Journal of Chromatography, 276 (1983) 385—394
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1754

DETERMINATION OF AMIKACIN IN SERUM BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ULTRAVIOLET DETECTION

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(First received January 20th, 1983; revised manuscript received April 7th, 1983)

SUMMARY

A procedure for the determination of amikacin in serum is described. The aminoglycoside is extracted from serum by using a disposable cation-exchange column. The eluate of this column is derivatized with 1-fluoro-2,4-dinitrobenzene and subsequently analysed by reversed-phase high-performance liquid chromatography with ultraviolet detection at 365 nm. The absolute recovery of amikacin by this procedure is 72%. Kanamycin is used as the internal standard. The sensitivitiy is 1 mg/l for amikacin with samples of 200 μl. Precision, expressed as the coefficient of variation, is about 3% in the therapeutic concentration range. The 2,4-dinitrophenyl derivative of amikacin is synthesized on a preparative scale by a new method and its structure is demonstrated to be the fully derivatized amikacin. The analysis of serum samples obtained in an in vivo experiment correlates well with the results from a microbiological assay.

INTRODUCTION

Previously it was shown that 2,4-dinitrophenylation of aminoglycosides produces derivatives with good chromatographic properties in reversed-phase high-performance liquid chromatographic (HPLC) systems and with sufficiently high absorbance at 365 nm to allow therapeutic drug monitoring [1—3]. Recently, a 2,4-dinitrophenylation method was described for amikacin [4]. The present paper reports also a method for quantitating amikacin in serum by means of precolumn derivatization with 1-fluoro-2,4-dinitrobenzene (FDNB), but with a simpler sample treatment and with the use of an internal standard. Furthermore, we succeeded in obtaining values for the absolute recovery of the determination.

0378-4347/83/$03.00

EXPERIMENTAL

Instrumentation

The chromatographic equipment was described previously [1]. Chromatography was performed on columns (30 cm × 3.9 mm I.D.) packed with LiChrosorb RP-18, particle size 10 μm (Merck, Darmstadt, G.F.R.). High-resolution proton nuclear magnetic resonance (H-NMR) spectra were recorded on a Bruker WP200 (Bruker, Rheinstetten, G.F.R.) at the Laboratory for Organic Chemistry, University of Utrecht, under supervision of Dr. de Bie. The National Institute of Public Health (RIV), Bilthoven, The Netherlands, did the microbiological assays, using an agar-well diffusion technique [5].

Materials

Unless mentioned otherwise, demineralized water was used. Ethanol, sodium hydroxide, sodium bromide and sodium sulphate decahydrate were of European Pharmacopoeia quality. Dimethyl sulphoxide (DMSO) was of British Pharmacopoeia quality. Acetic acid, acetone, FDNB, methanol and ethyl acetate were of analytical grade. Acetonitrile "zur Synthese" and tetrabutylammonium hydroxide (TBAH) 40% in water "zur Synthese" were from Merck. CM-Sephadex C-25 cation exchanger was from Pharmacia (Uppsala, Sweden) and kanamycin sulphate from Sigma (St. Louis, MO, U.S.A.). Ampoules (2 ml) of Amukin® containing the equivalent of 500 mg of amikacin as the sulphate salt, and amikacin base were obtained by courtesy of Pfizer (Rotterdam, The Netherlands). Weights of amikacin indicate weights of the chemical substance amikacin base, not microbiological potencies, unless otherwise indicated.

Pooled human serum was obtained from ambulatory patients, and was frozen and stored at −18°C within three days of collection.

Stoppered polypropylene centrifuge tubes of 1.5 ml capacity disposable glass pasteur pipettes of 5.4 mm I.D., glasswool and ampoules of 0.5 and 5 ml capacity were also used.

Procedures

Procedure A: HPLC determination of amikacin in serum. Remove most of the stem of a pasteur pipette and plug the remainder of the stem with glass wool. Incubate CM-Sephadex C-25 in a 0.2 mol/l sodium sulphate solution in water at room temperature for at least 24 h. Fill the pasteur pipette with sufficient of the Sephadex slurry to obtain a column height of 1.5 cm. Pipette 200 μl of the serum sample into a centrifuge tube and add 20 μl of a solution containing 250 mg/l kanamycin sulphate in water (internal standard). Vortex and dispense the contents of the centrifuge tube on top of the column. Elute the column with 2 ml of a solution containing 0.001 mol/l hydrochloric acid and 0.2 mol/l sodium sulphate (initial eluent). Discard the eluate. Elute the column with 250 μl of 0.05 mol/l sodium hydroxide. Discard the eluate (dead volume of the column). Elute the column with 1 ml of 0.05 mol/l sodium hydroxide and collect this eluate in a 5-ml ampoule. Add 2.5 ml of a solution of FDNB in methanol (30 g/l). The formation of a precipitate is observed, which redissolves upon mixing. Heat-seal the ampoule and place in boiling

water for 5 min. After cooling, break the seal and inject 150 μl into the chromatograph.

Prepare the mobile phase by mixing 470 ml of acetonitrile with 530 ml of water (both filtered through a 0.2-μm filter) and 1 ml of acetic acid, and deaerate ultrasonically. Pump the mobile phase at 2.5 ml/min and monitor the eluent at 365 nm.

Procedure B: synthesis of the 2,4-dinitrophenyl derivative of amikacin. Amukin (2 ml) was mixed with a sufficient quantity of sodium hydroxide solution (10 mol/l) to obtain a pH of 10. Then, 20 ml of DMSO were added. Alternately, FDNB and TBAH were added, guided by HPLC analysis of the reaction mixture after each addition. The final reaction mixture was diluted with 100 ml of a mixture of ethanol and water (25:75, v/v). The resulting precipitate was purified by dissolving in the smallest possible volume of a mixture of 630 ml of acetone and 370 ml of glass-distilled water.

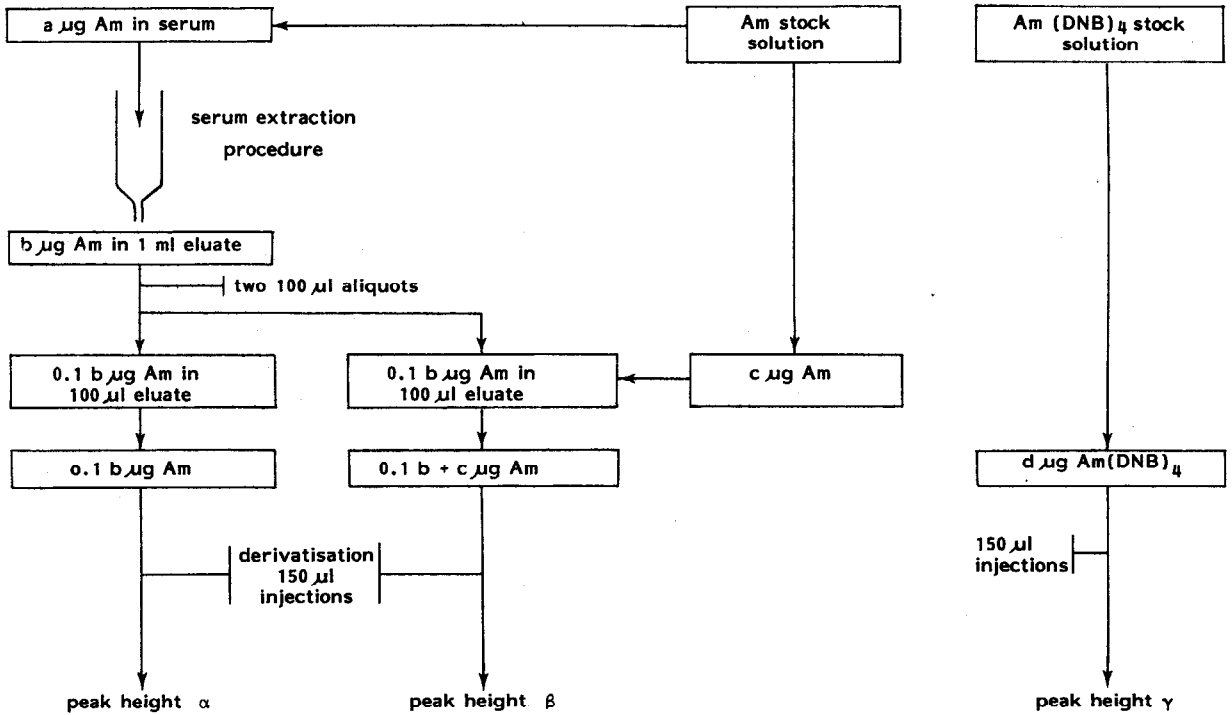
Aliquots of this solution were injected into the chromatograph, eluted with the same acetone–water mixture, and the appropriate fractions were collected. The combined fractions of repeated injections were diluted with glass-distilled water and extracted with ethyl acetate. The combined ethyl acetate extracts were evaporated and the residue was placed in a hygrostat, above a mixture of sodium bromide and water (2:1, w/w) until constant weight was obtained.

Procedure C: recovery measurements. Amikacin base was investigated for content of the chemical substance amikacin by non-aqueous titration [3]. Absence of related compounds was established by thin-layer chromatography [6].

Serum was spiked with an aqueous stock solution containing amikacin base and kanamycin sulphate, to a serum concentration of 16 mg/l of both antibiotics. Samples of 200 μl of this spiked serum were processed by means of a cation-exchange column as described under procedure A. The 1-ml eluate fraction which contained the aminoglycosides was collected and weighed in order to calculate the exact volume of the eluate. Two 100-μl portions were taken and to one aliquot 5 μl of the stock solution of amikacin and kanamycin were added (standard addition technique). After the addition of 250 μl of FDNB (30 g/l in methanol) both mixtures were derivatized and chromatographed. Also, samples of a solution of the purified amikacin derivative in the mobile phase were chromatographed. Serum extraction recoveries of amikacin and kanamycin, the derivatization yield of amikacin and the overall absolute recovery of amikacin were then calculated as shown in Fig. 1.

Procedure D: in vivo experiment and bioassay comparison study. A 69-kg healthy volunteer received 1 ampoule of Amukin by intramuscular injection. Blood samples were collected at regular time intervals and left in polypropylene tubes for some hours. The clot of erythrocytes was removed and the remaining serum samples were analysed by HPLC and by microbiological assay.

For both analytical techniques identical spiked serum samples were used for calibration.



Recovery of the serum extraction procedure $= \frac{b}{a} \cdot 100\% = \frac{c\,\alpha 1000}{a\,(\beta - \alpha)}\%$

Yield of derivatisation reaction $= \frac{(\beta - \alpha)\, d\, M_r(Am)}{\gamma\, c\, M_r(Am(DNB)_4)} \cdot 100\%$

Fig. 1. Scheme of the measurement and calculation of the serum extraction recovery and derivatization yield of amikacin. Am = amikacin, $Am(DNB)_4$ = amikacin derivative, M_r = moleculer weight.

RESULTS

Representative chromatograms are shown in Fig. 2. Identical chromatograms were obtained with serum samples from patients.

Linearity and precision

A calibration line was constructed. The results are summarized in Table I. Precision is reported in Table II.

Characterization of the derivative of amikacin

(1) Part of the high-resolution H-NMR spectrum of the derivative is shown in Fig. 3. In the H-NMR spectrum of the 2,4-dinitrophenyl group, doublets are expected for $H_{(3)}$ and $H_{(6)}$ because of the coupling between $H_{(3)}$ and $H_{(5)}$, and $H_{(6)}$ and $H_{(5)}$, respectively. $H_{(5)}$ is coupled with both $H_{(3)}$ and $H_{(6)}$, giving rise to quadruplets.

The H-NMR spectrum of the amikacin derivative shows four doublets of $H_{(6)}$ in the range 6.9–7.6 ppm and also four doublets of $H_{(3)}$ in the range 8.5—8.9 ppm. The four quadruplets of $H_{(5)}$ in the range 8.1—8.3 ppm are not completely resolved. Four 2,4-dinitrophenyl groups are therefore present,

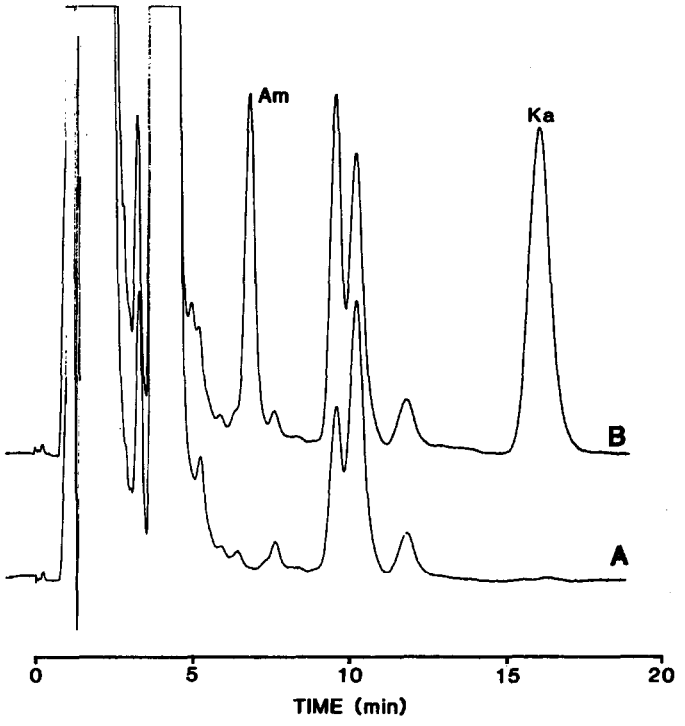


Fig. 2. HPLC of serum samples. Detector setting: 0.02 a.u.f.s. Chromatogram A is obtained with blank serum without internal standard. Chromatogram B is obtained with serum spiked with amikacin (16 mg/l) and with internal standard. Am = amikacin derivative, Ka = kanamycin derivative (internal standard).

TABLE I

STANDARD CURVE

Duplicate determinations were made on every concentration tested.

Amikacin concentration (mg/l)	PHR*	PHR/concentration
1	0.062	0.062
2	0.117	0.059
4	0.240	0.060
8	0.495	0.062
16	1.01	0.063
32	2.04	0.064
64	4.30	0.067

*Mean value of peak height ratio of derivatized amikacin to derivatized kanamycin (internal standard).

indicating that all four primary amino groups are derivatized. This was also concluded by Wong et al. [4] from other H-NMR data.

(2) A solution of the prepared derivative in the mobile phase was investigated for chromatographic purity by HPLC. Besides the major peak of the amikacin derivative, one minor peak was observed. The area of the peak of the amikacin derivative was 98.7% of the total peak area.

(3) The nitrogen content of the purified derivative was found to be 13.16% (S.D. = 0.04%, n = 2). The calculated nitrogen content for $C_{46}H_{51}N_{13}O_{29}$ is 14.57%. The purity of the prepared derivative was taken as

$$\frac{13.16}{14.57} \cdot 98.7\% = 89\%$$

TABLE II

WITHIN-RUN PRECISION

Amikacin concentration (mg/l)	Precision*	
	C.V. (%)	n
1	5.3	5
2	3.4	6
16	3.1	6
32	3.2	6
64	1.5	6

*C.V. = coefficient of variation of measured peak height ratios; n = number of determinations.

Recovery measurements

The results, corrected for the content of amikacin base and for the purity of the amikacin derivative, are given in Table III.

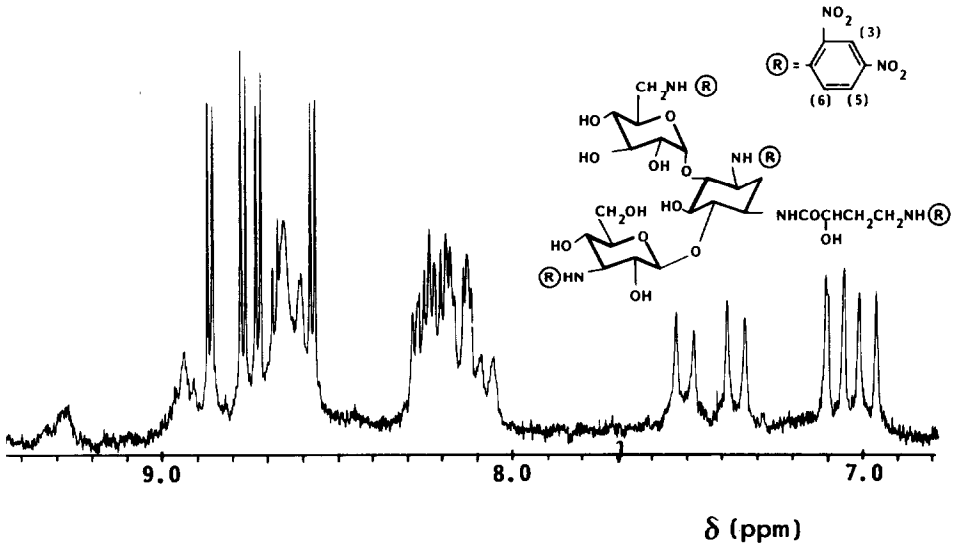


Fig. 3. Part of the 200 mHz H-NMR spectrum of the 2,4-dinitrophenyl derivative of amikacin. Chemical shift (δ) relative to tetramethylsilane. Conditions: saturated solution in DMSO-d_6 at 33°C. Assignment of peaks: see text. Inset: structure of the 2,4-dinitrophenyl derivative of amikacin.

TABLE III

RESULTS OF ABSOLUTE RECOVERY MEASUREMENTS

	Recovery (%)	n*	C.V. (%)
Serum extraction recovery of amikacin	95	7	6
Serum extraction recovery of kanamycin	92	8	9
Derivatization yield of amikacin	77	8	10
Overall recovery of amikacin	72	7	9

*n = number of determinations.

In vivo experiment and bioassay comparison study

The results are summarized in Table IV.

DISCUSSION

Serum extraction

Determinations of gentamicin, sisomicin and tobramycin in serum, by 2,4-dinitrophenylation were performed using a protein precipitation step with acetonitrile [1–3]. For amikacin, this failed because of the relatively high polarity of the 2,4-dinitrophenyl derivative of amikacin, making its separation from the peaks near the solvent front impossible.

The serum extraction procedure presented in this paper is a modification of

TABLE IV

RESULTS OF IN VIVO EXPERIMENT AND BIOASSAY COMPARISON STUDY

Serum level determinations by two methods on samples obtained after a single intramuscular dose of amikacin from one volunteer.
Linear regression analysis (HPLC values = X, microbiological values = Y): Y intercept = 0.02, standard deviation = 0.84; slope = 0.92, standard deviation = 0.05; r = 0.993.

Time after injection (h)	Amikacin concentration (mg/l by potency) found by	
	HPLC*	Microbiological assay**
0.5	16.2	14.3
1	24.2	23.7
1.5	24.8	22.1
2	21.2	18.5
3	13.8	13.7
5	6.1	6.0
8	2.4	1.8

*Standards and samples analysed once.
**Standards and samples analysed in duplicate.

the technique described by Anhalt and Brown [7]. In their procedure 0.2 mol/l sodium sulphate is used instead of 0.001 mol/l hydrochloric acid in 0.2 mol/l sodium sulphate as the initial eluent.

Eluting the cation-exchange column with neutral solutions sometimes yields low and irreproducible recoveries, due to the alkaline reaction of some serum samples. The amino groups of the aminoglycosides are deprotonated in alkaline solution, the cation exchanger will not retain these deprotonated aminoglycosides. Our findings were confirmed by Anhalt [8].

Derivatization

Tsuji et al. [9] and Helboe and Kryger [10] reported 2,4-dinitrophenylation of aminoglycosides in a water—methanol mixture in the presence of a borate buffer pH 9.0 with reaction temperatures and reaction times of 100°C and 45 min, and 60°C and 60 min, respectively. These authors reported no derivatization yields for their methods. We measured the derivatization yields for amikacin obtained by these two methods and found 86% and 90%, respectively. We also observed that derivatization, in sodium hydroxide solutions of concentrations ranging from 0.01 mol/l to 0.06 mol/l at 100°C for 5 min with the same reagent concentration, resulted in essentially the same derivatization yield, i.e. 92%. When this technique was applied to the eluate obtained after the serum extraction, somewhat lower derivatization yields were obtained, i.e. 77% (see Table III).

The only difference between the conditions in which these two results were obtained is the presence of a low concentration of residual sodium sulphate in the derivatization mixture obtained from the cation-exchange column. The negative influence of sodium sulphate upon the yield of the derivatization

reaction was confirmed by experiments in which increasing amounts of sodium sulphate were added to the derivatization mixture.

Chromatography

The 2,4-dinitrophenyl derivatives of amikacin and kanamycin show good chromatographic properties on RP-18 columns with mobile phases composed of water and acetone, or water and acetonitrile. However, only the acetonitrile–water mixture was useful as the mobile phase in the analysis of serum samples. Kanamycin is composed of three components: kanamycin A, the major component, and kanamycin B and C, the two minor congeners. As equimolar amounts of kanamycin and amikacin give rise to roughly equal peak areas, it may be concluded that the observed peak of kanamycin is the peak of the derivative of the major component of kanamycin, i.e. kanamycin A.

Preparative synthesis of the amikacin derivative

Bunnett and Hermann [11] showed that the reaction between FDNB and amino groups is very fast in DMSO. In this solvent amikacin base, FDNB and the derivative of amikacin are soluble. Amikacin sulphate is not soluble, so the salt was converted into the free base before the addition of DMSO.

We did not succeed in obtaining a "pure" derivative, because of its hygroscopic properties and our inability to find a suitable solvent for the recrystallization of the derivative. However, a derivative of known purity instead of a pure derivative also allowed measurements of the recoveries of the derivatization and determination. A chromatographic purification of the raw derivative was carried out using solvents without nitrogen in their molecular structure. So, the nitrogen content of the obtained purified derivative indicated its content of amikacin (2,4-dinitrophenyl)$_4$, the remainder being water.

CONCLUSIONS

With the proposed method, amikacin can be determined in 200-μl serum samples with sufficient accuracy, precision and sensitivity to make therapeutic drug monitoring possible.

The method described by Wong et al. [4] uses only 25 μl of serum, but in their approach a concentration step and several sample clean-up steps are required. A second difference with this method is the use of an internal standard, which is an important factor in the reliability of the assay [2].

ACKNOWLEDGEMENTS

The authors wish to thank Prof. Dr. A.W.M. Indemans and Prof. Dr. H.J. de Jong for helpful discussions, Dr. J. Renema, Dr. J. Meijer and Mr. D. Seijkens for carrying out and interpreting the NMR spectra, Ms. A. Rutgers for carrying out the antimicrobial assay and Mr. J. Teeuwsen for technical support.

Generous support has been given by Pfizer, Rotterdam, The Netherlands, by providing specimens of amikacin.

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