Journal of Chromatography, 305 (1984) 345—352
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1931

AN AUTOMATED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF AMINOGLYCOSIDES IN SERUM USING PRE-COLUMN SAMPLE CLEAN-UP AND DERIVATIZATION

LUDWIG ESSERS

Institute of Medical Microbiology, University of Essen, D-4300 Essen (F.R.G.)

(Received July 18th, 1983)

SUMMARY

An automated high-performance liquid chromatographic method for the determination of the aminoglycosides amikacin, dibekacin, gentamicin, netilmicin, sisomicin and tobramycin is described. The procedure involves sample clean-up by adsorption of the aminoglycosides on a pre-column, subsequent derivatization with *o*-phthalaldehyde and on-line separation of derivatives by column switching. A short cation-exchange column serving concurrently as a guard column in combination with a reversed-phase column was used for separation. Except for the determination of netilmicin an internal standard consisting of an aminoglycoside was used in each assay. The signals of the aminoglycosides determined were linear within the range of 1—16 mg/l serum. The inter-assay imprecision (n = 10) calculated as coefficient of variation was less than 6%. The results were obtained within 20 min after injection of the serum sample. Easy performance and flexibility make the procedure feasible for therapeutic drug monitoring.

INTRODUCTION

High-performance liquid chromatographic (HPLC) methods, when compared with other assays for the determination of gentamicin levels in serum, reveal excellent performance characteristics concerning linearity, accuracy, recovery and inter-assay precision [1, 2]. Another advantage is versatility, low running costs and a high degree of specificity. Nevertheless, HPLC procedures have not been widely introduced for drug monitoring of aminoglycosides in routine laboratories. A main reason for this is the fact that HPLC techniques for the analysis of aminoglycosides usually require labor-intensive sample preparation including deproteinization and derivatization to a fluorogenic or highly UV-absorbing compound.

0378-4347/84/$03.00

This paper describes an automated method for the assessment of aminoglycoside levels in body fluids using pre-column techniques for sample preparation and on-line separation and detection of phthalaldehyde derivatives.

EXPERIMENTAL

Instrumentation

The sample preparation device (see Fig. 1) consisted of two pumps (Model 410, Kontron, Zürich, Switzerland) for the delivery of rinsing buffer and derivatization reagent, two motor-driven six-port valves (Latek, Heidelberg, F.R.G.) switched by the integrator, a 40 × 4.6 mm stainless-steel column dry-packed with Bondapak C_{18} Corasil (37—50 μm, Waters, Milford, MA, U.S.A.) and a 250 × 4.6 mm pre-saturator column dry-packed with coarse-grained silica gel (Merck, Darmstadt, F.R.G.).

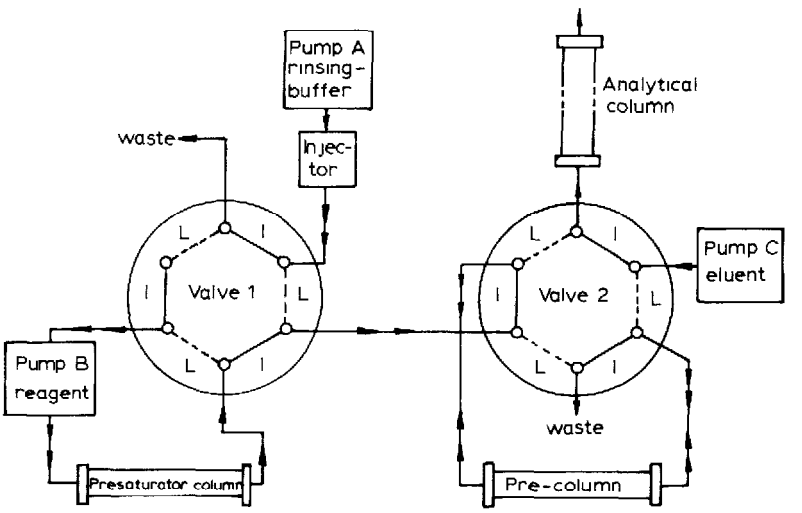

Fig. 1. Diagram of the pre-column switching system.

Chromatographic separations were performed with a Hewlett-Packard liquid chromatograph 1081 B equipped with a 7120 Rheodyne sample injector (20-μl loop, Rheodyne, Berkeley, CA, U.S.A.) and a temperature-controlled column compartment. The analytical column system consisted of a 60 × 4.6 mm column filled with Nucleosil SA, 5 μm particle size, adapted to a 125 × 4.6 mm column packed with Nucleosil C_{18} (5 μm). The packing material is available from Macherey & Nagel (Düren, F.R.G.). Pre-packed columns were purchased from Bischoff (Leonberg, F.R.G.).

The temperature of the analytical column was kept at 25°C. The column effluent was monitored by a Perkin-Elmer fluorimeter (Model 2000 (Perkin-Elmer, Norwalk, CT, U.S.A.) equipped with an 18-μl flow cell, a UG 1 excitation filter combined with a 10-mm diaphragm and a KV 418 filter at the emission site. Filters are available from Schott (Mainz, F.R.G.). The detector signal was recorded by an SP 4200 computing integrator (Spectra-Physics, Santa Clara, CA, U.S.A.).

Materials

Demineralized water "nanopure" grade was used. Methanol, 2-mercaptoethanol and sodium acetate were p.a. grade from Merck. *o*-Phthalaldehyde purissimum) was purchased from Serva (Heidelberg, F.R.G.). Amikacin sulphate (potency 749 μg/mg) was a gift from Grünenthal (Stolberg, F.R.G.), dibekacin sulphate (704 μg/mg) was from Pfizer (Karlsruhe, F.R.G.), gentamicin sulphate (606 μg/mg) was from Merck, gentamicin components C1 (620 μg/mg), C1A (788 μg/mg) and C2 (641 μg/mg) as well as netilmicin sulphate (581 μg/mg) were gifts from Schering (Bloomfield, NJ, U.S.A.), neamine base was kindly donated by Upjohn (Kalamazoo, MI, U.S.A.), sisomicin sulphate (594 μg/mg) was from Bayer (Leverkusen, F.R.G.) and tobramycin sulphate (928 μg/mg) was from Lilly (Indianapolis, IN, U.S.A.).

Standards were freshly prepared by spiking 950 μl of pooled serum from donors with 50 μl of each of the aminoglycosides to be determined and an internal standard solution (see Table I). Specimens were spiked with a stock solution of the internal standard (usually 10 μl per 200 μl of serum).

TABLE I

CHROMATOGRAPHIC ELUENTS USED FOR THE SEPARATION OF DIFFERENT AMINOGLYCOSIDES WITH THE AUTOMATED METHOD AND APPROPRIATE SUBSTANCES USED AS INTERNAL STANDARD

Aminoglycoside	Eluent	Internal standard
Amikacin	74% Methanol—water 0.25 *M* Sodium acetate, pH 7.4	Neamine
Dibekacin Gentamicin Sisomicin	80% Methanol—water 0.1 *M* Sodium acetate, pH 7.4	Tobramycin
Tobramycin	80% Methanol—water 0.1 *M* Sodium acetate, pH 7.4	Gentamicin C1A
Netilmicin	95% Methanol—water 0.2 *M* Sodium acetate, pH 5.0	Not yet found

Chromatographic conditions

The rinsing buffer for the sample preparation system was a 0.15 *M* sodium acetate buffer pH 7.4. The flow-rate was 1 ml/min.

Derivatizing reagent described by Maitra et al. [3] was pumped at a rate of 0.2 ml/min.

The mobile phase for the analytical column system (flow-rate 1 ml/min) varied depending on the aminoglycoside to be separated. Solvent composition and the appropriate internal standard can be read from Table I.

pH values were adjusted with concentrated nitric acid. Depending on the age of the column and the brand used for each column the amount of methanol and/or sodium acetate required may vary somewhat.

Solutions were passed through a 0.45-μm HVLP filter (Millipore, Bedford, MA, U.S.A.) and deaerated under reduced pressure immediately before use.

Procedure

Serum samples were centrifuged in 1.5-ml polypropylene cups (Eppendorf, Hamburg, F.R.G.) at 13,500 *g* (Haemofuge, Heraeus-Christ, Osterode, F.R.G.) for 1 min and spiked with the internal standard. With the injection of 20 μl of the serum sample the integrator switching the valves was started, whilst the right valve (Fig. 1) was in the "I" (inject) position and the left valve in the "L" (load) position. The sample was rinsed by pump A through the pre-column for 3 min. During this time derivatization reagent was recycled by pump B and the mobile phase used for separation was directly pumped through the analytical column system (pump C). After 3 min valve 1 was switched to the "I" position allowing the *o*-phthalaldehyde reagent to flow through the pre-column for 1 min. Then both valves were positioned to "L" for another 3 min. In this position the aminoglycloside derivative was eluted from the pre-column to the analytical column. By switching valve 2 to the "I" position the cycle was finished. The total time for sample preparation was 4 min; another 16 min were necessary for separation of the aminoglycoside derivatives from the blank.

RESULTS

As shown in Fig. 2, with the gentamicin components C1, C1A and C2, adsorption of aminoglycosides to reversed-phase material depends on the sodium acetate concentration added to the rinsing buffer of the pre-column. A compromise has been made with a concentration of 0.15 *M* sodium acetate

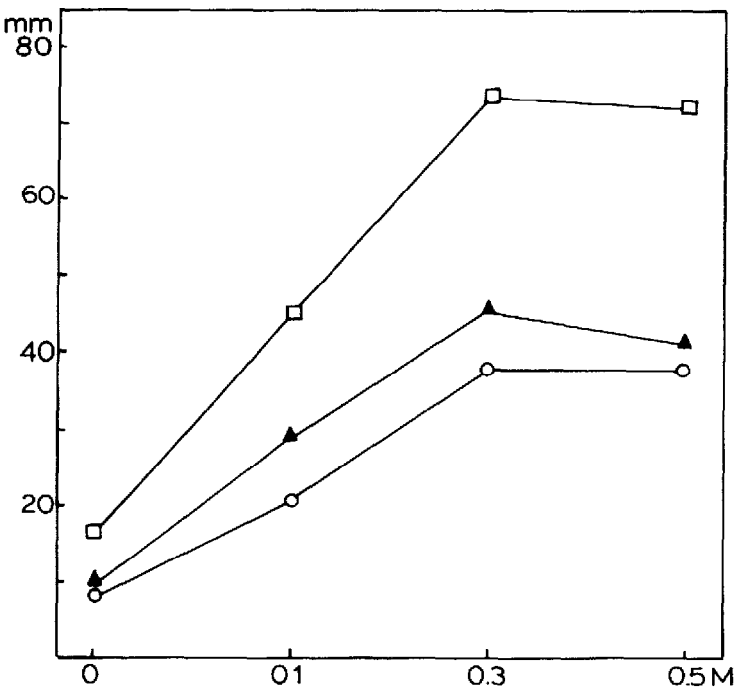

Fig. 2. Adsorption of gentamicin components C1 (□), C1A (▲) and C2 (○) dependent on the sodium acetate concentration of the rinsing buffer. Ordinate: peak height of derivatives eluted. Abscissa: acetate concentration

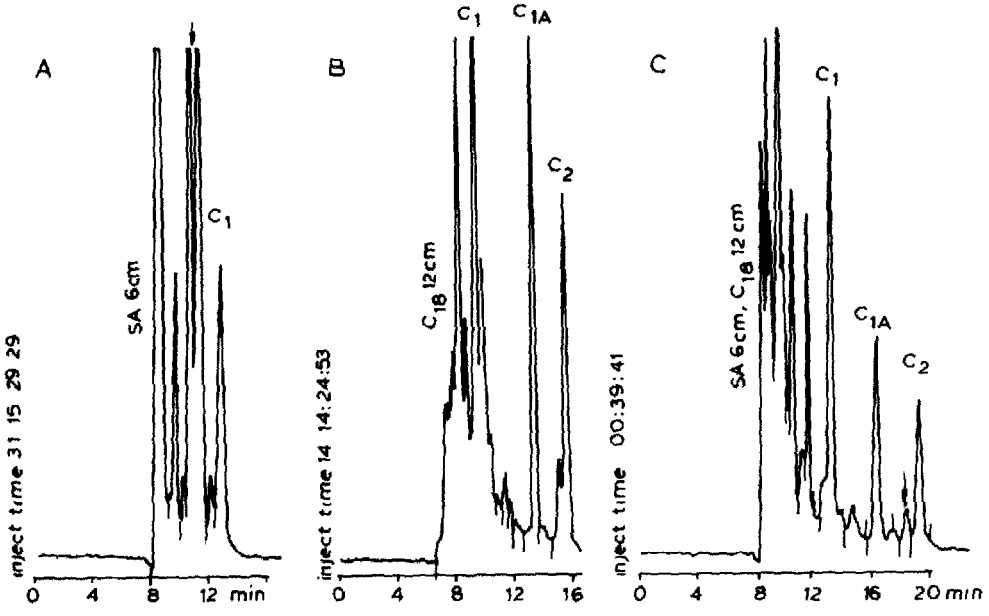

Fig. 3. Separation of gentamicin derivatives from blank peaks (A) short cation-exchange column (arrow indicates badly resolved C1A and C2 compounds); (B) reversed-phase column; (C) combination of both columns (arrow indicates C2A compound).

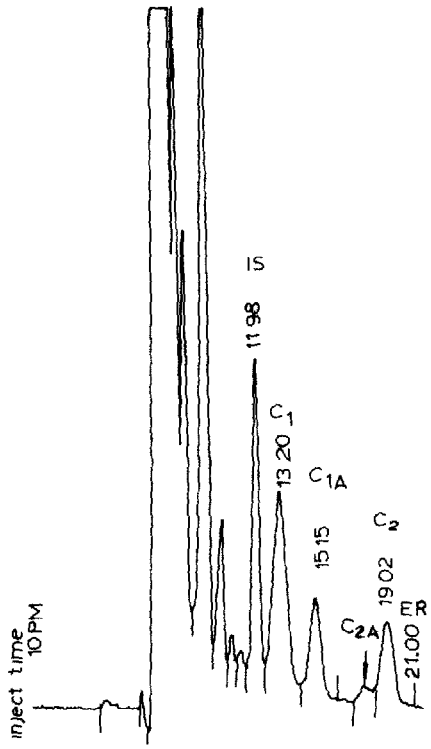

Fig. 4. Chromatogram of a serum sample containing 3.7 mg/l gentamicin spiked with 2.5 mg/ml tobramycin (internal standard, IS).

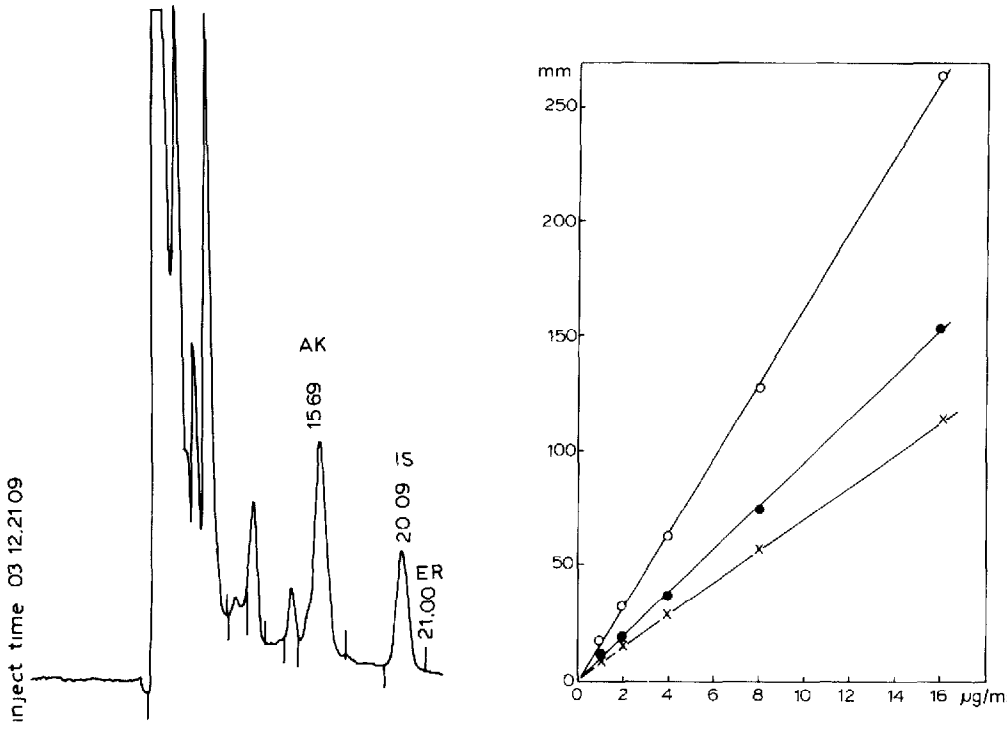

Fig. 5. Chromatogram of a serum sample containing 5 mg/l amikacin (AK) spiked with 2.5 mg/l neamine (internal standard, IS).

Fig. 6. Calibration curve of the automated system determined with the three gentamicin components. (○), C1: $Y = 16.6X - 1.6$, $r = 0.9998$. (●), C1A· $Y = 9.5X - 0.7$, $r = 0.9997$. (×), C2: $Y = 7.2X + 0.3$, $r = 0.9998$.

because amikacin adsorption was reduced at higher concentrations. The pH of the rinsing buffer has been adjusted to pH 7.4 to avoid precipitation of serum components. As indicated by protein determinations, more than 99% of serum proteins are eluted from the pre-column by rinsing for 3 min. Column pressure gradually rises but more than 100 samples can be handled with one pre-column packing.

Gentamicin C1 has a very short retention time compared to the other gentamicin components when eluted from reversed-phase material, resulting in a bad separation from blank peaks (Fig. 3B). The combination of a short cation-exchange column with a reversed-phase column can overcome this problem as the C1 component adsorbs more strongly to a cation-exchanger than gentamicin C1A and C2 (Fig. 3A and C).

Figs. 4 and 5 show typical chromatograms obtained with a serum sample containing gentamicin and amikacin, respectively, spiked with the appropriate internal standard.

Linearity of the analytical system is demonstrated with the calibration curve of gentamicin components (Fig. 6).

Intra-assay precision calculated by the coefficient of variation was determined for gentamicin and amikacin. Values ($n = 10$) were 5.9% at a gentamicin concentration of 4.7 mg/l and 4.9% at an amikacin concentration of 5.8 mg/l.

DISCUSSION

An automated method for the analysis of drugs by HPLC has been described by Roth et al. [4]. Their procedure cannot, however, be applied to aminoglycosides without modification because these molecules exhibit strong polarity and do not readily adsorb to reversed phases. The problem is solved at low cost by adding sodium acetate to the rinsing buffer which probably acts as an ion-pair reagent.

This is, as far as we know, the first time that pre-column derivatization with on-line separation has been tried for aminoglycoside analysis. Post-column derivatization systems have been described by Anhalt and Brown [5] and Mays et al. [6]. Even though the post-column procedure works reliably, some disadvantages should be mentioned: baseline noise is enhanced by pumping derivatization reagent through the detector; the flow cell may be soiled by reaction products; consumption of reagent is high; and reaction time can only be prolonged by increased dead volume resulting in additional peak broadening and loss of sensitivity. These flaws can be avoided by pre-column derivatization.

One problem of pre-column derivatization is the fact that alkaline *o*-phthaldehyde reagent (pH 10.4) is passed through the pre-column, leading gradually to destruction of the silica skeleton of the stationary phase. This effect can be minimized by passing the reagent through a pre-saturator column filled with coarse-grained silica gel. Efficient separation of four gentamicin components has been performed using ion-pair chromatography with alkylsulphonate [7] or an EDTA—methanol mixture [8] as the solvent.

This paper demonstrates that a high resolution of four gentamicin peaks is possible by the combination of a short cation-exchange column serving concurrently as a guard column with a reversed-phase column.

Variation of retention times caused by aging of the column packing or by different brands can be compensated by small changes of methanol or salt content of the eluent.

The HPLC method described has been integrated into a therapeutic drug monitoring program. Its main advantage is easy performance, reliability, flexibility and reduced running cost. The procedure has been applied with success to the aminoglycosides amikacin, dibekacin, gentamicin, netilmicin, sisomicin and tobramycin. The analysis time of 20 min seems to be acceptable as the number of samples sent to our laboratory is limited.

ACKNOWLEDGEMENTS

The author wishes to thank Mrs. E. Parolin and Mr. D. Kowczyk for their excellent assistance during the development of the method.

REFERENCES

1 C.J. Delaney, K.E Opheim, A.L. Smith and J.J. Plorde, Antimicrob. Agents Chemother., 21 (1982) 19.

2 L. Essers, Eur. J. Clin. Microbiol., 1 (1982) 367.
3 S.K. Maitra, T.T. Yoshikawa, J.L. Hansen, I. Nilsson-Ehle, W.J. Palin, M.C. Schotz and L.B. Guze, Clin. Chem., 23 (1977) 2275.
4 W. Roth, K. Beschke, R Jauch, A. Zimmer and F.W. Koss, J. Chromatogr., 222 (1981) 13.
5 J.P. Anhalt and S.D. Brown, Clin. Chem., 24 (1978) 1940.
6 D.L. Mays, R.J. Van Apeldoorn and R.G. Lauback, J. Chromatogr., 120 (1976) 93.
7 M. Freeman, P.A. Hawkins, J.L. Loran and J. Stead, J. Liquid Chromatogr., 2 (1979) 1305.
8 J. Marples and M.D.G. Oates, J. Antimicrob. Chemother., 10 (1982) 311.