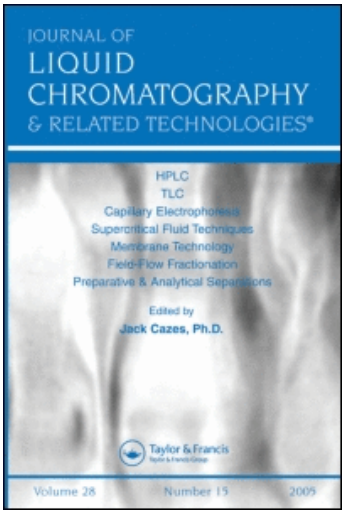

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TRACE ANALYSIS OF AMIKACIN IN COMMERCIAL PREPARATION BY DERIVATIZATION AND HPLC

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TRACE ANALYSIS OF AMIKACIN IN COMMERCIAL PREPARATION BY DERIVATIZATION AND HPLC

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ABSTRACT

A simple and sensitive liquid chromatographic method has been developed for the determination of amikacin by derivatization. The method is based on the derivatization of amikacin with derivatizing agent, 1-naphthoyl chloride, in pyridine at 30°C for 1 h. After derivatization reaction, a dimethylamine acetonitrile solution was added to the reaction mixture to eliminate the excess derivatizing agent. The derivative was analysed by HPLC on a Delta-Pak C_4 column with water-acetonitrile (15:85, v/v) as the mobile phase and detection at 295 nm. The parameters affecting the derivatization of amikacin, including reaction temperature, reaction time, and the amount of derivatizing agent, were investigated. The linear range of the method for the determination of amikacin was over 17-170 nmol/mL; the detection limit (signal to noise ratio = 5; injection volume, 20 μL) was 5 nmol/mL. Application of the method to the analysis of amikacin in commercial injections has proved satisfactory.

INTRODUCTION

Amikacin, an aminoglycoside antibiotic, is commonly administered parenterally for the treatment of gram-negative infections resistant to gentamicin, kanamycin, or tobramycin. Like the other aminoglycosides, amikacin has a comparably narrow safety margin. It's therapeutic plasma concentration is in the range of 8 to 16 μg/mL. Amakacin may cause both ototoxicity and nephrotoxicity in the patients with impaired renal function especially for a long-term therapy (1–3). Therefore, it is quite essential to assure the potency and content uniformity of amikacin in the pharmaceutical preparations.

Various methods have been reported for the determination of amikacin in various matrices including microbiological (4,5), high performance liquid chromatography (HPLC) (6–20), radioenzymatic (21,22), and immunoassays (23–26). The microbiological assay is inexpensive and simple, but may lack sensitivity and specificity because of interferences by other antimicrobial agents. The enzymatic and immunoassays can be specific and accurate, but they depend on the purity of the enzyme and the specificity of the antibodies. Cross-reactions are sometimes noted with the immunoassay kits. HPLC is the most widely used and accurate technique for the analysis of the aminoglycoside antibiotics in various formulations.

The structural formula of amikacin, shown in Figure 1, indicates that amikacin carries four primary amines, one primary OH group, one secondary amine, and seven secondary OH groups. Direct HPLC method for amikacin is not straightforward because the drug does not have a strong UV absorbing chromophore. Chemical derivatization can modify drugs to give efficient absorption in UV or visible wavelength and attain highly sensitive and selective determination of drugs by using HPLC. Derivatization with dansyl chloride (7), fluorescamine (8), 1-fluoro-2,4-dinitrobenzene (9–11), *o*-phthalaldehyde (12–17), 2,4,6-trinitrobenzene sulfonic

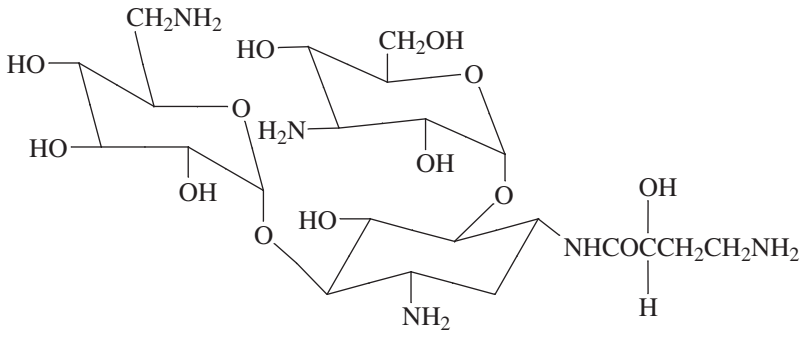

Figure 1. Chemical structure of amikacin.

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acid (18,19), and 3,5-dinitrobenzoyl chloride (20) have been described. However, it have been mentioned that derivatization with *o*-phthalaldehyde and 1-fluoro-2,4-dinitrobenzene resulted in unstable derivatives.

This work presents a simple, sensitive HPLC method for the determination of amikacin. The method is based on the chemical derivatization of amikacin with 1-naphthoyl chloride in pyridine. The applicability of the method to the analysis of amikacin in the commercial injections was also examined.

EXPERIMENTAL

Chemicals and Reagents

Amikacin sulfate, tobramycin sulfate, kanamycin sulfate, and vitamin K_1 (Sigma, St. Louis, MO, USA), 1-naphthoyl chloride (TCI, Tokyo, Japan), dimethylamine, pyridine, molecular sieves, and potassium hydroxide (E. Merck, Darmstadt, Germany), acetonitrile and other reagents were of analytical-reagent grade. Solutions of amikacin at various concentrations were prepared by dissolving a suitable amount of amikacin sulfate in 120 μL of alkaline solution and then diluted with pyridine to 25 mL because of its solubility. 1-Naphthoyl chloride (derivatizing agent) was also prepared in pyridine. Solutions of vitamin K_1 (internal standard, I.S.) and dimethylamine were prepared in acetonitrile.

HPLC Conditions

A Waters-Millipore LC system with a U6K injector and a Model 486 UV-Vis detector was used. A Delta-Pak C4 column (3.9 $\times$ 150 mm; 5 μm) and a mixed solvent of water-acetonitrile (15 : 85, v/v) at a flow-rate of 1.0 mL/min were used. The column eluate was monitored at 295 nm. The solvent was filtered with filter (Millipore, HVLP, 0.45 μm) under vacuum for degassing before use.

Optimization of the Derivatization Procedures

In order to establish the optimum conditions for amikacin analysis, the parameters affecting the derivatization of amikacin, including the amount of derivatizing agent, reaction temperature, and time, were studied. For the investigation, 170 nmol/mL of amikacin was used. The effects of those parameters were evaluated by peak-area ratio of the derivative to vitamin K_1 (I.S.).

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Optimized Derivatization Procedures

A 0.1 mL volume of amikacin solution was added to a 10-mL glass-stoppered test tube containing 0.1 mL of 2.2 M 1-naphthoyl chloride pyridine solution. The reaction mixture was shaken mechanically at 30°C in a thermostated water bath for 1h. At the end of the reaction, a 0.1 mL containing 1.5 M dimethylamine acetonitrile solution and 0.2 mL of 0.67 mM vitamin K_1 (I.S.) acetonitrile solution were added and mixed well. Then a 10 μL aliquot of the solution was analyzed by HPLC with UV detection at 295 nm.

Sample Preparation Procedure

Ten vials of amikacin injection (labeled amount 125 mg/mL) from a commercial source were accurately measured, separately. A quantity of the injections, equivalent to about 0.24 mmol of amikacin, was placed in a 10 mL volumetric flask containing 4.8 mL of 0.2 M KOH for neutralization and then diluted with water to volume. A 120 μL aliquot of the diluted solution was pipetted into a 25 mL volumetric flask and pyridine was added to volume. A 0.1 mL of the amikacin pyridine solution was pipetted into a 10 mL glass-stoppered test tube and derivatized by the procedure described under the Optimized Derivatization Procedures.

RESULTS AND DISCUSSION

There are four primary amines, one primary OH group, one secondary amine, and seven secondary OH groups in an amikacin molecule. In order to enhance the detection sensitivity, chromophoric groups might be introduced onto the NH_2 groups of the primary amine in amikacin with a derivatizing agent under a mild reaction condition to avoid the hydrolysis, degradation of the molecule, or production of many kinds of the adducts. As nucleophiles, the reactivity to these functional groups in the order is 1° NH_2 > 1° OH > 2° NH > 2° OH. A nucleophilic substitution takes places under a mild condition, which can't cause hydrolysis or degradation of the aminoglycoside antibiotics. We predicted that 1-naphthoyl chloride might only substitute all the 1° NH_2 groups due to reactivity and steric hindrance. A number of chromatographic parameters were investigated to optimize the separation in the shortest times. The composition of the mobile phase, stationary phase, and detection wavelength were varied to achieve optimum chromatographic and sensitive conditions. The optimized separation conditions were set as Experimental HPLC Condition. The effect of the tested parameters on the derivatization of amikacin was evaluated by the peak-area ratio of the resulting derivative to the vitamin K_1 (I.S.).

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Reaction Solvent

Because of the high polarity of aminoglycoside antibiotics, the solvent system commonly employed to solubilize them often contains large portions of water and alcohol which are not suitable in this case for derivatization. We considered using acetonitrile, N,N-dimethylformamide, dimethylsulfoxide, and pyridine as the reaction solvent. Dimethylsulfoxide can solubilize aminoglycoside antibiotics but it may react with 1-naphthoyl chloride. The solubility and reactivity of amikacin in acetonitrile and N,N-dimethylformamide are very bad, so they can't be used as the reaction solvent in this case. As a weak base ($Kb = 2.3 \times 10^{-9}$), pyridine can neutralize the hydrochloric acid that is generated during derivatization reaction; otherwise the accumulation of hydrochloric acid will bring about hydrolysis of glycosidic linkages of amikacin. It serves not only as a reaction medium, but also as a nucleophilic catalyst for derivatization reaction. The best solvent for the derivatization was found to be pyridine, amongst acetonitrile, N,N-dimethylformamide, dimethylsulfoxide, and pyridine.

Effect of the Amount of Derivatizing Agent

To optimize the amount of derivatizing agent for the derivatization of 170 nmol/mL of amikacin, different amounts of 1-naphthoyl chloride over a range 1.0-2.5 M under 1 h at 30°C, were examined. Figure 2 shows the effect of concentration of 1-naphthoyl chloride on the derivatization. The peak area of the amikacin-naphthoyl adduct was increased with an increase of 1-naphthoyl chloride, and

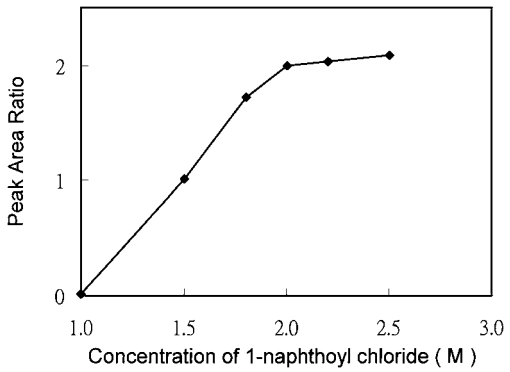

Figure 2. Effect of the amount of 1-naphthoyl chloride on the formation of the amikacin derivative. Reactions were carried out at 30°C for 1 h with 0.1 mL of 1.0–2.5 M 1-naphthoyl chloride pyridine solution as derivatizing agent.

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became constant in the range of 1-naphthoyl chloride concentrations of more than 2 M. An excess amount of 1-naphthoyl chloride, about 2.2 M, was used to compensate for possible consumption of the derivatizing agent by a small amount of water or coexisting components from the amikacin commercial injections in further applications.

Effects of Reaction Time and Reaction Temperature

The effects of reaction time at 30°C, 50°C, and 70°C on the derivatization of amikacin (170 nmol/mL) are shown in Figure 3. For derivatization at 30°C, a derivative of amikacin was observed and the highest yield of the derivative of amikacin was obtained in 60 min. After 90 min, other derivatives were produced and the major product (peak a in Figure 4) was decreased. The same situation occurred at 50°C and 70°C. A varying number of derivatized sites will cause different adducts for the same analyte under different reaction temperature and reaction time, resulting in multiple peaks. So after reaction, a dimethylamine acetonitrile solution was added to eliminate the derivatizing agent in order to prevent this complicated situation. A simpler HPLC chromatogram can be obtained for the derivatization of amikacin with 1-naphthoyl chloride under low reaction temperature rather than high temperature. Therefore, the reaction temperature and reaction time for the derivatization of amikacin was set at 30°C for 1 h. From the results, the concentration of derivatizing reagents, reaction time, and reaction temperature were fixed as follows: 2.2 M of 1-naphthoyl chloride, 1h and 30°C, respectively.

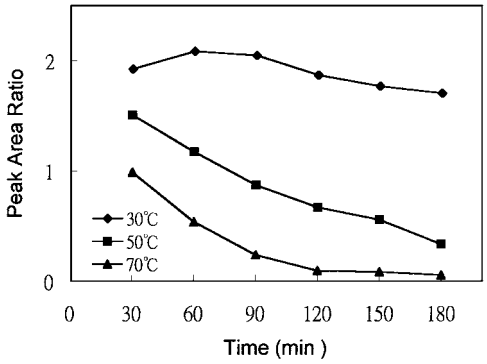

Figure 3. Effect of reaction temperature and reaction time on the formation of the amikacin derivative. Reactions were carried out at 30, 50, and 70°C, at varied reaction times with 0.1 mL of 2.2 M 1-naphthoyl chloride pyridine solution as derivatizing agent.

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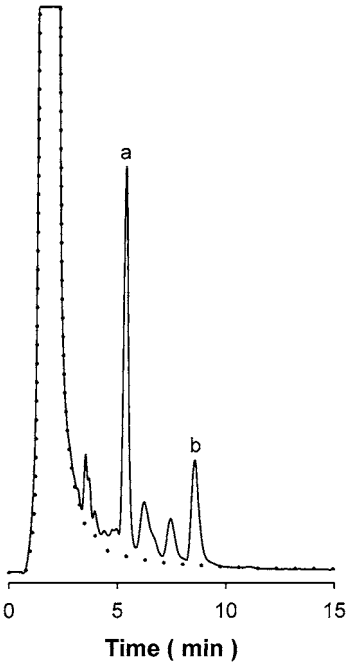

Figure 4. Composite HPLC chromatogram for the determination of amikacin (solid line) and reagent blank (dashed line). Peaks: a = the derivative of amikacin; b = internal standard. LC conditions : column, Delta-Pak C_4 (3.9 × 150mm; 5μm); mobile phase, water-acetonitrile (15 : 85, v/v); flow-rate, 1.0 mL/min; UV detection, 295 nm.

For this condition, the derivatization procedure was optimized to yield a maximum and a constant peak area of the derivative. The derivative of amikacin and I.S. were well-resolved using reversed-phase liquid chromatography with retention times of 5.1 and 8.3 min, respectively, as shown in Figure 4. The excess of the derivatizing agent and degradation products of the reagent were eluted with the solvent front.

Stability of the Amikacin Derivative

The relative stability of the amikacin derivative to I.S. at room temperature after derivatization and dimethylamine treatment was studied over a period of 24 h. No significant change in peak area ratio of the derivative to I.S. was found, indicating that the derivative is stable enough for routine HPLC analysis.

Table 1. Precision and Accuracy for the Determination of Amikacin

Concentration Known (μM)	Concentration (μM)	R.S.D. (%)	R. E. (%)
Intra-Day* **(n = 6)**			
170	170.77 ± 2.94	1.72	0.45
85	84.56 ± 1.70	2.01	−0.52
34	34.31 ± 0.57	1.67	0.91
17	17.24 ± 0.26	1.48	1.41
Inter-Day* **(n = 8)**			
170	170.63 ± 0.83	0.49	0.37
85	83.28 ± 2.30	2.76	−2.02
34	33.66 ± 0.71	2.11	−1.00
17	18.18 ± 1.05	5.75	6.94

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

Analytical Calibration

On the basis of the optimized conditions, we formulated the analytical procedure for amikacin determination as described in the Experimental Section. To validate the quantitative application of the method, four different concentrations of amikacin over the range 17–170 nmol/mL were evaluated. The calibration graph was established with the peak-area ratio of the derivative to I.S. as ordinate (y) vs. the amount of amikacin in nmol as abscissa (x). The linear regression equations were obtained as follows: $y = (-0.0723 \pm 0.0115) + (0.0129 \pm 0.0003)\ x$ for intra-day assay ($n = 6$, $r = 0.999$) and $y = (-0.0899 \pm 0.0175) + (0.0125 \pm 0.0005)\ x$ for inter-day assay ($n = 8$, $r = 0.999$). The results indicate that high linearity between y and x is attainable over the range studied. The lower detection limit is about 5 nmol/mL based on signal-to-noise ratio of 5. The relative standard deviation (R.S.D.) of the method based on the peak-area ratio for replicate determination of amikacin at 170, 85, 34, and 17 nmol/mL was studied. The results in Table 1 indicate that the intra-day R.S.D. ($n = 6$) of the analyte at four concentration levels was all below 2.1%; in parallel, the inter-day R.S.D. ($n = 8$) for the analyte at four concentration levels was all below 5.8%.

Selectivity of the Method

The selectivity of the method was briefly tested on the separation of a standard mixture of aminoglycoside antibiotics such as amikacin, tobramycin, gentamicin,

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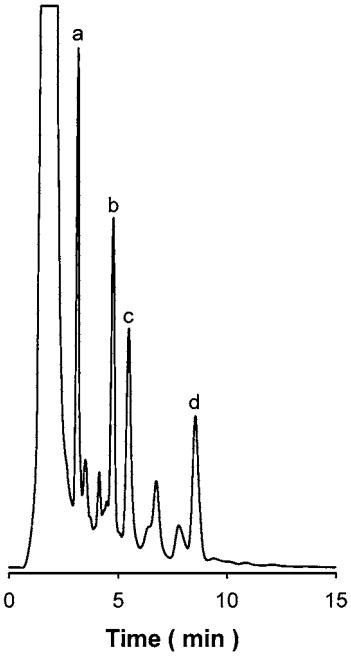

Figure 5. HPLC chromatogram for a standard mixture of three aminoglycosides, each at 170 μM. Peaks: a = tobramycin; b = kanamycin A; c = amikacin and d = vitamin K_1 (I.S.). LC conditions were the same as in Figure 4.

and kanamycin A, each at 17 nmol in 0.1 mL solution (170 nmol/mL). The aminoglycoside antibiotics mixture was derivatized according to the Optimized Derivatization Procedure. Under present HPLC conditions, a complete separation of all the aminoglycoside antibiotics tested was obtained as shown in Figure 5. None of the other commonly used aminoglycoside antibiotics (tobramycin, gentamicin and kanamycin A) were found to interfere in the procedures developed for amikacin.

Application

The proposed method was applied to the determination of amikacin in commercial injections of the content uniformity test usually required by an official pharmacopoeia. The results of amikacin in commercial injections are shown in Table 2. All the analytical values for amikacin fell within the range of 91-98% of the labeled content, which are acceptable by Pharmacopoeia for injection formulation. The chromatogram for analysis of amikacin in injection is shown in Figure 6.

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Table 2. Analytical Results for Content Uniformity of Amikacin Injections Obtained from Commercial Source

Injection[a]	Concentration Found[b] (mg/mL)	Percentage of Claimed Content[c] (%)
1	118.8 ± 12.1	95.0
2	122.4 ± 9.1	97.9
3	121.6 ± 13.0	97.3
4	121.9 ± 11.6	97.5
5	113.3 ± 13.7	90.6
6	113.7 ± 14.5	91.0
7	117.0 ± 7.1	93.6
8	120.7 ± 6.4	96.6
9	117.7 ± 9.6	94.2
10	114.9 ± 9.8	91.9
	Mean (%)	94.6
	S.D.	2.8

[a]Labeled concentration of amikacin in each injection is 125 mg/mL. [b]Mean ± S.D. of three replicate analyses. [c]Content uniformity test is used to check the variation of amikacin in each injection.

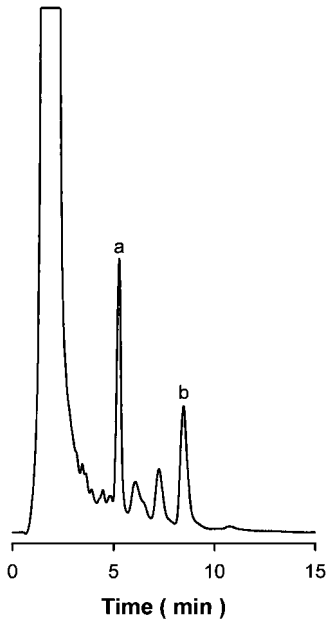

Figure 6. HPLC chromatogram for the determination of amikacin in injection. Peaks: a = the derivative of amikacin; b = internal standard.

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In conclusion, a simple and sensitive HPLC method based on the derivatization of amikacin with 1-naphthoyl chloride in pyridine has been established and optimized. Validation of the method for quantitation of amikacin showed that the method has high accuracy. The application of the method to commercial amikacin injections has proven satisfactory.

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