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Determination of amikacin in human plasma by high-performance capillary electrophoresis with fluorescence detection

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Abstract

A selective and reproducible high-performance capillary electrophoretic (HPCE) method for the quantification of amikacin (AMK), an aminocyclitol antibiotic, in human plasma, has been developed for use in clinical laboratory tests. The method involves ultrafiltration (UF) of plasma before derivatization with the fluorescence derivatization reagent 1-methoxycarbonylindolizine-3,5-dicarbaldehyde at room temperature for 15 min in the dark. An aliquot of the derivatives is directly introduced into the fused-silica capillary [75 cm (effective length)×50 μm I.D.] at the anode side by dynamic compression injection (50 hPa for 6 s). After electrophoresis with 40 m*M* SDS–20 m*M* phosphate–borate buffer (pH 7) in the micellar electrokinetic chromatography (MEKC) mode at 30 kV, the derivative had a retention time of 16.7 min and was detected by fluorescence intensity at 482 nm (with irradiation at 414 nm). The precision ($n=5$) of the method is 4.08 and 1.59% (C.V.) at the 50 and 100 μg AMK/ml plasma levels, respectively. Linearity ($r=0.998$) was established over the concentration range 5–100 mg of AMK/ml plasma and the detection limit (at a signal-to-noise ratio of 3) is 0.5 μg AMK/ml plasma. This assay method could potentially have wider application in the determination of other aminocyclitol antibiotics, such as arbekacin, dibekacin, kanamycin, in human plasma as well as of AMK.

Keywords: Amikacin

1. Introduction

Amikacin (AMK), an aminocyclitol antibiotic synthesized by chemical modification of kanamycin (KNM), was introduced in 1976 and has a broad spectrum activity against aerobic Gram-negative bacilli. Even now, it is often used clinically for Gram-negative bacillary infections resistant to other antibiotics such as methicillin-resistant *Staphylococcus aureus* (MRSA). Since clinical chemotherapy with aminocyclitol antibiotics is frequently associated with ototoxicity and nephrotoxicity, careful monitoring of blood levels is required especially when therapy is of long duration. About 20–30 years ago, the determination of plasma levels was performed by a microbiological method [1]; however, this method was time-consuming and was affected by the presence of other antibiotics. Later, high-performance liquid chromatographic (HPLC) technology was introduced which improved some of the problems such as the time needed, and the accuracy and sensitivity of the detection. Most of the methods reported to date involve pre-column derivatization of aminocyclitol antibiotics in ultrafiltrate (UF) plasma

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with 1-fluoro-2,4-dinitrobenzene (FDNB) prior to HPLC assay. FDNB has been used for the derivatization of sisomicin [2], gentamicin [2,3], neomycin [4], fortimicin [5] and AMK [6,7]. Although this method is excellent, the procedure is rather complicated. Significant interference due to the reagent is observed with derivatives unless the reagent is extracted prior to HPLC analysis or the molar excess of reagent is carefully limited. In order to perform derivatization of aminocyclitol antibiotics with FDNB, reaction conditions such as 60–85°C for 30–60 min are required. With regard to HPLC, there is a limit to the maintenance of column efficiency over long term usage. Repetitive analyses of many clinical samples are sometimes required. On the other hand, high-performance capillary electrophoresis (HPCE) has become an attractive separation technique due to its high resolution and its possibility to perform many analyses without decreasing resolution efficiency (as long as the capillary is not broken). This may be one of the best reasons for developing the HPCE method instead of the HPLC method for use in clinical laboratory studies.

In a previous paper [8], we described the new fluorescence derivatization reagent 1-methoxycarbonylindolizine-3,5-dicarbaldehyde (IDA) for amino groups and its adaptation to the analysis of amino acids [9]. In a series of studies we have tried to develop the assay method for use in clinical laboratory tests. The method involves derivatization of AMK with IDA in ultrafiltrate human plasma prior to HPCE separation using a system equipped with a fluorescence detector.

2. Experimental

2.1. Reagents and materials

A standard sample of amikacin base was donated by Bristol-Myers Squibb Japan (Tokyo, Japan) and the other aminocyclitol antibiotics were given to us by Meiji-Seika Kaisha (Tokyo, Japan). Other reagents were of HPLC grade or of the highest grade commercially available. All aqueous solutions were prepared using water purified with a Milli-Q purified system (Millipore, Tokyo, Japan). Dray IDA reagent (100 μg) tube was prepared and used in the present study. IDA was dissolved in ethyl acetate by sonication to make a 1.00 mg/ml solution. A portion (100 μg; 0.43 μmol) was transferred into a 0.6-ml polypropylene microcentrifuge tube. The solvent was carefully evaporated to dryness by placing the tubes in a desiccator under reduced pressure. The dry reagent tubes were capped and stored until use in a refrigerator in the dark. The reaction buffer was prepared according to the method described in a previous paper [9].

2.2. Apparatus

HPCE systems consisted of a Jasco Model CE-990 (Jasco, Tokyo, Japan) with an FP-920 fluorescence detector (Jasco) equipped with a capillary cell unit for HPCE or a CE-971 UV detector (Jasco) and a Model 807-IT data processor (Jasco). A fused-silica capillary tube (75 cm effective length×50 μm I.D.) was used throughout the work.

2.3. Sample preparation

An aqueous standard solution of AMK (1.0 mg/ml) was prepared and diluted with water to obtain the following concentrations; 500, 100, 50, 10, 5 and 1 μg/ml. A 10-μl volume of each aqueous AMK solution was added to 90 μl of human plasma to give final concentrations in the range of 0.1 to 100 μg/ml. These samples spiked with AMK were prepared just before use.

2.4. Preparation of ultrafiltrate (UF) plasma

Heparinized human blood was collected from five healthy adults (aged 23 to 35). Blood samples were immediately centrifuged at 1000 *g* for 10 min to give plasma. A portion (200 μl) of plasma or plasma spiked with AMK was transferred into an Ultrafree C3LCC tube (Millipore) and centrifuged at 2000 *g* for 20 min at 4°C to collect the ultrafiltrate (UF) plasma, containing all species with a molecular mass less than 5000.

2.5. Derivatization procedure

A 40-μl volume of reaction buffer was added to a dry reagent tube containing 100 μg (0.43 μmol) of

Table 1
Operation of the programmable injector for high-performance capillary electrophoresis

Step	Reservoir[a]		hPa	kV	min	Extension
	in	out				
1	1	1	2000	0	3.0	oven (40°C)
2	1	1	0	30	0.5	
3	2	1	50	0	0.1	
4	1	1	0	30	20.1	0.01 min (marker)

[a] 1=40 m*M* SDS–20 m*M* phosphate–borate buffer (pH 7), 2= IDA derivative solution.

IDA and then the tube was placed into an ultrasonic bath (Model UT-52, Sharp, Tokyo, Japan) for 30 s. After the addition of 20 μl of UF-plasma samples, the mixture was mixed well and left at room temperature for 15 min in the dark.

2.6. HPCE separation

Derivatives were directly introduced into the capillary tube from the anodic side by dynamic compression injection at 50 hPa for 6 s. The samples migrated electrophoretically in a carrier electrolyte, 40 m*M* SDS–20 m*M* phosphate–borate buffer (pH 7) in the micellar electrokinetic chromatography (MEKC) mode. The applied voltage was 30 kV throughout this work. The operation of the programmable injector of the HPCE system is shown in Table 1. Each electropherogram was recorded at 16 mV full-scale by monitoring the absorption at 280 nm or the fluorescence intensity (gain, ×100) at 482 nm (irradiation at 414 nm), respectively.

3. Results and discussion

3.1. Comparison of fluorescence detection and UV detection

To compare fluorescence detection and UV detection for the AMK derivative, the derivative was analyzed under the same HPCE conditions in a system that was equipped either with a fluorescence detector or with an UV detector. Fig. 1 shows a typical electropherogram of the standard AMK derivative in the UV detection mode (Fig. 1A) and in the fluorescence detection mode (Fig. 1B), respectively. The reagent did not interfere with fluorescence detection of the analyte and the reagent gave a minimal fluorescent response compared to absorbance. Furthermore, the peak-area response of IDA–AMK (retention time 17.6 min) using fluorescence detection was 1.7 times higher than that obtained with UV detection. From these results, it is clear that in this method fluorescence detection is better suited than UV detection for the determination of AMK.

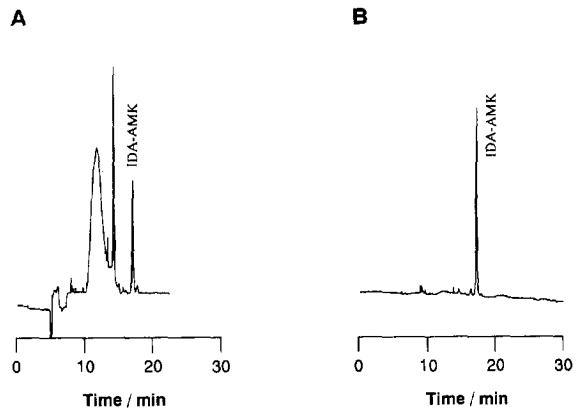

Fig. 1. Both electropherogarams of the amikacin derivative (IDA–AMK) were recorded at 16 mV full scale with (A) ultraviolet detection (λ=280 nm) and (B) fluorescence detection (λ_{ex}=414 nm, λ_{em}=482 nm), respectively. The other conditions of the HPCE systems are given in Section 2.

3.2. HPCE separation of AMK in human plasma

A 20-μl volume of human plasma spiked with AMK was analyzed after derivatization with IDA using HPCE with fluorescence detection. Typical electropherograms obtained with blank plasma and with plasma spiked at 10 and 100 μg/ml levels are shown in Fig. 2A–C, respectively. Although the peak corresponding to AMK (retention time of 17.6 min) was not sufficiently baseline resolved from neighboring peaks, the data processor integrated the areas under the peak by using a time program as a function of the baseline treatments (valley-to-valley).

In the case of other plasma samples collected from four healthy males, electropherograms similar to those shown in Fig. 2 were obtained. With this method, a plasma sample could be analyzed with a 20.6-min sample turnover using HPCE with fluorescence detection.

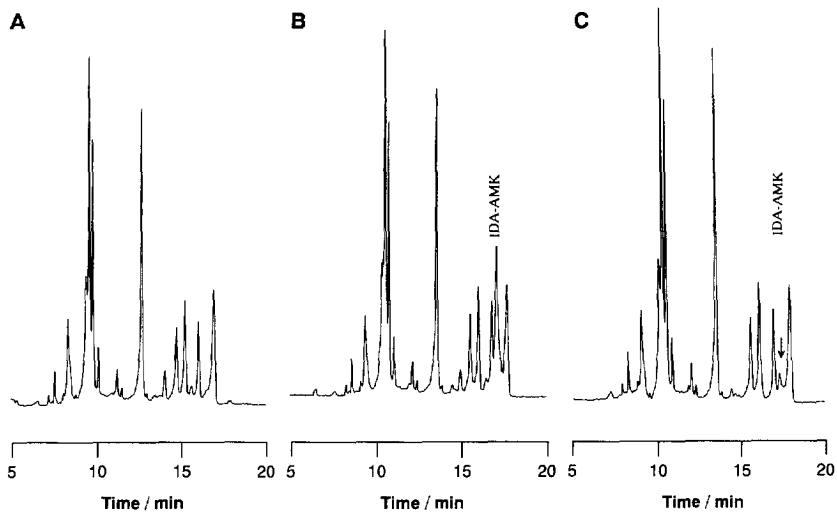

Fig. 2. Typical electropherograms of human plasma (A) blank, (B) spiked with AMK at 100 μg/ml and (C) at 10 μg/ml. The HPCE conditions are the same as in Fig. 1B.

3.3. Derivatization time-course of AMK with IDA

The derivatization time-course for AMK and IDA at room temperature in the dark was traced by measurement of the peak area of the AMK–IDA derivative separated by HPCE at 1, 5, 10, 20, 30, 40, 50 and 60 min after the start of the reaction by adding 20 μl of human UF-plasma spiked with AMK at a concentration of 100 μg/ml into 40 μl of the reagent solution in a tube containing 100 μg of IDA. The reaction profile is shown in Fig. 3. From these results it is clear that derivatization was completed within 10–20 min and that the derivative of AMK is relatively stable for 60 min. From this result, a derivatization time of 15 min was chosen.

3.4. Amount of IDA required for complete derivatization of AMK in human plasma

The amount of IDA required to ensure complete derivatization of AMK in human plasma was determined by derivatizing 20 μl of plasma spiked with AMK at a concentration of 100 μg/ml with varying amounts of IDA (0.215 to 0.86 μmol in a tube). The peak-area response of IDA–AMK showed a maximum at 0.43 μmol, with a plateau being reached with increasing amounts of IDA (Fig. 4). From this data, complete derivatization of AMK in 20 μl of human plasma was performed using more than 100 μg (0.43 μmol) of IDA.

3.5. Method validation

Reproducibility tests were carried out using plasma spiked at concentrations of 50 and 100 μg of

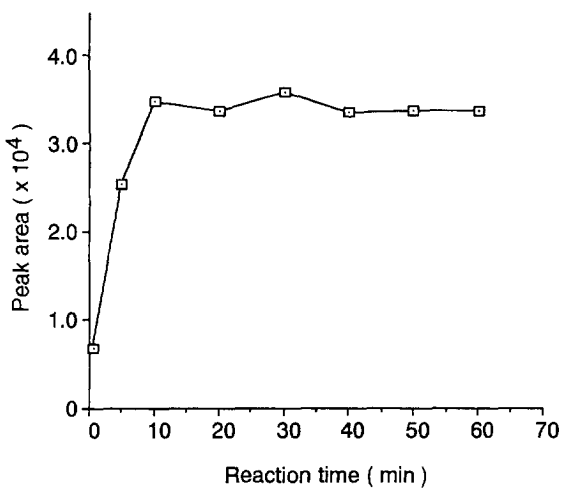

Fig. 3. Peak area of IDA–AMK as a function of the derivatization time at room temperature in the dark. A 20-μl volume of UF-plasma spiked with AMK at a concentration of 100 μg of amikacin/ml of plasma was analyzed by HPCE. The HPCE conditions used are the same as in Fig. 1B.

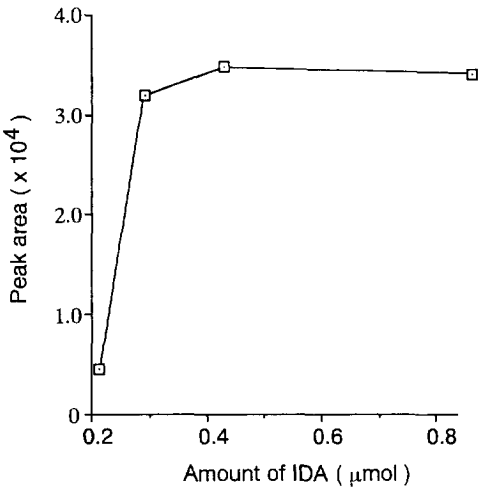

Fig. 4. Peak area of IDA–AMK as a function of the concentration of IDA. A 20-μl volume of ultrafiltrated plasma spiked with AMK at a concentration of 100 μg of AMK/ml of plasma was analyzed by HPCE. The conditions are the same as in Fig. 1B.

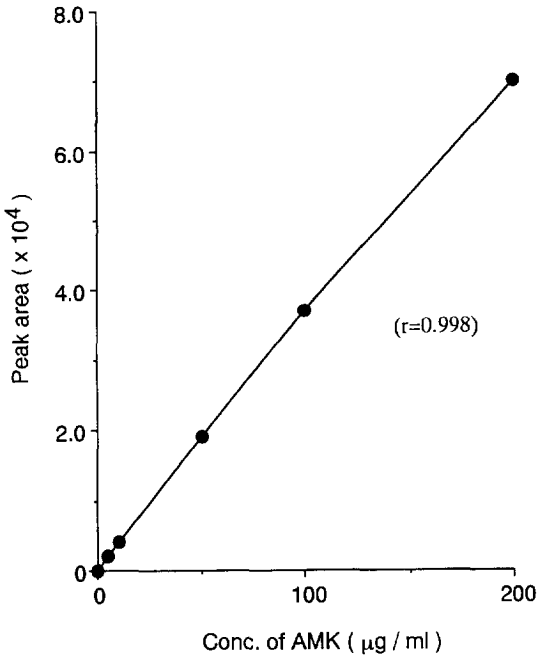

Fig. 5. Peak-area response of IDA–AMK as a function of AMK concentration by HPCE with fluorescence detection. The conditions are the same as in Fig. 1B.

AMK/ml, respectively. The data obtained with repetitive injections ($n=5$) are listed in Table 2. The detector response was linear (correlation factor $r=0.998$) over the range 5–200 μg/ml, as shown in Fig. 5 . The lower limit of quantitation and the detection limit (signal-to-noise ratio=3) were 5 and 0.5 mg/ml, respectively. These data made the determination of plasma levels of AMK in a therapeutic drug monitoring (TDM) study possible.

3.6. Other aminocyclitol antibiotics

Solutions containing the other aminocyclitol antibiotics ABK, DKB, KNM and a mixture of these (at a concentration of 100 μg/ml in water or plasma), were analyzed using the same procedure as that used for AMK. All derivatives have almost the same retention time on HPCE electropherograms. Fig. 6 shows a typical HPCE electropherogram obtained with a mixture of the antibiotics in water. Although the contents of the carrier electrolyte were varied, the peaks could not be separated. Fortunately, there are no cases where more than one aminocyclitol antibiotic is administered to a patient. Consequently, this assay method can be used for the determination of plasma levels of the other aminocyclitol antibiotics, ABK, DKB and KNM, as well as that of AMK.

With this method, determination of AMK in human urine is not possible because IDA derivatives of urine matrix components interfered with the measurement of the AMK derivative.

The chemical structure of IDA–AMK and the

Table 2
Assay reproducibility of human plasma spiked with AMK

Spiked plasma AMK (μg/ml)	Peak-area response ($\times 10^4$)						
	1	2	3	4	5	Average	C.V. (%)
50	1.91	2.03	1.81	1.95	2.01	1.94	4.08
100	3.71	3.78	3.62	3.65	3.51	3.70	1.59

The assay conditions for HPCE are as described in Fig. 1B.

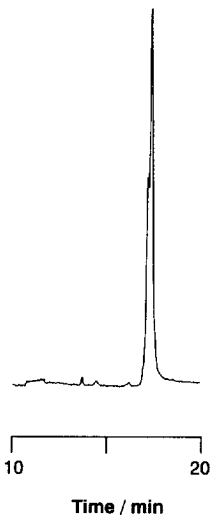

Fig. 6. Electropherogram of the derivatives of a mixture of arbekacin, dibekacin and kanamycin at 100 μg/ml. The HPCE conditions are the same as in Fig. 1B.

reaction mechanism of AMK with IDA have not been clear until now. The derivatives of AMK and ABK migrated at the same rate, even though AMK posseses four amino groups and ABK has five amino groups in a molecule. Possibly IDA does not react with all of the amino groups in a molecule of AMK (Fig. 7).

4. Conclusion

We have developed an assay method for the determination of amikacin in human plasma for use in clinical laboratory tests. The method involves ultrafiltration of plasma and derivatization with IDA prior to HPCE with fluorescence detection. Although the lower limit of quantitation (5 μg/ml) of this method is at the same level as that obtained with the other methods reported [6], the derivatization can be carried out using small amounts of plasma (50–100 μl). Possibly this procedure could be scaled down even further. The major advantages of this method are that it is simple and repeated analyses can be carried out without lowering the efficiency of resolution. Thus far we have analyzed more than 200 samples by injection without any trouble. A further advantage of this method is that it is applicable to other aminocyclitol antibiotics without having to alter the method.

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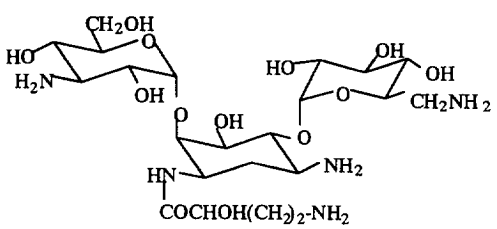
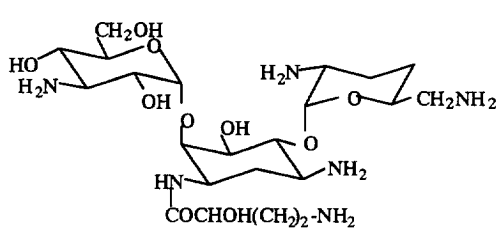


Amikacin (AMK)



Arbekacin (ABK)

Fig. 7. Chemical structures of amikacin and arbekacin.