Journal of Chromatography, 322 (1985) 321–331
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 17,461

PRE-COLUMN DERIVATIZATION OF AMINOGLYCOSIDES WITH 1-FLUORO-2,4-DINITROBENZENE

D. M. BARENDS*, J. S. BLAUW, C. W. MIJNSBERGEN, C. J. L. R. GOVERS and A. HULSHOFF
Department of Analytical Pharmacy of the Pharmaceutical Laboratory, University of Utrecht, Catharijnesingel 60, 3511 GH Utrecht (The Netherlands)
(First received September 13th, 1984; revised manuscript received December 5th, 1984)

SUMMARY

The reaction between tobramycin and 1-fluoro-2,4-dinitrobenzene was studied. This reaction shows a sharp pH optimum because, even under moderate alkaline conditions, derivatization of the aliphatic hydroxyl groups of tobramycin becomes an important side reaction. Phosphate and phthalate buffers also react with the derivatization reagent. Buffers comprising tertiary amines and hydrochloric acid are preferred.

INTRODUCTION

Pre-column derivatization of aminoglycosides with 1-fluoro-2,4-dinitrobenzene (FDNB) prior to high-performance liquid chromatography (HPLC) has become an important analytical technique for the assay of these antibiotics. The obtained derivatives possess high UV absorptivities at a favourable wavelength[1] and good chromatographic properties in both normal phase[2–5] and reversed-phase HPLC[1,4,6–8]. However, a drawback of FDNB is its toxicity[9]; it must be handled with protective gloves[10]. Also, difficulties have been reported in establishing optimum reaction conditions in the derivatization of aminoglycosides with FDNB and related nitrophenylation reagents[1,2,11]. In the present paper we describe the 2,4-dinitrophenylation of aminoglycosides with FDNB using tobramycin (Tb) (Fig. 1) as a model compound and water–acetonitrile (1:2, v/v) as the solvent. This solvent was chosen because the

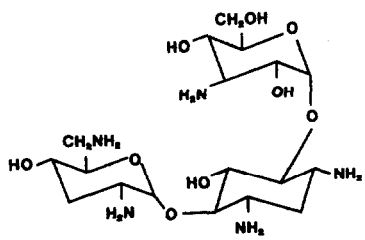

Fig. 1. Structure of tobramycin (Tb).

0021-9673/85/$03.30

use of a double volume of acetonitrile is a convenient deproteination method for serum samples prior to HPLC analysis in the therapeutic drug monitoring of aminoglycosides[1,6].

EXPERIMENTAL

Materials

Tobramycin (Tb) of known chemical composition was used[1]. The tobramycin derivative with all five amino groups 2,4-dinitrophenylated, $Tb(NDNB)_5$, and a tobramycin derivative with all five amino groups and one hydroxyl group 2,4-dinitrophenylated, $Tb(NDNB)_5(ODNB)$, were synthesized essentially as described previously[7]. Their structures were confirmed by high resolution proton NMR spectrometry indicating the presence of five and six 2,4-dinitrophenyl groups, respectively[7]. The chemical composition of these derivatives was determined by an elemental analysis for nitrogen[7].

Other chemicals used were of analytical reagent grade, except acetone which was of industrial quality (Shell Chemie) and acetonitrile and ethyldiisopropylamine which were "zur Synthese" (Merck).

Hydrogen-ion activity standards in water–acetonitrile (1:2, v/v)

A glass electrode/pH meter combination was calibrated at room temperature with aqueous pH standards. Thereafter, the electrode was immersed in a mixture of 100 parts of a solution of potassium hydrogen phthalate in water (0.0500 *M*), 15 parts of a solution of sodium hydroxide in water (0.100 *M*) and 230 parts of acetonitrile (all by volume). The readings of the pH meter at different temperatures of this mixture were as follows: 20°C, 6.95; 40°C, 6.95; 70°C, 6.92. The mixture was thereafter used as an apparent pH standard at 20, 40 and 70°C with the apparent pH values (pH*): pH^*_{20} 6.95; pH^*_{40} 6.95 and pH^*_{70} 6.92. The second pH* standard was a mixture of 10 parts of a solution of sodium tetraborate in water (0.010 *M*) and 20 parts of acetonitrile (both by volume). The pH* values assigned to this standard were: pH^*_{20} 11.12; pH^*_{40} 11.12 and pH^*_{70} 11.40.

The composition of the first pH* standard was chosen such that the glass electrode immersed in this pH* standard showed nearly zero voltage. The assumption can be made that a glass electrode/pH meter combination calibrated with the described pH^*_t standards follows the Nernst equation, *i.e.*, it shows a decrease of one pH^*_t unit for a ten-fold increase in hydrogen-ion activity[12].

HPLC

The reactions studied were followed by HPLC analysis of the reaction mixture, aliquots of which were injected directly. The chromatographic equipment has been described previously[13]. The columns were 30 cm × 3.9 mm I.D., laboratory packed with RP-18, particle size 10 μm (Merck). Quantitation of $Tb(NDNB)_5$ and $Tb(NDNB)_5(ODNB)$ was performed with a mobile phase of water–acetone–acetic acid (30:70:0.1, v/v). Detection was carried out at 365 nm and external standardization was used, the purified derivatives of known composition being available. A representative chromatogram is shown in Fig. 2A. Quantitation of FDNB was performed with a mobile phase of 0.05 *M* sodium phosphate buffer in water pH 7.5–

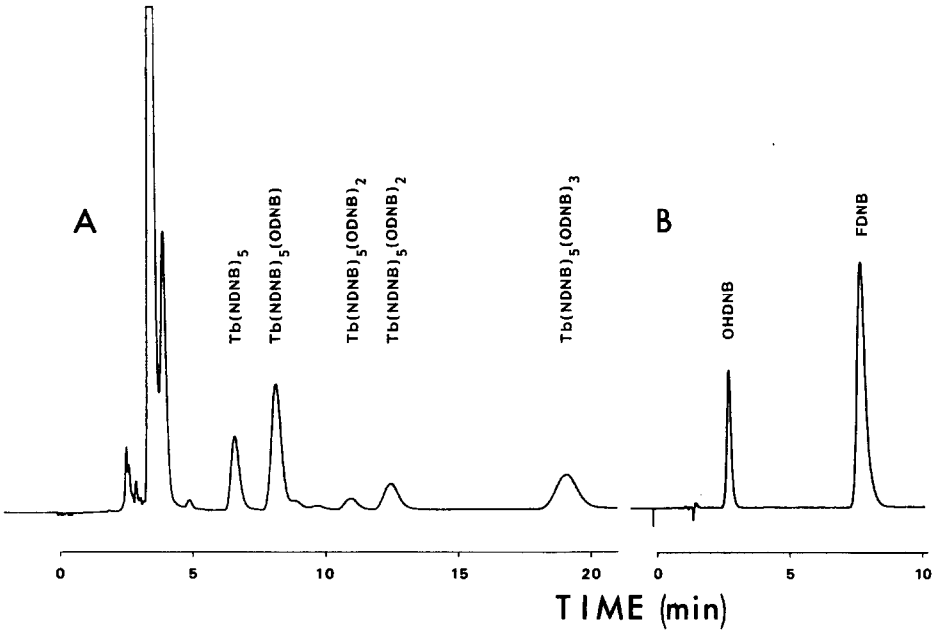

Fig. 2. Typical HPLC chromatograms of a mixture obtained from the reaction between FDNB and $Tb(NDNB)_5$ (see Fig. 3). Reaction conditions: initial concentration of $Tb(NDNB)_5$, $2.7 \cdot 10^{-5}$ *M*; initial concentration of FDNB, $5.7 \cdot 10^{-3}$ *M*; reaction temperature, 40°C; hydrogen-ion activity, pH^*_{40} 11; reaction time, 134 min. A, The derivatives of Tb. Chromatographic conditions: injection volume, 100 µl; flow-rate, 1.0 ml/min; detector setting, 1.0 a.u.f.s. B, FDNB and its hydrolysis product: OHDNB. Chromatographic conditions: injection volume, 5 µl; flow-rate, 1.3 ml/min; detector setting, 1.0 a.u.f.s. For other conditions see Experimental.

methanol (55:45, v/v). Detection was performed at 254 nm and external standardization was applied. A representative chromatogram is shown in Fig. 2B.

Kinetic experiments at constant hydrogen-ion activity (pH-stat)

The reactions studied at constant hydrogen-ion activity were carried out in a thermostatted glass reaction vessel. The hydrogen-ion activity of the reaction mixture was kept constant by the addition of sodium hydroxide solution; this was performed by an autotitration device (pH-stat, Metrohm E473, E415, E512) controlled by a combined glass electrode that was calibrated with the two pH* standards. All experiments were carried out in water–acetonitrile (1:2, v/v). Kinetic experiments were performed in duplicate. The following reactions were studied under pH-stat control.

Alkaline hydrolysis of FDNB (reaction III of Fig. 3). The hydrolysis of FDNB was studied at 40°C at pH^*_{40} 9, 10 and 11, the initial concentration of FDNB being about $5 \cdot 10^{-3}$ *M*.

Reaction between FDNB and Tb(NDNB)$_5$ (reactions IV and V of Fig. 3). The reaction between FDNB and $Tb(NDNB)_5$ was studied at 40°C at pH^*_{40} 9, 10 and 11. The initial concentrations of FDNB and $Tb(NDNB)_5$ were $5 \cdot 10^{-3}$ and $2 \cdot 10^{-5}$ *M*, respectively. At pH^*_{40} 10 this reaction was also followed with the initial concentrations of the reactants doubled or halved, to establish the order of the reaction.

Reaction between FDNB and Tb (reaction I of Fig. 3). The reaction between FDNB and Tb was followed at 40°C at pH^*_{40} 7, 8, 9, 10 and 11. The initial concentrations of Tb and FDNB were $2 \cdot 10^{-5}$ *M* and $5 \cdot 10^{-3}$ or $1 \cdot 10^{-2}$ *M*, respectively. At pH^*_{40} 9 this reaction was also followed with the initial concentration of Tb doubled or halved to establish the order of reaction. The reaction was also studied under pH-stat control at 70°C at pH^*_{70} 6, 7, 8 and 9.

Alkaline hydrolysis of Tb(NDNB)$_5$ (reaction VI of Fig. 3). The alkaline hydrolysis of Tb(NDNB)$_5$ was studied in solutions of different alkalinity at 40, 60 and 80°C.

Analytical derivatizations

Solutions containing Tb ($2 \cdot 10^{-5}$ *M*), FDNB ($5 \cdot 10^{-2}$ *M*), ethyldiisopropylamine (0.03, 0.10 or 0.40 *M*) and various amounts of hydrochloric acid were prepared and their pH^*_{20} values were measured. These samples were heated for 10 min at 70°C in glass ampoules. After cooling to room temperature, the pH^*_{20} value was measured again and the yield of Tb(NDNB)$_5$ was determined by HPLC.

RESULTS AND DISCUSSION

FDNB is known to react with primary and secondary amines, but also with aliphatic hydroxyl groups[14,15]. Possible reactions that could be involved in the derivatization of Tb with FDNB (see Fig. 3) are: I, the conversion of Tb into Tb(NDNB)$_5$; II, protonation of the amino groups of Tb in an acidic medium; III, hydrolysis of FDNB into 2,4-dinitrophenol (OHDNB); IV, the reaction between a hydroxyl group of Tb(NDNB)$_5$ and FDNB giving Tb(NDNB)$_5$(ODNB); V, further derivatization of hydroxyl groups of Tb(NDNB)$_5$(ODNB), producing derivatives of type Tb(NDNB)$_5$(ODNB)$_n$ ($n \geq 2$); VI, hydrolysis of Tb(NDNB)$_5$. The reactions I, II, III and VI could be studied separately.

Hydrolysis of Tb(NDNB)$_5$ (reaction VI)

At 40 and 60°C no decomposition of Tb(NDNB)$_5$ occurred. At 80°C some decomposition was observed, but only in strongly alkaline solutions. It was concluded that under the usual conditions encountered in the derivatization of Tb, Tb(NDNB)$_5$ is not appreciably hydrolyzed.

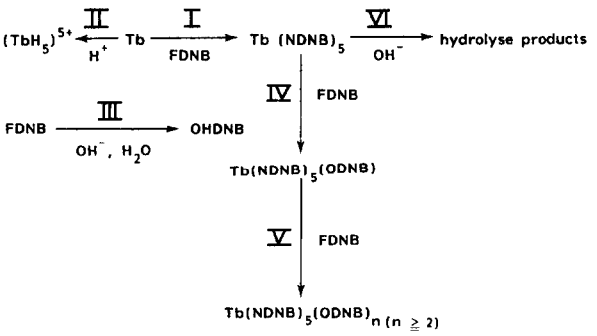

Fig. 3. Possible reactions involved in the derivatization of tobramycin with 1-fluoro-2,4-dinitrobenzene. For explanation of abbreviations see text.

TABLE I

RATE CONSTANTS (k) FOR THE REACTIONS INVOLVED IN THE DERIVATIZATION OF TOBRAMYCIN UNDER pH-STAT CONDITIONS AT 40°C

Numbers shown are the mean of two determinations unless otherwise indicated. For method of calculation of the rate constants from the experimental data see Appendix.

	Reaction		
	I	*III*	*IV*
	Apparent reaction order		
	2	*1*	*2*
	Units of k		
	l mol^{-1} min^{-1}	*min^{-1}*	*l mol^{-1} min^{-1}*
pH^*_{40} 7	0.41*	$3 \cdot 10^{-4}$***	***
pH^*_{40} 8	0.51*	$2 \cdot 10^{-4}$***	***
pH^*_{40} 9		$3 \cdot 10^{-4}$	$4 \cdot 10^{-2}$
pH^*_{40} 10		$1 \cdot 10^{-3}$	0.41§
pH^*_{40} 11		$6 \cdot 10^{-3}$	3.9

* Mean of four determinations.
** One determination only.
*** Too small to be measurable.
§ Mean of ten determinations, S.D. = 0.04 min^{-1}.

Hydrolysis of FDNB (reaction III)

Pseudo-first-order kinetics were observed under pH-stat conditions; the obtained rate constants are shown in Table I. The hydrolysis of FDNB is favoured by alkaline conditions. Assuming a ten-fold increase in hydroxyl-ion activity per pH^*_{40} unit, the results indicate reactions of FDNB with both hydroxyl ions and with the water molecules of the solvent, the latter reaction having a rate constant of about $3 \cdot 10^{-4}$ min^{-1} (Table II). It was concluded that no appreciable hydrolysis of FDNB takes place at $pH^*_{40} \leqslant 9$.

Reaction between Tb(NDNB)$_5$ and FDNB (reactions IV and V)

The reaction between the hydroxyl groups of Tb(NDNB)$_5$ and FDNB is fa-

TABLE II

SEPARATION OF THE RATE CONSTANTS FOR THE HYDROLYSIS OF FDNB INTO RATE CONSTANTS FOR THE REACTION OF FDNB WITH WATER (k_{H_2O}) AND FOR THE REACTION OF FDNB WITH HYDROXYL IONS (k_{OH^-})

Temperature: 40°C.

pH^*_{40}	k_{III}* (min^{-1})	k_{H_2O} (min^{-1})	k_{OH^-} (min^{-1})
$\leqslant 9$	$3 \cdot 10^{-4}$	$3 \cdot 10^{-4}$	0
10	$1 \cdot 10^{-3}$	$3 \cdot 10^{-4}$	$7 \cdot 10^{-4}$
11	$6 \cdot 10^{-3}$	$3 \cdot 10^{-4}$	$6 \cdot 10^{-3}$

* Observed first-order rate constant, see Table I.

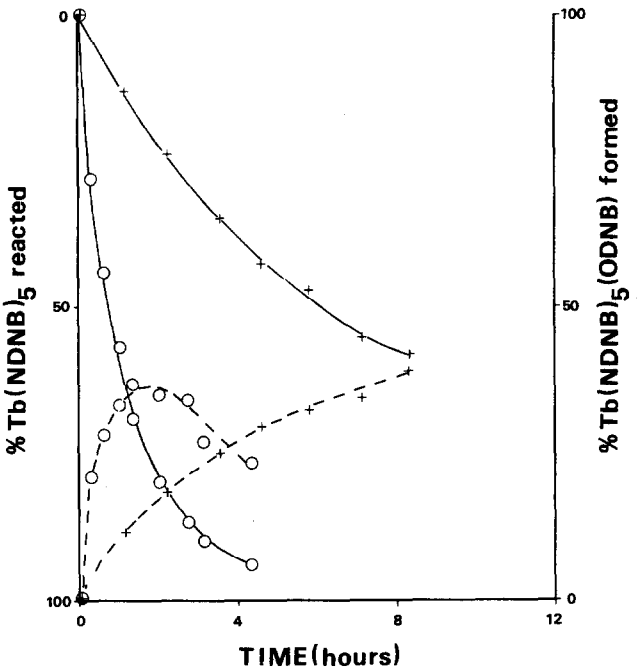

Fig. 4. Disappearance of $Tb(NDNB)_5$ (———) and formation of $Tb(NDNB)_5(ODNB)$ (– – – –) in the reaction between $Tb(NDNB)_5$ and FDNB at 40°C under pH-stat control with initial concentration of FDNB: $5.4 \cdot 10^{-3}$ *M*. +, pH^*_{40} 10; O, pH^*_{40} 11. Each point is the mean value from at least two different experiments.

voured by alkaline conditions. Fig. 4 shows some results. It is seen that at pH^*_{40} 11 the product of reaction IV, *i.e.*, $Tb(NDNB)_5(ODNB)$, disappears from the reaction mixture after its initial formation, indicating further derivatization of hydroxyl groups. This process is also evident in the chromatograms by the appearance of peaks eluted after $Tb(NDNB)_5(ODNB)$, see Fig. 2A. Based on the assumption that these peaks are due to derivatives of the type $Tb(NDNB)_5(ODNB)_n$ ($n \geqslant 2$) it was possible to assign formulae to the peaks in Fig. 2A by chromatography with simultaneous UV detection at the wavelengths of the maximum absorbance of the NDNB group and the ODNB group. This was carried out by a separate chromatographic analysis with detection at 292 and 380 nm, using acetonitrile–water (74:26, v/v) as the mobile phase. As can be seen from Fig. 2A, two isomers of gross structure $Tb(NDNB)_5(ODNB)_2$ are present and separated under the prevailing conditions.

For the reaction of $Tb(NDNB)_5$ with FDNB to give $Tb(NDNB)_5(ODNB)$ (reaction IV), second-order kinetics were expected with respect to the concentrations of FDNB and of $Tb(NDNB)_5$. This was confirmed by experiments at pH^*_{40} 10. Rate constants for reaction IV at different pH* values are shown in Table I. An increase of the pH^*_{40} value by one unit results in a ten-fold increase of the rate of reaction IV. These data suggest that $Tb(NDNB)_5$ molecules with one deprotonated hydroxyl group are the reactive species in the reaction of $Tb(NDNB)_5$ with FDNB. If the degree of deprotonation is low, the concentration of this ion is approximately linearly dependent on the reciprocal of the hydrogen-ion activity.

It was concluded that derivatization at the hydroxyl groups of $Tb(NDNB)_5$, and also of Tb, is negligible at $pH^*_{40} \leqslant 8$.

Conversion of Tb into $Tb(NDNB)_5$ (reaction I)

Both the reaction rate and the yield of $Tb(NDNB)_5$ depend on the pH^*_{40} value as shown in Fig. 5A. At pH^*_{40} 8 optimum conditions exist for the derivatization of Tb to $Tb(NDNB)_5$. Alkaline conditions give rise to derivatization at oxygen, whereas too acidic conditions cause protonation of the amino groups of Tb; protonated amino groups do not react with FDNB[16]. The kinetics of the overall reaction of Tb to $Tb(NDNB)_5$ were studied at pH^*_{40} 8, where side reactions are minimal. It was found that after an induction period the reaction was essentially second-order with respect to the concentrations of FDNB and Tb. Rate constants at pH^*_{40} 7 and 8 are shown in Table I.

To test the validity of the proposed reaction scheme (Fig. 3),, computer simulations of the yield of $Tb(NDNB)_5$ were constructed using the rate constants in Table I and the equations in the Appendix. For these simulations, two assumptions were made. (1) The predominant reactions are: Tb to $Tb(NDNB)_5$ (reaction I), $Tb(NDNB)_5$ to $Tb(NDNB)_5(ODNB)$ (reaction IV) and FDNB to OHDNB (reaction III). Only these reactions were taken into account in the simulations. (2) The rate constant for reaction I is the same at all pH^*_{40} values where deprotonation of the amino groups of Tb is complete, *i.e.*, at $pH^*_{40} \geqslant 8$. The latter assumption seems justified in view of studies by Bunnett and Hermann[16], who found that the pH de-

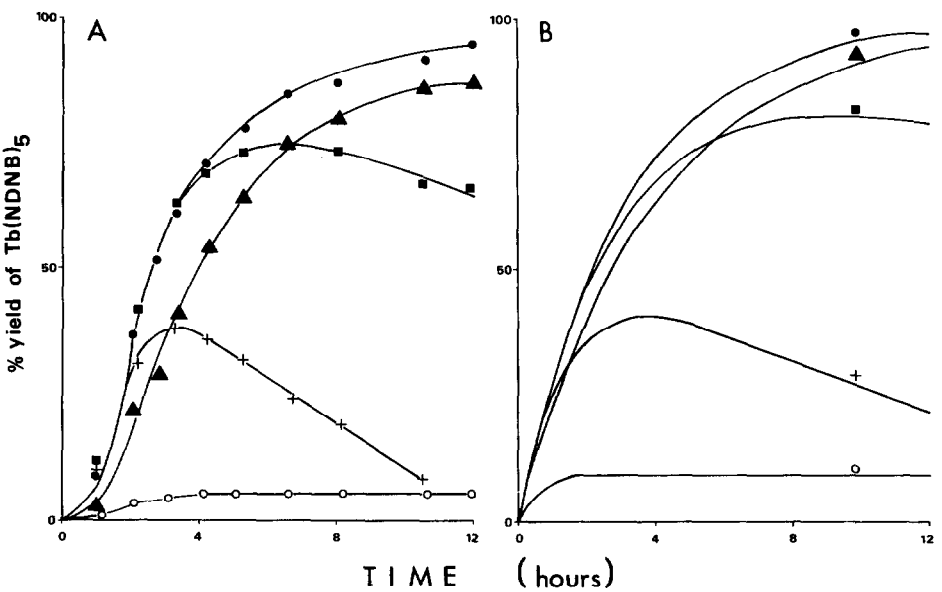

Fig. 5. Yields of $Tb(NDNB)_5$ in the derivatization of Tb with FDNB at 40°C under pH-stat conditions. Initial concentration of Tb, $2.2 \cdot 10^{-5}$ *M*; initial concentration of FDNB, $1.1 \cdot 10^{-2}$ *M*, except at pH^*_{40} 11 when the initial concentration of FDNB was $5.4 \cdot 10^{-3}$ *M*. pH^*_{40} 7 (▲), 8 (●), 9 (■), 10 (+) and 11 (○). A, Experimental data. Each point is the mean value of the yields obtained in two different experiments. B, Simulated profiles according to eqn. A8 (see Appendix) using the rate constants of Table I.

pendence of the rates of reaction of FDNB with amino acids can be entirely accounted for by the effect of pH on the ionization of the amino acids. Some results of the simulations are shown in Fig. 5B. The reasonable correspondence between the simulated and the experimentally determined yields indicates that the proposed reaction scheme and the assumptions used in the simulations are largely justified.

Optimum derivatization conditions

A distinct pH* optimum exists for the preparation of $Tb(NDNB)_5$. At 70°C this optimum was found to be situated around pH^*_{70} 8. The rate constant at pH^*_{70} 8 for reaction was found to be 3.9 l mol^{-1} min^{-1} (n = 2). Compared with the rate constant found at 40°C (Table I), this indicates that the reaction rate increases by a factor 2.0 for a 10°C rise in temperature.

We selected the following optimum derivatization conditions: temperature, 70°C; pH^*_{70} 8; initial concentration of FDNB, 10 g/l. These conditions should yield 90% conversion of Tb into $Tb(NDNB)_5$ within 10 min, with a minimum of side reactions.

Analytical derivatizations

To carry out the derivatization of Tb without the pH-stat assembly under the optimum derivatization conditions found, *i.e.*, pH^*_{70} 8, a suitable buffer system has to be used. Phthalic acid, phosphoric acid and tertiary aliphatic amines showed flat titration curves at pH^*_{70} 8 when titrated in the solvent at 70°C, indicating high buffer capacity. However, carboxylic acids and phosphoric acid react with FDNB[10,17,18], and indeed derivatization rates and yields in phthalate buffer and phosphate buffer were below the values found with the pH-stat experiments, leaving only tertiary amines as suitable buffer components. An additional advantage of the use of amines is their complete miscibility with the mixture of water and acetonitrile.

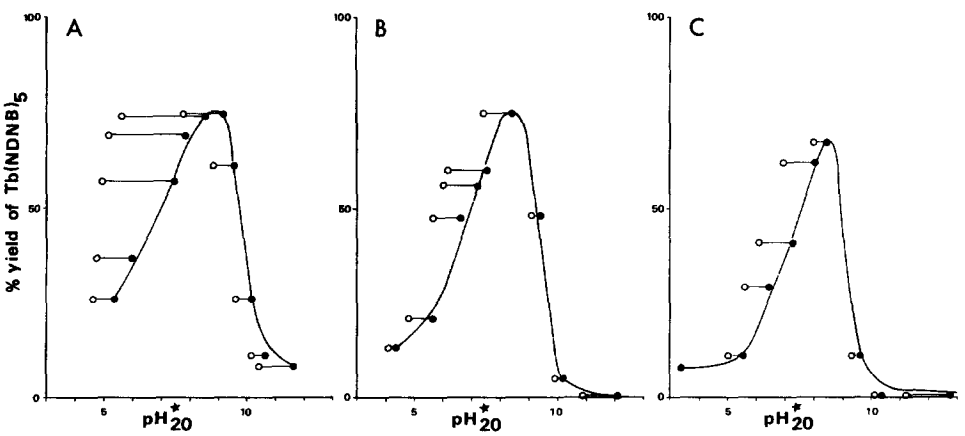

Fig. 6. Yields of $Tb(NDNB)_5$ as a function of initial pH^*_{20} (●) in the presence of ethyldiisopropylamine/HCl buffers. Also shown are pH^*_{20} values (○) after the derivatization for 10 min at 70°C. pH^*_{20} values of the same solution before and after derivatization are connected. Each point corresponds to one determination. Initial concentration of Tb, $2.4 \cdot 10^{-5}$ M; initial concentration of ethyldiisopropylamine, 0.03 M (A); 0.1 M (B) and 0.4 M (C). Hydrochloric acid was added to obtain the initial pH^*_{20} values shown.

Some results with ethyldiisopropylamine/hydrochloric acid buffers of different concentrations and initial pH^*_{20} values are shown in Fig. 6. As expected an optimum is found when the initial pH^*_{20} is 8.5. Under these conditions yields of 75% $Tb(NDNB)_5$ were obtained, together with about 10% of derivatives of the type $Tb(NDNB)_5(ODNB)_n$ ($n \geqslant 1$). The graphs of the yield of $Tb(NDNB)_5$ *versus* the initial pH^*_{20} (Fig. 6) become increasingly "sharp" with increasing buffer capacity, obviously because an unfavourable pH* is maintained more persistently at higher buffer capacities. On the other hand, the buffer capacity of the reaction mixture can be low if the reaction is carried out at the optimum pH*.

CONCLUSIONS

By use of FDNB, aminoglycosides can be converted fully into their N-2,4-dinitrophenyl derivatives with good yields in homogeneous systems in the presence of buffers composed of tertiary amines and hydrochloric acid. The reaction mixtures are directly injectable onto a reversed-phase HPLC system. However, elevated temperatures and/or a relatively high concentration of reagent are necessary to keep the reaction time within reasonable limits in a solvent comprised of water and acetonitrile. There is a sharp optimum in the hydrogen-ion activity for this derivatization reaction mainly due to the reactivity of the hydroxyl groups of the aminoglycosides with FDNB. In view of the critical reaction conditions, internal standardization is mandatory.

These conclusions will also be valid if this derivatization reaction is carried out in other solvents.

ACKNOWLEDGEMENTS

The authors wish to thank Professor A. W. M. Indemans, Professor H. J. de Jong and Dr. W. J. M. Underberg for helpful discussions, Dr. P. J. J. M. van Mil for help with the integration of the differential equations, Mr. O. A. G. J. van der Houwen, Mr. D. Seykens and Mr. J. Teeuwsen for help in data handling, recording proton-NMR spectra and technical support, respectively.

APPENDIX

Integrated rate equations

$[Tb(NDNB)_5]$, [FDNB], [Tb], and $[Tb(NDNB)_5]_0$, $[FDNB]_0$, $[Tb]_0$ refer to the concentration of $Tb(NDNB)_5$, FDNB and Tb at time t and zero time, respectively. Thus for reaction III we obtain

$$-\frac{d[FDNB]}{dt} = k_{III}[FDNB] \tag{A1}$$

$$[FDNB] = [FDNB]_0 \cdot \exp(-k_{III}t) \tag{A2}$$

where k_{III} is the slope of the plot of t (ordinate) *vs.* $\ln([FDNB]_0/[FDNB])$ (abscissa).

For reaction IV we can write

$$-\frac{d[Tb(NDNB)_5]}{dt} = k_{IV}\,[Tb(NDNB)_5]\,[FDNB] \tag{A3}$$

$$-\frac{d[FDNB]}{dt} = k_{III}\,[FDNB] \tag{A1}$$

$$[Tb(NDNB)_5] = [Tb(NDNB)_5]_0 \cdot \exp\left\{\frac{k_{IV}}{k_{III}} \cdot [FDNB]_0\,[\exp(-k_{III}t) - 1]\right\} \tag{A4}$$

where k_{IV} is the slope of the plot of $[FDNB]_0\,[1 - \exp(-k_{III}t)]/k_{III}$ (ordinate) *vs.* $\ln\{[Tb(NDNB)_5]_0/[Tb(NDNB)_5]\}$ (abscissa).

For Reaction I after an induction period

$$-\frac{d[Tb]}{dt} = k_I[Tb]\,[FDNB] \tag{A5}$$

where FDNB is in large excess over Tb. Under conditions that reaction IV is negligible, eqn. A5 becomes

$$-\frac{d[Tb]}{dt} = k_I\,[Tb]\cdot[FDNB]_0 \cdot \exp(-k_{III}t) \tag{A6}$$

$$[Tb(NDNB)_5] = [Tb]_0\left(1 - \exp\left\{\frac{k_I}{k_{III}} \cdot [FDNB]_0\,[\exp(-k_{III}t) - 1]\right\}\right) \tag{A7}$$

where k_I is the slope of the plot of $[FDNB]_0\,[1 - \exp(-k_{III}t)]/k_{III}$ (ordinate) *vs.* $\ln([Tb]_0/\{[Tb]_0 - [Tb(NDNB)_5]\})$ (abscissa).

In this calculation of k_I the experimental data pairs for high yields of $Tb(NDNB)_5$ have a very large influence on the slope of this plot and small errors in these data pairs cause large deviations. Consequently, only data pairs for yields of $Tb(NDNB)_5$ below 75% were used in the calculation of k_I.

Yield of Tb(NDNB)$_5$

$$\frac{d[Tb(NDNB)_5]}{dt} = k_I[Tb]\,[FDNB] \tag{A5}$$

$$-\frac{d\,[Tb(NDNB)_5]}{dt} = k_{IV}\,[Tb(NDNB)_5]\,[FDNB] \tag{A3}$$

$$-\frac{d\,[FDNB]}{dt} = k_{III}\,[FDNB] \tag{A1}$$

$$[\mathrm{Tb(NDNB)_5}] = \frac{k_\mathrm{I}}{k_\mathrm{IV} - k_\mathrm{I}} \cdot [\mathrm{Tb}]_0 \left(\exp \left\{ \frac{k_\mathrm{I}}{k_\mathrm{III}} \cdot [\mathrm{FDNB}]_0 \left[\exp \left(-k_\mathrm{III} t \right) - 1 \right] \right\} - \exp \left\{ \frac{k_\mathrm{IV}}{k_\mathrm{III}} \cdot [\mathrm{FDNB}]_0 \left[\exp \left(-k_\mathrm{III} t \right) - 1 \right] \right\} \right) \quad \text{(A8)}$$

REFERENCES

1 D. M. Barends, C. L. Zwaan and A. Hulshoff, *J. Chromatogr.*, 225 (1981) 417.
2 K. Tsuji, J. F. Goetz, W. VanMeter and K. A. Gusciora, *J. Chromatogr.*, 175 (1979) 141.
3 P. Helboe and S. Kryger, *J. Chromatogr.*, 235 (1982) 215.
4 L. T. Wong, A. R. Beaubien and A. P. Pakuts, *J. Chromatogr.*, 231 (1982) 145.
5 R. B. Binns and K. Tsuji, *J. Pharm. Sci.*, 73 (1984) 69.
6 D. M. Barends, C. L. Zwaan and A. Hulshoff, *J. Chromatogr.*, 222 (1981) 316.
7 D. M. Barends, J. S. Blauw, M. H. Smits and A. Hulshoff, *J. Chromatogr.*, 276 (1983) 385.
8 L. Elrod, L. B. White and C. F. Wong, *J. Chromatogr.*, 208 (1981) 357.
9 S. Thompson and O. P. Edmonds, *Ann. Occup. Hyg.*, 23 (1980) 27.
10 J. A. Ryan, *J. Pharm. Sci.*, 73 (1984) 1301.
11 P. M. Kabra, P. K. Bhatnager and M. A. Nelson, *J. Chromatogr.*, 307 (1984) 224.
12 P. P. Pashankov, P. S. Zikolov and O. B. Budevsky, *J. Chromatogr.*, 209 (1981) 149.
13 D. M. Barends, J. S. F. van der Sandt and A. Hulshoff, *J. Chromatogr.*, 182 (1980) 201.
14 H. J. Koeners, A. J. de Kok, C. Romers and J. H. van Boom, *Rec. Trav. Chim. Pays-Bas*, 99 (1980) 355.
15 D. J. Edwards, in K. Blau and G. S. King (Editors), *Handbook of Derivatives for Chromatography*, Heyden, London, 1977, p. 391.
16 J. F. Bunnett and D. H. Hermann, *Biochemistry*, 9 (1970) 816.
17 H. Kotake, K. Inomata, H. Kinoshita, K. Tanabe and O. Miyano, *Chem. Lett.*, (1977) 647.
18 R. Wittmann, *Chem. Ber.*, 96 (1963) 771.