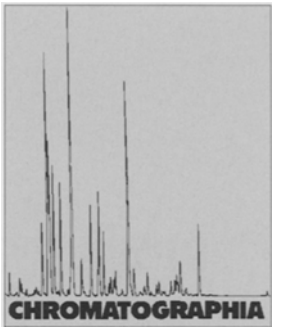
Trace Analysis of Amikacin in Human Plasma by High-Performance Liquid Chromatography



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Key Words

Column liquid chromatography
Amikacin in plasma
Naphthyl isothiocyanate derivatization

Summary

A simple and sensitive liquid chromatographic method is described for the determination of amikacin in human plasma. The amikacin is derivatized with 1-naphthyl isothiocyanate (NITC) at 70 °C. After derivatization, a methylamine acetonitrile solution is added to the reaction mixture to eliminate excess derivatizing agent. The resulting derivative was analysed by HPLC on a LiChroCART RP-C_{18} column with water-acetonitrile (57:43, *v/v*) mobile phase and detection at 230 nm. Parameters affecting derivatization of amikacin, including reaction temperature, reaction time and amount of derivatizing agent, were investigated. The linear range for the determination of amikacin in spiked plasma was over 10 – 52 nmol mL^{-1}; the detection limit (signal-to-noise ratio = 3; injection volume, 10 µL) was ca. 1 nmol mL^{-1}. The relative standard deviation was < 2.37% for intra-day assay (n = 6), 5.80% for inter-day assay (n = 6) and relative recovery was > 91%.

Introduction

Amikacin is an aminoglycoside antibiotic and commonly administered parenterally to treat infections caused by aerobic gram-negative bacteria. Amikacin is semi-synthesized from kanamycin B and developed to resolve drug resistance to gentamicin, kanamycin and tobramycin. It binds irreversibly to the 30S and 50S ribosomal subunits resulting in a defective, bacterial-cell membrane-synthesis of protein. Like other aminoglycosides, amikacin has a comparatively narrow safety margin. It may cause both ototoxicity and nephrotoxicity in patients, especially during long-term therapy. Its therapeutic plasma concentration is in the range: 8 – 16 µg mL^{-1}. Monitoring of amikacin levels in plasma is required for therapeutic and toxic control, hence, a simple, sensitive and specific method for trace analysis in plasma is essential [1 – 4].

Several methods, including microbiological assay [5 – 6], immunoassay [7 – 18] and chromatographic [19 – 35] methods, have been applied to the analysis of amikacin in various matrices. Microbiological assay is inexpensive and simple but time-consuming. Several parameters influence the accuracy of this method, such as test strain and presence of other antibiotics. Radioimmunoassay (RIA) methods [10 – 13] and fluorescence polarization immunoassay (FPIA) [14 – 18] are common assays for amikacin in human plasma. These methods are more specific and sensitive than microbiological assay, but they depend on suitable specific antibiotics. Cross-reactions are sometimes noted with the immunoassay kits. In addition, handling of radioactive materials and radioactive waste and high cost are inhibitory factors. HPLC is widely used and is the most accurate technique for the analysis of aminoglycoside antibiotics in various matrices.

The structure of amikacin, shown in Figure 1, indicates that amikacin has four primary amino groups, one primary OH group, one secondary amino group, and seven secondary OH groups. Due to lack of any chromophore in the molecule, direct HPLC of amikacin is not straightforward. Chemical derivatization can modify drugs as detector-oriented derivatives by reacting with a reagent to enhance detec-

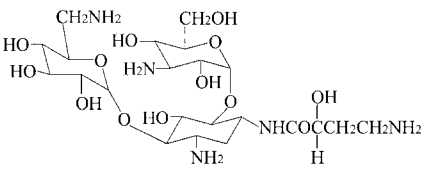

Figure 1. Structure of amikacin.

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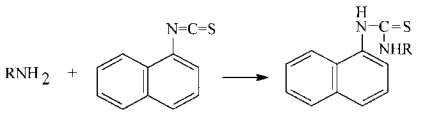

1- naphthyl isothiocyanate

Figure 2. Putative reaction scheme between amikacin and NITC.

tion sensitivity. To ensure amikacin has high UV absorption and enhanced chromatographic characteristics, derivatization is often applicable. Commonly used reagents are 2,4,6-trinitrobenzene sulfonic acid [22–24], 1-fluoro-2, 4-dinitrobenzene [25–27] and *o*-phthaldehyde [28–34]. However, the derivatives obtained with these agents were unstable.

In this paper, a simple plasma pretreatment and sensitive HPLC method is described for trace analysis of amikacin in human plasma. The method is based on chemical derivatization of amikacin with NITC in pyridine and the use of methylamine to eliminate excess NITC after derivatization. The proposed method also can be applied to other aminoglycoside antibiotics in clinical drug monitoring.

Experimental

Chemicals and Reagents

Amikacin was from Sigma (St. Louis MO, USA). 1-Naphthyl isothiocyanate (NITC) was from TCI (Tokyo, Japan). Naphthalene (internal standard, I.S.), methylamine and pyridine were from E. Merck (Darmstadt, Germany). Acetonitrile and other reagents were of analytical-reagent grade. Blank plasma from healthy donors was obtained from the Department of Transfusion Medicine, University of Kaohsiung Medicine. Solutions of amikacin at various concentrations were prepared by dissolving a suitable amount of amikacin in deionized water. The derivatizing agent, NITC, was fresh prepared in pyridine. Methylamine was prepared in acetonitrile.

HPLC Conditions

A Waters 717 plus autosampler, Model 486 UV-Vis detector, Beckman programmable solvent module 126 pump and system Gold software were used. A LiChroCART Rp_{18} column (55 × 4 mm; 3 μm) and water-acetonitrile (57:43, *v/v*) at 0.8 mL min^{-1} were used. The column eluate was monitored at 230 nm. The solvent was filtered through a Millipore, HVLP, 0.45 μm filter under vacuum for degassing before use.

Sample Preparation and Derivatization Procedures

Amikacin spiked plasma at various concentrations was prepared as follow: 0.4 mL human plasma was pipetted into a 10 mL glass-stoppered test tube, and 0.1 mL of an aqueous solution containing various amounts of amikacin were added to each tube to prepare final amikacin concentrations in plasma sample over the range 10–52 nmol mL^{-1}. Tubes were mixed for 10 s. A 0.5 mL sample of acetonitrile was added and mixed by vortexing for 1 min. Tubes were then centrifuged (1000 g) for 10 min. A 0.8 mL sample of supernate was transferred to a 10 mL glass-stoppered test tube and 0.3 mL NITC pyridine solution (80 mM) and 0.1 mL of naphthalene pyridine solution (1.25 mM) added. The reaction mixture was shaken for 1 h at 70 °C in a thermostatted water bath. After reaction, 0.1 mL methylamine acetonitrile solution (0.5 M) was added and the reaction mixture shaken for 5 min at 70 °C to eliminate excess derivatizing agent, then centrifuged at 1000 g for 5 min. A 10 μL aliquot of the supernate was injected into the HPLC for analysis.

Precision and Accuracy Test

The reproducibility and reliability of the proposed method were determined by extracting the amikacin from plasma, spiked at four different levels (10, 20, 30 and 52 nmol mL^{-1}), then treated according to the procedure described under Sample Preparation and Derivatization Procedure.

Results and Discussion

The structure of amikacin is shown in Figure 1 (see Introduction also). Since it lacks a chromophore 1-naphthyl isothiocyanate (NITC) was chosen as derivatization reagent for its high UV sensitivity. NITC can react with primary and secondary amines and alcohols. The reactivity of this agent towards primary amino groups seems greater than towards other groups. Primary amino groups can be added to isothiocyanate to give isothiourea derivatives hence the primary amino groups on amikacin react with NITC by addition to form naphthyl isothioureas. The putative reaction scheme for amikacin with NITC is illustrated in Figure 2. After derivatization, methylamine was added to eliminate excess NITC and stop further addition reactions. The effect of the tested parameters on the extraction-derivatization of amikacin was evaluated from the peak area ratios of the resulting derivative to the naphthalene (I. S.).

Effect of Organic Solvents

Because of the high polarity of aminoglycoside antibiotics, we considered using water-miscible organic solvents, e. g. N, N-dimethylformamide, dimethylsulfoxide, acetonitrile and pyridine for derivatization of extracted amikacin from spiked plasma. The solvent effect on the yield of amikacin derivative was studied according to the Derivatization Procedures. To prevent solvents from boiling, reaction temperatures were set lower than the respective boiling point of the solvents tested. Derivatization of amikacin with NITC in N, N-dimethylformamide or dimethylsulfoxide as reaction solvent leads to a more complicated chromatogram with interfering peaks. The solubility of NITC in acetonitrile is inadequate and a fronting peak of the amikacin derivative was obtained. Peak fronting is sometimes indicative of incomplete derivatization and hence failure of the assay method. The solubility of NITC in pyridine is very good. When pyridine is used as reaction solvent, complete derivatization of amikacin is assured. In this system, pyridine serves a dual role as a medium to maintain a basic reaction environment for the addition reaction and as a co-solvent for the non-polar amikacin-naphthyl adduct. It can improve the yield of amikacin-naphthyl adduct and ensure symmetrical chromatographic peaks. The results indicated that pyridine is the best solvent for derivatization of amikacin in human plasma.

Effect of Amount of Derivatizing Agent

To optimize the amount of derivatizing agent for amikacin in spiked plasma (52 nmol mL^{-1}), different amounts of NITC were examined. Over the range: 6–30 μmol NITC (0.3 mL, 20–100 mM) for 1 h at 70 °C required for derivatization to a maximum formation of the amikacin derivative is shown in Figure 3. The formation of the amikacin derivative increased with increasing derivatizing agent. Maximum formation of the derivative is attainable using NITC in amounts > 18 μmol. An excess amount of 24 μmol of NITC was used to compensate for possible consumption of derivatizing agent by water or other components of the human plasma. The result indicates that a suitable molar ratio of NITC to amikacin ≤ 350 is needed.

Effects of Reaction Temperature and Time

Amikacin has several reactive sites. It will give rise to different adducts at different reaction temperatures and reaction times, resulting in multiple peaks. Hence, after reaction, methylamine-acetonitrile solution was added to eliminate excess derivatizing agent to prevent problems in evaluating temperature and time effects on amikacin derivative formation. The effect of reaction time at 50 °C and 70 °C on derivatization of amikacin from spiked plasma (52 nmol mL^{-1}) is shown in Figure 4. At 70 °C, the formation of the amikacin derivative reached equilibrium in 1 h; whereas at 50 °C, equilibrium was not attained in 2 h and resulted in lower yield of derivative. A more complicated chromatogram was observed at a higher reaction temperature: 80 °C, but the yield of amikacin derivative was the same as at 70 °C in 1 h. Therefore, the reaction time and temperature were set at 1 h and 70 °C, respectively.

Elimination of Excess Derivatizing Agent

A large excess of derivatizing agent was usually used to speed up the derivatization reaction and achieve maximum formation of the derivative. A broad and tailing peak from the excess NITC, with a retention time > 40 min, was found. Methylamine and dimethylamine were examined to eliminate excess reagent after derivatization. Methylamine was better than dimethylamine. A more polar compound than the derivatizing agent was formed during treatment of the reaction mixture with methylamine acetonitrile solution at the end of derivatization. The resulting product of NITC and methylamine, with its more polar character, was eluted completely from a reversed-phase column prior to the amikacin derivative. Therefore, methylamine can stop derivatization of amikacin with NITC, simplify the chromatogram and shorten analysis time. Chromatograms obtained for amikacin extracted from plasma under optimized conditions are shown in Figure 5 (A) and (B). Figure 5 (A) shows the chromatogram of the plasma blank and Figure 5 (B) shows the chromatogram of amikacin extracted from spiked human plasma, respectively, and then derivatized. Peak a and peak b in Figure 5 represent the amikacin derivative and the I. S., respectively. There was no interference from the reagent blank with the resolution of the peak of amikacin derivative. The selectivity of the method was studied by spiking standard amikacin with other aminoglycoside antibiotics, including kanamycin A and tobramycin. The spiked plasma was analyzed according to procedure described under Sample Preparation and Derivatization Procedures sections. The amikacin-naphthyl derivative could be resolved from those of the other drugs, indicating that other aminoglycoside antibiotics did not interfere with the HPLC analysis of amikacin in this study. In consequence, the proposed method is specific and feasible for the analysis of amikacin in plasma for biological study or therapeutic drug monitoring.

Stability of the Amikacin Derivative

The relative stability of the amikacin derivative to I. S. at room temperature after derivatization and methylamine treatment was studied over a period of 14 h. No significant change in the ratio of the peak area of the derivative to I. S. was found, indicating that the derivative is stable enough in the reaction mixture for routine LC analysis.

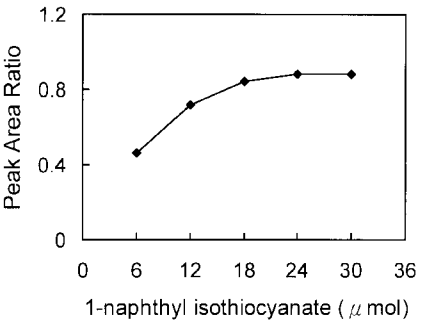

Figure 3. Effect of amount of 1-naphthyl isothiocyanate on formation of amikacin derivative. Reactions at 70 °C for 1 h with 6–30 μmol 1-naphthyl isothiocyanate pyridine derivatizing agent.

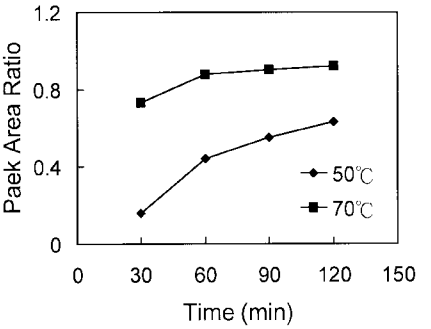

Figure 4. Effect of reaction temperature and reaction time on formation of amikacin derivative. Reactions were at 50 °C and 70 °C at varied reaction times with 0.3 mL 80 mM (24 μmol) 1-naphthyl isothiocyanate pyridine solution derivatizing agent.

Analytical Calibration

On the basis of the optimized conditions, we formulated an analytical procedure for amikacin determination as described under Experimental. To validate quantitative application of the method, four different concentrations of amikacin over the range 10–52 nmol mL^{-1} were evaluated. The calibration graph was established with the peak-area ratio of the derivative to I. S. as ordinate y; the amount of amikacin in nmol as abscissa x and the correlation coefficient as r. The linear regression equations were obtained as follows: $y = (-0.0498 \pm 0.0055) + (0.0180 \pm 0.0002)\ x$ for intra-day assay ($n = 6$, $r = 0.998$) and $y = (-0.0404 \pm 0.0087) + (0.0177 \pm 0.0003)\ x$ for inter-day assay ($n = 6$, $r = 0.998$). The precision (relative standard deviation, R. S. D.) of the slope of the calibration graphs for intra-day and inter-day analysis is always < 1.7%. The data indicate good linearity of the method. The detection limit (signal to noise ratio = 3) of amikacin was 1 nmol mL^{-1} in a 10 μL injection. The stability of amikacin in spiked plasma under

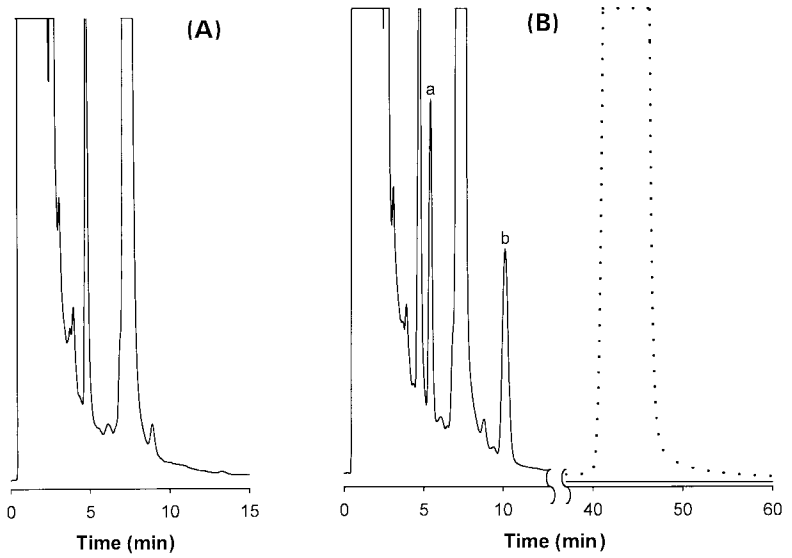

Figure 5. LC chromatograms for determination of amikacin in human plasma. (A) Plasma blank. (B) Amikacin in human plasma, with (solid line) and without (dashed line) methylamine treatment, after derivatization. Peaks: (a) amikacin derivative; (b) naphthalene (I. S.). LC conditions: LiChroCART Rp_{18} column (55 × 4 mm I.D.; 3 µm); mobile phase, water-acetonitrile (57:43, *v/v*); 0.8 mL min^{-1}; detection, 230 nm.

Table I. Precision and accuracy in analysis of amikacin-spiked human plasma.

Concentration known (µM)	Concentration found (µM)	R. S. D. (%)	R. E. (%)
Intra-day* (n = 6)			
52.00	52.68 ± 0.94	1.78	+1.31
30.00	28.87 ± 0.68	2.37	−3.76
20.00	19.55 ± 0.38	1.96	−2.28
10.00	10.96 ± 0.11	1.04	+9.55
Inter-day* (n = 6)			
52.00	52.62 ± 0.98	1.87	+1.20
30.00	29.73 ± 1.61	5.41	−0.91
20.00	19.08 ± 0.68	3.59	−4.59
10.00	10.83 ± 0.63	5.80	+8.29

* Intra-day data based on six replicate analyses and inter-day from six consecutive days.

Table II. Relative recoveries of amikacin in human plasma.

Sample	Amount spiked (µM)	Amount found[a] (µM)	Recovery (%)
1	0	N. D.	N. D.
	50.00	50.68 ± 0.55	101.36
	35.00	32.06 ± 0.95	91.60
	10.00	10.25 ± 0.36	102.50
2	0	N. D.	N. D.
	50.00	48.59 ± 0.87	97.18
	35.00	34.56 ± 0.85	98.74
	10.00	9.80 ± 0.52	98.00
3	0	N. D.	N. D.
	50.00	50.15 ± 0.42	100.30
	35.00	35.05 ± 0.30	100.14
	10.00	10.24 ± 0.25	102.40
4	0	N. D.	N. D.
	50.00	51.88 ± 0.64	103.76
	35.00	36.35 ± 0.53	103.86
	10.00	10.76 ± 0.08	107.60

[a] Mean ± S.D. of triplicate analyses. N. D. = not detected.

storage was also examined. Three different concentrations of amikacin at 10, 30 and 50 nmol mL^{-1} in spiked plasma were studied to assess the stability of the amikacin at 40 °C ± 2 °C. For each sample, determination of plasma amikacin was performed on days 0, 7, and 21. Statistical analysis of the results did not show any significant difference; therefore, amikacin is stable in plasma samples stored at 40 °C ± 2 °C for periods up to 21 days. This indicates favorable stability of amikacin in human plasma for analyses.

Precision and Accuracy

The reproducibility and reliability of the proposed method were assessed at three different concentrations of amikacin and evaluated as relative standard deviation (R. S. D.) and relative recovery, respectively. As shown in Table I, the precision of the method for amikacin spiked in human plasma was < 5.8% R. S. D. for both intra-day and inter-day assays. The relative recovery of amikacin, as shown in Table II, is > 91%, obtained from the calibration graph of plasma spiked with different amount of amikacin over the range 10–52 nmol mL^{-1}.

Conclusion

A simple and sensitive HPLC method based on the pre-column derivatization of amikacin in human plasma with 1-naphthyl isothiocyanate has been established and optimized. Validation of the method for quantitation of amikacin in spiked plasma showed that the method has high accuracy. The detection limit (signal-to-noise ratio, 3; injection volume, 10 µL) is ca. 1 nmol mL^{-1}. Further application of the method to the sensitive analysis of other aminoglycoside antibiotics is being carried out.

Acknowledgement

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