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Note

Influence of reaction parameters in the densitometry of gentamicin visualized with ninhydrin reagent

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The broad-spectrum antibiotic gentamicin C consists of three major components of very similar structures. The components have almost the same antibacterial activity ($C_1:C_{1a}:C_2 = 0.786:0.977:1.023$)[1,2] but there are some differences in their biological actions[1,2] and toxicological properties[3]. As a consequence, the determination of the component ratio is of great importance. ^{1}H NMR spectroscopy[4] yields only indirect information on the component ratio, suitable for quality control but insufficient, *e.g.*, for the assessment of clinical complaints. The FDA method, comprising a microbiological determination of the individual components after chromatographic separation and elution, is specific but rather time-consuming. The high-performance liquid chromatographic (HPLC) procedures reported recently[6] involve derivatization.
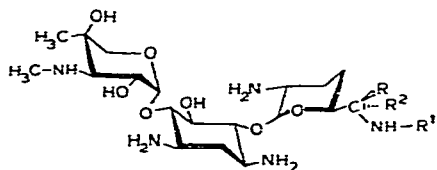
		R	R[1]	R[2]
gentamicin	C_1	CH_3	CH_3	H
	C_{1a}	H	H	H
	C_2	CH_3	H	H

A very rapid and simple densitometric quantitation of the separated gentamicin components after reaction with ninhydrin has been described by Wagman and co-workers[1,2] and Wilson *et al.*[7]. However, the components, exhibit different response for the bases, $C_{1a}:C_2:C_1$ relative response factors 1.000:0.578:0.518, and for the sulphate salts[1,2], 1.000:0.485:0.397. (The ratios of these factors are not even equal to the base: sulphate stoichiometry.)

There is no need to emphasize that exact knowledge of the relative response factors as well as of their confidence intervals, the latter being not reported in the literature, is essential when the method is used for the purpose of pharmaceutical quality control. Using thin-layer chromatography (TLC) and ninhydrin visualization

procedures described in the literature[1,2,7] we tried to determine the confidence intervals of the densitometric response factors. However, extremely high intraassay variances ($\pm$ 40%, $n = 20$) were observed, clearly indicating that the parameters of the *in situ* ninhydrin reaction had not been optimized.

In this paper the optimization of the gentamicin–ninhydrin *in situ* reaction, suitable for quantitative densitometric determination, as well as the modified relative response factors of the gentamicin C components are presented.

EXPERIMENTAL

Apparatus

Densitograms were recorded on an ERI-65 (Carl Zeiss Jena, Jena, G.D.R.) TLC scanner in a reflection mode, using a 510-nm filter. Peak areas were measured graphically.

Reagents

Pharmaceutical grade (658–687 IU/mg) gentamicin sulphates, authentic C_1, C_{1a} and C_2 components (all supplied by Chinoin, Budapest, Hungary) and analytical grade chemicals were used. The purity of ninhydrin was checked according to the literature[8]. The modified ninhydrin visualizing reagent was a freshly prepared solution of 1.0 g ninhydrin and 0.25 g cadmium acetate in a mixture of 10.0 ml acetic acid and 50.0 ml ethanol.

Chromatographic procedure

A 10-μl volume of a 10 mg/ml gentamicin solution (100 μg) and 10 μl of 1–60 mg/ml solutions of the gentamicin C_1, C_{1a} and C_2 components were applied on DC-Alufolien Kieselgel 60 (Merck Art. No. 5533) plates in a strip 15 mm long. After drying in ambient air the sheets were placed into the chromatographic chamber containing the developing solution [lower layer of chloroform–methanol–concentrated ammonia $(1:1:1 \text{ v/v})^{1,2,9}$]. After saturation for 1 h the development was started and it was stopped when the solvent front was approximately 150 mm from the start line. After drying at 120°C for 30 min, the sheets were sprayed with the ninhydrin reagent and heated again in a suitable oven at 120°C for a given time (see below).

RESULTS AND DISCUSSION

Our results shown in Figs. 1 and 2 indicate that heating procedures published for the ninhydrin–gentamicin *in situ* reaction (5 min at 105°C[1,2], 15 min at 135°C[7]) cannot be used for the quantitative densitometry. Comparing Figs. 1 and 2 it can be seen that the colour reaction of gentamicin C_{1a} with ninhydrin proceeds appreciably faster than those of the other two components. After heating for 1 h at 120°C the relative intensities are practically constant. For safety's sake, however, a heating time of 2 h is suggested. Under these circumstances, 30 parallel experiments were performed and the detector responses of the components, Y, relative to gentamicin C_{1a}, were determined (Table I). If the measured peak areas of each component are divided by the Y values and the sum of the areas taken in this way equated to 100%, the relative amounts of the components can be calculated.
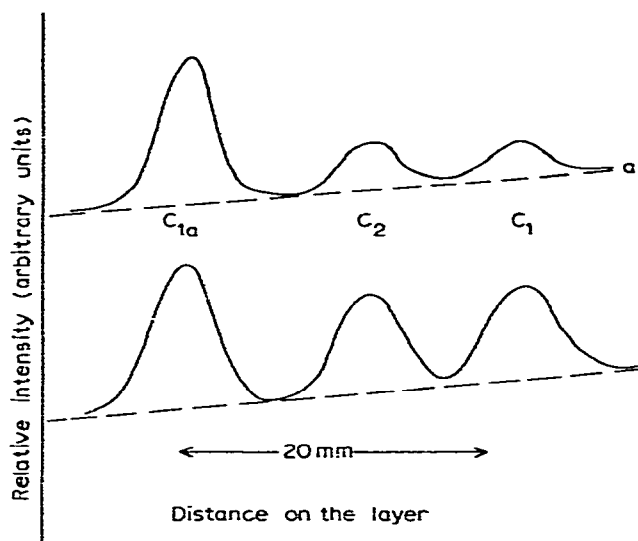
Fig. 1. Densitograms of gentamicin components visualized with ninhydrin reaction. Time of heating at 120°C after spraying the layer with the reagent: a, 10 min; b, 60 min.

For analyses of mixtures containing 20–50 % relative amounts of the individual components the relative standard deviations were 4.1 % for C_{1a} and C_2 and 5.3 % for C_1.

Table I represents the reliability of our modified densitometric method and its good correlation with the FDA procedure.

Our results seem to show that the environment of the amino group plays an important rôle in the rate of the reaction with ninhydrin. It should be noted that
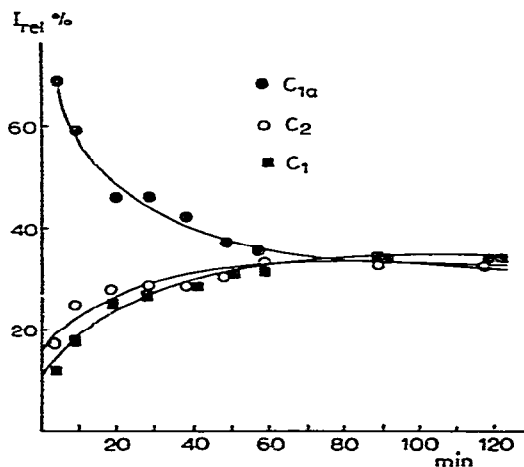

Fig. 2. The relative per cent intensity (I_{rel}) of the gentamicin components plotted against the time of heating at 120°C after spraying the chromatographed layer with ninhydrin reagent (sum of peak areas = 100 %).

TABLE I

DENSITOMETRIC DETERMINATION OF GENTAMICIN COMPONENT RATIO AND COMPARISON WITH THE FDA METHOD

The reliability of data is represented by their confidence intervals at the $P = 0.95$ level (n parallel experiments). Relative response factors of gentamicin components: $Y_{C_{1a}} = 1.00$; $Y_{C_2} = 0.85 \pm 0.04$; $Y_{C_1} = 0.86 \pm 0.05$ ($n = 30$). Measured quantities and observed data in relative per cent.

Sample	C_{1a}		C_2		C_1		n
	Meas.	Obs.	Meas.	Obs.	Meas.	Obs.	
I	50.8	48.0 ± 3.9	25.0	25.6 ± 4.0	24.3	26.4 ± 2.5	10
II	24.9	26.3 ± 1.9	35.0	34.5 ± 2.1	40.1	39.2 ± 4.5	11
III	29.5	30.9 ± 2.9	24.1	25.1 ± 2.3	46.4	43.9 ± 3.9	9

Sample	C_{1a}		C_2		C_1		n
	FDA	Dens.	FDA	Dens.	FDA	Dens.	
IV	23.9	22.4 ± 3.3	46.1	48.2 ± 1.8	30.0	29.8 ± 4.7	5
V	24.8	22.2 ± 1.4	48.0	50.4 ± 2.8	27.2	27.3 ± 2.4	5

qualitative results of Dutt and Teng Poh[10] indicated that the colour and intensity of spots of different substances visualized with ninhydrin reagent were dependent on the heating time. These data together with our results strongly suggest that several *in situ* ninhydrin reactions described in the 'iterature are insufficiently optimized for quantitative purposes.

ACKNOWLEDGEMENTS

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