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Short Communication

Fast pre-column derivatization of aminoglycosides with 1-fluoro-2,4-dinitrobenzene and its application to pharmaceutical analysis

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Introduction

Aminoglycosides neither absorb in the ultra-violet region, except for tail absorption at low wavelengths, nor undergo fluorescence. Therefore, for on-line detection after HPLC, either pre-column or post-column derivatization is necessary. The use of HPLC as an analytical method for therapeutic drug monitoring of aminoglycosides has been reviewed recently [1]. In pharmaceutical quality control, pre-column derivatization, with a label that shows ultra-violet absorption, is to be preferred. Nitrophenylation of aminoglycosides with 1-fluoro-2,4-dinitrobenzene (FDNB) [1–5] and 2,4,6-trinitrobenzene sulphonic acid has been used for this purpose [6].

The 2,4-dinitrophenyl derivatives of aminoglycosides show excellent chromatographic properties both on reversed-phase systems and normal-phase systems [1]. These derivatives are stable and possess high molar absorptivities at favourable wavelengths, making interferences by matrix compounds unlikely. The identity of these derivatives also has been established and methods have been described for their preparation, purification and chemical assay [7, 8]. Drawbacks of this pre-column derivatization include: the toxicity of the reagent, requiring the use of protective gloves [9], the slow reaction rate requiring heating and the high concentration of the reagent and the long reaction times required [10]. Also, it is necessary to optimize the acidity of the derivatization mixture to avoid attack of the hydroxyl groups of aminoglycosides by FDNB [10], which occurs under alkaline conditions.

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The present study aims to avoid some of these drawbacks by carrying out the derivatization in a solvent that accelerates the reaction. Also, a procedure is developed that minimizes the handling of FDNB.

Experimental

Materials

Tobramycin (Tb) and amikacin (Am) of known chemical content were used [7, 8]. Unless otherwise mentioned, concentrations of aminoglycosides are based on their chemical assay; if concentrations of aminoglycosides are intended to be expressed in terms of antibiotic potencies, this is indicated by the notation mg (potency)/ml.

The purified 2,4-dinitrophenyl derivatives of Tb and Am, $Tb(DNB)_5$ and $Am(DNB)_4$, respectively were available [7, 8]. Acetone was of technical quality (Shell Chemicals); it was filtered through a 0.2 μm filter (SM 116, Sartorius) before its use as a mobile phase component. All other reagents were of analytical reagent quality. Capillettor® micropipettes 1–5 μl and 0.5–2.5 ml (Clinicon) were used.

HPLC

A Solvent Delivery HPLC system (M6000A) with autosampler (WISP 710) and a UV detector (Model 440) set at 365 nm were used (all from Waters Assoc.). Separations were performed with a 30 cm × 3.9 mm i.d. RP18 column (10 μm particles, Merck). For the analysis of Tb the mobile phase was acetone–water–acetic acid (75:25:0.1, v/v/v); for the determination of Am: acetone–water–acetic acid (63:37:0.1, v/v/v). Chromatography was performed at room temperature at a flow of 1.0 ml/min.

Derivatization

The buffered solvent consisted of 0.10 M of boric acid, 0.24 M of mannitol and 0.02 M of sodium hydroxide in dimethylsulphoxide (DMSO)–water (80:20, v/v). Samples and standards were diluted with this buffered solvent to obtain final concentrations in the range 5–150 mg/l of aminoglycoside. Of the final dilutions, 2.5 ml were pipetted into autosampler vials and 5 μl of FDNB was added by means of a micropipette; for caution protective gloves should be worn. After closing and mixing the vials, they were heated together in a waterbath at 60°C for 5 min. After cooling to room temperature, the autosampler was programmed to inject 10 μl volumes of the reaction mixtures.

Results and Discussion

Choice of the solvent for the derivatization reaction

Previous work has shown that pre-column derivatization of aminoglycosides may be carried out using FDNB in 66% v/v acetonitrile [1] and that injection volumes of more than 100 μl can be introduced into the reversed-phase HPLC system without loss of performance. However, the derivatization reaction is rather slow in aqueous acetonitrile [10].

Bunnet and Hermann [11] measured the rate of the reaction between glycine and FDNB in mixtures of DMSO and water and mixtures of acetonitrile and water. The reaction was found to be much faster in solvents containing high concentrations of DMSO. Accordingly mixtures of DMSO and water were investigated as possible solvents for the pre-column derivatization of aminoglycosides with FDNB. In order to minimize

the handling of FDNB, derivatization mixtures should be injected without further dilutions or liquid transfers after its addition. Thus, portions of a reaction mixture containing a high content of DMSO need to be injected into the chromatographic system. Therefore the compatibility of DMSO–water mixtures with subsequent HPLC analysis was investigated. Purified $Tb(DNB)_5$ was dissolved in mixtures of DMSO and water and these mixtures chromatographed using a range of injection volumes. Typical results are summarized in Fig. 1.

Also, the reproducibility of injecting 10 μl portions of purified $Tb(DNB)_5$ and $Am(DNB)_4$, dissolved in DMSO 80% v/v was measured and found to be acceptable. It was concluded that 10 μl injections of derivatization mixtures composed of DMSO 80% v/v are possible without unacceptable loss of analytical performance.

Optimization of the derivatization reaction

Tobramycin was chosen as the model compound and the derivatization in DMSO 80% v/v was studied, varying the apparent pH (pH*), the initial concentration of FDNB, the reaction time and temperature [10]. These experiments were performed in a non-buffered solvent, using a pH-stat [10]. The reaction conditions were optimized with respect to high derivative yield, short reaction time and clean chromatograms. Thereafter, several acids and bases were dissolved in DMSO 80% v/v and titration curves determined with sodium hydroxide or hydrochloric acid as titrate, also dissolved in DMSO 80% v/v. A mixture of boric acid and mannitol, titrated with sodium hydroxide showed a flat titration curve at the optimal pH* selected from the pH-stat measurements. The mixture of sodium hydroxide, boric acid and mannitol, dissolved in DMSO 80% v/v was found to be the most suitable buffered solvent (for composition: see Experimental section).

The chosen derivatization conditions give rise to a high yield of the $Tb(DNB)_5$, 86% and for $Am(DNB)_4$ 98%. If the reaction conditions are subjected to small changes essentially the same yields are obtained, indicating the conditions are not very critical (Table 1).

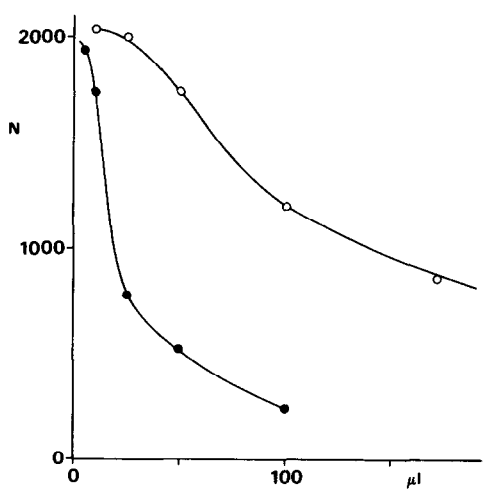

Figure 1
Column efficiency (N) versus injection volume for $Tb(DNB)_5$ dissolved in DMSO 80% v/v (●). Also shown are the results obtained with $Tb(DNB)_5$, dissolved in the mobile phase (○).

Table 1
Derivatization yields of tobramycin under various conditions. Temperature: 60°C

pH^*_{20}	µl FDNB/2.5 ml reaction mixture	Time (min)	Yield (%)
8.3	5	5	86
8.3	10	5	86
8.3	5	15	86
8.9	5	5	83
7.8	5	5	86
7.8	10	15	85

pH scale defined by the following two pH* standards [9]: pH^*_{20} 6.97; phthalic acid 1.2 mM, potassium hydrogen phthalate 25 mM in DMSO 80% v/v. pH^*_{20} 4.73; potassium tetraoxalate 9.9 mM in DMSO 80% v/v.

Table 2
Repeatability and linearity of the determination of tobramycin (Tb) and amikacin (Am)

Tb concentration (mg/ml)	c.v. (%)	pH/Tb concentration	Am concentration (mg/ml)	c.v. (%)	pH/Am concentration
137	3.5	5.50	108	1.6	0.360
27.3	1.4	5.68	21.7	1.7	0.369
5.41	0.4	5.57	4.32	5.2	0.358

Peak height (pH) measurements. Five derivatizations and assays were carried out for each test concentration. Each derivatization mixture was injected once. Repeatability is expressed as the coefficient of variation (c.v.).

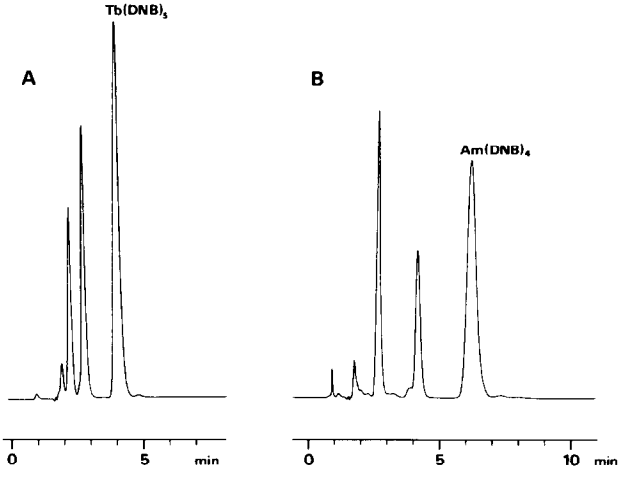

Figure 2
HPLC of the 2,4-dinitrophenyl derivatives of tobramycin (Tb) and amikacin (Am) after derivatization in DMSO 80% v/v. Peaks eluting in front of $Tb(DNB)_5$ and $Am(DNB)_4$ are excess FDNB and its hydrolysis product: 1-hydroxy-2,4-dinitrobenzene [7]. A: a chromatogram obtained from a derivatization mixture containing the equivalent of 68 mg/l Tb. Attenuation: 0.1 a.u.f.s.; B: A chromatogram obtained from a derivatization mixture containing the equivalent of 114 mg/l Am. Attenuation: 0.5 a.u.f.s.

Analytical evaluation

Representative chromatograms of the derivatization products of Tb and Am are shown in Fig. 2 and the quantitative characteristics of the assay are presented in Table 2.

A commercial injection solution containing Tb with a declared concentration of 40 mg (potency)/ml of Tb (as the sulphate salt) was analysed. The result of a microbiological assay was: 41.4 mg (potency)/ml, with 95% confidence interval: 40.6–42.1 mg (potency)/ml. The result of the HPLC assay was 40.7 mg/ml (n = 3). Similarly a commercial injection solution containing Am, with declared concentration: 250 mg (potency)/ml of Am, was analysed to yield a value of 264 mg/ml Am (n = 3).

Conclusions

A simple and rapid derivatization technique is described for the liquid chromatographic assay of amikacin and tobramycin. Because of the structural similarity of aminoglycosides it is possible that derivatization with 1-fluoro-2,4-dinitrobenzene may be applied to the assay of other aminoglycosides.

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