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Sensitive liquid chromatography assay for the determination of amikacin in human plasma

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Abstract: A selective LC method with on-line post-column derivatization is described for the determination of amikacin in biological fluids. Chromatography was performed on a reversed-phase column, using pentane sulphonic acid as an ion-pairing reagent. For the analysis of biological fluids, amikacin and the internal standard tobramycin were extracted using an ion exchanger (Sephadex). Following complete removal of plasma proteins, the aminoglycosides were eluted with alkaline sodium sulphate solution and injected into the chromatograph. After chromatographic separation the eluent was mixed with the derivatization reagent (*o*-phthalaldehyde and mercaptoethanol in borate buffer pH 10.4) in a reaction coil at 50°C. Detection was performed by fluorescence (excitation: 340 nm, emission: 418 nm). The overall run time was 8 min, at a flow rate of 1.2 ml min^{-1}. The limit of quantification was 25 ng ml^{-1} for amikacin in plasma.

Keywords: *Amikacin; HPLC assay; post-column derivatization; o-phthalaldehyde; plasma; tobramycin.*

Introduction

Aminoglycosides, such as amikacin, are used for the treatment of bacterial infections [1]. After systemic administration therapeutic plasma concentrations range from 2 to 20 μg ml^{-1} [2, 3]. Liquid chromatography is complicated by the fact that the drug does not have any significant UV absorbance or fluorescence. Hence, some kind of pre- or post-column derivatization is needed to render a detectable derivative. Pre-column derivatization with 2,4-dinitrofluorbenzene [4] or *o*-phthalaldehyde [5] resulted in unstable derivatives, which precluded the use of an autosampler. Hence, a post-column approach, as described by Anhalt using *o*-phthalaldehyde [6], was thought to be more promising, because the fluorescent product is formed on-line and detected rapidly. However, the method as described was not sensitive enough to detect amikacin levels in the nanogram range. Although such low concentrations are not relevant for systemic administration of amikacin, they can be a useful parameter in the evaluation of locally administered drug, for instance in pulmonary amikacin therapy, which results in high local concentrations at the site of drug administration, but only low systemic levels in blood.

Experimental

Chemicals and reagents

All chemicals were of analytical grade, except acetic acid, *o*-phthalaldehyde and mercaptoethanol, which were of reagent grade. Tobramycin, Triton-X-100, CM Sephadex (C25), mercaptoethanol and Amberlite IRC 50 were used as received from Sigma Chemical Co. (St. Louis, MO, USA). Pentane sulphonic acid, sodium sulphate, boric acid, potassium hydroxide, acetic acid (99.7%) and *o*-phthalaldehyde were purchased from Fisher Scientific (Springfield, NJ, USA). Amikacin was a gift from Bristol Myers Squibb (New York, NY, USA).

Instrumentation

Reversed-phase liquid chromatography was performed using a Constametric III G pump, an automatic injector (ISS 100, Perkin Elmer Corp. Norwalk, CT, USA), a pre-column (Du Pont 50 × 4.5 mm i.d. packed with Zorbax® ODS Chromatography Packing, E.I. DuPont De Nemours & Co., Wilmington, DE, USA) and a Nucleosil C-18 column (10 μm, 150 × 4.6 mm i.d., Keystone Scientific, State College, PA, USA). A diagram of the experimental settings is shown in Fig. 1. A second

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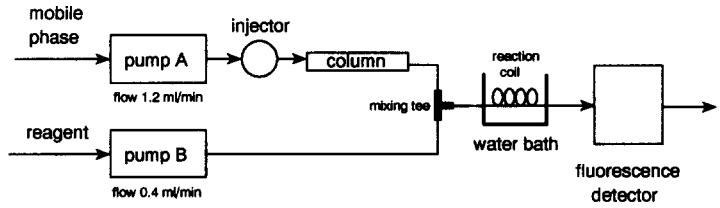

Figure 1
Schematic diagram of the LC system.

Constametric pump transported the reagent to a tee-piece, where the column eluent was mixed with reagent. A stainless steel capillary between reagent pump and mixing tee was necessary to generate a back pressure and allow a smooth flow of the reagent. The back pressure was used to increase the life time of the pump seals. The reaction took place in a Teflon reaction coil (i.d. 0.034", 1.0 m) which was heated by a Haake D1 waterbath. A Perkin Elmer 650-10S fluorescence detector (Perkin Elmer, Norwalk, CT, USA) was set at 340 nm excitation and 418 nm emission wavelength. The fluorescent output was recorded by a HP 3392 A integrator (Hewlett-Packard, Avondale, PA, USA).

Chromatographic conditions

o-*Phthalaldehyde reagent.* To a solution of 800 mg *o*-phthalaldehyde in 10 ml methanol were added 2 g Triton-X-100 and 1.2 ml mercaptoethanol and the solution was mixed. Borate buffer (1 l; 0.4 M boric acid, 0.38 M KOH, pH 10.4, filtered through 0.45 μm filter) was added under shaking. The flow was set to 0.4 ml min^{-1} and created a pressure of 900 psi.

Mobile phase. A solution of 0.2 M sodium sulphate, 0.02 M sodium pentane sulphonate and 1 ml acetic acid in 1 l distilled water (pH 3.3) was filtered through a Teflon filter (0.45 μm). The flow rate of 1.2 ml min^{-1} resulted in a pressure of 1500 psi.

Preparation of plasma samples

Amikacin and the internal standard were extracted from plasma using ion-exchange gel chromatography. CM Sephadex (4 g) was suspended in 48 ml 0.2 M sodium sulphate solution for at least 24 h. An aliquot of the Sephadex suspension (1.5 ml) was sandwiched between two Millipore cotton filters in the barrel of a disposable syringe (9 mm i.d.). After washing with 2 ml sodium sulphate solution, 500 μl plasma and 50 μl tobramycin solution (4 μg ml^{-1} in water) were pipetted onto the column. The column was eluted 3 times with 2 ml sodium sulphate solution and once with 500 μl alkaline sodium sulphate solution (0.2 N sodium sulphate, 0.01 N sodium hydroxide). The eluates were discarded. After addition of 350 μl alkaline sodium sulphate solution, the eluate was collected and the ion concentration was adjusted to the mobile phase by adding 38 μl ion-pair concentrate (2 M sodium sulphate, 0.2 M sodium pentanesulphonate and 10 ml acetic acid per litre distilled water). The injection volume was 40 μl.

Results and Discussion

During the assay development optimum conditions for maximum sensitivity were determined. Post-column derivatization was evaluated using reaction coils of various length at various temperature settings and flow rates.

The length of the reaction coil was important to ensure sufficient time for complete reaction and temperature equilibration. However, if the coil is too long, peak broadening and degradation of the reaction product may occur [6]. In order to achieve a high reaction yield with minimum decrease in resolution, the length of the coil was chosen to be 1.0 m, thus preventing temperature fluctuations in the reaction coil which could have led to changes in reaction yield.

The effect of increasing temperatures on the detector response is shown in Fig. 2. Highest peak areas and best reproducibility were observed at 50°C. Lower temperatures resulted in incomplete reaction, whereas the lower yield at temperatures higher than 60°C was probably due to temperature dependent fluorescence, as described for many fluorescent probes [7].

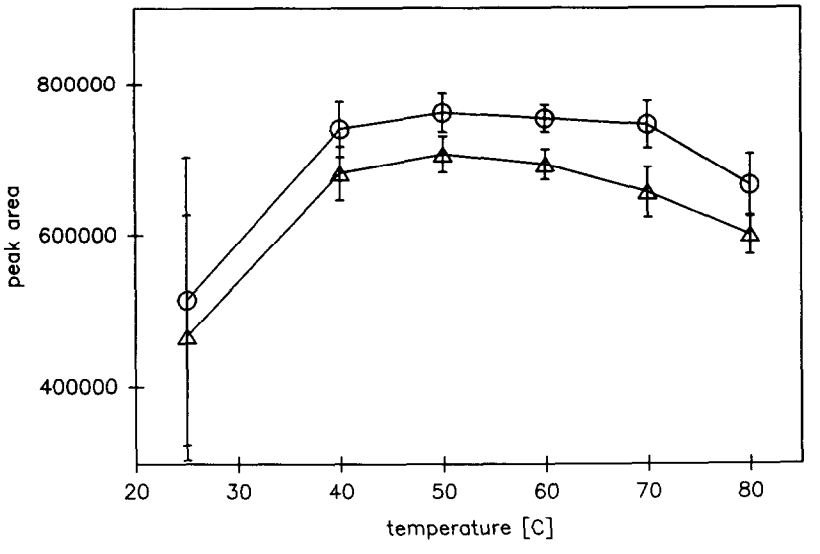

Figure 2
Peak areas of amikacin and tobramycin as a function of temperature.

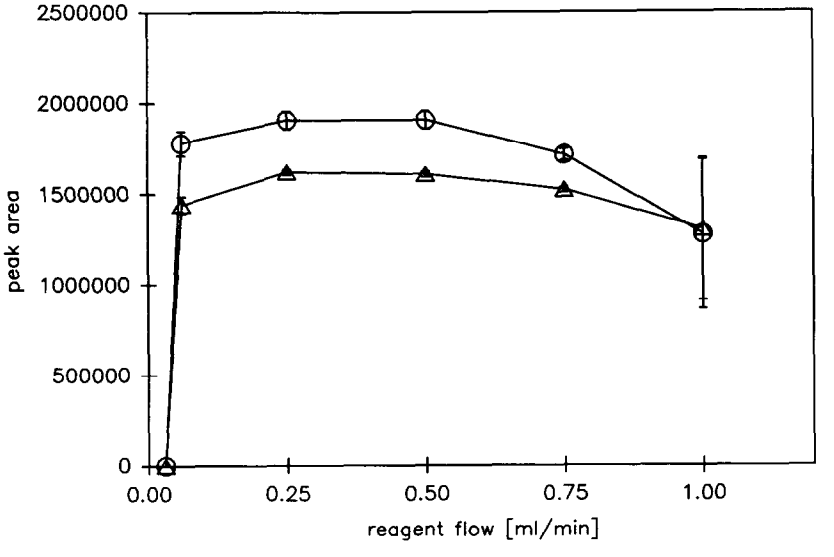

Figure 3
Peak areas of amikacin and tobramycin as a function of reagent flow.

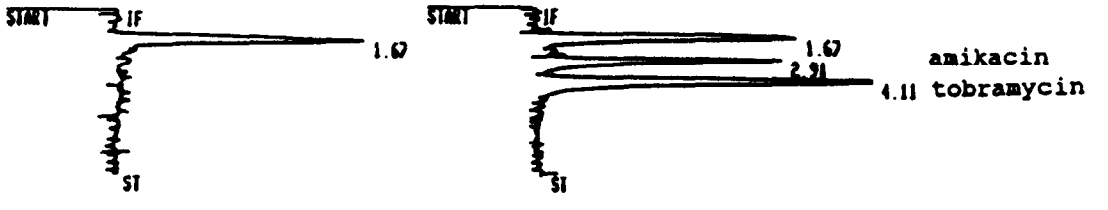

Figure 4
Representative chromatogram of blank and amikacin (100 ng ml^{-1}) containing plasma samples, spiked with internal standard tobramycin (200 ng ml^{-1}).

Increased degradation of the fluorescent reaction product may also be possible. As shown in Fig. 3, the optimum reagent flow was between 0.3–0.5 ml min^{-1}. A reagent flow of 0.06 ml min^{-1} did not result in any signals, whereas flow rates above 0.5 ml min^{-1} gave lower peak areas due to the dilution of the eluent by the reagent. Hence, the flow rate was set to 0.4 ml min^{-1}. Figures 2 and 3 also show that small changes in temperature and flow rate will have little effect on the peak area.

Intra- and interday variability were determined in the low concentration range. Results obtained from intraday assays are shown in Tables 1 and 2. The interday results were obtained at 5 different days with freshly prepared reagents. Although the precision and accuracy were not ideal for concentrations below 100 ng ml^{-1}, the assay was still useful to get a valid estimate. The limit of quantification with a RSD of less than 30% was 25 ng ml^{-1}. Calibration curves were linear over the concentration range 25–2000 ng ml^{-1}.

Table 1
Intraday assay variability (n = 5)

Actual concentration (ng ml^{-1})	Measured concentration (ng ml^{-1})	RSD (%)
25	32	28.4
50	53	17.2
100	122	12.2
250	262	8.7
500	526	5.6
750	740	4.8
1000	1022	7.4
1500	1520	3.9
2000	2100	6.3

Table 2
Interday assay variability (n = 5)

Actual concentration (ng ml^{-1})	Measured concentration (ng ml^{-1})	RSD (%)
25	26	25.1
50	42	23.4
100	110	14.2
250	270	7.9
500	487	3.2
750	780	7.7
1000	1017	6.2
1500	1590	4.5
2000	2020	6.0

The optimization of the instrument setting and the extraction procedure enabled the development of a sensitive assay based on the original method described by Anhalt [6]. The method described here allowed the determination of concentrations in plasma that are more than an order of magnitude less than those described previously [8]. The assay is currently used in this laboratory to monitor low concentration plasma profiles after local administration of amikacin.

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