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Note

British Pharmacopoeial gentamicin sulphate component ratio test by high-performance liquid chromatography

The effect of derivative breakdown on final result

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Gentamicin sulphate is an aminoglycoside antibiotic complex, consisting of four major components, designated C1, C1a, C2 and C2a[1–5]. The structures of these components have been determined spectroscopically[6]. Since the C-complex components may differ in antibiotic activity and in toxicity, pharmacopoeial monographs on gentamicin contain tests which limit their relative ratios. In the United States Pharmacopoeia (U.S.P.) procedure[7], three components (C1, C1a and C2) are separated by paper chromatography and are determined by microbiological assay. Until recently, the British Pharmacopoeia (B.P.)[8] utilised an NMR method. The 1983 B.P. Addendum[9] introduced a reversed-phase high-performance liquid chromatographic (HPLC) method[10] in which limits are applied to the relative ratio of the normalised C1, C1a, and combined C2 + C2a peak areas.

The B.P. procedure requires that gentamicin is derivatised prior to HPLC. Primary amine groups of the gentamicin components are reacted with *o*-phthalaldehyde (OPA) and mercaptoacetic acid in borate buffer at pH 10.4 to yield 1-alkylthio-2-alkylisoindole derivatives. These provide strong chromophores for UV detection at 330 nm. Although derivatisation occurs under alkaline conditions, the derivatised components are chromatographed under acidic conditions (pH 4–5). Since isoindoles are generally highly reactive compounds[11–13], concern was voiced as to the stability of these derivatives during the HPLC procedure.

In this study, the stability of gentamicin derivatives was determined under the alkaline conditions required for derivatisation and under the acidic conditions used for chromatography. It was envisaged that any derivative breakdown during chromatography would be time-dependent and that component derivatives with long retention times (C2 and C2a) would be particularly affected by degradation prior to detection. Since this could influence the peak area ratios obtained by this method,

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the effect on the final result of changing the HPLC analysis time was investigated by varying the flow-rate of the mobile phase.

EXPERIMENTAL

Apparatus

A Gilson 302 pump, a Cecil CE2112 variable-wavelength UV detector and a Rheodyne 7125 loop-valve (20 μl) were used. Peak area data was obtained with a Trilab computer (Trivector Scientific).

Chemicals

Gentamicin sulphate BP was from Roussel Laboratories Ltd. All other chemicals and solvents were obtained from BDH (Poole, U.K.).

Stability of derivatives diluted with borate buffer, pH 10.4, and with mobile phase

A 0.0624% (w/v) solution of gentamicin sulphate was derivatised with OPA in accordance with the B.P. method[9]. The derivative solution was then diluted 1:5 with (a) 0.04 *M* borate buffer, pH 10.4, or (b) mobile phase used in the HPLC procedure, 0.02 *M* sodium heptanesulphonate in water–glacial acetic acid–methanol (25:5:70, v/v) (final pH of this dilution = 4.57). Both dilutions were protected from light and maintained at ambient temperature (22°C). At intervals of 0, 30 and 60 min after preparation, samples of both dilutions were analysed by the HPLC procedure of the B.P.[9], using a 12.5 cm × 5 mm I.D. column packed with Hypersil C_{18} (5 μm particle size) and UV detection at 330 nm.

Effect of HPLC analysis time on normalised peak area ratio

Solutions of gentamicin sulphate (0.0624%, w/v) were derivatised in accordance with the B.P. procedure[9]. Freshly prepared derivatives were immediately chromatographed under B.P. conditions, but using a 25.0 cm × 5 mm I.D. column (packed with 5-μm Spherisorb ODS) to increase resolution at high flow-rates. The flow-rate was varied over the range 0.25–3.0 ml min^{-1} for successive injections of freshly prepared derivatives.

RESULTS AND DISCUSSION

Stability of derivatives diluted with borate buffer, pH 10.4, and with mobile phase

Gentamicin derivatives diluted with borate buffer, pH 10.4, for up to 60 min prior to HPLC analysis showed no significant decrease in peak area (Table Ia). Under the alkaline conditions used for derivatisation, all four gentamicin component derivatives are therefore stable. When diluted with acidic mobile phase, the peak area of all four component derivatives decreased with time. Changes in peak area over the time intervals studied are expressed as a percentage in Table I. Decreases in peak area varied between components, and over a 60-min period ranged from 16.7% in the case of the C1 derivative peak area to 43.7% with the C1a derivative peak (Table Ib).

Since the on-column situation would not be one in which excess derivatisation reagent and all gentamicin components would be intimately mixed, simple dilution

TABLE I

HPLC PEAK AREAS AND PERCENTAGE CHANGE IN PEAK AREAS OF OPA-GENTAMICIN DERIVATIVE SOLUTION DILUTED IN 0.04 *M* BORATE BUFFER, pH 10.4 AND MOBILE PHASE, ANALYSED AT INTERVALS OF 0, 30 AND 60 min FROM TIME OF DILUTION

Peak area is expressed in arbitrary units.

Time after dilution (min)	*Peak area (percentage change in peak area)*				
	C_1	C_{1a}	C_{2a}	C_2	*Total area*
Borate buffer, pH 10.4					
0	6321	5031	2378	6576	20 306
30	6334 (+0.2)	5052 (−0.4)	2436 (+2.4)	6770 (+3.0)	20 592 (+1.4)
60	6387 (−0.5)	5027 (−0.1)	2425 (+2.0)	6804 (+3.5)	20 543 (+1.2)
Mobile phase, pH 4.57					
0	6526	4380	2249	6184	19 339
30	5890 (−9.8)	3361 (−23.3)	2048 (−8.9)	5646 (−8.7)	16 945 (−12.4)
60	5439 (−16.7)	2466 (−43.7)	1756 (−21.9)	5141 (−16.9)	14 802 (−23.5)

experiments of the type described could not be used to determine exact rates of derivative breakdown during chromatography. Further experiments were required in which the retention time of the component derivatives on the column was varied by altering the flow-rate of the mobile phase.

Effect of HPLC analysis time on derivative breakdown and normalised peak area ratios

Variation of the mobile phase flow-rate between 0.25 and 3.0 ml min^{-1} produced HPLC analysis times ranging from approximately 10 to 100 min. The retention times and corresponding normalised peak areas of gentamicin derivatives, obtained at different flow-rates are shown in Table II. These data are presented graphically in

TABLE II

EFFECT OF CHROMATOGRAPHIC ANALYSIS TIME ON GENTAMICIN COMPONENT RATIOS OBTAINED BY THE B.P. PROCEDURE

Flow-rate (ml min^{-1})	*Component retention time (t_R)*				*Normalised peak areas (%)*			
	C_1	C_{1a}	C_{2a}	C_2	C_1	C_{1a}	C_{2a}	C_2
3.0	2.5	6.4	8.5	9.6	29.6	22.0	14.3	34.1
2.0	3.7	9.5	12.5	14.2	29.7	22.7	15.0	32.6
1.5	4.8	12.0	15.8	17.9	30.5	22.1	14.1	33.2
1.0	7.5	18.9	24.9	28.2	30.3	23.7	13.9	32.1
0.5	14.9	37.0	48.6	54.9	32.3	20.9	15.0	31.8
0.25	28.5	69.1	90.2	101.7	33.3	20.6	13.2	30.8
		Extrapolated to t_R = 0:			29.4	22.9	14.6	33.1
		B.P. limits:			25–50	15–40	20–50	

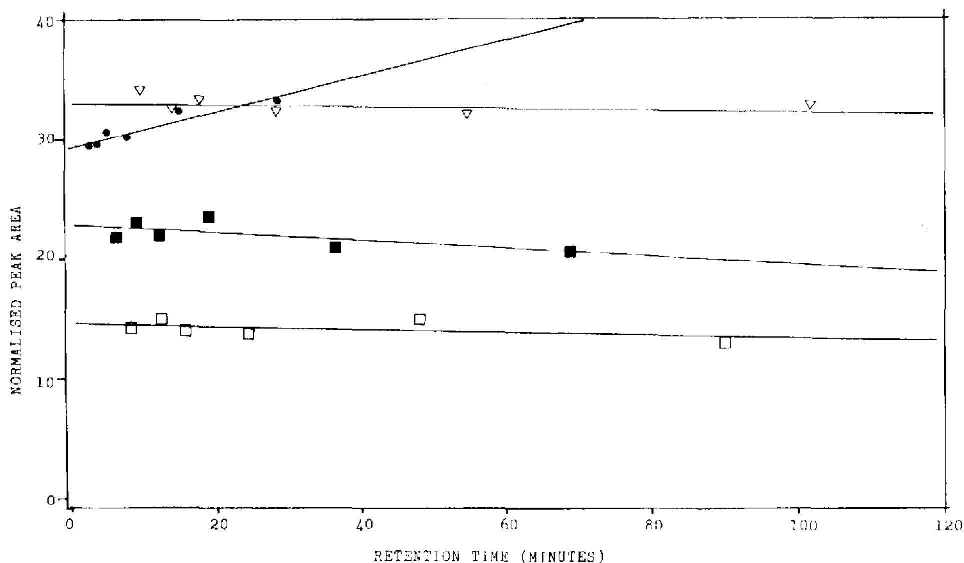

Fig. 1. Plot of normalised peak area ratio for gentamicin C-Complex components against HPLC retention time. ●, Component C_1; ■, component C_{1a}; □, component C_{2a}; ▽, component C_2.

Fig. 1, where extrapolation to zero-time yields the true value of normalised peak area ratios without any contribution from derivative breakdown. The extrapolated "zero-time" peak area ratios are included in Table II together with the B.P.[9] limits.

The normalised peak areas obtained at different flow-rates (Table II) were used to calculate the effect of HPLC analysis time on the accuracy of normalised peak areas. As the HPLC retention time increased, the normalized peak areas of the C1a, C2a and C2 components decreased while that of the C1 component increased. These observations were attributed to derivative breakdown during chromatography. The error associated with the normalised peak areas could be decreased by reducing the HPLC analysis time. In our experience with the B.P.[9] method, adequate resolution has been achieved with analysis times of 20–30 min. In this study an analysis time of 30 min (flow-rate = 1 ml min^{-1}) gave errors of +3.1%, +3.5% and −3.6% for the respective C1, C1a and C2a + C2 normalised peak areas. These errors would be insignificant except in the most borderline of cases.

This study highlights potential difficulties in the HPLC analysis of complex drug compounds requiring pre-column derivatisation. In the case of gentamicin, it is possible to circumvent these problems by limiting the HPLC analysis time. Alternatively, the use of normalised peak areas could be eliminated from this method by the use of a gentamicin reference compound, the composition of which would be determined by pharmacopoeial laboratories using the graphical approach described in this report. It is our opinion that one of these simple modifications should be incorporated into the B.P.[9] procedure, which in all other respects is a valuable step forward in the quality control of gentamicin for pharmaceutical use.

REFERENCES

1 J. P. Anhalt, F. D. Sancilio and T. McCorkle, *J. Chromatogr.*, 153 (1978) 489.
2 K. M. Byrne, A. S. Kershner, H. Maehr, J. A. Marquez and C. P. Schaffner, *J. Chromatogr.*, 131 (1977) 191.
3 A. H. Thomas and S. D. Tappin, *J. Chromatogr.*, 97 (1974) 280.
4 W. L. Wilson, G. Richard and D. W. Hughes, *J. Chromatogr.*, 78 (1973) 442.
5 G. H. Wagman, J. A. Marquez, J. V. Bailey, D. Cooper, J. Weistein, R. Tkach and P. Daniels, *J. Chromatogr.*, 70 (1972) 171.
6 R. T. Parfitt, D. E. Games, M. Rossiter, M. S. Rodgers and A. Weston, *Biomed. Mass Spectrom.*, 3 (1976) 232.
7 *United States Pharmacopoeia*, U.S.P. Convention Inc., Rockville, IL, 20th Revision, 1980.
8 *British Pharmacopoeia*, Vol. 1, H.M. Stationery Office, London, 1980, p. 208.
9 *British Pharmacopoeia*, Addendum, H.M. Stationery Office, London, 1983, p. 235.
10 M. Freeman, P. A. Hawkins, J. S. Loran and J. A. Stead, *J. Liq. Chromatogr.*, 2 (1979) 1305.
11 E. Chacko, J. Bornstein and D. J. Sardella, *Tetrahedron Lett.*, 13 (1977) 1095.
12 S. S. Simons and D. F. Johnson, *J. Org. Chem.*, 43 (1978) 2886.
13 E. Chacko, J. Bornstein and D. J. Sardella, *Tetrahedron*, 35 (1979) 1055.