*Journal of Chromatography*, 574 (1992) 93–99
*Biomedical Applications*
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6162

# High-performance liquid chromatographic assays for the quantification of amikacin in human plasma and urine

Eugene A. Papp*, Catherine A. Knupp and Rashmi H. Barbhaiya

*Department of Metabolism and Pharmacokinetics, Bristol-Myers Squibb Pharmaceutical Research Institute, Bristol-Myers Squibb Company, P.O. Box 4755, Syracuse, NY 13221-4755 (USA)*

(First received July 2nd, 1991; revised manuscript received September 24th, 1991)

## ABSTRACT

Amikacin, an aminoglycoside antibiotic, is frequently coadministered with penicillins and broad-spectrum cephalosporins to synergize the activity of these agents. Sensitive, selective and reproducible high-performance liquid chromatographic assays have been developed for the quantification of amikacin in plasma and urine collected from human subjects. The plasma method involves the ultrafiltration of plasma prior to derivatization. An aliquot of plasma ultrafiltrate or urine is mixed with dimethyl sulfoxide and tris(hydroxymethyl)aminoethane followed by derivatization of amikacin with 1-fluoro-2,4-dinitrobenzene at 58°C for 30 min. The reaction mixture is then injected directly onto a reversed-phase $C_{18}$ column preceded by a guard column. The column is eluted with a mobile phase containing acetonitrile and 2-methoxyethanol in 1% Tris buffer. Amikacin derivative is detected at 340 nm. The methods were applied for the analysis of amikacin in plasma and urine samples from volunteers receiving amikacin and cefepime, a fourth-generation cephalosporin, in a clinical pharmacokinetic drug interaction study.

## INTRODUCTION

Amikacin, an aminoglycoside antibiotic, is a semi-synthetic derivative of kanamycin. Due to its broad spectrum of acitivity against aerobic gram-negative bacilli, it is commonly administered parenterally for gram-negative bacillary infections. Coadministation of aminoglycoside antibiotics with penicillins or cephalosporins exerts a synergistic effect in killing bacteria [1–4]. It has been shown in *in vitro* experiments that aminoglycosides are inactivated by penicillins such as carbenicillin and ticarcillin [5]. Since clinical therapy with aminoglycosides is frequently associated with toxic side-effects, accurate quantitation of circulating drug levels is a necessity. Over the years, microbiological assays, radioenzymatic assays, radioimmunoassays, high-performance liquid chromatographic (HPLC), gas chromatographic (GC) and mass spectrometric techniques have been used for the quantification of aminoglycosides in biological samples. Maitra *et al.* [6] published an extensive review of these methods. The most practical methods for the analysis of aminoglycosides involve the use of derivatizing reagents, such as *o*-phthalaldehyde (OPA), dansyl chloride, fluorescamine and 1-fluoro-2,4-dinitrobenzene (FDNB), followed by HPLC separation. FDNB has been used for the derivatization of neomycin [7], gentamicin [8], sisomicin [8], fortimicin [9] and amikacin [10] in plasma samples. Amikacin has also been derivatized with OPA, followed by HPLC analysis coupled with fluorescence detection [11]. Drawbacks of this latter method include the relatively large volume of sample required for analysis (1 ml) and labor-intensive sample preparation. The assay also is relatively limited in terms of sensitivity (1 μg/ml) and linear range (1–15 μg/ml).

Amikacin can be coadministered with cefepime, a fourth-generation cephalosporin, for the treatment of serious infections. A clinical interaction study was designed to investigate the effect of concurrent administration of amikacin and ce-

0378-4347/92/$05.00 

fepime on the pharmacokinetics of these two antibiotics. In support of this study, it was necessary to develop assays in human plasma and urine that were suitable for the analysis of amikacin in the presence of cefepime. The advantages of the HPLC assays for amikacin described in this report include greater sensitivity than was reported in previous publications and simple and rapid sample preparation.

## EXPERIMENTAL

### *Reagents and materials*

The amikacin and cefepime were obtained from Bristol-Myers Squibb Pharmaceutical Research Institute (Syracuse, NY, USA). The purity of the reference compound was greater than 99%. Human plasma, containing sodium heparin, was purchased from Cocalico Biologicals (Reamstown, PA, USA) or obtained from volunteers on site. Human urine was obtained from volunteers on site. The following reagents and chemicals were purchased commercially: glacial acetic acid and acetonitrile from Fisher Scientific (Fairlawn, NJ, USA); dimethyl sulfoxide and 2-methoxyethanol from Burdick and Jackson (Muskegon, MI, USA); tris(hydroxymethyl)aminoethane and 1-fluoro-2,4-dinitrobenzene from Aldrich (Milwaukee, WI, USA); tetrahydrofuran from J. T. Baker (Phillipsburg, NJ, USA); ethyl alcohol from Quantum Chemical (Newark, NJ, USA). An Amicon Centrifree micropartition system unit and Centriflo Membrane Cones CF25 were from Amicon Division, W. R. Grace (Beverly, MA, USA). All chemicals were HPLC- or reagent-grade quality.

### *Equipment*

The HPLC system consisted of a Waters Model 590 solvent delivery pump, a Waters Model 710B autosampler, a Waters Model 441 ultraviolet–visible absorbance detector, equipped with a mercury lamp and a 340-nm filter (Waters Chromatography Division, Millipore, Milford, MA, USA) and interfaced through an analog-to-digital converter to a Model HP-3357 laboratory automation system (Hewlett Packard, Cupertino, CA, USA). Other equipment included a Model IEC HN-SII centrifuge with a fixed-head 6/84 rotor (Damon/IEC Division, International Equipment, Needham Height, MA, USA), a HPLC column heater (Bio-Rad, Richmond, CA, USA) and a Model DB-3 Dri-Block heater (Techne, Princeton, NJ, USA).

### *Sample preparation and processing*

The amikacin assay standards, the quality control samples and the stability samples were prepared in heparinized human plasma or human urine. The assay standards contained only amikacin. Standard solutions were also prepared in heparinized plasma ultrafiltrate to assess recovery. Some of the quality control samples contained amikacin only, and some contained both amikacin and cefepime. The plasma and urine storage stability samples were spiked with both amikacin and cefepime. The urine storage stability samples were divided into two groups. The samples in group 1 were buffered with 200 m*M* pH 4.25 sodium acetate buffer (one part urine to two parts buffer) and were used for the analysis of cefepime. The samples in group 2 were not buffered and were used for the analysis of amikacin. All samples were divided into aliquots and stored at −70°C for up to 65 days for plasma and 55 days for urine.

### *Plasma ultrafiltration*

Plasma, 400 μl, was transferred to an Amicon Centrifree Micropartition system unit and centrifuged at 800 *g* relative centrifugal force for 50 min.

### *Derivatization of amikacin*

A 200-μl aliquot of plasma ultrafiltrate or urine was mixed in a screw-capped tube with 200 μl of 1% (w/v) water solution of tris(hydroxymethyl)aminoethane, 400 μl of dimethyl sulfoxide and 400 μl of 1.5% (w/v) 1-fluoro-2,4-dinitrobenzene in 95% ethanol. The capped tube was incubated at 55°C in a dri-block heater and mixed at 5-min intervals for 30 min. After incubation, the sample was cooled at room temperature for 30 min. A 200-μl aliquot was transferred to a micro WISP vial insert and analyzed as described under *Chromatographic conditions*.

*Chromatographic conditions*

The plasma and urine samples were analyzed using a 300 mm × 3.9 I.D. $C_{18}$ μBondapak analytical column packed with 10 μm particles, preceded by a 25 mm × 4 mm I.D. precolumn packed with $C_{18}$/Corasil, 37–50 μm particles (Waters Chromatography Division). The analytical column temperature was maintained at 58°C. The injection volume was 10 μl from both processed matrices. The mobile phase consisted of acetonitrile–2-methoxyethanol–tetrahydrofuran–glacial acetic acid–tris(hydroxymethyl)-aminoethane (1% aqueous solution) (41:4.52:4.24:0.21:50, v/v). The pH of the mobile phase was 7.00 ± 0.02 and was delivered at a programmed flow-rate of: 0–8.5 min at 1.0 ml/min; 8.5–19.0 min at 0.5 ml/min; 19.0–25 min at 3.0 ml/min; 25–30 min at 1.0 ml/min.

*Data processing*

The analog output of the UV–VIS detector was digitized by an analog-to-digital converter and recorded by the HP-3357 HP laboratory automation system [12]. The standard curve was computed by using the least-squares linear regression method for the peak height on concentration for the standards. Each standard was weighted by the inverse of its nominal concentration. The outlier rejection for the standards was done by using the procedure of Prescott [13]. The unknown sample concentration was determined by inverse prediction from the linear regression line.

*Assay validation*

The validation of the analytical methods include documentation of assay linearity, accuracy, precision, lower limit of quantitation (LLQ), selectivity, recovery and stability of amikacin in the presence of cefepime during storage. Chromatograms of blank and spiked samples were inspected to assess the degree of interference with the peak of interest by endogenous matrix components. Urine and heparinized plasma from ten people, blank and spiked at the LLQ level, were processed. The accuracy and precision were assessed at the upper and lower quartile of the standard curve range. Accuracy was defined as the deviation of the mean observed concentrations from nominal, expresssed as a percentage of the nominal concentration. The intra-assay precision was calculated as the percentrage relative standard deviation (R.S.D.) for each concentration. The data from the analysis on three different days were evaluated by a one-way ANOVA. The inter-assay precision (R.S.D.) was calculated according to the equation $100[(\mathrm{TrMS}-\mathrm{EMS})/n]^{0.5}/\mathrm{GM}$, where TrMS, EMS and GM refer to treatment mean square, error mean square and grand mean, respectively.

The stability of amikacin and cefepime during storage at −70°C was evaluated using spiked plasma and urine samples. The samples were assayed for amikacin as described previously and for cefepime according to the methods described in ref. 14. The stability of the amikacin derivative, dinitrophenylamikacin (DNP-A), was evaluated at 25°C by repeatedly injecting, with an autosampler, a processed sample over a 41-h time period. The percentage recovery of amikacin from plasma was determined by comparing the slope of standard curve prepared in plasma to the slope of a standard curve prepared in plasma ultrafiltrate.

*Collection of clinical samples*

The pharmacokinetics and the extent of urinary excretion of amikacin in the presence of cefepime have been investigated in a Phase I drug interaction study. Patients received, by intravenous infusion at a constant rate over a 30-min interval, doses of 300 mg amikacin alone, or 2000 mg of cefepime and 300 mg of amikacin concurrently. Serial blood and urine samples were collected after the intravenous infusion. The plasma and urine samples were analyzed the same way as described previously.

## RESULTS AND DISCUSSION

The structures of amikacin and cefepime are presented in Fig. 1. Typical chromatograms obtained from blank plasma, plasma spiked with amikacin and a sample from a patient receiving amikacin are shown in Fig. 2. The retention time for DNP-A is approximately 16.5 min. The detector response was linear over the range of 0.5–75 μg/ml. The lower limit of quantitation was es-

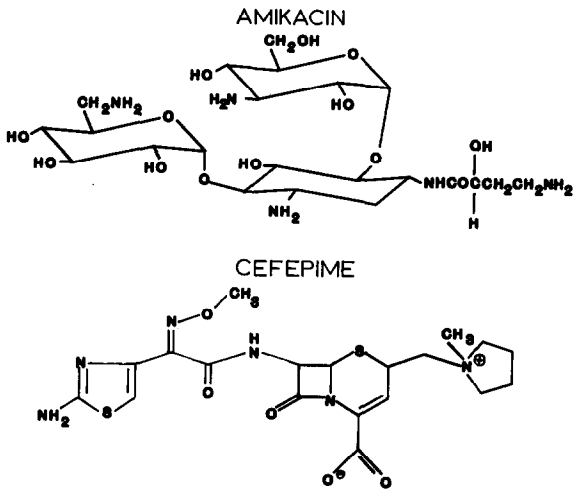


Fig. 1. Structures of amikacin and cefepime.

tablished at 0.5 μg/ml. The mean predicted concentration for the LLQ samples was 0.53 μg/ml with a 7.5% (R.S.D.) and 5.4% accuracy. There was no evidence of any endogenous plasma materials or the reagents causing significant interference with the quantification of amikacin. The intra-assay accuracy and precision results are presented in Table I. The inter-assay (between-day) precision was 3.8% R.S.D. at the concentration of 2.7 μg/ml and 2.8% R.S.D. at the concentration of 52.0 μg/ml of amikacin in plasma. Although this method can only detect free (non-protein-bound) amikacin, the recovery of amikacin was 96.9%, demonstrating minimal protein binding. Amikacin was found to be stable in the presence of cefepime in plasma when stored at −70°C for at least 65 days as presented in Table II. The DNP-A is stable in the injection media at 25°C for up to 41 h. This is in sharp contrast to the report by Wong *et al.* [10], which demonstrated that purification of DNP-A resulted in rapid degradation of the derivative with time.

Representative chromatograms obtained from a blank, spiked and patient urine sample are shown in Fig. 3. The retention time for DNP-A is 16.5 min. The detector response was linear over the range of 10–500 μg/ml. The lower limit of quantitation was established at 10 μg/ml. The mean predicted concentration for the LLQ samples was 11.0 μg/ml with an 8.7% R.S.D. and 10.0% accuracy. There was no evidence of any endogenous urine substances causing significant

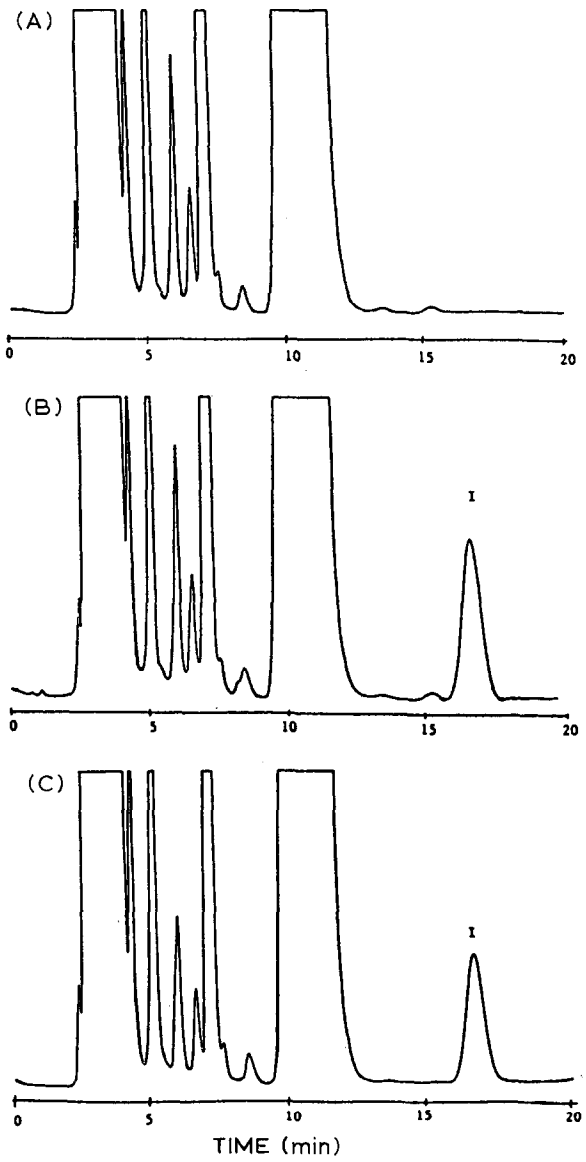


Fig. 2. Representative chromatograms of human plasma. (A) Blank plasma; (B) plasma standard spiked with amikacin (I) at 30 μg/ml; (C) patient plasma.

interference at the retention time of amikacin. The intra-assay accuracy and precision data are shown in Table I. Between-day precision results at the concentrations of 20.3 and 406.0 μg/ml were 0.1 and 6.6% R.S.D., respectively. Since urine samples are derivatized directly, without any extraction procedures, the recovery estimation was not needed. The stability of amikacin in the presence of cefepime in urine (Table II) and the stability of DNP-A in the injection media are equivalent to that observed for plasma.

TABLE I

ACCURACY AND PRECISION RESULTS FOR THE ANALYSIS OF AMIKACIN IN HUMAN PLASMA AND URINE

| Matrix[a] | Cefepime concentration (μg/ml) | | | R.S.D. (%) | Deviation from nominal (%) |
|---|---|---|---|---|---|
| | Nominal | Predicted | S.D. | | |
| Plasma | 2.7 | 2.8 | 0.092 | 3.3 | 3.7 |
| | 52.0 | 54.9 | 1.32 | 1.9 | 5.6 |
| Urine | 20.3 | 19.1 | 0.36 | 0.1 | −5.9 |
| | 406.0 | 419.2 | 8.80 | 2.1 | 3.3 |

[a] A total of ten replicates from each concentration were assayed.

TABLE II

STABILITY OF AMIKACIN (A) AND CEFEPIME (C) IN PLASMA AND URINE AT −70°C

| Duration of storage (days) | Mean nominal concentration (A/C) (μg/ml) | Mean predicted concentration (A/C) (μg/ml) | Deviation from nominal (A/C) (%) | R.S.D. (A/C) (%) |
|---|---|---|---|---|
| *Plasma* | | | | |
| 0 | 45/45 | 42.8/47.2 | −4.9/4.9 | 0.2/5.2 |
| | 10/10 | 9.6/9.9 | −4.0/−1.0 | 1.6/1.3 |
| | 2/2 | 1.9/2.0 | −5.0/0.0 | 0.7/3.4 |
| | 5/45 | 4.9/41.6 | −2.0/−7.6 | 0.0/5.8 |
| | 45/5 | 44.0/5.1 | −2.2/2.2 | 1.0/3.3 |
| 20 | 45/45 | 44.9/45.7 | −0.2/1.6 | 0.5/3.7 |
| | 10/10 | 10.1/9.8 | 1.0/−2.0 | 4.7/5.1 |
| | 2/2 | 2.1/1.9 | 5.0/−5.0 | 9.0/4.0 |
| | 5/45 | 5.4/42.5 | 8.0/−5.6 | 10.7/0.1 |
| | 45/5 | 43.3/4.9 | −3.8/−2.0 | 5.0/8.8 |
| 65 | 45/45 | 42.7/46.0 | −5.1/2.2 | 6.9/2.6 |
| | 10/10 | 9.3/10.0 | −7.0/0.0 | 3.7/2.3 |
| | 2/2 | 1.8/1.9 | −10.0/−5.0 | 6.2/1.2 |
| | 5/45 | 4.5/44.9 | −10.0/−0.2 | 1.0/0.2 |
| | 45/5 | 43.9/5.6 | −3.0/12.0 | 6.9/0.7 |
| *Urine* | | | | |
| 0 | 450/450 | 404.0/466.4 | −10.2/3.6 | 1.9/0.1 |
| | 100/100 | 96.4/106.7 | −3.6/6.7 | 3.3/0.1 |
| | 20/20 | 16.1/21.2 | −19.6/5.9 | 0.8/0.4 |
| | 20/450 | 17.1/473.9 | −14.4/5.3 | 0.3/0.1 |
| | 450/20 | 447.3/21.3 | −0.6/6.5 | 0.0/0.0 |
| 22 | 450/450 | 452.5/425.3 | 0.5/−5.5 | 3.2/5.6 |
| | 100/100 | 103.1/102.2 | 3.1/2.2 | 2.3/10.1 |
| | 20/20 | 19.4/20.2 | −2.2/0.9 | 4.2/0.9 |
| | 20/450 | 18.4/493.0 | −8.0/9.6 | 0.5/10.2 |
| | 450/20 | 466.1/20.1 | 3.6/3.9 | 1.1/3.0 |
| 55 | 450/450 | 388.3/421.0 | −13.7/−6.4 | 3.0/9.0 |
| | 100/100 | 87.4/107.6 | −12.6/7.6 | 0.5/1.5 |
| | 20/20 | 16.7/21.6 | −16.4/7.8 | 0.1/5.6 |
| | 20/450 | 17.0/480.3 | −15.0/6.7 | 1.5/1.0 |
| | 450/20 | 422.6/20.9 | −6.1/4.4 | 1.6/0.4 |

Analyses of amikacin in plasma and urine samples collected during a clinical interaction study have been performed. Fig. 4 is a representative plasma concentration *versus* time profile for amikacin in a subject receiving 300 mg amikacin alone and in combination with 2000 mg cefepime. Quantifiable levels of amikacin were detected for up to 8 h after each treatment. The maximum mean plasma concentration was 27.4 μg/ml when amikacin was administered alone and 24.6 μg/ml when administered with cefepime. The apparent elimination half-life was 2.3 h, whether amikacin was administered alone or with cefepime. The mean urinary recovery for amikacin was 92.1% when administered alone and 94.9% when administered with cefepime. The concurrent administration of amikacin with cefepime had no significant effect on any of the pharmacokinetic parameters of amikacin or cefepime.

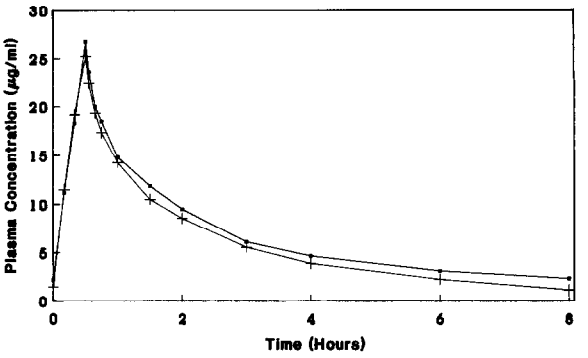


Fig. 4. Plasma levels of amikacin in one volunteer after intravenous infusion of amikacin alone (+) and simultaneous infusion with cefepime (−).

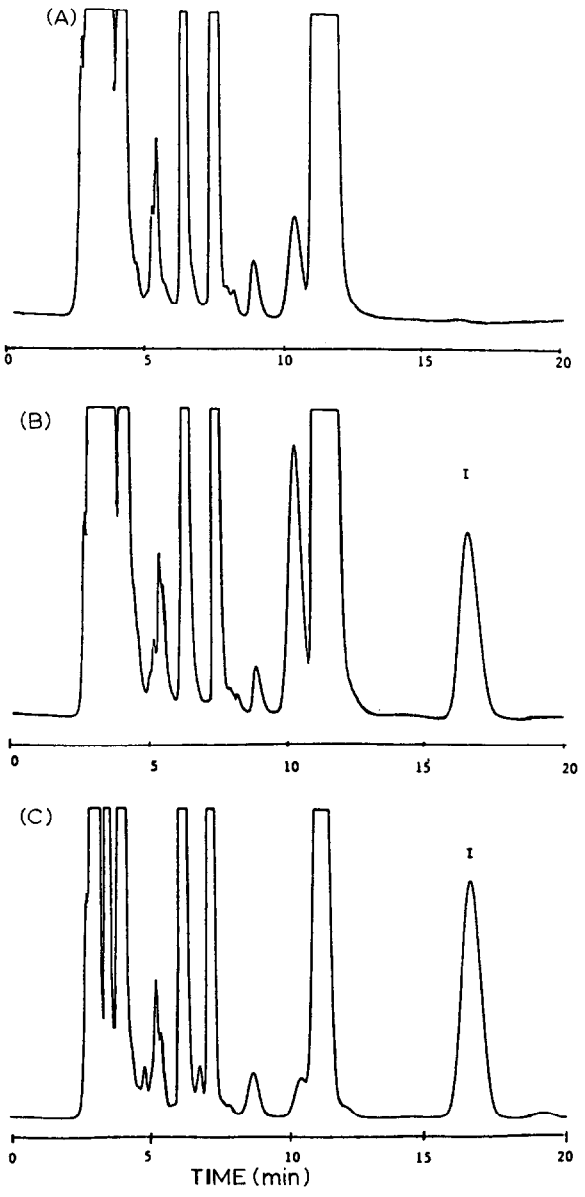


Fig. 3. Representative chromatograms of human urine. (A) Blank urine; (B) urine standard spiked with amikacin (I) at 75 μg/ml; (C) patient urine.

The advantage of these assays over previously published methods is that the sample preparation is minimal, and no loss or degradation of the derivatized amikacin occurs due to sample purification [10]. Plasma concentrations as low as 0.5 μg/ml of amikacin can be determined. This represents an improvement in assay sensitivity, as compared to many other reported methods. However, a recently published method by Wichert *et al.* [15] is capable of detecting 25 ng/ml amikacin in plasma. It became apparent during the development of the plasma assay that the concentration ratio of 2-methoxyethanol to tetrahydrofuran in the mobile phase selectively affected the retention time of the DNP-A peak. Therefore, the DNP-A peak could be eluted from the analytical column at a position where the interference was minimal in the chromatogram. There was considerable amount of material that eluted from the analytical column after the derivatized amikacin peak. To avoid the accumulation of these materials on the analytical column and

to eliminate the interference from injection to injection, the flow-rate of the mobile phase was increased to 3 ml/min for several minutes. The presence of cefepime in the sample did not interfere with the quantification of amikacin. Cefepime is degraded during the derivatization of amikacin and the degradation products elute in the solvent front.

In conclusion, the assays for the quantitation of amikacin in plasma and urine are sensitive, selective, accurate, precise and reproducible.

## REFERENCES


1 J. Klastersky, F. Meunier-Carpenter and J. M. Prevost, *Am. J. Med. Sci.*, 273 (1977) 157.
2 G. W. White, J. B. Malow, W. M. Zimelis, H. Pahlavanzadeh, A. P. Panwalker and G. G. Jackson, *Antimicrob. Agents Chemother.*, 15 (1979) 540.
3 T. O. Kurtz, D. J. Winston, D. A. Breeckner and W. J. Martin, *Antimicrob. Agents Chemother.*, 20 (1981) 239.
4 A. E. Brown, Q. Quesada and D. Armstrong, *Antimicrob. Agents Chemother.*, 21 (1982) 592.
5 B. S. Iyengar, V. Kumar, T. P. Wunz and W. A. Remers, *J. Med. Chem.*, 29 (1986) 611.
6 S. K. Maitra, T. T. Yoshikawa, L. B. Ouze and M. C. Schotz, *Clin. Chem.*, 258 (1979) 1361.
7 K. Tsuji, J. F. Goetz, W. Van Meter and K. A. Gusciora, *J. Chromatogr.*, 175 (1979) 141.
8 D. M. Barends, C. L. Zwaan and A. Hulshoff, *J. Chromatogr.*, 222 (1981) 316.
9 L. Elrod Sr., L. B. White and C. F. Wong, *J. Chromatogr.*, 208 (1981) 357.
10 L. T. Wong, A. R. Beaubien and A. P. Pakuts, *J. Chromatogr.*, 231 (1982) 145.
11 S. K. Maitra, T. T. Yoshikawa, C. M. Steyss, L. B. Guze and M. C. Schotz, *Antimicrob. Agents Chemother.*, 14 (1978) 880.
12 R. H. Farmen, J. F. Muniak and K. A. Pittman, *Drug Inf. J.*, 21 (1987) 141.
13 P. Prescott, *Technometrics*, 17 (1975) 129.
14 R. H. Barbhaiya, S. T. Forgue, W. C. Shyu, E. A. Papp and K. A. Pittman, *Antimicrob. Agents Chemother.*, 31 (1987) 55.
15 B. Wichert, H. Scheier and H. Derendorf, *J. Pharm. Biomed. Anal.*, 9 (1991) 251.