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Journal of Chromatography B, 780 (2002) 349–354

JOURNAL OF CHROMATOGRAPHY B

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Trace analysis of tobramycin in human plasma by derivatization and high-performance liquid chromatography with ultraviolet detection

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Received 3 April 2002; received in revised form 15 July 2002; accepted 8 August 2002

Abstract

A simple and sensitive high-performance liquid chromatographic (HPLC) method is established for the trace determination of tobramycin in human plasma by derivatization. The method is based on the chemical derivatization of aminoglycoside antibiotic, tobramycin in human plasma, with 1-naphthyl isothiocyanate (NITC) in pyridine at 70 °C. After derivatization reaction, a methylamine/acetonitrile solution was added to the reaction mixture to eliminate the excess derivatizing agent and shorten the analysis time. The resulting derivative was separated using a Purospher® STAR RP-18e column and a water–acetonitrile (50:50, v/v) mobile phase (detection at 230 nm). Optimization conditions for the derivatization of tobramycin were investigated by HPLC. The linear range for the quantitation of tobramycin in spiked plasma was over 0.93–9.34 mg/l; the detection limit (signal-to-noise ratio=3; injection volume, 10 μl) was about 0.23 mg/l. The relative standard deviation was less than 2.1% for intra-day assay (n=6) and 5.2% for inter-day assay (n=6) and relative recoveries were found greater than 99%.

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Keywords: Tobramycin

1. Introduction

Tobramycin, an aminoglycoside antibiotic, is commonly administered parenterally for the treatment of serious gram-negative bacillary infections. The bactericidal activity of tobramycin is accomplished by binding irreversibly to 30*S* and 50*S* ribosomal subunits resulting in a defective protein. Like the other aminoglycosides, tobramycin has a comparably narrow safety margin. The therapeutic plasma concentration of tobramycin is in the range of 4–8 mg/l and may cause severe ototoxicity and nephrotoxicity in a long-term therapy [1–3]. Therefore, the monitoring of tobramycin levels in plasma is required for therapeutic and toxic control.

For the measurement of tobramycin concentrations, immunoassays [4–13], spectrophotometric [14–17] and chromatographic [18–30] methods have been introduced. The fluorescence polarization immunoassay (FPIA) [8–13] and HPLC [18–30] are the most widely used techniques for the analysis of tobramycin in various matrices. In FPIA, cross-re-

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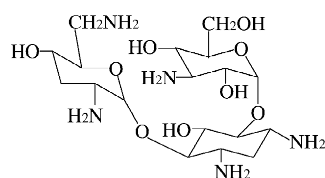
PII: S1570-0232(02)00544-5

actions are sometimes noted and the reagents are expensive. The immunoassay method also would be inappropriate for determination of the potential presence of degradation product or impurities.

The structure of tobramycin, shown in Fig. 1, indicates that tobramycin has five primary amines, one primary OH group and four secondary OH groups. Due to the low chromophore in the molecule, direct HPLC method for tobramycin is not straightforward. In order to increase the UV absorptivity of the tobramycin molecule, a derivatization is often applied. The commonly used derivatizing agents are 3,5-dinitrobenzoyl chloride [21], 1-fluoro-2,4-dinitrobenzene [22–24], 2,4,6-trinitrobenzene sulfonic acid [25], and *o*-phthalaldehyde [26–29]. However, derivatization with these agents resulted in unstable derivatives or complicated procedures. There are many functional groups in the tobramycin molecule. In order to enhance the detection sensitivity and obtain a stable derivative, chromophoric groups were introduced onto the primary amino groups of tobramycin via a derivatizing agent. The reaction took place under mild conditions to avoid hydrolysis and/or degradation of the molecule. Owing to the fact that primary amino groups can be added to isothiocyanate to give a stable isothiourea derivative, 1-naphthyl isothiocyanate (NITC) was chosen as the derivatizing agent.

In this paper, a simple plasma pretreatment and sensitive HPLC method is described for the trace analysis of tobramycin in human plasma. We used 1-naphthyl isothiocyanate (NITC) as the derivatizing agent and methylamine to eliminate excess NITC after derivatization. The proposed method can also be applied to other aminoglycoside antibiotics in clinical drug monitoring studies.



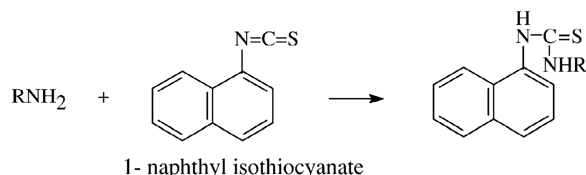


Fig. 1. Putative reaction scheme for tobramycin with NITC.

2. Experimental

2.1. Chemicals and reagents

Tobramycin was purchased from Sigma (St Louis, MO, USA). 1-Naphthyl isothiocyanate (NITC) and anthracene (internal standard, I.S.) were from TCI (Tokyo, Japan). Methylamine and pyridine were purchased from 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 tobramycin at various concentrations were prepared by dissolving a suitable amount of tobramycin in deionized water. The derivatizing agent, NITC, was freshly prepared in pyridine (80 m*M*). Methylamine was prepared in acetonitrile (0.48 *M*).

2.2. HPLC conditions

A Waters 717 plus autosampler, a Model 486 UV–Vis detector, a Beckman system Gold programmable solvent module 126 pump and system Gold software were used. A Purospher® STAR RP-18e column (55×4 mm; 3 μm, Merck) and water–acetonitrile (50:50, v/v) at a flow-rate of 1.3 ml/min were used. The column eluate was monitored at 230 nm. The solvent was filtered (Millipore, HVLP, 0.45 μm) under vacuum for degassing before use.

2.3. Sample preparation and derivatization procedure

A 0.4-ml volume of human plasma was pipetted into a 10-ml glass-stoppered test-tube, and 0.1 ml of aqueous solution containing various amounts of tobramycin was added to each tube to prepare final tobramycin concentrations in plasma samples over the range of 0.93–9.34 mg/l. The tubes were mixed

for 10 s. A 0.5-ml aliquot of acetonitrile was added and mixed by vortexing for 1 min. The tubes were then centrifuged (1000 *g*) for 10 min. A 0.8-ml aliquot of supernatant was transferred to a 10-ml glass-stoppered test-tube. Then 0.3 ml of NITC (derivatizing agent)/pyridine solution (80 m*M*) and 0.1 ml of anthracene/acetonitrile solution (250 μ*M*) (I.S.) were added. The reaction mixture was shaken for 1 h at 70 °C in a thermostated water bath. After reaction, 0.1 ml methylamine/acetonitrile solution (0.48 *M*) was added and the reaction mixture was 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 supernatant was injected into the HPLC system for analysis.

2.4. Precision and accuracy test

The reproducibility and reliability of the proposed method were determined by extracting the tobramycin from plasma, spiked with five different levels of tobramycin (0.93, 2.33, 4.65, 6.98 and 9.34 mg/l), then derivatizing according to the procedure described above. The relative recovery of tobramycin from the plasma was tested by spiking with three different concentrations of tobramycin (1.86, 5.58 and 8.37 mg/l) and treated as described in Section 2.3.

3. Results and discussion

3.1. Chemical derivatization

The structure of tobramycin is shown in Fig. 1. It lacks any chromophores capable of a general and reliable signal in the UV region but does contain functional groups. Because of this, a direct HPLC analysis of tobramycin using UV detection is not straightforward. Chemical derivatization can modify drugs to give efficient absorption in the UV or visible wavelength range. Consequently, a detector-oriented chemical derivatization method was chosen for analysis of tobramycin in plasma. The primary amino groups on tobramycin react with NITC by addition to form naphthyl isothiourea derivatives. The putative reaction scheme for tobramycin with NITC is illustrated in Fig. 1.

3.2. Effect of amount of derivatizing agent

To establish the optimum amount of derivatizing agent, NITC, for conversion of tobramycin in spiked plasma, different amounts of NITC over the range 3–30 μmol NITC (0.3 ml, 10–100 m*M*) in pyridine were reacted with tobramycin (9.34 mg/l, 70 °C, 1 h). The parameter was evaluated by computing the peak area ratio of the resulting tobramycin derivative to the I.S. anthracene. The amount of NITC required for derivatization is shown in Fig. 2. An excess amount of NITC (24 μmol, 0.3 ml, 80 m*M*), was used to compensate for possible consumption of the derivatizing agent by water or coexisting components present in human plasma. Because an excess of derivatizing agent was added to speed up the reaction, a broad NITC peak was found in this study. We used methylamine to remove the excess reagent after derivatization.

3.3. Effects of reaction temperature and time

Tobramycin has many functional groups that can react with NITC. This causes the formation of different adducts under different sets of reaction temperatures and times, resulting in the formation of a complicated chromatogram. The effects of reaction time at 50 and 70 °C on the derivatization of

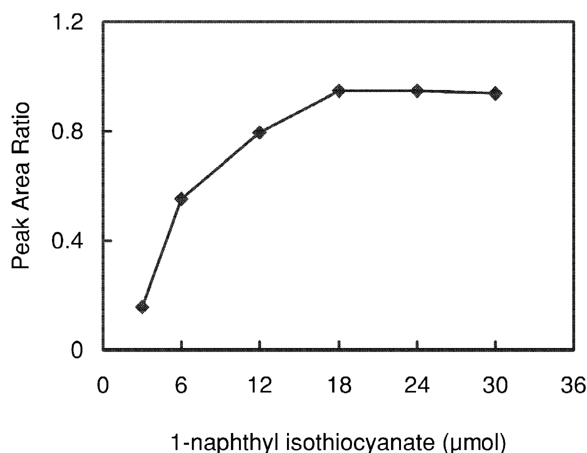


Fig. 2. Effect of the amount of 1-naphthyl isothiocyanate on the formation of the tobramycin derivative. Reactions were carried out at 70 °C for 1 h with 0.3 ml of 10–100 m*M* 1-naphthyl isothiocyanate pyridine solution as derivatizing agent.

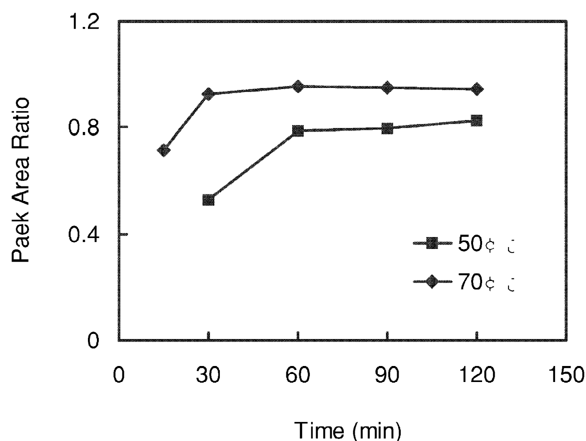


Fig. 3. Effect of reaction temperature and reaction time on the formation of the tobramycin derivative. Reactions were carried out at 50 and 70 °C at varied reaction times with 0.3 ml of 80 m*M* 1-naphthyl isothiocyanate pyridine solution as derivatizing agent.

tobramycin from spiked plasma (9.3 mg/l) is shown in Fig. 3. For derivatization at 70 °C, the formation of the tobramycin derivative reached equilibrium in 0.5 h, whereas the reaction at 50 °C reached equilibrium in 1 h and resulted in a lower yield of the tobramycin derivative compared with 70 °C.

3.4. Selectivity of the method

A typical chromatogram for the analysis of tobramycin extracted from plasma is illustrated in Fig. 4B. Peaks 1 and 2 represent the tobramycin derivative and the I.S., respectively. The large peak and the smaller peak presented by the dashed line in Fig. 4B are NITC and reagent impurity, respectively. They can react with methylamine. So, 48 μmol of methylamine was added to eliminate the excess NITC and its impurity at the end of derivatization. There was no interference from the reagent blank with the tobramycin derivative (Fig. 4A). The selectivity of the method was studied by spiking standard tobramycin solutions with other aminoglycoside antibiotics, including kanamycin A, kanamycin B, gentamycin and amikacin. The spiked plasma was analyzed according to the procedure described above (Section 2.3). The tobramycin-naphthyl derivative could be resolved from those of the other drugs, indicating that other aminoglycoside antibiotics did not interfere with the HPLC analysis of tobramycin in this study. A change in the composition of the mobile phase from water–acetonitrile (50:50, v/v) at a flow-rate of 1.3 ml/min to water–acetonitrile (57:43, v/v) at a flow-rate of 0.8 ml/min allows four aminoglycoside antibiotics to be separately identified in the chromatogram. The elution order is amikacin, kanamycin A, kanamycin B and then tobramycin. Gentamicin was not observed under these conditions. A comparison of the retention times of derivatives of tobramycin and amikacin under these chromatographic conditions suggests that the naphthyl-tobramycin adduct is more hydrophobic than the naphthyl-amikacin adduct. Tobramycin and amikacin have five and four primary amino groups, respectively, that can react with NITC. Cephalosporin antibiotics are usually combined with aminoglycoside antibiotics such as tobramycin for treatment of serious infections. To study the effects of interference, standard cefamandole and cefotaxime solutions (200 mg/l) were spiked in plasma samples that already contained 9.34 mg/l of tobramycin. No additional peak was observed in this chromatogram. This indicates that the proposed method is specific and feasible for the analysis of tobramycin in plasma for therapeutic drug monitoring.

3.5. Analytical calibration

To examine the quantitative application of the proposed method, five different concentrations of tobramycin over the range 0.93–9.34 mg/l were evaluated. The linear regression equations were obtained as follows: $y=(-0.0215\pm0.0040)+(0.1027\pm0.0011)x$ for intra-day assay ($n=6$, $r=0.999$) and $y=(-0.0264\pm0.0051)+(0.1064\pm0.0028)x$ for inter-day assay ($n=6$, $r=0.998$); y is the peak-area ratio of the derivative to I.S., x is the amount of tobramycin in mg/l and r is the correlation coefficient. The data indicate good linearity of the proposed method. The precision (relative standard deviation, RSD) of the slope of the calibration graphs for intra-day and inter-day analysis is 1.0 and 2.6%, respectively. The detection limit (signal-to-noise ratio=3) of tobramycin was 0.23 mg/l in 10 μl of injection. The stability of tobramycin in spiked plasma under storage was also examined. The concentration of tobramycin at 9.34 mg/l in spiked plasma was studied to assess the stability of the

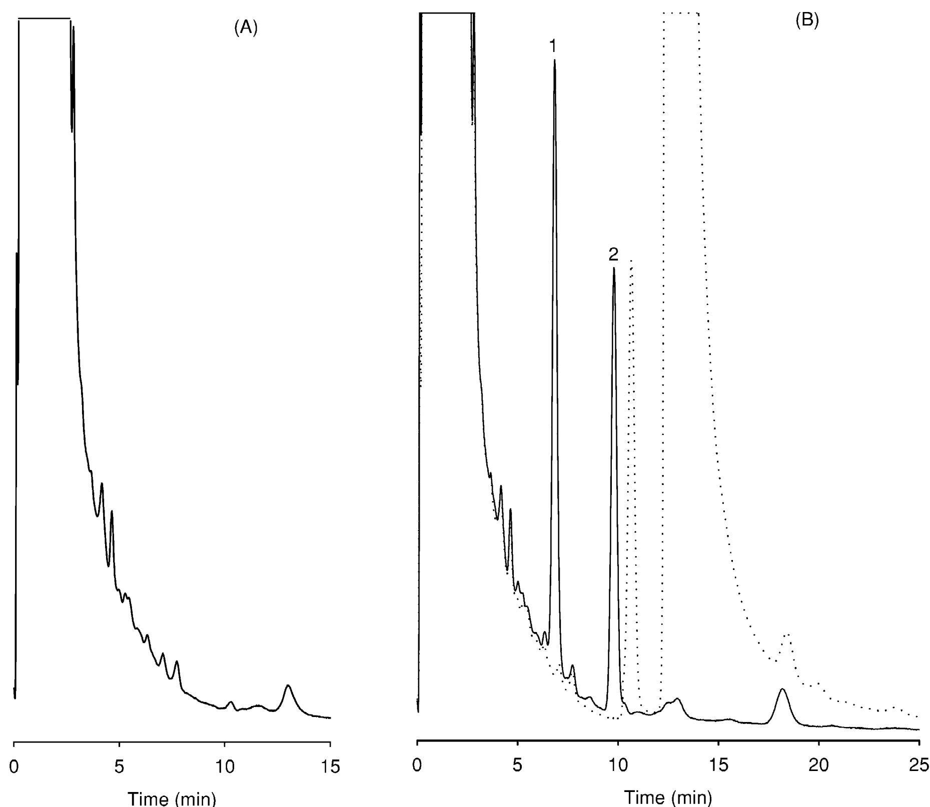


Fig. 4. HPLC chromatograms for determination of tobramycin in human plasma. (A) Plasma blank. (B) Tobramycin in human plasma with methylamine treatment (solid line) and without methylamine treatment (dashed line) after derivatization. Peaks: 1, tobramycin derivative; 2, anthracene (I.S.). HPLC conditions: Purospher® STAR RP-18e column (55×4 mm I.D.; 3 μm); mobile phase, water–acetonitrile (50:50, v/v); flow-rate, 1.3 ml/min; detection, 230 nm.

tobramycin at 0±2 °C. Determination of tobramycin concentration in plasma was carried out on days 1, 3, 5, 10 and 15. Statistical analysis of the results did not show any significant difference; therefore, tobramycin is stable in plasma samples stored at 0±2 °C for periods up to 15 days.

3.6. Precision and accuracy

The reproducibility and reliability of the proposed method were assessed at five different concentrations of tobramycin and evaluated as RSD and relative recovery, respectively. As shown in Table 1, the precision of the method for tobramycin spiked in human plasma were all less than 5.2% RSD for both intra-day and inter-day assays. The relative recovery of tobramycin, as shown in Table 2, is more than 99%, which was obtained from the calibration graph constructed from plasma spiked with different amounts of tobramycin over the range of 0.93–9.34 mg/l.

4. Conclusions

A simple and sensitive HPLC method based on the pre-column derivatization of tobramycin in human plasma with the derivatizing agent, NITC, has been established and optimized. Validation of the method for quantitation of tobramycin showed that the method has high accuracy. The method can be applied to tobramycin analysis in clinical drug monitoring studies.

Table 1
Precision and accuracy for the analysis of tobramycin spiked in human plasma

Concentration known (mg/l)	Concentration found (mg/l)	RSD (%)	RE (%)
Intra-day[a] ($n=6$)			
0.93	0.96±0.02	2.1	3.0
2.33	2.40±0.04	1.7	3.0
4.65	4.59±0.09	2.0	−1.3
6.98	6.71±0.14	2.1	−4.1
9.34	9.52±0.06	0.6	1.9
Inter-day[a] ($n=6$)			
0.93	0.97±0.05	5.2	5.0
2.33	2.34±0.04	1.7	0.6
4.65	4.62±0.08	1.7	−0.6
6.98	6.83±0.17	2.5	−2.2
9.34	9.47±0.10	1.0	1.4

RE represents relative error.
[a] Intra-day data were based on six replicate analyses and inter-day were from six consecutive days.

Table 2
Relative recoveries of tobramycin in human plasma

Sample	Amount spiked (mg/l)	Amount found[a] (mg/l)	Relative recovery (%)
1	1.86	1.92±0.04	103.2
	5.58	5.72±0.05	102.5
	8.37	8.34±0.06	99.6
2	1.86	1.94±0.04	104.3
	5.58	5.57±0.07	99.9
	8.37	8.46±0.08	101.2
3	1.86	1.85±0.02	99.5
	5.58	5.83±0.09	104.5
	8.37	8.74±0.12	104.4

[a] Mean±SD of triplicate analyses.

Acknowledgements

The authors are grateful to the National Science Council, ROC, for financial support of the work (NSC 91-2113-M-037-003).

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