Journal of Chromatography, 593 (1992) 69–72
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 2476

High-performance liquid chromatography method for the determination of aminoglycosides based on automated pre-column derivatization with *o*-phthalaldehyde

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ABSTRACT

Aminoglycosides, such as amikacin, kanamycin, tobramycin and gentamycin, are often determined, after derivatization, by high-performance liquid chromatography with UV detection. The aim of this work was to develop a sensitive and precise automated method to determine amikacin in pharmaceutical formulations from a stability study. A liquid chromatograph fitted with an autosampler, a diode-array detector set at 340 nm and a C_{18} column was used. The method uses an automated pre-column derivatizing with *o*-phthalaldehyde for compounds containing derivatizable primary amino groups. The derivatization is fast at ambient temperatures, improving the precision and sensitivity (0.5 μg/ml), and there is a wide linearity range.

INTRODUCTION

The use of high-performance liquid chromatography (HPLC) as an analytical method for therapeutic drug monitoring of aminoglycosides [1–7] and their determination in pharmaceutical formulations [8–12] has been reported previously. As aminoglycosides do not have a suitable UV absorption or fluorescence emission for on-line detection after separation by HPLC, either pre- or post-column derivatization is necessary for their determination.

Various liquid chromatography procedures have been reported for the determination of amikacin in pharmaceuticals [7,11] using pre-column derivatization with 1-fluoro-2,4-dinitrobenzene [7] or 2,4,6-trinitrobenzenesulphonic acid [11]. These reagents have slow reaction rates and require heating for the derivatization to occur. *o*-Phthalaldehyde (OPA) is one of the most commonly used reagents for the derivatization of aminoglucosides, mainly due to the fact that the reaction is relatively fast, even at ambient temperatures. However, OPA-derivatized aminoglycosides are unstable [3] and differing delay times after the derivatization affect the precision of the method. This method aims to avoid some of the drawbacks by using an automated derivatization injection procedure. This automated method is fast, simple and shows good precision and sensitivity compared with manual methods. It has been used for the determination of amikacin in pharmaceutical solutions from a stability study.

EXPERIMENTAL

Chemicals and reagents

Amikacin sulphate, kanamycin acid sulphate, sodium heptane sulphonate and OPA were all obtained from Sigma (St. Louis, MO, USA). Mercaptoethanol was of synthesis grade (Merck, Schuchardt, Hohenbrunn, Germany). Methanol (HPLC grade) was supplied by Scharlau (Barcelona, Spain). Glacial acetic acid and potassium hydroxide

0021-9673/92/$05.00

were of analytical reagent grade (Merck, Darmstadt, Germany).

Chromatographic system

Apparatus. The HPLC analyses were performed using a modular liquid chromatographic system consisting of a 6000A pump from Waters, (Milford, MA, USA) and a Model 1000S diode-array detector from Applied Biosystems (Foster City, CA, USA), together with a Promis II autosampler from Spark-Holland (Emmen, Netherlands) or a Rheodyne 7125 manual injector. Data collection and reduction were performed using a computing integrator program (Nelson Analytical 2600 version 5; Perkin-Elmer Nelson System).

Column and mobile phase. Separations were performed using a C_{18} reversed-phase column (Hypersil ODS 3 μm; 15 × 0.40 cm, Tracer). The mobile phase consisted of a mixture of acetic acid–heptanesulphonate–methanol (4.5:22.5:73, v/v/v). The chromatographic separation was performed isocratically at ambient temperature at a flow-rate of 0.4–0.5 ml/min and UV detection at 340 nm was used.

Derivatizing reagent

Potassium borate buffer was prepared from boric acid (24.7 g) and potassium hydroxide (21.3 g) dissolved in 900 ml of water. A pH range of 10.38–10.42 was acceptable and adjustment was not usually necessary. The solution was diluted to 1.0 l. The reagent was prepared by dissolving OPA (100 mg) in 1 ml of methanol, followed by the addition of 200 μl of mercaptoethanol and then diluting to 20 ml with potassium borate buffer. The solution was prepared weekly and stored in an air-tight amber bottle at 4°C.

Derivatization procedures

Manual pre-column derivatization (MAN) was performed by adding 100 μl of the OPA solution to 100 μl of the amikacin standard solution. At exactly 2 min after this addition (measured by a chronometer), 25 μl were injected into the chromatograph.

Automatic pre-column derivatization (AUT) was performed by programming the autosampler (provided with a special cassette) to take 100 μl each of the OPA and amikacin solutions, waiting for 2 min and then injecting 25 μl of the mixture into the chromatograph.

RESULTS

The representative chromatogram of amikacin and kanamycin (internal standard) is given in Fig. 1.

Derivatization products

The amikacin solution generated two peaks [retention times (t_R) 6.20 and 6.80 min] giving qualitatively identical spectra by diode-array detection (Fig. 2).

When different reaction times (1,2,3 and 5 min) were investigated with OPA, the first peak decreased with the reaction time and the second increased. The increase was less than 1.5% between 2 and 5 min of reaction time. The results indicate that the first peak (t_R = 6.2 min) is a product of incomplete derivatization; only the second amikacin peak (t_R = 6.8 min) was used for the determination.

Method validation

Linearity was determined in the concentration range 2–100 μg/ml (AUT), resulting in a coefficient

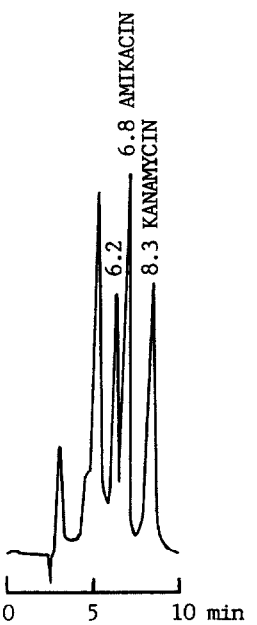

Fig. 1. Typical chromatogram for a sample of 50 μg/ml amikacin and kanamycin (internal standard) solution. The peak with t_R = 6.2 min is the incompletely derivatized amikacin. Flow-rate = 0.5 ml/min.

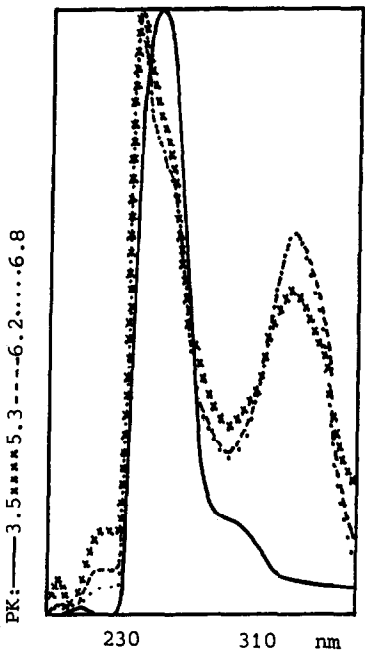

Fig. 2. "On-the-fly" spectra of the two peaks using diode-array detection. --- = First amikacin peak; ··· = second amikacin peak (used for determination); ——— = excess of OPA reagent.

of regression of $r = 0.998$, and the range 5–100 μg/ml (MAN), giving $r = 0.994$.

Fluorimetric detection severely restricted the linear range to 2–32 and 1–16 μg/ml [2,5] compared with this UV detection method.

TABLE I

INTRA- AND INTER-DAY PRECISION: COMPARISON OF AUTOMATED (AUT) AND MANUAL (MAN) DERIVATIZATION

Concentration (μg/ml)	n	Coefficient of variation (%)	
		MAN method	AUT method
Intra-day precision:			
5	5	11.5	2.1
25	5	6.8	0.3
45	5	2.4	2.0
55	5	9.6	3.9
100	5	8.7	4.2
Inter-day precision:			
45	5	22.0	7.4
50	5	18.6	5.3
55	5	17.8	6.2

The limit of quantitation, defined as the lowest concentration that can be determined with a coefficient of variation (C.V.) less than 15%, was about 5 and 0.5 μg/ml for the MAN and AUT methods, respectively.

Intra-day precision, calculated by the C.V., was determined for the MAN and AUT methods at five different concentrations. The values ($n = 5$) were 2.4–11.5% for the MAN and 0.3–4.2% for the AUT methods. The day-to-day C.V. ranged from 17.8 to 22.0% for the MAN and 5.3 to 7.4% for the AUT methods (Table I).

DISCUSSION

Liquid chromatography with post-column derivatization using OPA is well suited to the determination of aminoglycosides in pharmaceutical formulations [6]. However, the post-column reaction method shows some disadvantages [5]: baseline noise is enhanced due to pumping the derivatization reagent through the detector; the flow cell may be soiled by the reaction products; the consumption of reagent is high; and the reaction time can only be increased by the increased column volume, resulting in additional peak broadening and loss of sensitivity.

The critical point for the pre-column derivatization with OPA has been the need to carefully control the reaction time to provide reproducible results. In the method presented here, the reaction is automatically controlled, improving the precision and sensitivity. The derivatization is fast at ambient temperatures and the linearity range wide.

The procedure has been successfully applied to the determination of the aminoglycosides amikacin, gentamycin, tobramycin and kanamycin.

CONCLUSIONS

This automated method, as a result of its precision, limit of quantitation, capacity and simplicity, offers a suitable procedure for the determination of compounds containing derivatizable primary amino groups.

ACKNOWLEDGEMENTS

We acknowledge the technical assistance of Carolina Sevillano.

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