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## Note

# High-performance liquid chromatographic analysis of gramicidin, a polypeptide antibiotic

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Gramidicin (Gdin), a polypeptide antibiotic isolated from tyrothricin, has previously been fractionated into three major components by countercurrent distribution (CCD)[1–3] and by droplet countercurrent chromatography (DCCC)[4]. There were strong indications that each of the major components, designated Gdin A, Gdin B and Gdin C, actually consists of two closely related components, designated valine-gramicidin ([Val]-Gdin) and isoleucine-gramicidin ([Ile]-Gdin)[5].

The gramicidins are linear N-acylated pentadecapeptide-ethanolamides with their terminal amino groups blocked by a formyl group. The structures of the individual components which have been elucidated are shown in Fig. 1[6–13].

HCO–Y–Gly–L-Ala–D-Leu–L-Ala–D-Val–┐
┌D-Leu–Z–D-Leu–L-Trp–D-Val–L-Val–┘
└L-Trp–D-Leu–L-Trp–NH$(CH_2)_2$OH

| | Y | Z | Mol.wt. |
|---|---|---|---|
| [Val]-Gdin A: | L-Val | L-Trp | 1882 |
| [Ile]-Gdin A: | L-Ile | L-Trp | 1896 |
| [Val]-Gdin B: | L-Val | L-Phe | 1843 |
| [Ile]-Gdin B: | L-Ile | L-Phe | 1857 |
| [Val]-Gdin C: | L-Val | L-Tyr | 1859 |
| [Ile]-Gdin C: | L-Ile | L-Tyr | 1873 |

Fig. 1. Structure of the gramicidin components.

This paper describes a high-performance liquid chromatographic (HPLC) method that provides an efficient analytical separation of the gramicidin components. The method is used to determine the ratio of components in the World Health Organization (WHO) international reference preparation of gramicidin[14] and to study the effect of recrystallizations on ratio of components.

## EXPERIMENTAL

### *Materials*

The WHO international reference preparation of gramicidin (and the U.S. Food and Drugs Administration internal working standard) is identical with crystalline gramicidin lot No. 27 produced by H. Lundbeck (Copenhagen, Denmark)[14].

After an initial determination of the ratio of components this was used as the HPLC working standard.

For the identification and quantification of components in the working standard, relatively pure components were available from a CCD separation performed by Dr. Lyman C. Craig of the Rockefeller Institute, New York[14].

A non-purified gramicidin was used as starting material for the recrystallization experiment.

*Recrystallizations*

A saturated solution was prepared in 96% ethanol by gentle heating, and was left in a refrigerator for 48 h. The crystals were filtered off and dried in a vacuum oven at 60° for 5 h. Eight consecutive crystallizations were performed.

*High-performance liquid chromatography*

A DuPont 830 liquid chromatograph was used, with a DuPont 837 spectrophotometer detector operated at a wavelength of 220 nm. The column was a 25 cm × 2.1 mm I.D. stainless steel column prepacked with Zorbax ODS (DuPont, Hitchin, Great Britain), which is a microspheroidal silica packing with a chemically bonded octadecyl stationary phase (particle size 5 $\mu$m). The column was operated at 60° and at a pressure of 2000 p.s.i.

The mobile phase was a mixture of analytical grade methanol and a 0.005 *M* aqueous solution of ammonium sulphate in the ratio 74:26. Samples were dissolved in the mobile phase and introduced into the column by means of a six-port valve with a 10 $\mu$l loop corresponding to an injected amount of 1 $\mu$g gramicidin.

## RESULTS AND DISCUSSION

*The reference preparation*

A typical chromatogram of lot No. 27 is shown in Fig. 2, and as expected from CCD and DCCC data, the elution sequence turned out to be:

[Val]-Gdin C
[Ile]-Gdin C
[Val]-Gdin A
[Ile]-Gdin A
[Val]-Gdin B
[Ile]-Gdin B

↓ increasing lipophilicity

The quantitative determination of components showed that lot No. 27 contains 78% w/w Gdin A, 14% w/w Gdin C, and 8% w/w Gdin B. The amount of [Ile]-Gdin in each of these is from 10 to 15% w/w. These values are listed in Table I and compared with values calculated on the basis of an amino-acid analysis[14] and the basic structures shown in Fig. 1. A very good agreement is observed.

*The recrystallization experiment*

It has been observed that the gramicidin components show quantitative differences in their antibacterial activity, and it is doubtful whether reliable micro-

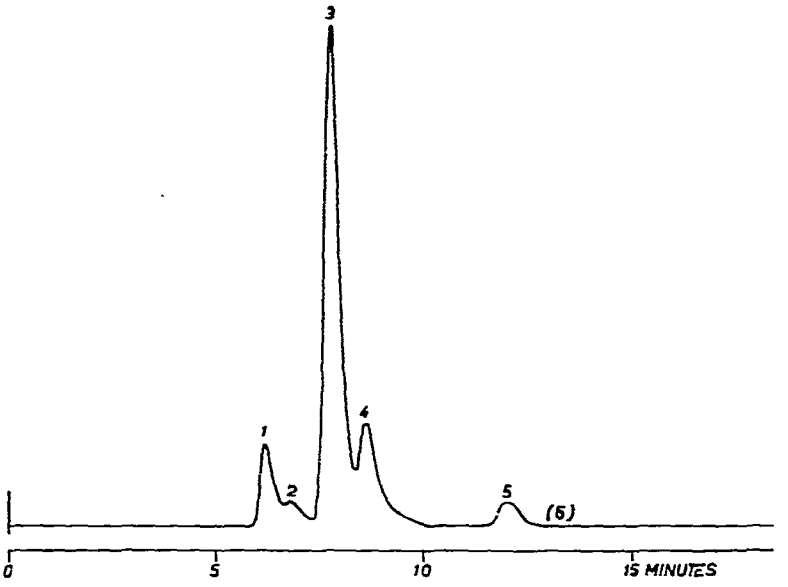


Fig. 2. Crystalline gramicidin lot No. 27. Chromatogram showing the separation of: [Val]-Gdin C (1), [Ile]-Gdin C (2), [Val]-Gdin A (3), [Ile]-Gdin A (4), [Val]-Gdin B (5), [Ile]-Gdin B (6).

biological assays can be performed if the sample and the standard preparation have different component ratios. Consequently it is of great importance that the influence of manufacturing procedures, such as recrystallizations, is known.

The ratios of components found in the amorphous starting material and in the crystalline fractions obtained are shown in Fig. 3, from which it appears that the ratios are influenced considerably by the first three or four crystallizations. Additional recrystallizations have little effect. It also appears that pure Gdin A cannot be obtained in this way. The influence of recrystallizations on melting points and solubilities of gramicidin has previously been reported[15].

*Other applications*

The HPLC method described is not restricted to gramicidin analysis. It is also

TABLE I

CRYSTALLINE GRAMICIDIN LOT No. 27 HPLC RESULTS COMPARED WITH CALCULATED VALUES BASED ON AMINO-ACID ANALYSIS AND THE BASIC STRUCTURE OF THE COMPONENTS (Fig. 1)

| *Components* | *Quantitative determination by HPLC (% w/w)* | *Calculated on the basis of amino-acid analysis* (% w/w)* |
|---|---|---|
| [Val] + [Ile]-Gdin A | 78 | 79** |
| [Val] + [Ile]-Gdin B | 8 | 7 |
| [Val] + [Ile]-Gdin C | 14 | 14 |
| [Ile]-Gdin A + B + C | 10–15 | 14 |

* 0.98% Ile, 1.31% Tyr, 0.58% Phe (% w/w as free amino acids).
** Calculated as difference.

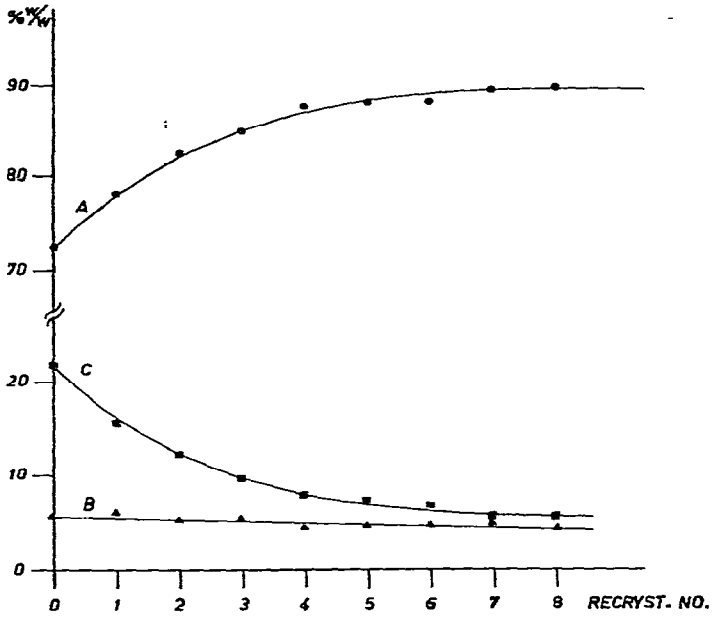


Fig. 3. Relationship between the ratio of components (Gdin A, Gdin B, and Gdin C) and the number of recrystallizations performed.

applicable to tyrothricin (gramicidin and tyrocidin), and to any intermediate between fermentation broth and crystalline gramicidin.

Fig. 4 shows a chromatogram of an extract of a fermentation broth. Several peaks are seen in the tyrocidin area, and it appears that a fermentation broth contains considerably more Gdin C than a crystalline gramicidin.

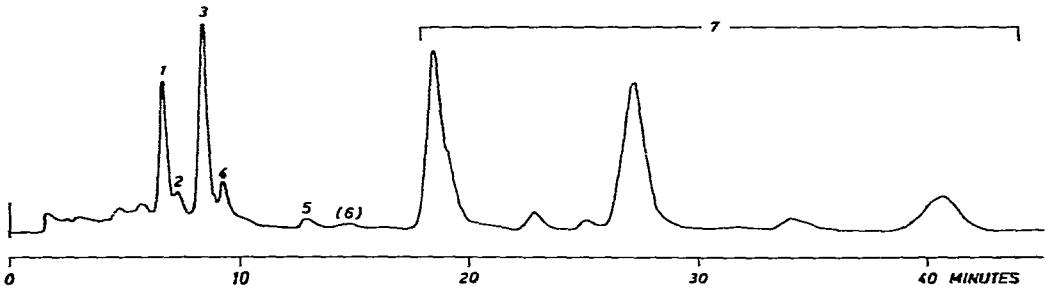


Fig. 4. Chromatogram of a fermentation broth showing gramicidins (1–6) (Fig. 2) and tyrocidins (7).

## CONCLUSION

HPLC has provided a rapid and powerful tool for the component analysis of gramicidin and its intermediates. From an analytical point of view HPLC is considerably more efficient than earlier applied CCD- and DCCC-separations. The separation of gram-size samples would, however, require a very large and costly

increase in scale, and from a preparative point of view CCD- and DCCC-separations are still to be preferred.

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