ELSEVIER

Journal of Chromatography B, 675 (1996) 295–302

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Sensitive fluorimetric determination of gentamicin sulfate in biological matrices using solid-phase extraction, pre-column derivatization with 9-fluorenylmethyl chloroformate and reversed-phase high-performance liquid chromatography

D.A. Stead*, R.M.E. Richards

School of Pharmacy, The Robert Gordon University, Schoolhill, Aberdeen AB9 1FR, UK

Received 28 May 1995; revised 31 July 1995; accepted 24 August 1995

Abstract

A high-performance liquid chromatographic method is described for the determination of gentamicin in bacterial culture medium or plasma with increased sensitivity and improved separation of the C_1 component. Gentamicin was extracted from the biological matrix with high efficiency using carboxypropyl (CBA)-bonded silica. Derivatization with 9-fluorenylmethyl chloroformate (FMOC-Cl) followed by C_{18} reversed-phase chromatography allowed the fluorimetric detection of gentamicins C_1, C_{1a} and C_2. A fourth component, considered to be gentamicin C_{2a}, was partially resolved from the C_2 peak. Optimal conditions for the extraction and derivatization of gentamicin are described. The detection limit was below 50 μg/l, the assay was linear to 5 mg/l and showed good reproducibility. It is concluded that pre-column derivatization with FMOC-Cl substantially improves the analysis of gentamicin compared with present methods based on reaction with *o*-phthaldialdehyde.

Keywords: Gentamycin sulfate

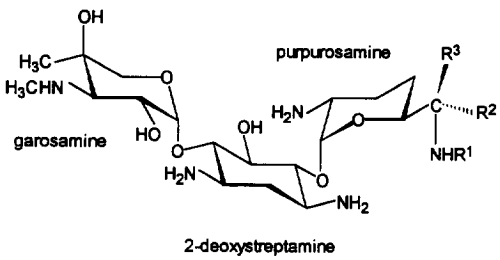
1. Introduction

Gentamicin, an aminoglycoside produced by *Micromonospora purpurea*, has a broad spectrum of antibacterial activity and is widely used for the treatment of serious infections. Careful monitoring of plasma gentamicin levels are indicated because of its narrow therapeutic range and the danger of toxic side-effects, particularly in patients with renal failure. Gentamicin is a complex mixture consisting of three major components C_1, C_{1a} and C_2, and several minor components including C_{2a} and C_{2b} (Fig. 1). The composition can vary considerably, but most commercial preparations have been shown by high-performance liquid chromatography (HPLC) to contain 30–45% C_1, 12–30% C_{1a}, 19–43% C_2 and 7–14% C_{2a} [1].

Numerous methods for the determination of gentamicin and other aminoglycosides have been developed, based on microbiological [2], enzymatic [3–6], radioimmuno- [7], fluoroimmuno- [8], gas chromatographic [9] or HPLC assays [1,10–23].

*Corresponding author.

0378-4347/96/$15.00
SSDI 0378-4347(95)00355-X



Gentamicin	R^1	R^2	R^3
C_1	CH_3	H	CH_3
C_{1a}	H	H	H
C_2	H	H	CH_3
C_{2a}	H	CH_3	H
C_{2b}	CH_3	H	H

Fig. 1. Structures of the gentamicin C components.

Only the chromatographic techniques are capable of quantifying individual components of the gentamicin complex and of avoiding interferences from other antibiotics which may be present. The analysis of gentamicin by HPLC is complicated for two reasons. Firstly, the major components differ in structure only in the degree and orientation of methylation of the purpurosamine moiety (Fig. 1). Secondly, gentamicin has no ultraviolet (UV) or visible absorption and cannot be detected by these conventional techniques without derivatization. Although gentamicin has been analysed using electrochemical [18], refractive index [19], and mass spectrometric detection [23], most workers have used pre- or post-column derivatization for fluorescence or UV detection. Reagents which have been used include *o*-phthaldialdehyde (OPA) [10,11,13], dansyl chloride [12], fluorescamine [14], 1-fluoro-2,4-dinitrobenzene [17] and 2,4,6-trinitrobenzenesulphonic acid [20].

In general, pre-column derivatization with a fluorescent reagent allows for the simplest and most sensitive analysis, and OPA has been adopted as the reagent of choice for the analysis of gentamicin (British Pharmacopoeia 1993). However, this method is not without its drawbacks. Firstly, the OPA reagent is unstable and has to be prepared regularly. Secondly, the elution of the OPA derivatives has been found to be affected by the concentration of inorganic cations in the HPLC mobile phase [21]. It was concluded that the effect resulted from an interaction between residual silanol groups on the column support and secondary amine groups on the gentamicin derivatives. Since gentamicin C_1 (and C_{2b}) has two secondary amines whereas the other components have only one, the elution order was observed to vary depending on the type of column support and mobile phase composition used. The authors noted that this could be an important source of error in the method, leading to misidentification of peaks in the gentamicin complex, particularly since pure standards of the individual components are not generally available.

In order to study the in vitro effects of gentamicin on bacterial cell cultures, either alone or in combination with other antibacterial drugs, it was necessary to develop an HPLC assay suitable for measuring gentamicin at subinhibitory levels in the media used for bacterial growth, in particular Iso-sensitest broth, which is recommended for bacterial susceptibility tests [24]. We initially tested pre-column derivatization with OPA-mercaptoacetic acid [13] but found that the sensitivity was inadequate and also that the C_1 component could not be resolved from the reagent front. Substituting 2-mercaptoethanol [11] or 3-mercaptopropionic acid [22] as the thiol gave a 20- to 30-fold increase in fluorescence response with OPA, but the problem of the early elution of the C_1 derivative remained. 9-Fluorenylmethyl chloroformate (FMOC-Cl) has been shown to be a suitable reagent for the fluorescent labelling of both primary and secondary amines [25,26]. We describe the complete derivatization of the amino groups of gentamicin using FMOC-Cl, with analysis of the derivatives by reversed-phase HPLC. This procedure allows the fluorimetric detection of the individual gentamicin components C_1, C_{1a} and C_2 (and tentatively C_{2a}), well separated from the reagent front.

A further consideration was the extraction of gentamicin from the matrix, which is necessary to eliminate potentially interfering components prior to derivatization. Existing methods rely either on laborious liquid–liquid extraction [12,14] or on the use of small columns individually prepared from silicic acid [11] or cation-exchange resins [10,16].

Commercial disposable solid-phase extraction (SPE) columns are available with a variety of chemically-bonded silica phases and these provide the most convenient and reliable means of performing sample clean-up prior to HPLC analysis. However, we have found only two reports of such SPE columns used in aminoglycoside analysis; octadecyl (C_{18})-bonded silica for gentamicin from urine [15] and cyanopropyl (CN)-bonded silica for the extraction of isepamicin from plasma [28]. The former was not applied to plasma and still required a liquid–liquid extraction step while the latter gave unacceptably low recoveries (5%). Because of the strongly basic nature of the aminoglycosides and the success of existing extraction procedures based on cation-exchange media containing carboxylic acid functional groups, it was logical to use carboxypropyl (CBA)-bonded SPE columns for the extraction of gentamicin from the biological matrix. A simple, efficient and reliable procedure using this technology is described. The extraction step has been optimized for subsequent pre-column derivatization with FMOC-Cl for the sensitive fluorimetric analysis of gentamicin by HPLC.

2. Experimental

2.1. Chemicals and reagents

Gentamicin sulfate (labelled potency 648 μg/mg) was obtained from Sigma (Poole, UK). Authentic standards of gentamicin C_1, C_{1a} and C_2 were kindly provided by Schering-Plough Research Institute (Kenilworth, NJ, USA). 9-Fluorenylmethyl chloroformate (FMOC-Cl) was supplied by Sigma. Acetonitrile and methanol were obtained from Rathburn (Walkerburn, UK). Water was glass-distilled and further purified for HPLC with a Millipore Milli-Q system. Iso-sensitest broth was obtained from Oxoid (Basingstoke, UK). SPE columns (3 ml) packed with 100 mg Isolute CBA-bonded silica (Jones Chromatography, Hengoed, UK) were supplied by Crawford (Strathaven, UK). Phosphate buffers were prepared by mixing appropriate volumes of 20 m*M* sodium dihydrogen phosphate and sodium hydrogen phosphate solutions. Borate buffers were prepared by adjusting the pH of boric acid solutions with potassium hydroxide (45%, w/v).

2.2. High-performance liquid chromatography

The HPLC system comprised a Waters Model 510 pump, a Rheodyne Model 7125 valve fitted with a 100-μl loop, a stainless-steel column (200×4.6 mm I.D.) slurry packed with 3-μm ODS Hypersil (Shandon HPLC, Runcorn, UK). Detection was by a Varian Model 2070 spectrofluorometer (excitation 260 nm, emission 315 nm) connected to a Goertz Metrawatt Model SE120 chart recorder for manual peak-height measurement.

The mobile phase, unless stated otherwise, was acetonitrile–water (90:10, v/v), degassed prior to use by vacuum filtration through a 0.2-μm filter. Gentamicin–FMOC derivatives were separated at ambient temperature using a flow-rate of 1.0 ml/min.

2.3. Sample pre-treatment

A CBA-bonded SPE column was wetted with 1 ml methanol and conditioned with 1 ml phosphate buffer (20 m*M*, pH 7.4). An aliquot (1 ml) of Iso-sensitest broth or plasma containing the analytes was applied to the column, which was then washed with 2 ml phosphate buffer followed by 4 ml borate buffer (0.2 *M*, pH 9.0). The column was dried by application of a volume of air (30 ml) then eluted into a 2-ml glass vial with 1 ml acetonitrile–borate buffer (0.2 *M*, pH 10.5) (1:1, v/v) followed by a further volume of air.

2.4. Derivatization procedure

Eluate (1 ml) was adjusted to pH 8.9 by the addition of 0.2 ml boric acid (0.8 *M*) and derivatized with 0.2 ml FMOC-Cl (2.5 m*M* in acetonitrile) at ambient temperature for 15 min. The reaction was stopped by adding 25 μl glycine (0.1 *M*) and, after a further 2 min, a volume (50 μl) of the reaction mixture was injected into the chromatograph.

In experiments performed during the optimization of derivatization conditions, 0.5 ml gentamicin (1.0 mg/l in 0.37 *M* borate buffer, pH 8.9) was mixed

with 0.5 ml FMOC-Cl (1.0 m*M* in acetonitrile) in a 2-ml glass vial and reacted as above.

3. Results and discussion

3.1. Optimization of derivatization conditions

Gentamicin sulfate (1.0 mg/l) was reacted with FMOC-Cl, as described above, but the pH, reaction time, concentrations of acetonitrile, borate and FMOC-Cl were independently varied in turn. The effects of these parameters on the peak heights of the three major components of gentamicin are shown in Fig. 2, Fig. 3, Fig. 4, Fig. 5, Fig. 6.

Optimal reaction of gentamicin with FMOC-Cl occurred at pH 7.5–9.0 and was complete after 10 min. Maximal yields of the derivatives were detected following reaction in 50% (v/v) acetonitrile, and at concentrations of borate and FMOC-Cl above 50 m*M* and 1 m*M*, respectively.

The reaction of FMOC-Cl with amines is shown in Fig. 7. Because the individual pure components C_1 and C_{1a} both gave a single peak by HPLC, it was assumed that all five amine groups of the gentamicins were completely derivatized. This was further supported by the order of elution of the components on reversed-phase HPLC (Fig. 8), which corresponded with the degree of methylation of their purpurosamine moieties (Fig. 1). If the secondary amine groups had not been derivatized by FMOC-Cl, then the C_1 component would be expected to elute first, as was the case when using OPA. Gentamicin components C_1, C_{1a} and C_2 were identified by comparison of their retention times with those of the authentic standards chromatographed individually. The identity of C_{2a} could not be confirmed because no authentic standard was available. Analyses of gentamicins C_1 and C_{1a} using peak-area estimation showed that the response factors for each component were identical. This is not the case for the OPA

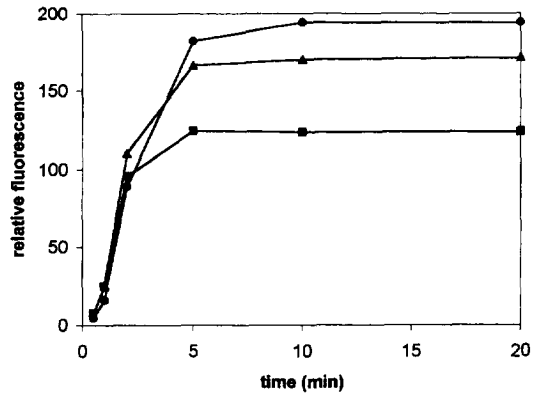


Fig. 3. Time course for the reaction of gentamicin with FMOC-Cl. Gentamicin (1.0 mg/l) in 0.4 *M* borate buffer pH 8.0 (3.0 ml) was derivatized with 1 m*M* FMOC-Cl in acetonitrile (1.5 ml). Aliquots (0.6 ml) were removed at intervals into vials containing glycine (25 μl) and stored at 4°C before analysis. Legend: ●=C_1, ■=C_{1a}, ▲=C_2.

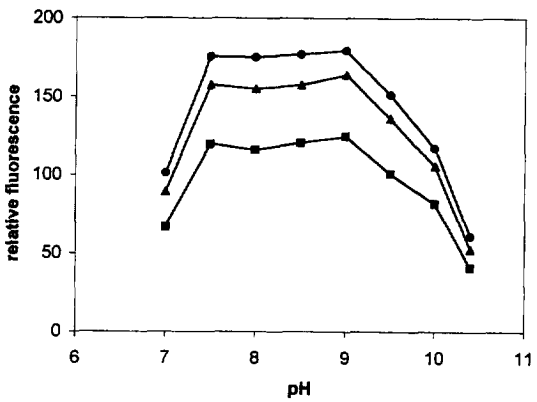


Fig. 2. Effect of pH on the reaction of gentamicin with FMOC-Cl. Conditions as in Section 2 except 0.4 *M* borate, 33% v/v acetonitrile and 10 min reaction time. Legend: ●=C_1, ■=C_{1a}, ▲=C_2.

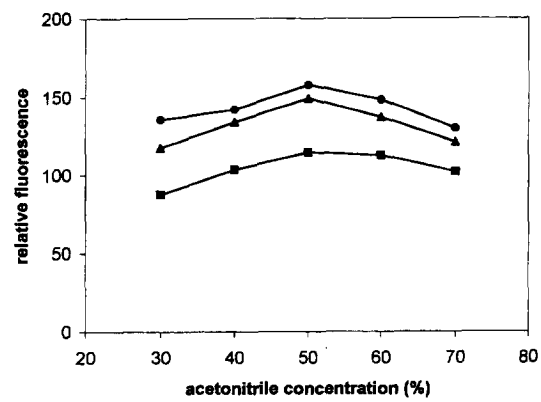


Fig. 4. Effect of acetonitrile concentration on the reaction of gentamicin with FMOC-Cl. Conditions as in Section 2 except 0.53 *M* borate, 0.5 m*M* FMOC-Cl and 10 min reaction time. Legend: ●=C_1, ■=C_{1a}, ▲=C_2.

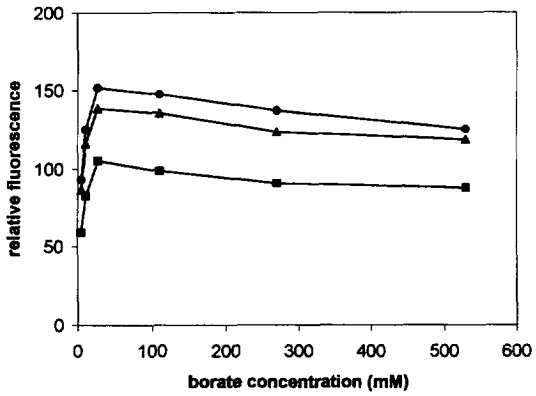


Fig. 5. Effect of borate buffer concentration on the reaction of gentamicin with FMOC-Cl. Conditions as in Section 2 except 0.5 m*M* FMOC-Cl and 10 min reaction time. Legend: ●=C_1, ■=C_{1a}, ▲=C_2.

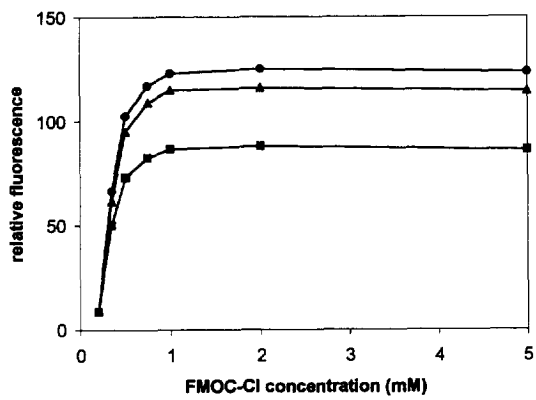


Fig. 6. Effect of FMOC-Cl concentration on the reaction of gentamicin with FMOC-Cl. Conditions as in Section 2. Legend: ●=C_1, ■=C_{1a}, ▲=C_2.

derivatives, which have been shown to have markedly different response factors [1].

The pH dependence of the reaction (Fig. 2) was consistent with that reported for amino acids [26] but gentamicin required a longer time to reach completion (Fig. 3). Similar conditions have been reported recently for the derivatization of melanotropin potentiating factor [27] and selegiline [29] using FMOC-Cl.

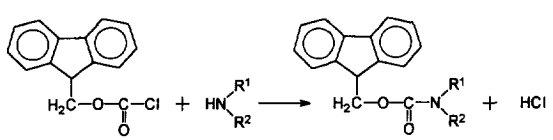


Fig. 7. Reaction of amines with FMOC-Cl.

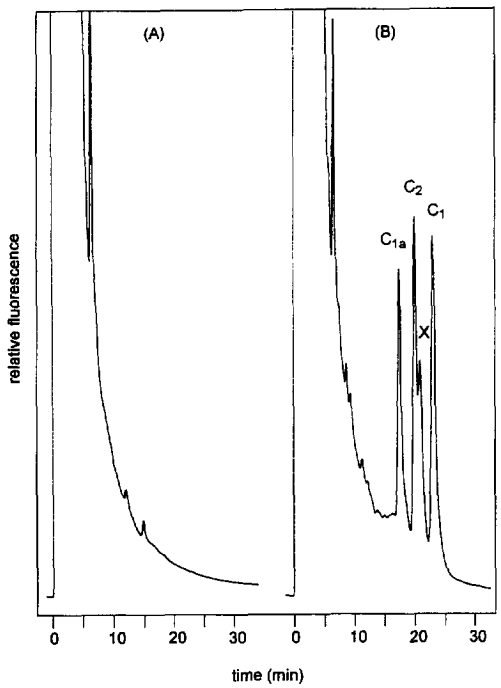


Fig. 8. HPLC of gentamicin in Iso-sensitest broth. Iso-sensitest broth, (A) blank or (B) spiked with 1.0 mg/l gentamicin, was extracted and analysed as described, except the HPLC mobile phase which was acetonitrile–water (87:13, v/v). Detector settings; gain ×1, attenuation 32. Peak X is considered to be gentamicin C_{2a}.

It was important to maintain a concentration of acetonitrile of around 50% (v/v) in the reaction mixture (Fig. 4). FMOC-Cl was precipitated if the proportion of acetonitrile fell below 30%, while gentamicin sulfate is not soluble in acetonitrile and is probably unavailable for reaction at high acetonitrile concentrations.

The necessity for adequate buffering, predictable since hydrogen ions are released during the derivatization reaction, was clearly demonstrated (Fig. 5). A sufficiently high concentration of FMOC-Cl was also required for the reaction to proceed efficiently (Fig. 6). In practice, this was limited at 1 m*M* because FMOC-Cl concentrations above this increased the reagent excess and had a deleterious effect on the quantification of low levels of gentamicin (see Section 3.3).

Table 1
Recovery of gentamicin extracted from biological matrices

Gentamicin component	Recovery (%)	
	Iso-sensitest broth	Plasma
C_1	84.0	96.8
C_{1a}	88.3	99.0
C_2	87.4	97.8
C_{2a}	84.0	93.9

3.2. Optimization of extraction conditions

Preliminary experiments showed that gentamicin could be extracted from the biological matrix at pH 6–8 using CBA-bonded silica and eluted from the solid phase at pH>9.5. Washing with 2 ml phosphate buffer removed most of the interfering matrix components, but it was necessary to introduce a second wash of 4 ml borate buffer (pH 9.0) to completely remove all residual interferences. The use of an eluent containing 50% (v/v) acetonitrile increased the efficiency of elution (possibly due to improved wetting of the partially dried solid phase) but also allowed the final reaction volume to be kept as low as possible while maintaining the optimum acetonitrile concentration for derivatization. Because the pH required for elution of gentamicin (>10) was higher than that for optimal derivatization (<9), it was necessary to adjust the pH of the eluate from 10.5 to 8.9 by the addition of 0.2 vol. boric acid (0.8 *M*) prior to the addition of FMOC-Cl.

The recoveries of gentamicin extracted from 1 mg/l spiked Iso-sensitest broth or plasma, calculated by comparison with a 1 mg/l solution of gentamicin in eluent which was derivatized directly, are shown in Table 1.

Table 3
Reproducibility of the HPLC assay of gentamicin in Iso-sensitest broth

Gentamicin component	Imprecision (C.V. %)[a]			
	Intra-day		Inter-day	
	0.2 mg/l	1.0 mg/l	0.2 mg/l	1.0 mg/l
C_1	8.6	6.4	6.3	7.9
C_{1a}	6.0	5.9	8.9	5.7
C_2	7.0	5.8	5.5	5.0
C_{2a}	5.8	4.3	8.9	6.0

[a]Coefficient of variation (n=5).

3.3. Linearity, reproducibility and sensitivity

Calibration lines for each of the major components C_1, C_{1a}, C_2 and C_{2a} were obtained by triplicate analyses of spiked solutions of gentamicin in Iso-sensitest broth at concentrations of 0.2, 0.5, 1.0, 2.0 and 5.0 mg/l as shown in Table 2. In this experiment the HPLC mobile phase was acetonitrile–water (87:13, v/v). Close correlations with the linear regression equations were observed for all four components. Analyses of spiked solutions at higher gentamicin concentrations indicated that while there was slight deviation from linearity above 5 mg/l, accurate quantification was possible up to 20 mg/l (results not shown).

Reproducibility of the assay in Iso-sensitest broth was assessed: (i) intra-day by five consecutive analyses of 0.2 mg/l and 1.0 mg/l gentamicin solutions; and (ii) inter-day by analysis of the same solutions on five separate occasions. The HPLC mobile phase was acetonitrile–water (87:13, v/v). The results are shown in Table 3.

The lower limit of detection was estimated by

Table 2
Calibration lines for the HPLC assay of gentamicin in Iso-sensitest broth

Gentamicin component	Linear regression equation ($y=a+bx$)		
	Slope, b (S.D.)[a]	Intercept, a (S.D.)	Correlation, r^2
C_1	118.2 (2.6)	−2.5 (3.9)	1.0000
C_{1a}	92.9 (2.4)	−3.7 (2.3)	1.0000
C_2	120.7 (1.8)	−4.7 (3.9)	0.9999
C_{2a}	72.7 (4.2)	−3.3 (5.2)	0.9999

Results based on peak heights (mm) with detector settings; gain ×1, attenuation 32.
[a]Standard deviation (n=3).

Table 4
Comparison of pre-column derivatization HPLC assays for gentamicin

Derivative	Extraction method	Sensitivity (mg/l)	Imprecision (C.V. %)			I.S.	Ref.
			Intra-day	Inter-day	Level (mg/l)		
FMOC	CBA-Bonded silica	<0.05	4.3–6.4	5.0–7.9	1.0	None	[a]
OPA	Silicic acid	ND	5.1–7.5	ND	1.0	None	[11]
OPA	Amberlite CG50	0.5	ND	5.8	2.0	TOB	[16]
OPA	2-step liquid–liquid	0.5	6.0–14.4	<8.0	0.5	TOB	[15]
OPA[b]	CM-Sephadex	0.06	6.8	7.2	0.2	None	[22]
Dansyl	3-Step liquid–liquid	1.0	2.1–3.0	6.6–7.6	6.9–8.7	None	[12]
FDNB[c]	CM-Sephadex	1.0	5.3	ND	1.0	KAN	[17]
Fluorescamine	2-Step liquid–liquid	1.0	<3.5	<2.0	5.9–19.1	None	[14]

Abbreviations: C.V.=coefficient of variation; I.S.=internal standard; ND=not determined; TOB=tobramycin; FDNB=1-fluoro-2,4-dinitrobenzene; KAN=kanamycin.
[a]Present communication.
[b]Analyte was sisomicin.
[c]Analyte was amikacin.

analysing Iso-sensitest broth spiked with gentamicin at progressively lower concentration. Although peaks corresponding to the derivatives were detectable well above the instrument noise level from a 5 μg/l gentamicin solution, the practical sensitivity was between 10 and 50 μg/l because of the difficulty of measuring peaks accurately on a steeply sloping baseline at such low levels. The analyte peaks could be separated further from the reagent excess by increasing slightly the proportion of water in the mobile phase, but at the expense of longer analysis times. Alternatively, it may be possible to remove most of the reagent excess using a post-derivatization extraction step [27,30,31] should even higher sensitivity be required. However, reaction of the reagent excess with an amino acid such as glycine and direct injection of the reaction products (FMOC–glycine being eluted in the first part of the chromatogram) gives more than adequate sensitivity for most practical purposes.

Table 4 provides a comparison of the analytical performance of the present method with those reported for other pre-column derivatization HPLC assays for aminoglycosides, showing the improved sensitivity which has been obtained, without loss in reproducibility.

4. Conclusions

A procedure has been developed for the analysis of gentamicin sulfate in biological matrices using solid-phase extraction, derivatization with FMOC-Cl and reversed-phase HPLC. The advantages of pre-column derivatization with fluorescence detection are retained, but in addition FMOC-Cl provides greater sensitivity, stability, and improved separation of the major C_1 component compared with existing OPA methods. This is probably explained by the reaction of FMOC-Cl with both the primary and secondary amines of gentamicin. If the same number of fluorescent groups are introduced into each component, then their response factors are expected to be equal; this has been confirmed for gentamicins C_1 and C_{1a}. The use of peak-area quantification in the present assay is therefore likely to be more accurate for the determination of the composition of gentamicin preparations than existing methods based on OPA derivatization. The procedure has been applied to the analysis of gentamicin in Iso-sensitest broth and plasma following a simple, efficient and reliable clean-up step using CBA-bonded SPE columns.

References

[1] P.J. Claes, R. Busson and H. Vanderhaeghe, J. Chromatogr., 298 (1984) 445.
[2] L.D. Sabath, J.I. Casey and P.A. Ruch, J. Lab. Clin. Med., 78 (1971) 457.
[3] P. Noone, J.R. Pattison and D. Samson, Lancet ii, (1971) 16.
[4] M.J. Haas and J. Davies, Antimicrob. Agents Chemother., 4 (1973) 497.

[5] E. Ten Krooden and J.H. Darrel, J. Clin. Pathol., 27 (1974) 452.
[6] J.M. Broughall and D.S. Reeves, J. Clin. Pathol., 28 (1975) 140.
[7] W.A. Mahon, J. Ezer and T.W. Wilson, Antimicrob. Agents Chemother., 3 (1973) 585.
[8] R.A.A. Watson, J. Landon, E.J. Shaw and D.S. Smith, Clin. Chim. Acta, 73 (1976) 51.
[9] J.W. Mayhew and S.L. Gorbach, J. Chromatogr., 151 (1978) 133.
[10] J.P. Anhalt, Antimicrob. Agents Chemother., 11 (1977) 651.
[11] S.K. Maitra, T.T. Yoshikawa, J.L. Hansen, I. Nilsson-Ehle, W.J. Palin, M.C. Schotz and L.B. Guze, Clin. Chem., 23 (1977) 2275.
[12] G.W. Peng, M.A.F. Gadalla, A. Peng, V. Smith and W.L. Chiou, Clin. Chem., 23 (1977) 1838.
[13] M. Freeman, P.A. Hawkins, J.S. Loran and J.A. Stead, J. Liq. Chromatogr., 2 (1979) 1305.
[14] S.E. Walker and P.E. Coates, J. Chromatogr., 223 (1981) 131.
[15] J. D'Souza and R.I. Ogilvie, J. Chromatogr., 232 (1982) 212.
[16] J. Marples and M.D.G. Oates, J. Antimicrob. Chemother., 10 (1982) 311.
[17] D.M. Barends, J.S. Blauw, M.H. Smits and A. Hulshoff, J. Chromatogr., 276 (1983) 385.
[18] T. Getek, A. Haneke and G. Selzer, J. Assoc. Off. Anal. Chem., 66 (1983) 172.
[19] G. Inchauspé and D. Samain, J. Chromatogr., 303 (1984) 277.
[20] P. Gambardella, R. Punziano, M. Gionti, C. Guadalupi and G. Mancini, J. Chromatogr., 348 (1985) 229.
[21] J.E. Lacy, R.T. Parfitt and M.G. Rowan, Int. J. Pharm., 43 (1988) 111.
[22] R. Tawa, K. Koshide and S. Hirose, J. Chromatogr., 425 (1988) 143.
[23] T.A. Getek, M.L. Vestal and T.G. Alexander, J. Chromatogr., 554 (1991) 191.
[24] The Oxoid Manual of Culture Media Ingredients, Oxoid, Basingstoke, 5th ed., 1982, p. 147.
[25] H. Anson Moye and A.J. Boning, Jr., Anal. Lett., 12 (1979) 25.
[26] S. Einarsson, B. Josefsson and S. Lagerkvist, J. Chromatogr., 282 (1983) 609.
[27] J.R.E. Lewis, J.S. Morley and R.F. Venn, J. Chromatogr., 615 (1993) 37.
[28] J.A. Maloney and W.M. Awni, J. Chromatogr., 526 (1990) 487.
[29] R. La Croix, E. Pianezzola and M. Strolin Benedetti, J. Chromatogr. B, 656 (1994) 251.
[30] M.T. Rosseel, A.M. Vermeulen and F.M. Belpaire, J. Chromatogr., 568 (1991) 239.
[31] A. Roux, G. Blanchot, A. Baglin and B. Flouvat, J. Chromatogr., 570 (1991) 453.