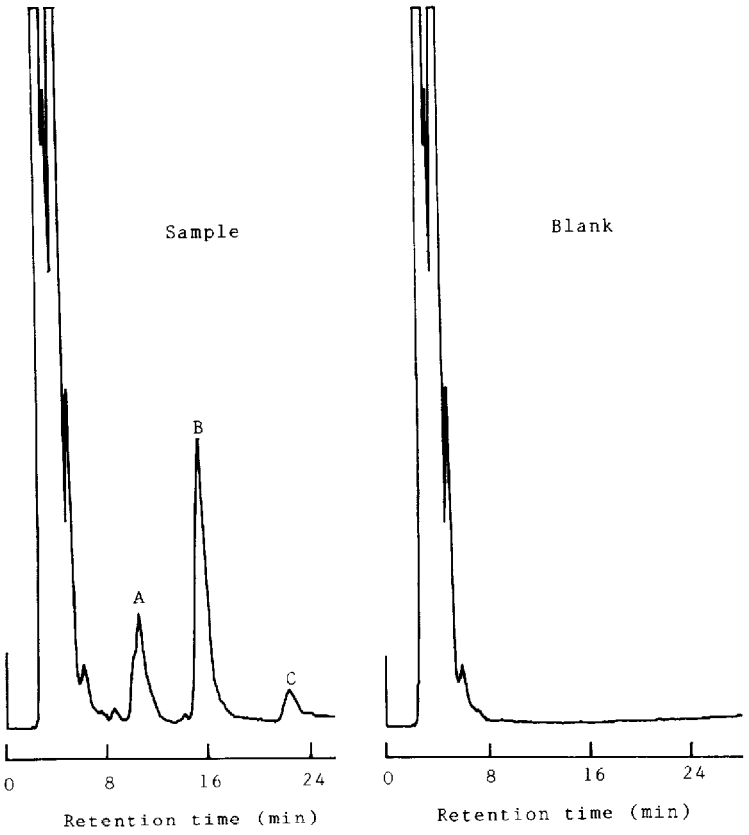

Fig. 2. HPLC of the OPA-β-MP derivatives of a standard solution of sisomicin (50 μg/l) and a blank solution. Peaks A, B and C are the main products of the derivatization of sisomicin. Separation conditions as described under Experimental.

products were determined using different OPA concentration, pH, temperature and solvent composition. The results were assessed from one peak height in the chromatogram (peak B in Fig. 2) with a constant time interval after the derivatization reaction.

Solvent composition is one of the most important factors contributing to the stability of the primary amine derivatives using OPA–thiol systems. The various amino acid derivatives with OPA–β-ME, as reported previously by Simons and Johnson [8], were generally least stable in aqueous solution, and also gave a constant relative fluorescence for at least 1 h after formation if the solvent was primarily methanol or 95% ethanol. In this study, methanol, ethanol and propanol were used and we investigated their stabilizing effects on the sisomicin derivative with OPA–β-MP. Fig. 3 shows that the fluorescent response (peak height) was most stable with 50% methanol under the conditions used, although the maximal response was slightly slower as the methanol content increased. With 50% ethanol the derivative gave a more intense fluorescence than in methanol, but after a few hours unknown fluorescent products from OPA–β-MP were formed, which had the same retention time as one of the sisomicin derivatives. With 50% propanol analogous products were obtained from the reagents.

It is reported that the optimal pH range for reaction of primary amines with OPA–thiol is 8.5–10.5 [17,18]. Fig. 4 illustrates the time dependence of fluorescence intensity on pH in the range 7.0–12.0. The increase in fluorescence intensity above pH 10.0 may be due to the formation and co-elution of the fluorescent product from OPA–β-MP as described above. Cohn and Lyle [19] previously reported that under alkaline conditions the sulphydryl group of reduced glutathione (GSH) might be expected to form a hemimercaptal with OPA and that the fluorescence intensity was maximal at a pH approximately equal to the pK_a of the sulphydryl in GSH. The unknown fluorescent product in this study may be formed by an analogous mechanism to that for OPA and GSH, considering that the pK_a of the sulphydryl in β-MP is 10.24 [20].

As it has been reported that an excess of OPA significantly increases the destabilizing effect for the isoindole of amino acids [13,21,22], we varied the concentration of OPA in this reaction mixture, the concentrations of sisomicin and β-MP being maintained at 50 μg/l and 1 mmol/l, respectively. Excess β-MP did not affect the stability of the derivatives, as reported for the OPA derivatization with other thiols [13]. The result is shown in Fig. 5. The optimal concentration of OPA was 0.1 g/l.

The reaction times to give a constant fluorescence intensity were 60, 40 and 10 min at 20, 30 and 60°C, respectively, under the conditions used. At 30 and 60°C, however, intensely fluorescent products were also formed from the OPA–β-MP reagents and interfered in the determination of sisomicin by the present HPLC method. We found that these products could be extracted by use of dichloromethane, ethyl acetate or chloroform as the extraction solvent. For example, when 5 ml of dichloromethane was added to 1 ml of the reaction solution followed by shaking for 5 min, more than 90% of the fluorescent products were removed (Fig. 6), and the sample solution gave a constant fluorescence intensity for more than 4 h at 30°C and 1 h at 60°C.

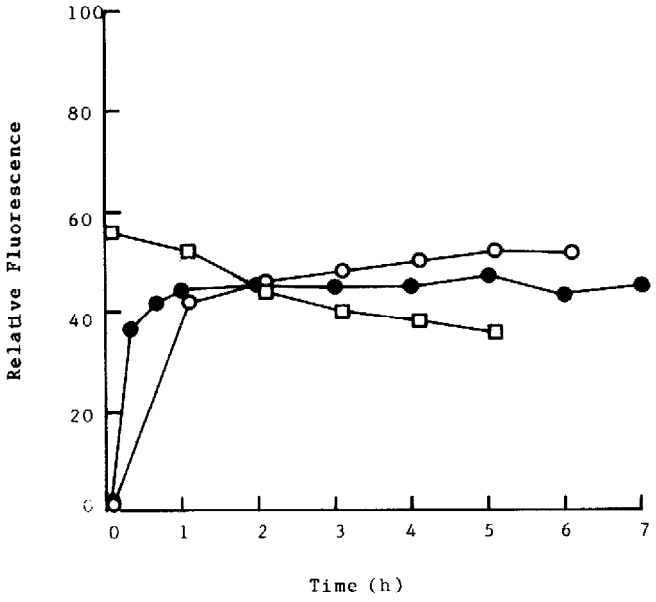

Fig. 3. Effect of methanol concentration on the stability of sisomicin derivatives with OPA-β-MP. Methanol: (□) 5%; (○) 50%; (●) 60%. Concentration of OPA, 0.1 g/l; temperature, 20°C; 50 mmol/l KH_2PO_4-borate buffer, pH 9.0. Other conditions as described under Experimental.

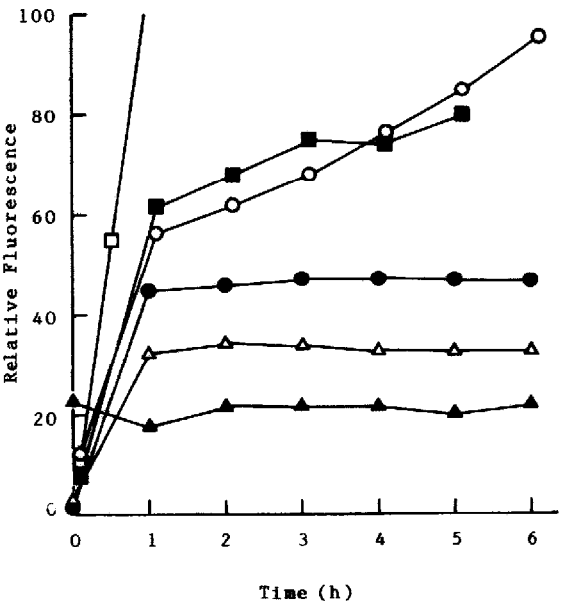

Fig. 4. Effect of the reaction pH on the stability of sisomicin derivatives with OPA-β-MP. 50 mmol/l buffer, pH: (▲) 7.0; (△) 8.0; (●) 9.0; (○) 10.0; (■) 11.0; (□) 12.0. Temperature, 20°C; concentration of OPA, 0.1 g/l.

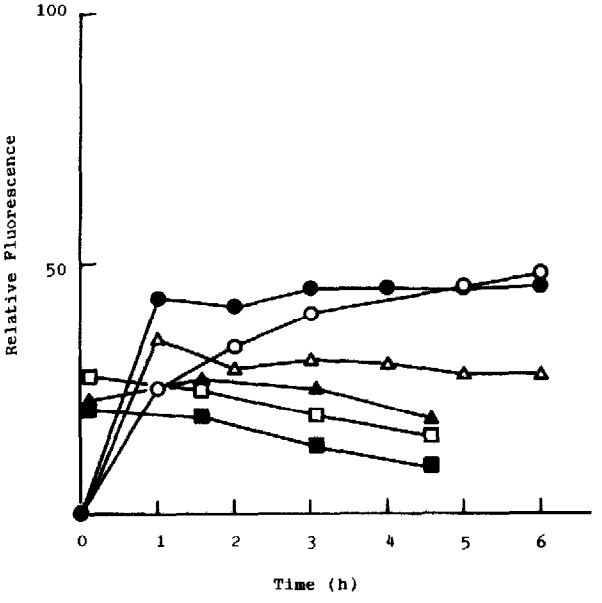

Fig. 5. Effect of OPA concentration on the stability of sisomicin derivatives. Temperature, 20°C; 50 mmol/l KH_2PO_4-borate buffer, pH 9.0. Concentration of OPA: (○) 0.05; (●) 0.1; (△) 0.2; (▲) 0.5; (□) 1.0; (■) 2.0 g/l. Other conditions as described under Experimental.

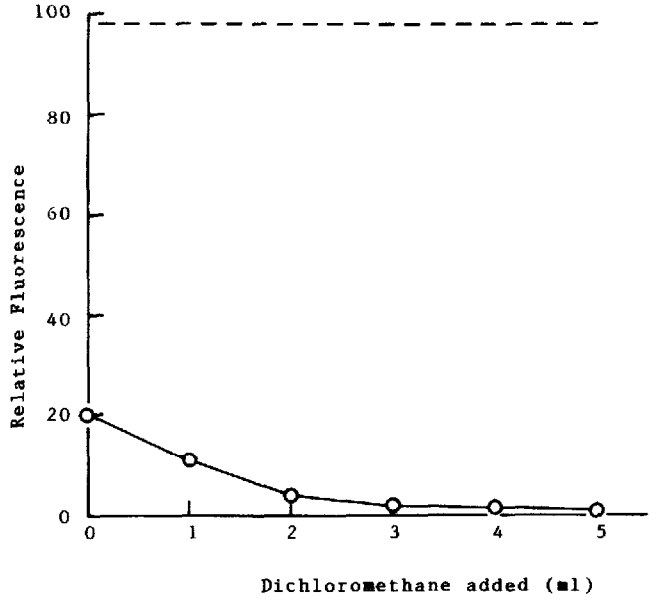

Fig. 6. Extraction of the reagent products with dichloromethane. Shaking time, 5 min; 1 ml of the derivative solution of sisomicin was used. Broken line, peak level of sisomicin (50 μg/l) derivative under the conditions used.

Analytical application of OPA–β-MP

The potential usefulness of the derivatization with OPA–β-MP was investigated for the HPLC analysis of sisomicin in plasma. Rabbit plasma samples of 200 μl spiked with sisomicin were analysed using the RP-HPLC system. The linear calibration equation was $y=0.940x-1.592$ ($r^2=1.000$) in the range 136–900 pg of sisomicin per 40-μl injection of plasma samples (5 ml). As little as 0.06 μg of sisomicin per 1 ml of plasma could be detected (signal -to-noise ratio $\geqslant 2$). However, the sensitivity of detection per 1 ml of plasma might be improved by some modifications to the preparation of the plasma samples. For five plasma samples spiked with 0.2 μg/ml of sisomicin, the recovery was 97.1 ± 6.6% (mean ± S.D.) with a within-run coefficient of variation (C.V.) of 6.8% and a day-to-day C.V. of 7.2%. None of the other commonly used Ags (amikacin, dibekacin, bekanamycin and ribostamycin) were found to interfere in the procedures developed for sisomicin, because the retention times of amikacin, bekanamycin and dibekacin were <5 min and that of ribostamycin was 50 min under the HPLC conditions used.

Plasma samples spiked with sisomicin were also allowed to react for 40 min at 30 °C and 1 ml of the reaction mixture was shaken with 5 ml of dichloromethane. The linear calibration equation was $y=1.571x-2.592$ ($r^2=0.995$) in the range 0.208–1.250 ng of sisomicin per 40-μl injection of plasma samples (5 ml). As little as 0.08 μg of sisomicin per 1 ml of plasma could be detected (signal-to-noise ratio $\geqslant 2$). For five plasma samples spiked with 0.26 μg/ml sisomicin the recovery was 111 ± 8.0% (mean ± S.D.) with a within-run C.V. of 5.5% and a day-to-day C.V. of 8.0%.

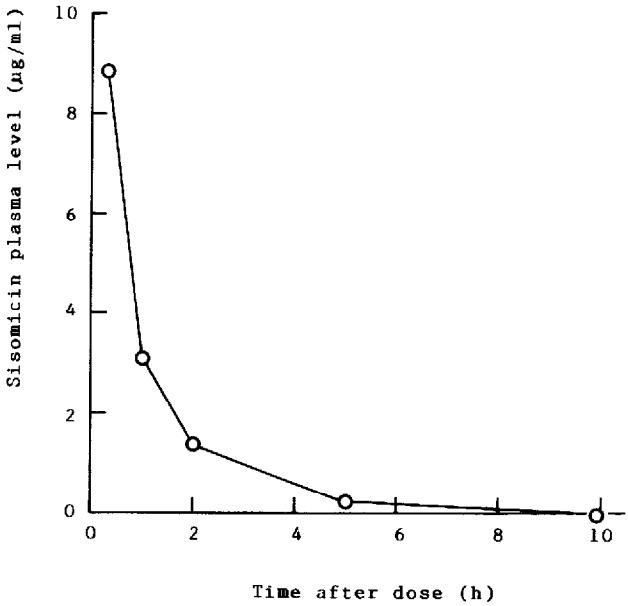

Fig. 7. Plasma profile of sisomicin from a healthy rabbit following an intramuscular injection of 1 mg/kg body weight. Each value is the mean of three determinations.

In both instances it was observed that the regression lines passed below the origin. This may arise from the plasma protein binding of sisomicin, which cannot be neglected at lower concentrations [23,24].

The concentrations of sisomicin in plasma samples obtained in the in vivo experiment were also calculated from the calibration graph. A typical time profile of sisomicin in rabbit plasma after a subcutaneous injection of 1 mg/kg of sisomicin is illustrated in Fig. 7.

CONCLUSION

RP-HPLC with OPA pre-column derivatization offers advantages over conventional methods for Ags. For satisfactory HPLC analysis, the Ags derivatives must be stable long enough to be analysed. In this study trials were made of the OPA derivatization of sisomicin using β-MP as an alternative thiol to β-ME, and showed the importance of solvent composition and OPA concentration for the stability of the fluorescent sisomicin derivatives, i.e., the stability of the derivatives may be increased by adding methanol and minimizing the excess of OPA used. OPA–β-MP has potential usefulness for the determination of low levels of Ags in microlitre samples and it is well suited to performing pharmacokinetic studies in systems where the sample volume is restricted because in human studies a fingerprick rather than a venepuncture is often used for blood collection.

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