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DETERMINATION OF AMIKACIN IN MICROLITRE QUANTITIES OF BIOLOGICAL FLUIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING 1-FLUORO-2,4-DINITROBENZENE DERIVATIZATION*

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SUMMARY

Pre-column derivatization of amikacin with 1-fluoro-2,4-dinitrobenzene in 25 μl of guinea pig plasma or human serum produced a stable chromophore which was measured by UV detection after rapid separation on normal-phase or reversed-phase high-performance liquid chromatography systems. The reversed-phase system, selected for routine analysis due to instability of the normal-phase column, consisted of an Ultrasphere-ODS C18 column preceded by a guard column, and used acetonitrile—water (68 : 32) as the mobile phase. A high degree of linearity was found in the range of 2—64 μg/ml with a coefficient of variation averaging less than 5%.

INTRODUCTION

Amikacin is a semisynthetic derivative of kanamycin and has a broad spectrum of activity against aerobic gram-negative bacilli. Because the amikacin molecule has fewer points susceptible to enzymatic attack than have most other aminoglycosides [1] it is often used clinically for infections resistant to gentamicin or tobramycin. Its ototoxicity [2] and nephrotoxicity [3], however, require careful monitoring of blood levels especially in treating life-threatening infections in patients with impaired renal function or when therapy is of long duration [4].

Amikacin serum or plasma levels are most often determined by a microbiological method [5] but this is time consuming and can be affected by the pres-

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ence of other antibiotics. Other techniques which are more specific and rapid include radioenzymatic assay [6], radioimmunoassay [7], gas–liquid chromatography [8] and high-performance liquid chromatography (HPLC) [9]. Due to equipment availability a HPLC technique was the most suitable for our purposes. The published HPLC method for amikacin [9] used fluorescence detection of an *o*-phthalaldehyde derivative, and needed 1 ml of serum for analysis. Our experiments with guinea pigs required frequent sampling of amikacin blood levels from the same animal and therefore a micro-technique was essential.

1-Fluoro-2,4-dinitrobenzene (FDNB) has been used as a reagent for the derivatization of amino function groups for a number of years [10]. Recently, HPLC using FDNB derivatization has been described for neomycin [11], gentamicin [12], sisomicin [12] and fortimicin C [13]. The 2,4-dinitrophenyl (DNP) derivative formed is stable and can be measured using UV detection. This technique appeared suitable for the measurement of amikacin levels since this aminoglycoside has four primary amino functional groups. The ability to measure by UV detection is an advantage since a high-performance liquid chromatograph with a UV detector is rapidly becoming standard equipment in clinical laboratories.

In this report we describe a sensitive micro-technique for the determination of amikacin in plasma which was developed for ototoxicity studies in guinea pigs. Since no interfering peaks are detectable in human serum even in the presence of several other drugs, this technique is readily adaptable for the clinical monitoring of amikacin levels.

MATERIALS AND METHODS

Reagents

Amikacin base was donated by Bristol-Myers Pharmaceutical Group (Candiac, Quebec, Canada). 1-Fluoro-2,4-dinitrobenzene, 98% pure, was obtained from Aldrich (Milwaukee, WI, U.S.A.). All other chemicals were reagent grade and obtained from commercial sources.

Normal-phase chromatographic system

A Varian-Aerograph Series 4100 liquid chromatograph equipped with a positive displacement pump capable of developing a pressure of 34.5 MPa (5000 p.s.i.) was used as the solvent delivery system. Flow-rate was always maintained at 1 ml/min. Samples were injected using a Valco loop (125 μl) injection valve. UV absorbance was monitored at 360 nm with a variable-wavelength UV detector (Varian Vari-Chrom, Model VUV-10) connected to a strip chart recorder (Linear Instruments). Attenuation was set at 0.01, 0.02 or 0.05 a.u.f.s. for a 100-mV full scale deflection. Chromatographic separation was achieved with a 200 × 4.6 mm stainless-steel column packed with 5-μm Spherisorb silica (Phase Separations, Queensferry, Great Britain) using a high-pressure (34.5 MPa) balanced-density slurry technique [14]. The mobile phase consisted of the lower layer of a mixture of chloroform–methanol–glass distilled water–acetic acid (214:35:20:1) passed through a Whatman 2V filter and purged with nitrogen.

Reversed-phase chromatographic system

The solvent pump and UV detection systems were the same as that used in the normal-phase system. Samples were introduced using a Valco loop injection valve (CV-10-UHP$_a$-N60) equipped with a 50-μl loop. A Beckman Instruments (Montreal, Canada) column (Ultrasphere-ODS C18, 250 × 4.6 mm, particle size 5 μm) preceded by a guard column (RP-18, 30 × 4.6 mm, particle size 5 μm, Brownlee Labs., Santa Clara, U.S.A.) was used for chromatographic separation. The mobile phase consisted of acetonitrile—glass-distilled water (68:32) passed through a Whatman 2V filter and purged with nitrogen.

Sample preparation

Stock solutions were prepared by adding amikacin base to glass-distilled water to give concentrations ranging from 0.080 to 2.560 mg/ml. Spiked samples were prepared by adding 25 μl of stock solution to 975 μl of guinea pig plasma or human serum to give final concentrations in the range of 2 to 64 μg/ml. A 25-μl aliquot of spiked or blank plasma or serum was placed in a 0.3-ml Reacti-Vial (Pierce, Rockford, IL, U.S.A.) together with 10 μl of 0.1 *M* borate buffer (pH 9.3). After 100 μl of methanol were added to precipitate the plasma proteins, the vials were vortexed, sealed and centrifuged at 2000 *g* for 5 min (IEC Clinical Centrifuge, Damon/IEC, Needham, MA, U.S.A.). A 75-μl aliquot of supernatant was transferred to a 1.0-ml Reacti-Vial and 10 μl of FDNB in methanol (180 mg/ml) were added. The vials were sealed, incubated for 30 min at 80°C in a dry bath (Fisher Scientific, Isotemp Model 145) and cooled to room temperature in a freezer.

Normal-phase chromatography of samples

Chloroform (500 μl) was added to the sample vials which were then vortexed for 1 min and centrifuged at 2000 *g* for 5 min. Aliquots (125 μl) of the chloroform layer were injected onto the column.

Cleanup of samples and reversed-phase chromatography

The contents of the sample vials were evaporated with nitrogen until an oily residue remained and then 6—8 mg of binder-free silica gel (MN-Kieselgel N, Macherey, Nagel and Co., Düren, G.F.R.) were added. After introducing a 600-μl aliquot of diethyl ether the vials were shaken (Burrell Wrist Action Shaker, Burell Corporation, Pittsburgh, PA, U.S.A.) for 5 min, vortexed and then centrifuged for 5 min at 2000 *g*. The diethyl ether was discarded by decanting and the washing procedure repeated once more with 600 μl of diethyl ether, then with 400 μl of 0.2 *M* acetate buffer (pH 4.0). Vortexing without prior shaking was all that was needed for the final two washes. The derivative was then extracted by vortexing the silica gel pellet with 300 μl of mobile phase. After centrifuging for 5 min, 50-μl aliquots of the liquid above the silica gel pellet were injected onto the column.

RESULTS AND DISCUSSION

HPLC separation

The amikacin derivative has a retention time of approximately 6 min in the

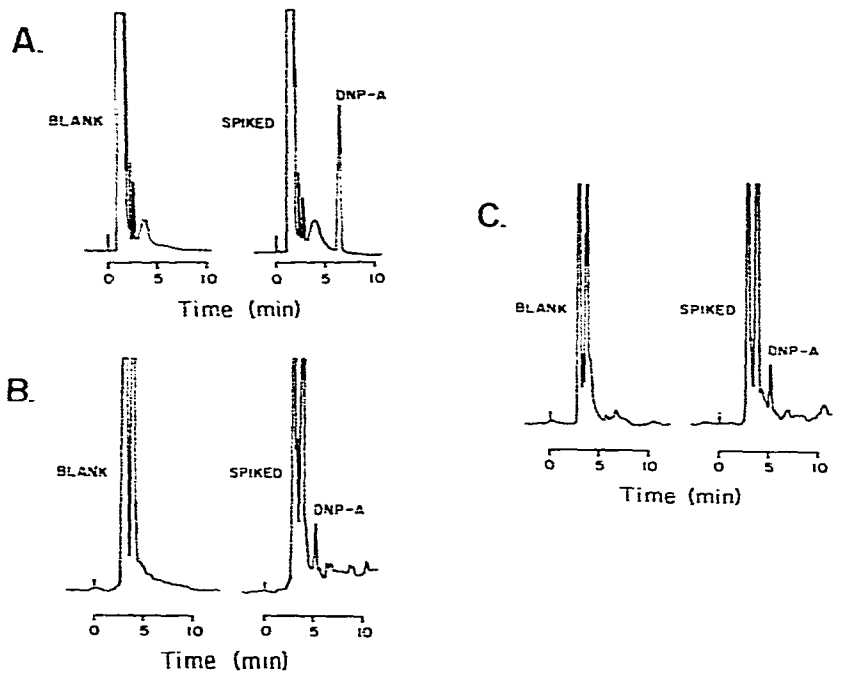

Fig. 1. Typical chromatograms at 0.01 a.u.f.s. of blank and spiked guinea pig plasma (A and B) and human serum (C). The normal-phase HPLC system was used for (A) and the reversed-phase system for (B) and (C). Concentrations of amikacin were 16 (A) and 4 (B and C) μg/ml. DNP-A = DNP-amikacin.

normal-phase system (Fig. 1A). Guinea pig plasma contains a few endogenous peaks which elute in less than 5 min and therefore do not interfere with the analysis. Although the normal-phase system provided adequate sensitivity, it had two major drawbacks. The first was retention time instability from day to day due to extreme sensitivity to the water content of the mobile phase and the long time required to fully equilibrate the column to new batches of mobile phase. The second drawback was that the retention time of the column started to deteriorate after about 100 samples. Since the packing material was found to take on a yellow colour the deterioration of the column could have been due to the presence of unreacted FDNB in the samples. For these reasons, a reversed-phase system was developed together with an extensive cleanup procedure. Guinea pig plasma and human serum chromatograms produced with the reversed-phase system are shown in Fig. 1B and C. Apart from the amikacin derivative peak with a retention time of 5.1 min, all other major peaks occur with the solvent front. FDNB, which has a retention time of 7.0 min is either absent or occurs as a tiny peak. The other minor peaks which follow the DNP-amikacin (DNP-A) peak are eluted by 11 min, and therefore samples can be run about 7 min apart by allowing these peaks to come out in the solvent front of the next sample. These minor peaks probably arise from degradation products of compounds in the solvent front since they do not appear if the sample is chromatographed immediately after the cleanup procedure (e.g., the chromatogram of blank guinea pig plasma in Fig. 1B). The cleanup procedure appears

somehow to catalyze this degradation process since derivatized samples can be stored in the freezer several days prior to extraction without an increase in the size of the peaks following DNP-A. For this reason, chromatograms should be run within 2—3 h of the cleanup procedure, especially for low levels of amikacin. The derivatization procedure however, can be carried out several days in advance without loss of DNP-A since it is stable for at least one week. To date, 150 samples have been injected with no appreciable loss of sensitivity or alteration of retention time. A gradual increase in back pressure occurred with usage but this was eliminated after the guard column was replaced. This reversed-phase system is thus now used routinely for the analysis of amikacin.

Derivatization of amikacin

The optimal conditions for the formation of DNP-amikacin were determined by investigating the effects of pH, FDNB concentration, reaction time and temperature on the derivatization of aqueous amikacin solutions.

A number of buffer systems (acetate, triethanolamine HCl, tris(hydroxymethyl)-aminomethane, bicarbonate, borate) were tested and borate buffer at pH 9.3 was found to give the maximum yield of product. The effect of borate buffer concentration on DNP-amikacin peak height was also examined for

TABLE I

EFFECT OF BORATE BUFFER STRENGTH ON PEAK HEIGHT OF AMIKACIN DERIVATIVE

Concentration of borate buffer aliquot (*M*)	*n*	Peak height* ± S.D. (mm at 0.02 a.u.f.s., normal-phase system)
0.016	3	90.6 ± 3.09
0.032	2	108 ± 6.06
0.064	3	112 ± 0.62
0.128	3	114 ± 3.76

*Plasma samples spiked with amikacin (64 μg/ml) were used.

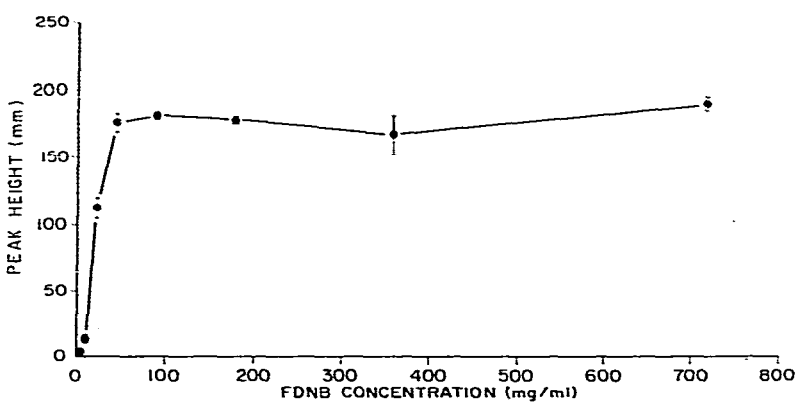

Fig. 2. Peak height (mm at 0.05 a.u.f.s., normal-phase system) of DNP-amikacin as a function of FDNB concentration. Amikacin concentration was 200 μg/ml in guinea pig plasma. Vertical bars are standard deviation. $n = 3$.

guinea pig plasma (Table I). Peak height plateaued above 0.032 *M*. A borate buffer concentration of 0.1 *M* was selected for routine analysis.

The amount of FDNB required to ensure complete derivatization was determined by incubating a high concentration of amikacin (200 μg/ml) in guinea pig plasma with varying FDNB concentrations (Fig. 2). The peak height of the amikacin derivative approached a maximum at a FDNB concentration of 45 mg/ml and leveled off with increasing concentrations. The concentration selected for routine analysis was 180 mg/ml, which gives a molar ratio of 283 for each amikacin primary amino group at a drug concentration of 200 μg/ml.

The effect of reaction time on the peak height of DNP-amikacin was examined at 80°C (Fig. 3). The reaction was judged to be maximal by 10 min but 30 min was selected for routine analysis to ensure stable results. The formation of the amikacin derivative was also examined at 60°C and 100°C. At the lower temperature the reaction was found to proceed too slowly whereas at the higher temperature the problem of solvent evaporation was encountered.

An attempt was made to determine recovery but we were unable to obtain an authentic derivative of high enough purity for this calculation.

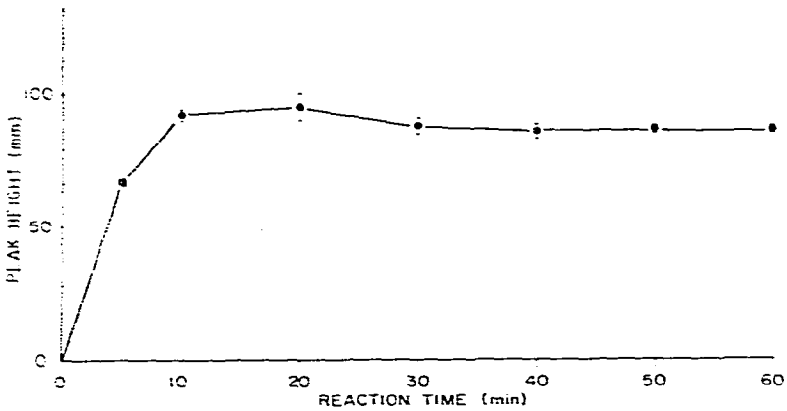

Fig. 3. Peak height (mm at 0.05 a.u.f.s., normal-phase system) of DNP-amikacin as a function of reaction time at 80°C. Amikacin concentration was 100 μg/ml in guinea pig plasma. Vertical bars are standard deviation. $n = 3$.

Cleanup of derivative

Samples injected onto the reversed-phase columns after derivatization without cleanup contained a number of large interfering peaks. Also, the column quickly deteriorated possibly due to unreacted FDNB. Since the amikacin derivative was soluble only in semi-polar solvents such as methanol, partitioning was unsuccessful. Hence silica gel was used to retain the derivative while more and less polar compounds were washed out with suitable solvents. Washing the derivatized sample with diethyl ether reduced unwanted peaks, especially that arising from excess FDNB, without reducing the size of the DNP-amikacin peak. Washing with acetate buffer (pH 4.0) greatly reduced other unwanted peaks, especially the large solvent front peak (arising partly from dinitrophenol). The derivatized amikacin peak was also reduced by about 20% but since this did not appear to affect precision, the reduction in peak size seemed

a fair price to pay for a cleaner chromatogram. The amount of silica gel used in the cleanup process to retain the derivatized amikacin was not critical in the range 5—15 mg per vial. Amounts of silica gel below this range caused a reduction in the size of the amikacin derivative peak. Amounts greater than 10 mg increased the amikacin derivative peak slightly but also greatly increased the size of contaminant peaks. Hence, we used an amount of silica gel in the range of 6—8 mg for routine analysis.

Characterization of derivative

The number of DNP substitutions on the DNP-amikacin derivative was examined by proton magnetic resonance (PMR) spectroscopy (Fig. 4). The PMR spectrum of underivatized amikacin shows two doublets between 5 and 6 ppm arising from the two anomeric carbon protons. The PMR spectrum of the DNP-amikacin derivative shows the presence of aromatic protons of the DNP groups

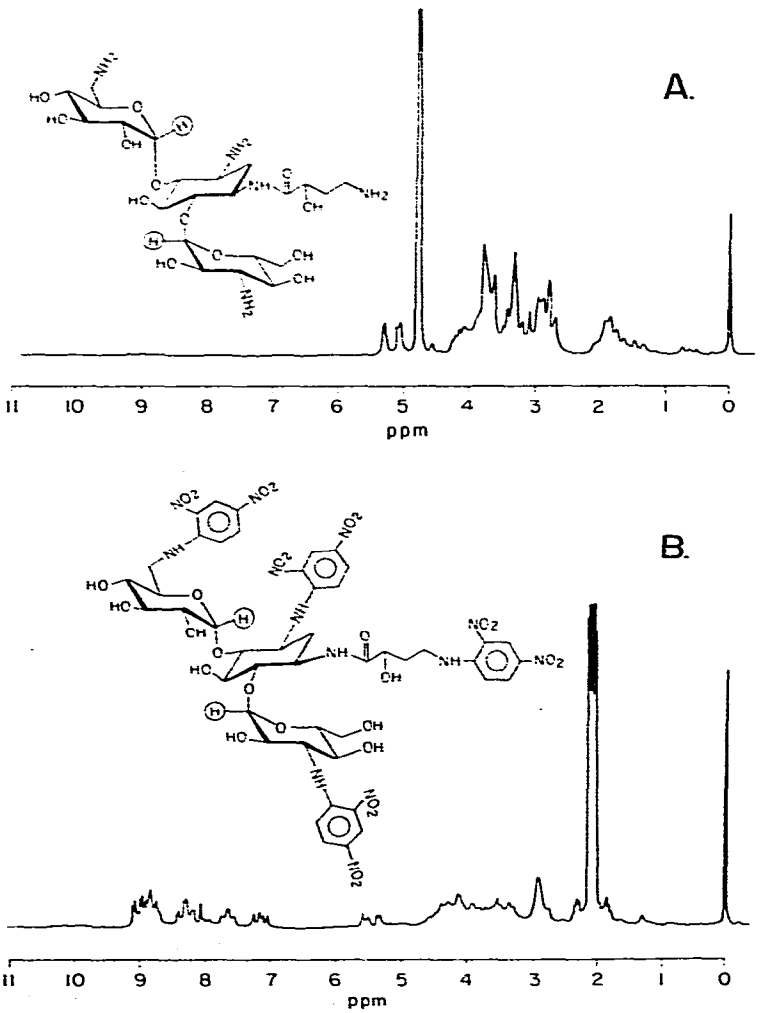

Fig. 4. PMR spectra of underivatized amikacin (A) and DNP-amikacin (B).

between 7 and 9.1 ppm. The ratio of the combined area of the aromatic proton peaks to that of the anomeric proton peaks was 12:2 suggesting that all four primary amino groups of amikacin were substituted. Differing solubility properties required that DNP-amikacin be run in deuterated acetone as opposed to underivatized amikacin which was run in deuterated water.

Quantitation

Spiked plasma samples, each containing one of seven different concentrations of amikacin were analyzed using the reversed-phase technique. The results are summarized in Table II. The standard curve was linear in the range of 2—64 μg/ml for guinea pig plasma and the mean coefficient of variation was less than 5%. Although regression analysis shows the standard curve to possess a high degree of linearity (r = 0.9996), it has been a consistent finding with all the

TABLE II

STANDARD CURVE OF AMIKACIN ADDED TO GUINEA PIG PLASMA

Amikacin added (μg/ml)	No. of samples	Peak height ± S.D. (mm at 0.02 a.u.f.s.)	Coefficient of variation (%)
2	4	5.75 ± 0.57	9.9
4	4	11.5 ± 0.42	3.6
8	4	23.6 ± 0.60	2.6
16	4	49.2 ± 1.14	2.3
32	3	96.9 ± 0.81	0.8
48	4	152.2 ± 2.83	1.9
64	4	204.9 ± 2.34	1.1

Mean coefficient of variation = 3.17%.
Y-intercept = −1.92 (95% confidence limits are −3.10 and −0.73).
Slope = 3.21.
Correlation coefficient = 0.9996.

TABLE III

ACCURACY OF REVERSED-PHASE METHOD USING PEAK HEIGHT COMPARISON WITH 32 μg/ml STANDARD*

T (Theoretical concentration in μg/ml)	E (Estimated concentration from peak height comparison)	Accuracy (%) $\left(\frac{E-T}{T} \times 100\right)$	E' (Estimated concentration using eqn. 1)**	Accuracy (%) $\left(\frac{E'-T}{T} \times 100\right)$
2	1.90	−5.1	2.02	+1.0
4	3.79	−5.3	4.02	+0.4
8	7.80	−2.6	8.19	+2.4
16	16.26	+1.0	16.78	+4.9
48	50.25	+4.7	48.48	+1.0
64	67.66	+5.7	63.15	−1.3

*Data taken from mean peak height values in Table II.
**$(E') = (E) \times$ (C.F.). The value of Z used in eqn. 1 was 0.936.

amikacin standard curves which we have run (with both normal-phase and reversed-phase techniques) that the relationship between peak height and plasma concentration is actually slightly curvilinear. One indication of this is the observation that the regression line passes slightly below the origin (i.e., the 95% confidence limits of the *Y*-intercept do not include zero). The slight curvature is shown more clearly in Table III when estimates of amikacin concentrations are made by comparing sample peak heights with the peak height obtained for a concentration of 32 μg/ml, using the mean data in Table II. Although deviations from theoretical values are less than 6%, concentrations below 32 μg/ml tend to be underestimated whereas those greater than 32 μg/ml tend to be overestimated. The slight curvature may arise from the small amount of plasma protein binding of amikacin [15] which would be proportionately more important at lower concentrations. Accuracy can be improved (Table III) by multiplying the first estimate by the following correction factor (C.F.) which partially compensates for the curvature:

$$\text{C.F.} = (\text{P.H.})_s/[Z(\text{P.H.})_s + (1 - Z)(\text{P.H.})_u] \quad (1)$$

where $(\text{P.H.})_s$ is the mean peak height of 32 μg/ml amikacin standards, $(\text{P.H.})_u$ is the peak height of the unknown sample and Z is an empirical constant whose magnitude is inversely related to the degree of curvature. The value of Z was determined to be 0.936 ± 0.025 (mean ± S.D.) using the best fit data from four separate standard curves.

Specificity

The following drugs were added to human serum at high or higher than clinical concentrations and were not found to produce peaks which would interfere with the reversed-phase technique for amikacin analysis: ampicillin (20 μg/ml), chlorpromazine (500 ng/ml), diazepam (500 ng/ml), digoxin (2 ng/ml), doxycycline (15 μg/ml), gentamicin sulfate (10 μg/ml), sodium heparin (3 I.U./ml), imipramine (1 μg/ml) and phenobarbital (30 μg/ml).

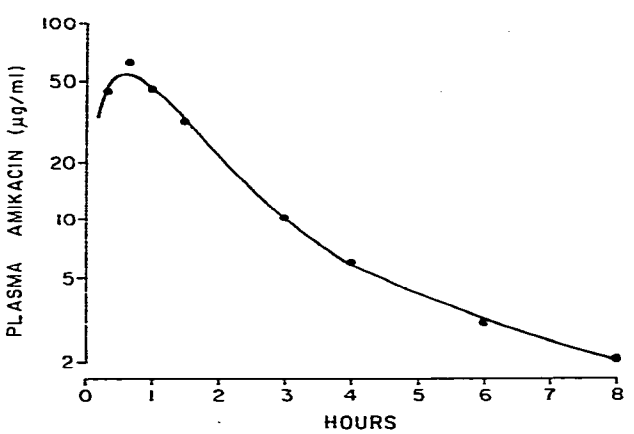

Fig. 5. Amikacin in guinea pig plasma after subcutaneous injection (50 mg/kg). The curve is drawn according to the best fit equation to a two-compartment open model as determined by computer analysis.

Application

The time profile of amikacin in plasma from a guinea pig after a subcutaneous injection of 50 mg/kg is shown in Fig. 5. The data were fitted to a two-compartment open model with a computer program run in a NOVA 800 computer (Data General Corporation, Southboro, MA, U.S.A.). The program used an iterative technique to minimize the squared deviations between the curve and the data values. The equation for the best fit curve was determined to be:

$$C_p = -271.3e^{-2.24t} + 259.9e^{-1.36t} + 11.4e^{-0.22t} \quad (2)$$

where C_p is the plasma amikacin concentration at time t. The β half-life was found to be 3.14 h which is similar to 2.79 h reported for human adults after an intramuscular injection of 500 mg of amikacin [16]. The α half-life for the guinea pig was 0.51 h which compares to 0.25 h found in children [17] and 2.77 h in the human adult study [16].

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