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ISOLATION OF GENTAMICIN C COMPOUNDS FROM CULTURE FILTRATES OF *MICROMONOSPORA PURPUREA*

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SUMMARY

A liquid chromatographic method was developed for the isolation of gentamicin C compounds from commercial fermentation products in order to monitor health hazards (oto- and nephrotoxicity). Chromatography was carried out on silica gel 60 (15–40 μm) with a medium-pressure chromatographic system, employing methanol–25% ammonia solution (85:15, v/v) and methanol–chloroform–25% ammonia solution (20:10:5, v/v) as mobile phases. The eluted fractions were neutralized with 1.0 *M* hydrochloric acid, concentrated *in vacuo* and desalted by gel filtration. It was possible to demonstrate by ^{1}H NMR spectroscopy and high-performance liquid and thin-layer chromatography that the separated fractions contained components C_1, C_{1a} and C_2 in purities of more than 95%.

INTRODUCTION

Gentamicin is a broad-spectrum, basic, water-soluble aminoglycoside antibiotic mixture produced by *Micromonospora purpurea*[1–4]. The isolation of the gentamicins from fermentation broth can be performed by several methods[5–15] and, depending on the procedure, it is possible to extract the whole antibiotic complex or some of its components. Thirteen gentamicin compounds have been isolated and identified. The most important method is extraction by ion-exchange chromatography on cation- and anion-exchange resins, such as Amberlite IRC-50, CG-50, CG-120 and Dowex 1X2. It is possible to isolate the three major components C_1, C_{1a} and C_2 on cellulose powder[6,7], whereas the other C compounds (C_{2a} and C_{2b}) can be extracted by Craig counter-current distribution and represent only 4% of the total C complex[11,12]. Column chromatography on silica gel has been employed for the isolation of the major gentamicin components A, B, B_1 and X[8]. The separation of the gentamicin C compounds on silica gel was not satisfactory[15].

The aim of this investigation was to develop a medium-pressure liquid chromatographic (MPLC) method on silica gel for the isolation of pure major gentamicin components.

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EXPERIMENTAL

Materials and reagents

Gentamicin samples were obtained from Merck (Darmstadt, F.R.G.) and Serva (Heidelberg, F.R.G.). Purified components were made available as standards by G. H. Miller (Schering , Bloomfield, NJ, U.S.A.). The USP gentamicin standard (microbiological activity, 663 μg/mg; C_1, 36.9%; C_2, 31.6%; and C_{1a}, 31.5%) was obtained from United States Pharmacopeial Convention (Rockville, MD, U.S.A.). All other chemicals employed were supplied by Merck or Baker (Gross-Gerau, F.R.G.).

MPLC equipment

The chromatographic equipment consisted of a Büchi B-681 pump (Büchi, Göppingen, F.R.G.) with an injection valve (2-ml loop) and a Büchi B-684 fraction collector. Columns (23 × 2.6 cm I.D.) were packed in the laboratory with silica gel 60 (15–40 μm) for column chromatography (Merck).

Mobile phases

Mobile phase A consisted of methanol–25% ammonia solution (85:15, v/v) and mobile phase B consisted of methanol–chloroform–25% ammonia solution (20:10:5, v/v/v), both pumped through the column at flow-rate of 25 ml/min.

Detector system

The detection of the gentamicins was carried out by high-performance thin-layer chromatography (HPTLC) and derivatization with ninhydrin. A volume of 6 μl of each eluted fraction was applied with microcaps (Drummond, Broomall, U.S.A.) to an HPTLC silica gel plate (10 × 20 cm, Merck). After one-dimensional development with a solvent system consisting of chloroform–methanol–32% ammonia solution (10:8:5, v/v/v), the spots were detected by ninhydrin derivatization by dipping the well dried plate into a 0.2% (w/v) methanolic ninhydrin solution and heating for 10 min at 110°C.

MPLC procedure

Gentamicin sulphate (1 g) was dissolved in 6 ml of water–methanol (5:1; v/v) and a 2.0-ml volume of this solution was injected into the chromatographic system. After passage of 200 ml of mobile phase, the effluent was collected in fractions of 20 ml.

Gel filtration

The eluted fractions were neutralized with 1.0 *M* hydrochloric acid and concentrated in *vacuo*; 200-mg samples of the residues obtained were dissolved in 2 ml of water and purified by gel chromatography on Bio-Gel P-2 (450 × 22 mm) (Bio-Rad Labs., Munich, F.R.G.).

^1H NMR spectrometry

The spectrometry investigations were carried out in deuterium oxide with sodium 3-trimethylsilylpropionate-2,2,3,3-d_4 as internal reference (Bruker AM 400 Multinucleus Fourier NMR apparatus, 400 MHz) (Bruker, Karlsruhe, F.R.G.).

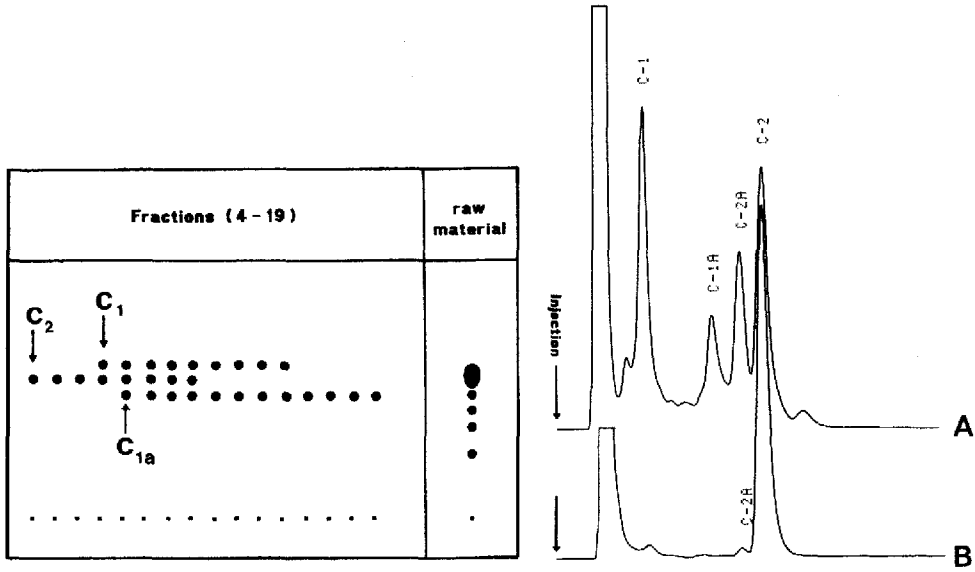

Fig. 1. Elution of the gentamicin components with mobile phase A in comparison with those of the raw material and pure components.

Fig. 2. HPLC of (A) the gentamicin C complex and (B) the eluted C_2 fraction according to Weigand and Coombes[16].

RESULTS AND DISCUSSION

With mobile phase A C_2 was eluted first and could be isolated from fractions 4–6 (Fig. 1). Fractions 7–11 were collected, neutralized, concentrated *in vacuo* and chromatographed again under the same conditions. Mobile phase B was then employed to isolate C_1 and C_{1a} from the concentrated extract of fractions 12–19.

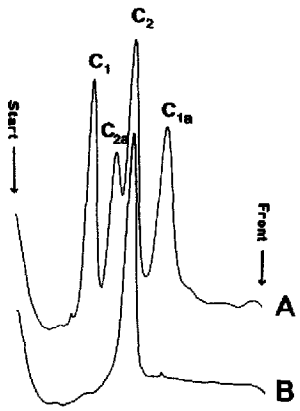

Fig. 3. HPTLC of (A) the gentamicin C complex and (B) the eluted C_2 fraction according to Kunz and Jork[35].

Under these conditions, the elution sequence changed ($C_1 \rightarrow C_2 \rightarrow C_{1a}$) and C_1 could be completely separated from C_{1a}.

The purity of the separated components was monitored by high-performance liquid chromatography (HPLC), HPTLC and ^1H NMR spectroscopy. Fig. 2 illustrates the HPLC separation of the gentamicin C complex (A) and the eluted C_2 fraction (B), carried out by the method of Weigand and Coombes[16]. This HPLC procedure was the most satisfactory[17–35]. C_1 and C_{1a} were of a comparable degree of purity (results not shown). Analogous results were obtained by HPTLC (see Figure 3)[35]. ^1H NMR spectroscopy showed no evidence of any contamination of the isolated C compounds.

In conclusion, it has been possible to isolate the three major components of the gentamicin complex in high purity with one chromatographic system by combining two mobile phase systems.

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