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The effects of column packing material and inorganic cations on the separation of fluorescent *o*-phthalaldehyde derivatives of gentamicin by high-performance liquid chromatography

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Summary

Reported differences in the order of elution of fluorescent *o*-phthalaldehyde derivatives of gentamicins C_1, C_{1a} and C_2 on apparently similar reversed-phase HPLC systems are explained. The differences arise from the relatively higher sensitivity of the κ' value of the gentamicin C_1 derivative to the presence of cations in the eluting solvent. This effect also depends on the commercial source of the bonded ODS phase used and is inversely related to the surface coverage of the silica with alkyl chains. The evidence suggests that this phenomenon results from an interaction between the residual silanol groups on the column support and secondary amino groups on the gentamicin derivatives, the C_1 derivative differing from the C_{1a} and C_2 derivatives in having two rather than only one such group.

Introduction

Many workers have used high-performance liquid chromatography (HPLC) of the fluorescent isoindole derivatives formed by reaction of *o*-phthalaldehyde, a thiol and a primary amine as a means of separating and quantifying the individual components of commercial gentamicin (Maitra et al., 1977; Freeman et al., 1979; Kraisintu et al., 1982; Marples and Oates, 1982; D'Souza and Ogilvie, 1982). Such a method was adopted by the British Pharmacopoeia (BP 1980 addendum, 1983), but subsequently discontinued (BP 1980 addendum, 1986).

However, reports in the literature reveal that workers using apparently similar reversed-phase HPLC systems observe different orders of elution for the major components. Thus Maitra et al. (1977) and Marples and Oates (1982) reported the order gentamicin C_1, C_{1a}, C_2 whereas D'Souza and Ogilvie (1982) and Kraisintu et al. (1982) reported the order gentamicin C_{1a}, C_2, C_1. The latter authors were the first to note this phenomenon though they were unable to explain it fully. More recently Claes et al. (1984) compared a number of HPLC methods and speculated that the

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reported differences in elution order might reflect partial derivatisation of the gentamicin C_{1a} and C_2 components.

In the present paper we report that a combination of column packing type and ionic strength, and types of cation in the eluting solvent may account for these discrepancies. This variation is related to the participation of two different modes of retention in the HPLC of the gentamicin derivatives.

Materials and Methods

HPLC analyses were carried out with a Laboratory Data Control Minipump and Fluoromonitor III using 150 × 5 mm columns packed with chemically bonded octadecylsilane reversed-phase packing materials. The packings used were Spherisorb ODS 5μm (Phase Separations), Zorbax ODS 7 μm (Du Pont U.K.) and Hypersil ODS 5 μm (Shandon Southern). Separations by Spherisorb and Hypersil were carried out at ambient temperature whereas the Zorbax column was thermostatically maintained at 40 °C. The mobile phase consisted of methanol/water (80 : 20) and the ionic strength was varied by addition of salts to the aqueous component before mixing. The pH of the aqueous component was varied over a range of pH 3.5–10.5 by means of an ammonia–acetic acid buffer. In the pH experiments the ionic strength of the buffer solutions was adjusted to 0.075 with potassium iodide. The mobile phase was degassed before use and the flow rate was 1.3 ml/min.

Gentamicin samples were derivatised prior to injection according to the method of Kraisintu et al. (1982) but omitting the internal standard. Gentamicin sulphate was supplied by Nicholas International, standard gentamicin components were isolated by the method of Kraisintu et al. (1982) and netilmicin was a gift from Kirby-Warrick Pharmaceuticals. HPLC grade methanol was used and all other reagents were analytical grade.

Results and Discussion

Fig. 1 shows the effect on the kappa (κ') values of each of the components of gentamicin C and

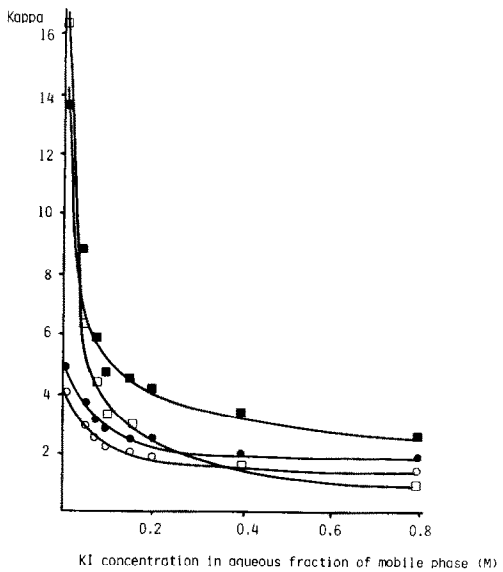


Fig. 1. Effect of potassium iodide concentration upon the retention of netilmicin and gentamicin C_1, C_{1a} and C_2 by a Spherisorb S5 ODS column. ○, gentamicin C_{1a}; ●, gentamicin C_2; □, gentamicin C_1; ■, netilmicin.

netilmicin of changing the concentration of potassium iodide in the aqueous phase of the eluting solvent using a column packed with Spherisorb S5 ODS. In the absence of salt all the components were irreversibly bound on the column but on increasing the concentration, gentamicin C_1 and netilmicin behaved differently from gentamicins C_{1a} and C_2 resulting in a change in the order of elution at high salt concentration. It was notable that increasing the ionic strength only affected the behaviour of gentamicins C_{1a} and C_2 at very low salt concentration whereas the κ' values for gentamicin C_1 and netilmicin continued to vary over a wide range of concentrations. The concentration at which $\kappa' C_1 = \kappa' C_{1a}$ provides a convenient reference point for comparing the behaviour of other salts and column packing materials. Table 1 shows the values for this "C_1/C_{1a} crossover point" obtained with various salts using the Spherisorb column. The choice of salt was restricted by considerations of solubility and stability in the 20% aqueous methanol eluting solvent but it is clear that the choice of cation had an important influence upon crossover point whereas the anions were of lesser importance. Fig. 2 shows

TABLE 1

"C_1/C_{1a} crossover points" on Spherisorb ODS

Salt	Crossover point (M) [a]
$BaCl_2$	0.08
KI	0.40
KSCN	0.40
NaI	0.80
$NaNO_3$	0.80
LiCl	1.40

[a] Concentration of salt in aqueous component of elution solvent.

that the effects of the different cations on the κ' values of gentamicin C_1 follow a similar pattern but that the magnitude of the effect varies in the order: $Ba^{2+} > K^+ > Na^+ > Li^+$. This phenomenon has been considered typical of systems in which cationic ion exchange is a major mechanism of binding (Karger et al., 1973).

If an ion exchange mechanism is operating, it is unlikely that the octadecyl side-chains of the stationary phase are involved. However, underivatised silanol groups on the silica support might possess such activity. Accordingly the behaviour of different chemically bound ODS packing

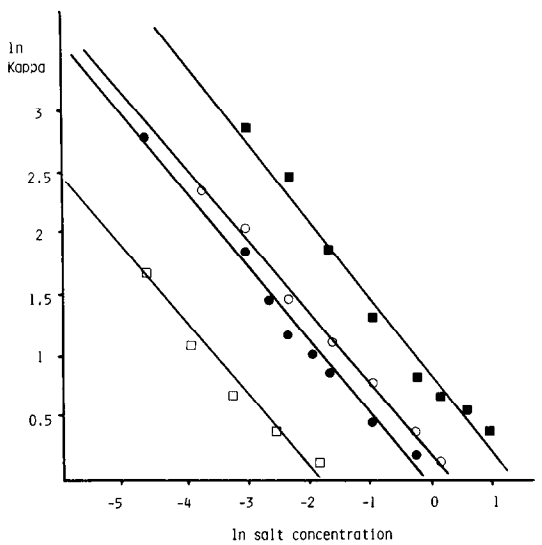


Fig. 2. Ln κ' against ln salt concentration for gentamicin C_1 on a Spherisorb S5 ODS column. ■, LiCl; □, $BaCl_2$; ●, KI; ○, NaI.

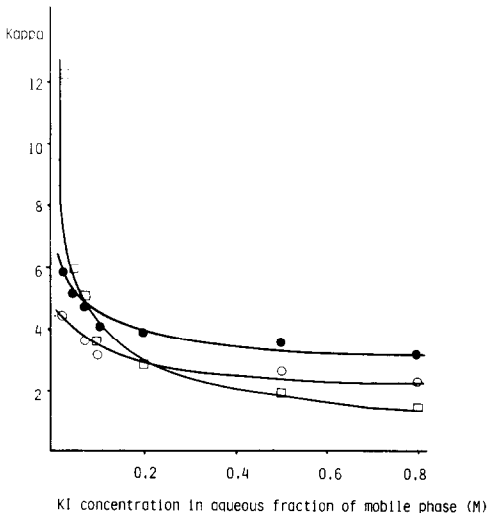


Fig. 3. Effect of potassium iodide concentration upon the retention of gentamicin C_1, C_{1a} and C_2 by a Zorbax ODS 7 μm column. ○, gentamicin C_{1a}; ●, gentamicin C_2; □, gentamicin C_1.

materials was examined. According to manufacturer's data Spherisorb ODS has a carbon loading of 7% with some end capping of free silanol groups, Zorbax ODS has a carbon loading of 16% with no end capping and Hypersil has 9% carbon loading with extensive end capping. Surface coverage with ODS chains are 1.36, 2.50 and 3.52 μmol/m^2, respectively. Thus, using this last criterion, Spherisorb might be expected to show the greatest and Hypersil the least effect due to interaction of the solute with unreacted silanol groups. The effects of increasing KI concentration on the κ' values of the gentamicin components on columns of Zorbax and Hypersil are shown in Figs. 3 and 4, respectively. Comparison with Fig. 1 shows a qualitatively similar effect but with C_1/C_{1a} crossover values of 0.4 M on Spherisorb, 0.2 M on Zorbax and 0.004 M on Hypersil. Thus, as predicted, the contribution of the ion exchange-like effect on the κ' value of gentamicin C_1 decreases from Spherisorb to Zorbax and is almost negligible on Hypersil. It is also notable that Hypersil is the only column on which irreversible binding does not occur at zero salt concentration.

Several of the published separations of gentamicin *o*-phthalaldehyde derivatives use tripotassium EDTA rather than potassium iodide.

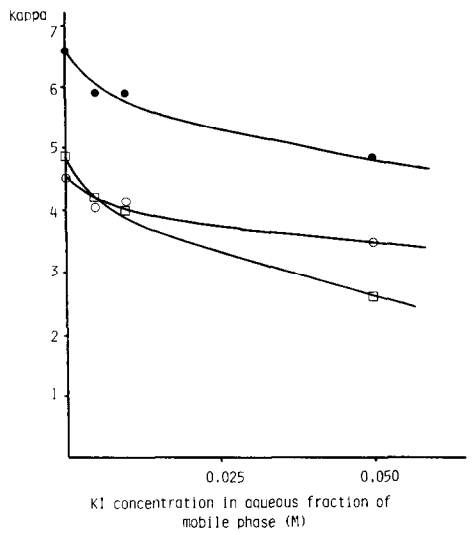


Fig. 4. Effect of potassium iodide concentration upon the retention of gentamicin C_1, C_{1a} and C_2 by a Hypersil ODS 5 μm column. ○, gentamicin C_{1a}; ●, gentamicin C_2; □, gentamicin C_1.

Fig. 5 shows the effect of K_3EDTA on the κ' values obtained on a Zorbax column and by comparison with Fig. 3 shows a similar qualitative effect but with a much higher C_1/C_{1a} crossover point. Thus it appears that the complex EDTA anion may reduce the effectiveness of K^+ in nullifying the ion exchange effect.

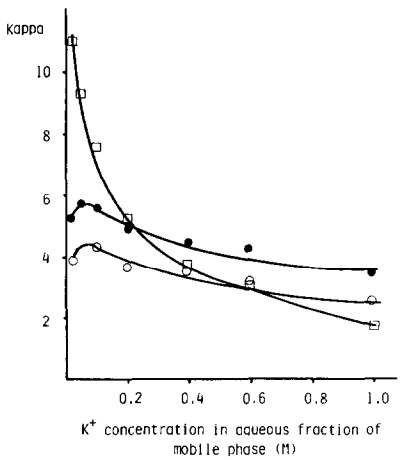


Fig. 5. Effect of tripotassium EDTA concentration upon the retention of gentamicin C_1, C_{1a} and C_2 by a Zorbax ODS 7 μm column. ○, gentamicin C_{1a}; ●, gentamicin C_2; □, gentamicin C_1.

We conclude therefore that the retention of gentamicin-OPA derivatives on bonded-phase ODS stationary phases departs from that which might be predicted by solvophobic theory (Horvath and Melander, 1977; Horvath et al., 1977) in the following ways. Firstly, in the absence of ions in the eluting solvent all the gentamicin derivatives show a degree of ionic interaction with the support. Secondly, on increasing the cation concentration, gentamicin C_{1a} and C_2 derivatives rapidly come to conform with the behaviour predicted by solvophobic theory whereas gentamicin C_1 continues to show retention behaviour characteristic of ionic interactions over a wide range of cation concentrations. These effects are most prominent with those stationary phases possessing a relatively large proportion of underivatised silanol groups. Many other ionisable compounds have been shown to behave in an anomalous manner on bonded phase HPLC systems and this has been attributed to a dual site mechanism of retention involving the ODS chains and the residual silanol groups (Melander et al., 1980; Sokdowski and Wahlund, 1980; Bij et al., 1981; Tilly Melin et al., 1979).

Table 2 shows the parameters obtained by fitting our data into the two-site Eqn. 1 of Bij et al. (1981).

$$\frac{[A]}{\kappa_0' - \kappa'} = \frac{1}{\kappa_2' \cdot K_A} + \frac{[A]}{\kappa_2'} \qquad (1)$$

TABLE 2

Calculated dual-site parameters with KI as masking agent

Column	Component	κ_0' [a]	κ_2'	r
Spherisorb	C_{1a}	4.60	3.25	1.000
	C_2	5.30	3.58	1.000
	C_1	25.0	24.2	1.000
Zorbax	C_{1a}	4.60	2.43	0.995
	C_2	6.51	3.37	0.997
	C_1	25.0	23.87	1.000
Hypersil	C_{1a}	4.52	1.65	0.945
	C_2	6.57	2.24	0.981
	C_1	4.89	3.07	0.973

[a] This value was estimated for Spherisorb and Zorbax columns by extrapolation of a plot of $1/\kappa'$ against salt concentration.

TABLE 3

Comparison of estimated residual silanol groups with silanophilic influence on retention

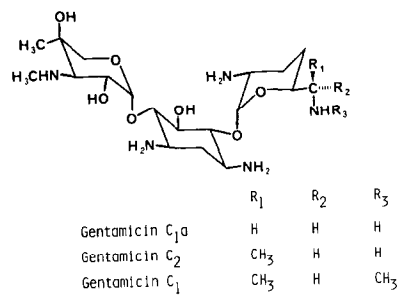
Column	Surface coverage (μmol · m^{-2})	% residual silanols calculated from manufacturer's data	% silanophilic influence on retention		
			C_{1a}	C_2	C_1
Spherisorb	1.36	70	71	68	97
Zorbax	2.50	46	53	52	96
Hypersil	3.52	24	37	34	63

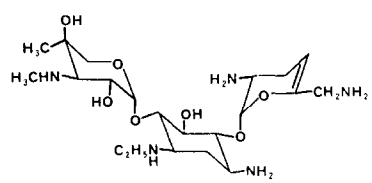
where [A] is concentration of masking agent; κ_0' is the capacity factor in absence of agent; K_A is binding constant of agent to silanol groups; and κ_2' is capacity factor due to silanophilic binding in absence of agent.

The Hypersil column shows less adherence to the equation than the other two columns which may indicate the dual-site model to be less applicable to the retention on this column.

The κ_2' term can be used to calculate the percentage contribution of silanophilic binding present in the retention mechanism by dividing κ_2' by κ_0'. Table 3 compares this value with the estimated residual silanol groups on the columns, calculated assuming a maximum of 4.5 μmol · m^{-2} for silanol groups available for reaction with bonding groups (Unger, 1979). It is observed that gentamicin C_{1a} and C_2 are retained by a silanophilic mechanism in direct proportion to the estimated residual silanol groups present on the packing. However, for gentamicin C_1 the silanophilic mechanism appears to dominate the total retention especially in the case of the Zorbax and Spherisorb columns. This could explain the selective action of salts on the retention of gentamicin C_1 on these columns.

The gentamicins have the chemical structures shown in Fig. 6 and the OPA derivatisation reaction used here replaces primary amino groups with the isoindole moiety shown (Simons and Johnson, 1976). We have established that under the reaction conditions used in this work all the primary amino groups on the gentamicins react with OPA (Lacy, 1984). Thus the only basic groups available for interaction with the silanol groups will be the secondary amino groups of which gentamicins C_{1a} and C_2 possess one and gentamicin C_1 has two. Netilmicin, which behaves like gentamicin C_1 (Fig. 1) also possesses two secondary amino functions. The secondary amino group of gentamicins C_{1a} and C_2 (which gentamicin C_1 also possesses) has been reported to have a pK_a of 6.2 (Zhelyazkov et al., 1976). This rather low value for a secondary amino group may well be due to the presence of hydroxy groups on the adjacent carbon atoms. The additional secondary amino functions on both





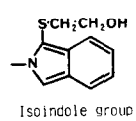


Fig. 6. Chemical structures of netilmicin, gentamicin C_1, C_{1a} and C_2 and their isoindole derivatives.

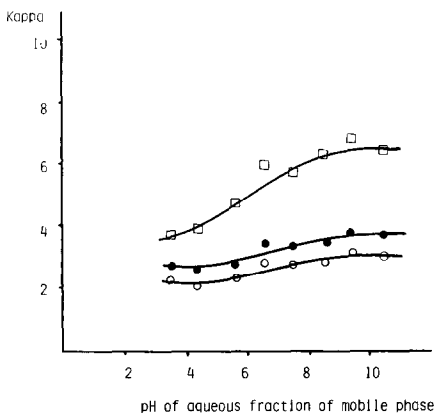


Fig. 7. Effect of variation of the pH of the aqueous fraction of the mobile phase upon retention of gentamicin C_1, C_{1a} and C_2 by a Spherisorb S5 ODS column. ○, gentamicin C_{1a}; ●, gentamicin C_2; □, gentamicin C_1.

gentamicin C_1 and netilmicin are not in this environment and might be expected to show more basic character. Moreover, the single basic group on gentamicin C_{1a} and C_2 derivatives might well be masked in certain conformations of these rather flexible molecules whereas it is unlikely that both secondary amino groups on gentamicin C_1 would be masked simultaneously. Some support for this possible masking effect is provided by Fig. 7 which shows the variation of κ' with pH at constant ionic strength. The solvophobic theory would predict that for an ionisable solute the retention would be greater for the unionised form and less for the ionised form resulting in a sigmoid curve. All 3 gentamicin components show this general sigmoid form with higher κ' at high pH but for gentamicins C_{1a} and C_2 the magnitude of this effect is very small suggesting that the ionisable group is masked.

We consider that the differences in HPLC behaviour of gentamicin-OPA derivatives observed by several workers as indicated in the introduction to this paper arise from the action of two mechanisms of separation, one solvophobic and the other involving ionic interactions with underivatised silanol groups. Gentamicin C_1 differs from C_{1a} and C_2 in the relative importance of these two mechanisms. Indeed, Essers (1984) has shown that gentamicin C_1 is eluted after C_{1a} and C_2 on a purely cation exchange column. The extent to which either mechanism predominates when using a reversed-phase column is dependent upon the proportion of free silanol groups on the stationary phase and upon the presence of cations in the eluting solvent.

It is noteworthy that the HPLC system used here has some similarities to that adopted by the British Pharmacopoeia (BP 1980 addendum, 1983) which was based on the method of Freeman et al. (1979). That method used mercaptoacetic acid instead of mercaptoethanol in the derivatisation reaction and an acidic mobile phase with sodium heptanesulphonate as counter ion. The BP specified the use of an ODS bonded stationary phase but did not specify any manufacturing source – it simply stated that Hypersil was suitable. Although it might be expected that the use of a counter ion would reduce any ion exchange effect the use of ODS columns other than Hypersil might be an important source of inaccuracies in the method. This problem is particularly significant since the general lack of pure standards of gentamicins C_1, C_{1a} and C_2 might lead to misidentification of peaks in mixtures of the 3.

The method of Freeman et al. utilises UV absorption for detection and thus is less suitable than the more sensitive fluorescence methods to be adapted for the determination of gentamicin in biological fluids. In such applications, proximity of the gentamicin C_1 peak to the solvent front and its poor separation from blank peaks may lead to difficulties in quantification. Essers (1984) overcame this problem by inserting a short cation exchange column before the reverse-phase column to increase the retention of gentamicin C_1 though this did not change the elution order. Clearly, with the present system it would be possible to manipulate the salt concentration in the elution buffer to elute the gentamicin peaks in positions free from interference by blank peaks.

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