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Review

High-performance liquid chromatographic analysis of aminoglycoside antibiotics

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Abstract

A simple precolumn derivatisation method for the determination of aminoglycoside antibiotics (AGs) is described. The stability of the *o*-phthalaldehyde (OPA) derivatives of the AGs obtained using β-mercaptopropionic acid (β-MP) was investigated by reversed-phase HPLC. 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 of 136–900 μg of sisomicin per injected volume (40 μl). As little as 0.06 μg of sisomicin per 1 ml of plasma could be detected with a signal-to-noise ratio ≥2. The method was also applied to whole blood samples from rabbit after a subcutaneous injection of 1 mg/kg of the AGs, using dried blood spots (DBS) on filter-paper punched discs. The detection limits of sisomicin and netilmicin in the DBSs on punched discs (10.1 μl of whole blood) were 0.053 and 0.50 μg per ml of whole blood, respectively (signal-to-noise ratio ≥2). The method permits a simple collection of blood at the microlitre level and should prove particularly useful for monitoring the AGs in blood at therapeutic levels in geriatric and paediatric patients and could be also used for the preclinical study of the AGs blood levels of a number of mice or rats without killing. An RP-HPLC method using an on-line clean-up procedure for large sample-volume analysis of serum AGs is also described.

Keywords: Reviews; Derivatisation, LC; Aminoglycosides; Antibiotics

Contents

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1. Introduction

Aminoglycoside antibiotics (AGs) are widely used against aerobic gram-negative bacillary infections, but in treating life-threatening infections in patients with impaired renal function or when the therapy is of long duration, their potential ototoxicity and nephrotoxicity require the careful monitoring of AGs concentrations in blood, because of their narrow therapeutic index [1–3]. To assure therapeutic serum concentrations and to minimize these toxicities, frequent and careful monitoring of serum AG levels is essential for a considerable time after administration. Various methods have been reported for the determination of AGs in serum, including microbiological assay, radioenzymic assay, homogeneous enzymic immunoassay [4]. High-performance liquid chromatographic (HPLC) procedures have also been reported for the determination of AGs as the most sensitive approach, although most require either pre or postcolumn derivatisation [5]. Although postcolumn derivatisation is adequate, it requires special reaction chambers as part of the chromatographic system and so is dedicated to one type of analysis. Precolumn derivatisation offers greater efficiency and higher sensitivity than the postcolumn technique, but little has been published. In many investigations using precolumn derivatisation, the AGs have been labelled with 1-fluoro-2,4-dinitrobenzene (FDNB) [6–8] or related nitrophenylation reagents [9–11] for ultraviolet (UV) detection. The drawback of using FDNB or related reagents is their toxicity and difficulties in establishing the optimal derivatisation conditions, although HPLC with UV detection is well suited for routine analysis in clinical laboratories for therapeutic drug monitoring.

This minireview attempts to summarize the fluorometric precolumn derivatisation of the AGs and the developments in the HPLC separation of the serum AGs sample and the application to monitoring the AGs concentration in microliter whole blood from the clinical point of view.

2. Precolumn derivatisation of the aminoglycoside antibiotics (AGs)

The derivatisation reaction of AGs with *o*-phthalaldehyde (OPA)-β-mercaptoethanol (β-ME), which yields intensely fluorescent 1,2-disubstituted isoindole derivatives [12], is more sensitive, but it is impossible to use it for precolumn derivatisation because of the instability of the derivatives [13], so it has been most frequently used for postcolumn fluorescent derivatisation [14–16]. It is suggested that increasing the steric bulk of the thiol side-chain should enhance the stability of the OPA derivatives of various primary amines. Kucera and Umagat [17] found that the OPA-β-mercaptopropionic acid (β-MP) adduct of alanine gave good stability and fluorescence response and this reagent system has been developed for the sensitive HPLC of individual amino acids in biological fluids [18]. Here, an investigation is described of the stability of the derivatives of sisomicin (Fig. 1), a dehydro analogue of the AGs, gentamicin C_{1a}, by OPA-β-MP and its application as a precolumn derivatisation reagent for the microdetermination of sisomicin in plasma by reversed-phase HPLC (RP-HPLC) [19–21].

The three substances giving rise to peaks A, B and C (Fig. 2) of which retention times were 10, 16 and 23 min, respectively, with the RP system used, were separated for the derivatisation of sisomicin with OPA-β-MP, although their structures could be elucidated with certainty. The optimal conditions for fluorescent derivatisation and the stability of the 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 derivatisation 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 [12], 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. The stabilizing effects of methanol, ethanol and propanol on the sisomicin derivative with OPA-β-MP were investigated. The fluorescent response (peak height) was most stable with 50% aqueous methanol under the conditions used, although the maximal response was slightly slower as the methanol content increased. With 50% aqueous

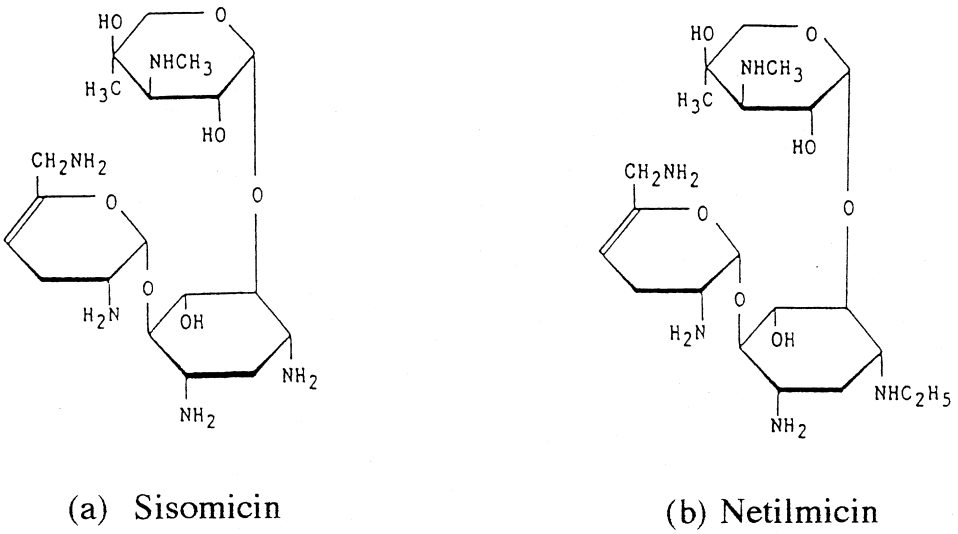

Fig. 1. Structures of sisomicin and netilmicin.

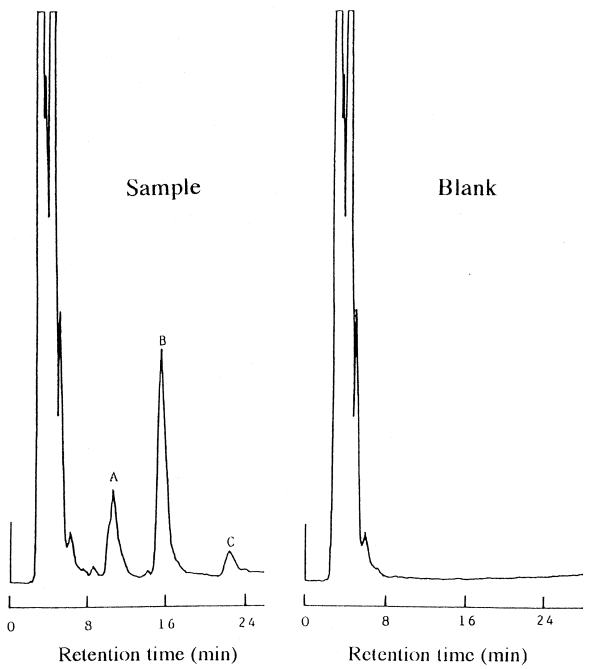

Fig. 2. HPLC of the OPA-ß-MP derivatives of a standard solution of sisomicin (50 μg/l) and a blank solution. Peaks A, B and C are the main products of the derivatisation of sisomicin. Separation conditions as described in Ref. [20].

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% aqueous propanol the 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 [22,23], and the time dependence of fluorescence intensity on pH in the range 7.0–12.0 was investigated. Above pH 10.0, the fluorescence intensity was increased due to the formation and coelution of the fluorescent product from OPA-β-MP. Cohn and Lyle [24] 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]. It has been reported that an excess of OPA significantly increases the destabilizing effect for the isoindole of amino acids [25–27]. Excess β-MP did not affect the stability of the derivatives, as reported for the OPA derivatisation with other thiols. 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 HPLC method. 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, and the sample solution gave a constant fluorescence intensity for more than 4 h at 30°C and 1 h at 60°C.

The potential usefulness of the derivatisation 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 μg 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 ≥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 relative standard deviation (R.S.D.) of 6.8% and a day-to-day R.S.D. 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 ≥2). For five plasma samples spiked with 0.26 μg/ml of sisomicin, the recovery was 111±8.0% (mean±S.D.) with a within-run R.S.D. of 5.5% and a day-to-day R.S.D. of 8.0%. 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 [28,29]. 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. 3.

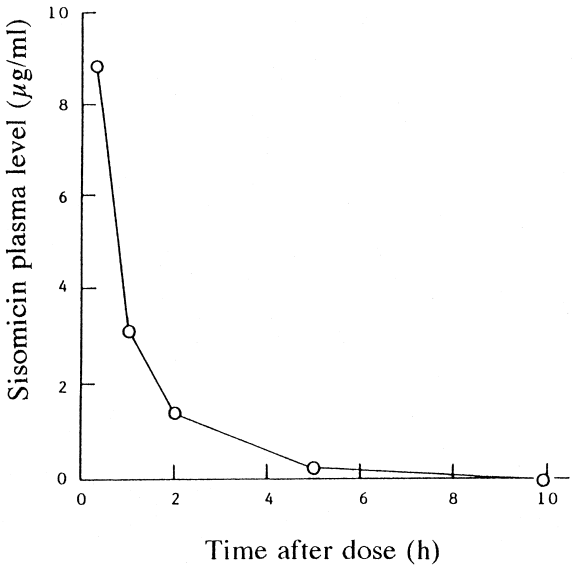

Fig. 3. Plasma profile of sisomicin from a healthy rabbit following an intramuscular injection of 1 mg/kg of body weight. Each value is the mean of three determinations. Reproduced from Ref. [20].

3. Application to the monitoring of AGs in whole blood

Monitoring the serum concentration of AGs has been considered to be helpful in achieving suitable therapeutic ranges and avoiding undesirable side-effects. Multiple sample collection by finger pricking is advantageous for paediatric patients, where venipuncture is often difficult or impossible to perform on multiple occasions. Capillary sampling has been used interchangeably with venous sampling for the determination of theophylline, phenytoin and tobramycin and various biochemical and haematological

measurements. The micro-volume techniques make it possible to assay small-volume samples, collected, by a finger lancet puncture. The dried blood spot (DBS) method has been used in diagnostic screening [30–32], and it has an advantage in terms of multiple sample collection from paediatric patients [33,34] and small animals, e.g., mouse or rat. Simpler methods, e.g., fluorescence polarisation immunoassay, may also be applied to the determination of AGs in filter-paper, although the sensitivity of detection is lower, so the blood volume in the DBS would need to be increased [4]. The methodology presented here combines the advantage of microsampling, inherent in blood collection on filter-paper, with an accurate, precise and selective HPLC method for the determination of AGs in serum, which is particularly useful in paediatrics and for patients with renal failure where sample size is of major concern [19,35]. Also, as some AGs on filter-paper are stable for up to ten days (at ambient temperature or 35°C) [4], the DBS can be easily handled and stored in the laboratory.

The elution of sisomicin or its 1-*N*-ethyl derivative, netilmicin from the DBS was several factors, including the nature of the solvent and the duration and the method of elution. Distilled water, physiological saline and 60% (v/v) ethanol had no effect on the elution of sisomicin or netilmicin; 0.5 *M* Na_2HPO_4 solution (pH 8.7) was more effective than 0.05 *M* KH_2PO_4–borate buffer (pH 9.0) for the elution of sisomicin by ultrasonication, as shown in Table 1, although the haemoglobin was also released from the DBS. The optimum elution of sisomicin was checked with 500 μl of 0.5 *M* Na_2HPO_4 solution by monitoring the recovery of sisomicin as a function of time, by gently shaking the tubes in a water bath (37 and 50°C) or by ultrasonication (Fig. 4). Thirty minutes of ultrasonication ensured good recoveries of sisomicin, and also ultrasonication for more than 40 min denatured the protein in the eluates; this was utilized as a standard elution technique for the other AGs. Using a paper punch as a means of quantification of the volume of blood, it was necessary to analyse the blood in the punched disc (5-mm diameter) of filter-paper. To obtain information on the volume of blood and the reproducibility, six filter-papers were punched in duplicate. The resulting twelve discs were extracted with 0.5 *M* Na_2HPO_4 solution as described above and the eluates were measured by spectrophotometry based on the absorption of the haemoglobin at 575 nm. The blood volume in these filter-paper discs was found to average 10.1 μl with a standard deviation of 0.33 μl ($n=6$), based on the calibration graph for the whole

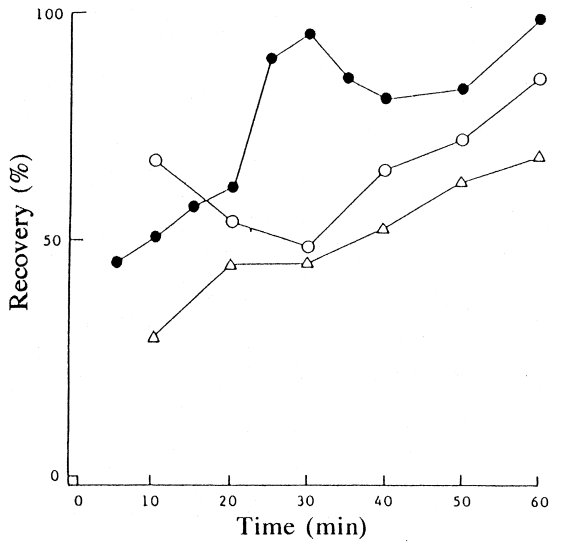

Fig. 4. Efficiency of extraction methods: effect of shaking in a water bath at 37°C (△) or 50°C (○) and ultrasonication (●) for various times on the recovery of sisomicin (7.4 μg/ml) in a DBS punched disc (5-mm diameter). The extraction was carried out in a glass tube containing 500 μl of 0.5 *M* Na_2HPO_4 solution (pH 8.7). Reproduced from Ref. [21].

Table 1
Influence of solvents on the extraction of sisomicin from the DBS[a]

Solvent	*n*	Recovery (mean±S.D.) (%)
0.05 *M* KH_2PO_4–borate buffer (pH 9.0)	3	68.9
0.5 *M* Na_2HPO_4 (pH 8.7)	6	93.6±8.5

[a] The concentration of sisomicin in the DBS was 7.4 μg/ml. Samples were treated in 500 μl of the solvent by ultrasonication for 30 min. Reproduced from Ref. [21].

blood. The intra-assay coefficient of variation for the blood volume in twelve discs was thus 3.3%.

Sisomicin and netilmicin derivatives of OPA-β-MP were well resolved and free from apparent interferences in blood. The relationship between the peak heights and the volume of sample injected was linear over the range 200–1000 μl for 5.0 μg/ml sisomicin in the DBSs. The calibration graphs for sisomicin and netilmicin in the DBS were linear over the concentration ranges 0.1–7.4 μg/ml ($y=0.049+0.334x$, $r^2=0.999$) and 1.0–10.0 μg/ml ($y=-0.552+0.306x$, $r^2=0.998$), respectively. The limits of detection of the assay were 0.053 μg/ml for sisomicin in whole blood on the DBS and 0.5 μg/ml for netilmicin with a signal-to-noise ratio ≥2. Both detection limits are sufficient for monitoring these drugs in serum, considering that the physiological levels of AGs in patients are in the range 4–30 μg/ml. The higher detection limit for netilmicin may possibly be due to the lower fluorescence intensity or lower stability of the OPA derivative of netilmicin than that of sisomicin under the conditions used [36]. Spiked DBS samples, each containing different concentrations of sisomicin or netilmicin, were analysed by the overall assay technique using the punched discs. The results are summarized in Table 2, where the mean recoveries were calculated from the calibration graph using the DBS samples. There were also no significant differences between the recoveries of sisomicin in DBS and plasma samples prepared from blood containing sisomicin concentrations from 2.0 to 20.0 μg/ml, assuming that the ratio of the slopes of the calibration graphs obtained for the DBS and the plasma samples was 2.3 (DBS samples, $y=-0.409+0.384x$, $r^2=1.000$; plasma samples, $y=-0.340+0.872x$, $r^2=1.000$) and that the haematocrit value in whole blood is about 50% (v/v) [36]. None of the other commonly used AGs were found to interfere in the determination of sisomicin, but astromicin and dibekacin produced peaks that interfered with netilmicin.

Monitoring the serum concentration of AGs has been considered to be helpful in achieving suitable therapeutic ranges and avoiding undesirable side-effects. Multiple sample collection by finger pricking is advantageous for paediatric patients, where venipuncture is often difficult or impossible to perform on multiple occasions. The DBS method has been used in diagnostic screening [30–32], and it has an advantage in terms of multiple sample collection from paediatric patients [33,34] and small animals, e.g., mouse or rat. However, the applicability of the method is limited, and no report has been published concerning determination of AGs in dried whole blood spotted on filter paper. This method was used for sample collection for therapeutic drug monitoring and pharmacokinetic studies [37].

The blood concentration of sisomicin versus time data obtained after single-dose administration in rats were employed to estimate one-compartment pharmacokinetic parameters, as summarized in Table 3. The calculation of two-compartment pharmacokinetic parameters was also examined using the above concentrations of sisomicin. The disposition curves of sisomicin in blood showed a biexponential decline at a dose of 10 and 30 mg/kg. There were

Table 2
Analytical recoveries of sisomicin and netilmicin from the DBS on the punched disc

AG	Added (A) (μg/ml)	n	Recovered (R) (μg/ml)	Accuracy[a] (%)	R.S.D.	
					Intra-assay	Inter-assay
Sisomicin	0.74	6	0.69±0.052	−6.8	7.5	
	2.0	6	2.0±0.18	0.0	9.5	
		5	2.1±0.22	5.0		10.5
Netilmicin	5.0	8	5.2±0.72	4.0	13.7	
	10.0	5	10.2±0.89	2.0	8.7	
		6	10.3±1.69	3.0		
	25.0	8	24.7±2.10	−1.2	8.3	16.4
	50.0	6	48.3±2.30	−3.4	4.8	
	75.0	5	79.7±2.75	6.3	3.5	

[a] $[(R-A)/A]\cdot 100$. Reproduced from Ref. [21].

Table 3
One-compartment pharmacokinetic parameters of sisomicin after single-dose administration in rats

Dose (mg/kg)	Rat no.	AIC[a]	SS[b]	K_a (h^{-1})	K_e (h^{-1})	V_c (l/kg)
10	1	5.93	0.98	11.334	1.057	0.645
10	2	8.44	1.50	6.615	0.495	0.556
10	3	21.63	9.32	2.288	1.163	0.532
30	4	21.85	14.04	5.252	1.254	0.455
30	5	12.52	2.96	9.885	0.985	1.163
30	6	15.73	5.06	10.487	0.986	1.220

[a] An information criterion.
[b] Sum of squares. Reproduced from Ref. [35].

small but significant differences between individual rats (Table 4). Mice or rats have been utilized for toxicity studies, safety studies, absorption, distribution, metabolism or excretion studies in the initial stages of development of various drugs. For the determination of drug levels in serum, blood samples are usually collected from the heart or retro-orbit sinus of mice or rats under adequate anaesthesia and assayed. It has been believed that blood cannot be collected more than twice from a mouse or a rat without serious damage to the animal. The concept of monitoring the concentration in blood using the DBS technique arises from the practical problems of obtaining samples that are suitable for microassay in small animals, so as to avoid the need to kill a number of mice or rats just for preclinical or toxicity studies of blood levels. By applying DBS, a definitive time-course assay for pharmacokinetic studies of AGs in individual rats is feasible. The data summarized in Tables 3 and 4 suggest that it will be possible to obtain pharmacokinetic parameters for various AGs in rat plasma, although individual differences in pharmacokinetic parameters were found. If more rats were used, mean pharmacokinetic parameters of the AGs could be established more precisely. The principal problems with the punched disc are to ensure constant blood volume for different samples and consistent elution of blood constituents from the punched disc. However, these factors can be corrected for by measurement of the haemoglobin values. In a clinical study in paediatric patients, this method gave an excellent linear correlation with the concentrations of AGs in plasma [37]. Therefore, it is considered that the presented HPLC method coupled with this sampling method is also suitable for use in preclinical studies of AGs with rats.

4. Determination of the serum AGs using on-line clean-up procedure

A sensitive analysis of the AGs by HPLC with column-switching after precolumn derivatisation using OPA and thioglycolic acid (TGA) has been developed [16]. However, the deproteinisation steps

Table 4
Two-compartment pharmacokinetic parameters of sisomicin after single-dose administration in rats

Dose (mg/kg)	Rat No.	AIC[a]	SS[b]	K_{12} (h^{-1})	K_{21} (h^{-1})	K_a (h^{-1})	K_e (h^{-1})	V_c (l/kg)
10	1	9.93	0.98	0.19	7.11	10.788	1.092	0.624
10	2	−3.15	0.11	0.66	0.28	3.541	0.548	0.355
10	3	24.47	7.90	−0.29	−0.15	2.017	1.737	0.451
30	4	22.96	8.67	1.71	0.21	3.34	0.936	0.287
30	5	−1.14	0.16	−0.21	−0.39	6.077	1.767	0.890
30	6	19.73	5.06	1.214	0.965	10.872	0.977	1.227

[a] An information criterion.
[b] Sum of squares. Reproduced from Ref. [35].

in both methods are very time-consuming and unsuitable for routine laboratory analysis. A precolumn venting method, which was developed by Wahlund et al. [38] can remove the protein and other interfering substances in serum by the use of a precolumn. The present method of coupling a back-flush procedure with the column-venting method in a HPLC system using commercially available instruments, appears to be particularly appropriate for direct injection of a serum sample of large volume [39–41].

When a large-volume serum sample was passed through a membrane filter of 0.22- or 0.45-μm, the sisomicin derivative could not be determined, although in the case of a small volume of the sample the procedure was effective. Anhalt's method [16] using carboxymethyl (CM)-Sephadex can also eliminate most of the protein in serum, but this method gave poor reproducibility and is more tedious. The reduced plate height (h) was calculated from the theoretical plate height (H) and the particle diameter (d_p) as $h=H/d_p$, where H was calculated from the column length (L) and the plate number (N) as $H=L/N$, where N was calculated from the retention time and the base width. Ch values define the h value changes at the start to ten measurements, that is, they indicate the stability of the precolumn. When the Ch value is lower, the support is more suitable for the precolumn. Table 5 shows the average of peak heights and the peak efficiency of the analytical column when using the precolumn filled with various supports with different particle sizes and different numbers of residua] silanol groups. When a support with large particle size was placed in the precolumn, the analytical column gave greater peak heights than in the case of small particle size. In general, the interparticle channels are wider so that denatured proteins tend to adhere to the particle surfaces to a lesser extent, so the stability is greater for precolumns with large particles. Advidsson, et al. pointed out that this beneficial effect of the stability of large particles in the precolumn can only be utilized for less demanding separations [42]. However, the precolumn was then used for the pretreatment of serum samples, residual silanol groups should be taken into account as well as particle size in evaluating the stability of the precolumn. To investigate the effect of the residual silanol groups, two kinds of supports, Nucleosil C_{18} (5-μm) and Chemcosorb ODS-H (7-μm), were examined. Chemcosorb ODS-H has a small amount of residual silanol groups, compared with Nucleosil C_{18}. As shown in Table 5, the efficiency of the analytical column was

Table 5
Influences of the support in the precolumn on the stability of the analytical column[a]

	VR (ml)[b]	$W_{1/2}$ (mm)[c]	N[d]	L (cm)[e]	H[f]	d_p (μm)[g]	h[h]	Ch (%)[i]	P.H. (μV)[j]	R.S.D. (%)[k]
Starting value										
Nucleosil $5C_{18}$	4.5	17.4	1.1	5	46	5	9174	–	–	–
Chemcosorb 7–ODS-H	3.1	11.3	1.2	5	41	7	5857	–	–	–
Chemcosorb 30-ODS-H	5.1	39.9	0.3	5	188	30	6267	–	–	–
After 10 times										
Nucleosil $5C_{18}$	3.0	13.3	0.8	5	61	5	12248	25.1	401	6.96
Chemcosorb 7-ODS-H	3.4	12.7	1.1	5	43	7	6211	5.7	343	6.53
Chemcosorb 30-ODS-H	6.2	49.2	0.3	5	196	30	6521	3.9	441	4.93

[a] Injection volume was 1 ml.
[b] Elution volume.
[c] Half width.
[d] Plate number.
[e] Column length.
[f] The theoretical plate height.
[g] Particle diameter.
[h] Reduced plate height.
[i] The change width of h after 10 injections.
[j] The mean of peak heights for 10 samples.
[k] The relative standard deviation. Reproduced from Ref. [40].

enormously stabilized by the use of the precolumn filled with Chemcosorb ODS-H. Nucleosil C_{18} was less stable and acquired a yellowish color, probably caused by strongly retained lipid material on the support (this was not observed with Chemcosorb ODS-H). Consequently, the stability of the support may be highly dependent on the amount of residual silanol groups. Therefore, two points have to be considered in adopting a precolumn for the pretreatment of biological fluids: (1) particle diameter should be chosen so as to exclude interfering substances as early as possible from the precolumn and (2) packing materials should be chosen to give stable efficiency of the analytical column. Although monohydric alcohols are utilized to regulate the retention of the desired peak, they tend to denature protein. On the other hand, polyhydric alcohols can be used to prevent the denaturation of proteins in serum. In this study, ethylene glycol was adopted as an organic modifier in the rinsing buffer. The contents of ethylene glycol in the rinsing buffer were varied to examine clean-up of serum samples. The void peaks were reduced with an increasing amount of ethylene glycol up to 40%, but addition of over 60% caused elution of the sisomicin derivatives from the precolumn and also increased the column back-pressure owing to the high viscosity. A solution of 30% ethylene glycol in 0.05 *M* phosphate buffer (pH 7) and 10% ethylene glycol in counter-ion solution containing 80% methanol were employed as the mobile phases for rinsing and elution, respectively. Addition of ethylene glycol to the rinsing buffer improved the durability of this system and more than 50 samples could be analyzed. A typical chromatogram obtained by this system is shown in Fig. 5. The mean recovery of sisomicin (5 μg/ml) from serum using the present method was 97.5% ($n=10$) and the standard deviation was ±2.07. A small amount might have been adsorbed by protein [42]. When amounts of 0.5 to 5 μg/ml of sisomicin spiked into serum were injected, the calibration curve for the peak height (y) versus the concentration of sisomicin (x) was $y=-0.694+16.753x$ ($r^2=0.9992$; $n=8$). The relative standard deviations (R.S.D.s) of 5 and 0.25 μg/ml were 4.44% and 4.65%, respectively. The variation at higher concentratrons was thought to be due to the concentration procedure on the column rather than errors in the injected volume. On the other hand, the variation in peak height at lower concentrations may be dependent on the base-line noise of the detector. The detection limit of sisomicin in serum was 62.5 n/ml with an injection volume of 1 ml (Table 6).

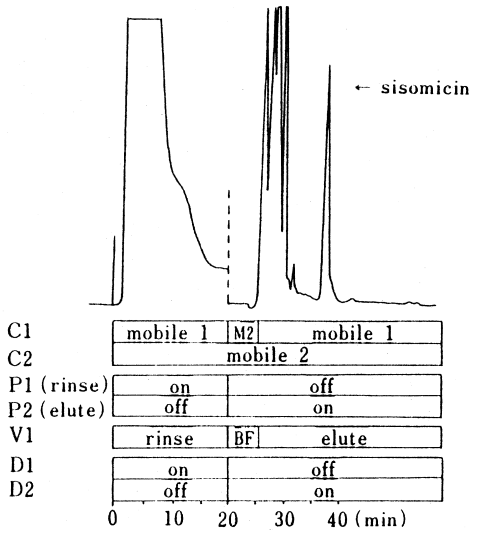

Fig. 5. Typical chromatogram of a serum sample containing 0.25 μg/ml sisomicin with the sample pretreatment system. M2, mobile phase 2; BF, back-flush mode. Reproduced from Ref. [40].

5. Conclusion

A RP–HPLC with precolumn derivatisation using OPA offers advantages over conventional methods for AGs. For satisfactory HPLC analysis, the AGs derivatives must be stable long enough to be analysed. Some trials were made of the OPA derivatisation 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. The HPLC analysis using OPA-β-MP as the precolumn derivative reagent has potential usefulness for the determination of low levels of Ags in microlitre samples and it is well suited to performing pharmacokinetic studies in the AGs monitoring system where the sample volume is restricted be-

Table 6
Analytical data for serum samples with the pretreatment system[a]

	Concentration of sisomicin		
	5 μg/ml	0.25 μg/ml	
P.H.	899 V	44.5 V	
S.D.	39.92	2.07	
R.S.D.	4.44%	4.65%	
Limit of detection			62.5 ng/ml
Mean recovery			97.50%

[a] The injection volume was 1 ml. Reproduced from Ref. [35].

cause in human studies a fingerprick rather than a venipuncture is often used for blood collection.

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