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Capillary Electrophoresis analysis of gentamicin sulphate with UV detection after pre-capillary derivatization with 1,2-phthalic dicarboxaldehyde and mercaptoacetic acid

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Abstract

A selective, sensitive, and rapid pre-capillary derivatization method for determination of the multicomponent aminoglycoside antibiotic gentamicin is described. The derivatization reagents 1,2-phthalic dicarboxaldehyde and mercaptoacetic acid were used and the thioisoindole derivative was UV detected at 330 nm. A central composite experimental design was performed to optimize selectivity and derivatization conditions. Baseline separation of gentamicin C_1, C_{1a}, C_2, C_{2a}, C_{2b}, sisomicin and several minor components was achieved with a background electrolyte containing 30 m*M* sodium tetraborate, 7.5 m*M* β-cyclodextrin and 12.5% (v/v) methanol at pH 10. Quantitative analysis was performed and illustrated the potential use of capillary electrophoresis for the identification and quantitation of gentamicin as an alternative to methods prescribed in the United States Pharmacopeia and European Pharmacopoeia.

Keywords: Experimental design; Derivatization, electrophoresis; Central composite design; Gentamicin sulfate; Antibiotics

1. Introduction

Gentamicin is a complex mixture of broad spectrum aminoglycoside antibiotics produced by the fermentation of *Micromonospora purpurea* [1]. It is administered to patients suffering from potentially life-threatening bacterial infections. The narrow therapeutic index necessitates a constant blood monitoring, as excess dosage is potentially ototoxic and nephrotoxic. It has four major components C_1, C_{1a}, C_2, and C_{2a} (Fig. 1). Several minor components like sisomicin [2], gentamicin C_{2b}, also known as sagamicin [3,4] and dihydroxyC_{2a} (Antibiotic JI-20B) which is a precursor of C_{2a}, C_2 and C_1 are known to exist. At first only gentamicin C_1, C_{1a} and C_2 were considered as the main components but it has been shown that gentamicin samples contain a considerable amount of C_{2a}.

Analysis of gentamicin sulphate is difficult and most challenging because it is a multicomponent mixture with lack of UV chromophore. Numerous analytical methods have been used to assay gentamicin [5]. These methods initially employed paper chromatography to determine the content of gentamicin C_1 and C_2 [6] and later C_{1a} was separated [7]. The paper chromatographic method followed by microbiological assay of the separated components were adopted as the official US Food and Drug Administration (FDA) protocol [8]. Subsequently, additional investigation by paper chromatography and TLC confirmed the presence of several minor components in gentamicin samples [9,10]. Ion-ex-

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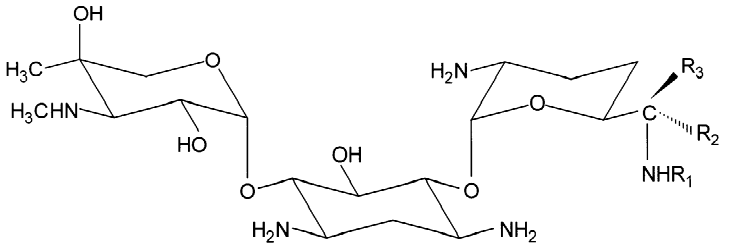

	R_1	R_2	R_3
Gentamicin C_1	CH_3	H	CH_3
Gentamicin C_2	H	H	CH_3
Gentamicin C_{1a}	H	H	H
Gentamicin C_{2a}	H	CH_3	H
Gentamicin C_{2b}	CH_3	H	H

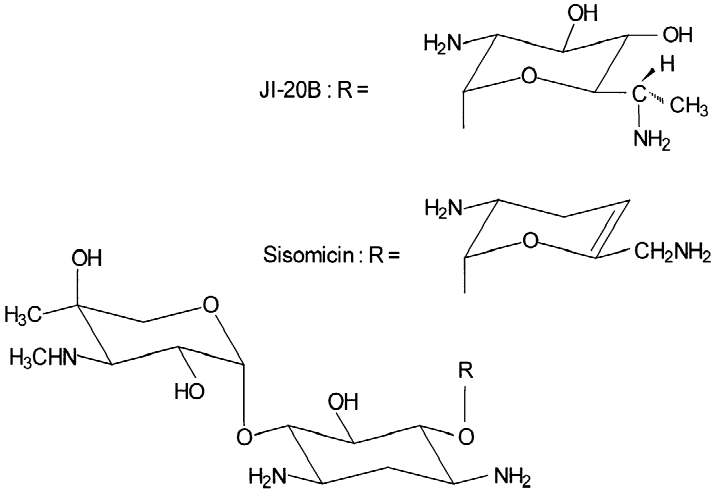

Fig. 1. The chemical structure of gentamicin components.

change chromatography separated the major components of gentamicin, which was then detected with a conductivity bridge [11]. Thomas and Tappin [12] employed ion-exchange column chromatography with optical rotation detection for gentamicin analysis. The first utilization of an ion-pairing reagent with a reversed-phase (RP) column for gentamicin analysis was reported by Anhalt [13].

Detection of gentamicin was accomplished with fluorescence detection after post-column derivatization with 1,2-phthalic dicarboxaldehyde (OPA) [13,14]. Similarly, liquid chromatography (LC) with

pre-column derivatization by OPA [15] and dansylchloride [16] followed by fluorescence detection was performed for gentamicin analyses.

Freeman et al. [17] employed pre-column derivatization with OPA and mercaptoacetic acid (MAA) reagents followed by UV detection at 330 nm. They reported that the C_{2a} component represents a significant proportion of gentamicin antibiotic. Claes et al. [18] utilized ion pair LC and pre-column derivatization with UV detection at 350 nm for analysis of C_{2a} component. Other gentamicin analyses were accomplished with pre-column derivatization by 2,4,6-trinitrobenzenesulphonic acid [19]. Seidl and Nerad [20] used isocratic ion-exchange chromatography with post-column OPA reaction followed by fluorescence to detect C_{1a}, C_1, C_2, C_{2a} and C_{2b} components. The separation order of this ion-exchange chromatography was C_{1a}, C_2, C_{2a}, C_{2b} and C_1 [20].

Inchauspé and Samain [21] were able to separate several aminoglycoside antibiotics by using perfluorinated carboxylic acid as an ion-pairing reagent in RPLC with refractive index (RI) detection. The elution order was C_{1a}, C_2 and C_1 and one unknown component.

Previous studies have shown that LC with pulsed electrochemical detection (PED) is useful for detecting gentamicin sulphate components without the need for derivatization [22,23]. However, electrochemical detection even in the pulsed mode suffers from some stability problems and some experience to obtain good repeatability is required [23].

Mass spectrometry (MS) of gentamicin sulphate has been reported by Rosenkranz et al. [24], Parfitt et al. [25]. Plasma desorption MS [26] and atmospheric pressure ionization MS with corona discharge [27] was applied for analysis of several aminoglycoside antibiotics including gentamicin sulphate.

Traditional separation techniques such as LC use considerable amounts of expensive and environmentally hazardous organic solvents. Safe disposal or recycling results is an additional cost. Over the past few decades microseparation techniques such as capillary electrophoresis (CE) and capillary electrochromatograpy (CEC) have offered a substantial revolution over the drawbacks imposed by the previous methods. However, the miniaturized techniques are not yet fully utilized. Currently, there are very few papers, which have reported the analysis of aminoglycosides by these novel techniques. The first major work on the use of CE in the study of aminoglycosides utilized indirect UV detection at low pH under reversed polarity [28]. Studies conducted by Hoffstetter-Kuhn et al. [29] and Flurer [30,31] utilized the formation of negatively charged complexes between the hydroxyl groups of carbohydrates and borate for direct UV detection at 195 nm.

Capillary zone electrophoresis (CZE) with indirect UV detection coupled with Micellar electrokinetic capillary chromatography (MECC) analysis of aminoglycosides have also been reported by Ackermans et al. [28].

The United States Pharmacopeia (USP) [32] and the European Pharmacopoeia (Ph. Eur.) [33] prescribe a RPLC method with pre-column derivatization with OPA to determine the composition of gentamicin. In this system however, the resolution between C_{2b} and C_1 is insufficient to determine composition based on peak areas.

Chemical derivatization of aminoglycosides with CE analysis remains a potential area for future research. In this work we report the results of CE analysis of gentamicin using pre-capillary derivatization with OPA and MAA and UV detection at 330 nm as an alternative to methods prescribed in the USP and Ph. Eur.

2. Experimental

2.1. Reagents, samples and reference standards

Sodium tetraborate decahydrate, β-cyclodextrin (β-CD), 1,2-phthalic dicarboxaldehyde and mercaptoacetic acid were obtained from Acros Organics (Geel, Belgium), methanol HPLC grade from Rathburn (Walkerburn, UK), 2-propanol Chromasolv from Riedel-de Haën (Seelze, Germany), sodium hydroxide pellets from BDH (Poole, UK), boric acid from Vel (Leuven, Belgium), picric acid from UCB (Brussels, Belgium). The gentamicin components C_1, C_{1a}, C_2 and C_{2a} were obtained from Pierrel (Capua, Italy), gentamicin C_{2b} was provided by Kyowa Hakko Kogyo (Tokyo, Japan) and sisomicin by the European Pharmacopoeia Laboratory (Strasbourg, France). Gentamicin commercial samples were ob-

tained from Wuxi Pharmaceuticals (Wuxi, China) and Dopharma, Netherlands. All solutions were made with ultrapure Milli-Q water (Millipore, Milford, MA, USA) and filtered with a 0.2 μm filter (Euroscientific, Lint, Belgium).

3. Instrumentation

Method development was performed on a Spectraphoresis-ultra CE instrument controlled by PC 1000 software version 3.5.1 (Thermoseparation Products, Fremont, CA, USA). The pH measurements were performed on a Consort C831 Multichannel analyzer (Turnhout, Belgium). Uncoated fused-silica capillary was obtained from Composite Metal Services (Hallow, UK).

3.1. Preparation of reagent buffer

The reagent buffer was freshly prepared by dissolving 520 mg of OPA in 2 ml of methanol and about 15 ml of 30 m*M* boric acid previously adjusted with 8 *M* potassium hydroxide to give a pH of 10.4, and 1040 μl of MAA was added. The resulting solution was adjusted to pH 10.4 using 8 *M* potassium hydroxide solution. The volume was made to 20 ml with boric acid previously adjusted to pH 10.4 to make 194 m*M* of OPA and 750 m*M* mercaptoacetic acid. Freshly prepared reagent was used throughout this study as in aqueous solution OPA is unstable despite the fact that Zhang and Yeung [34] described the use of a much older solution of OPA stored at 4°C for 48 h and 3 days of storage are allowed in the Ph. Eur. [33]. More peaks which are not coming from gentamicin components were obtained when old reagent solution was used.

3.2. Preparation of running buffer

The electrophoretic mobility of solutes changes with the pH and ionic strength of the buffer [35]. Thus to ensure consistent results the pH meter Consort C831 was calibrated before each measurement with buffers prescribed by the Ph. Eur. [33]. The buffers for CE experiments were prepared as follows: sodium tetraborate and β-cyclodextrin were dissolved in about 80 ml of water. The pH was adjusted to 10.0 by 1.0 *M* sodium hydroxide solution. To this solution 12.5 ml of methanol was added making up the volume to 100.0 ml with water to contain 30 m*M* tetraborate, 7.5 m*M* β-cyclodextrin and 12.5% (v/v) methanol.

3.3. Sample preparation and derivatization

All reference and sample solutions were prepared by dissolving 2 mg/ml in water and were stored at room temperature. Sample 10.0 ml, 5.0 ml of isopropanol and 4.0 ml of the reagent were thoroughly mixed. The volume was made to 25.0 ml with isopropanol. The mixture was heated in a thermostated water bath at 40°C for 5 min and cooled prior to injection using iced water. For quantitative analysis the gentamicin was dissolved in a 0.344 mg/ml solution of picric acid (internal standard).

3.4. Electrophoretic conditions

The capillary was conditioned every morning and whenever the buffer system was changed. This was performed with 0.1 *M* sodium hydroxide for 5 min, water for 5 min both at 60°C and running electrolyte for 5 min at 25°C, and with a new capillary a 1.0 *M* sodium hydroxide wash was added in the beginning. In the experimental design and quantitative experiments a 2 min wash with 0.1 *M* NaOH, 1 min with water and 1 min with buffer was instituted between runs and whenever a different buffer was used to ensure repeatable results. See Table 1 for CE conditions used.

3.5. Experimental design

Screening and optimization of the selectivity and derivatization conditions was performed by experimental design and multivariate analysis using Modde 4.0 software (Umetri, Umea, Sweden). Important factors were determined by a screening experiment and optimized by a response surface modeling (RSM) [36]. The screening experiment was carried out as a two level full factorial design with four variables ($k=4$) with three centre points ($n=3$) giving a total of $2^k+n=19$ runs. The most important factors were selected for further optimization by a central composite response surface

Table 1
CE conditions used for analysis of gentamicin sulphate

Parameters and conditions used			
(i) Uncoated fused-silica capillary			
Internal diameter	50 μm	Total length	40 cm
External diameter	375 μm	Effective length	33.7 cm
(ii) Hydrodynamic sample introduction			
Injection pressure	0.81 p.s.i.[a]	Injection time	4 s
(iii) Separation conditions			
Voltage	15 kV	Temperature	25°C
Run time	20 min	Current generated	47 μA
(iv) Concentration of analytes and reagents			
Gentamicin	2.0 mg/ml	Reagents	194 m*M* OPA
Picric acid (IS)	0.344 mg/ml		750 m*M* MAA
(v) Background electrolyte			
Sodium tetraborate	30 mM	Methanol	12.5% (v/v)
β-Cyclodextrin	7.5 mM	pH	10.0

[a] 1 p.s.i. = 6894.76 Pa.

modeling experiment. The factors levels which showed to be optimal in the screening experiments were set as centre point values in the RSM experiment to get description around this area. The central composite design face-centered (CCF) permitted the response surface to be modeled by fitting a second-order polynomial model with a number of experiments equal to $2^k + 2k + n$, where $(k = 3)$ is the number of variables and $(n = 3)$ is the number of extra points at the centre of the design which makes a total of 17 experiments. In particular the CCF consists of points of a full factorial design $(2^k + n)$ which have been augmented with $2k$ star points to enable this model estimate the response curvature plot. The star points are located at the centre and both extreme levels of the experimental domain [36,37].

The statistical relationship between a response Y and the experimental variables X_i and X_j can be described by the Taylor's series (expansion):

$$Y = \beta o + \Sigma \beta_i X_i + \Sigma \beta_{ij} X_i X_j + \Sigma \beta_{ii} X_i^2 + E$$

where β = regression coefficient and E = overall experimental error [36].

The linear coefficient for the experimental variables, β_i describes their quantitative effect in the model. The cross product, β_{ij} will measure the interaction effect between the variables and the square term $\beta_{ii} X_i^2$ will describe non-linear effect on response. The 95% confidence limits are expressed by using error bars. A regression coefficient smaller than the error bar interval shows the variation of the response caused by changing the variable is smaller than the experimental error. Therefore, the effect of variable change is considered insignificant when compared to the response.

4. Results and discussion

4.1. CE Method development

Method development was performed with a commercial sample of gentamicin sulphate. A typical electropherogram obtained under optimized conditions (Table 1) is shown in Fig. 2. The reagent did not co-migrate with gentamicin components.

The influence of different electrophoretic parameters known to influence selectivity and resolution was investigated. The average migration times were used for the calculation of selectivity factors. Gentamicin C_{1a}, C_{2a} and C_2 were chosen as critical peaks whose separation was set as target as the

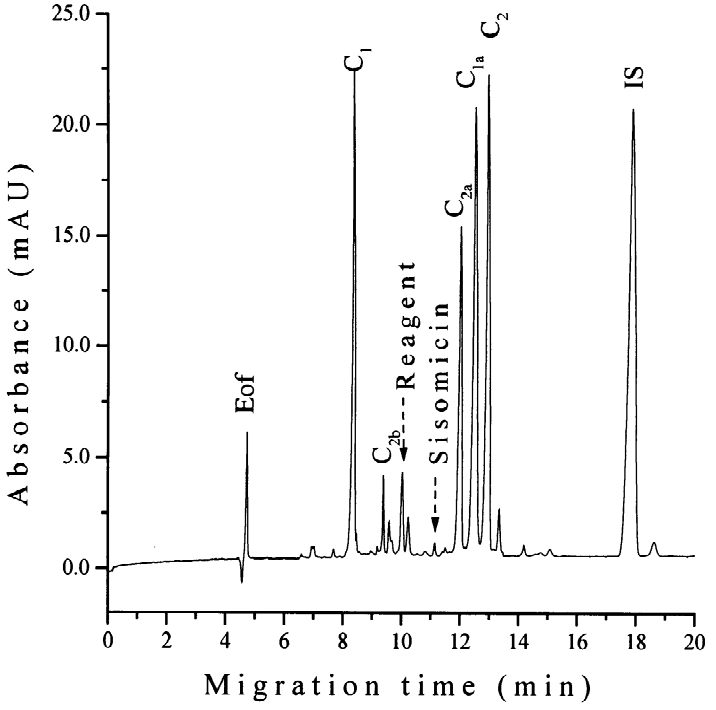

Fig. 2. A typical electropherogram of a commercial sample of gentamicin sulphate. See Table 1 for CE conditions applied. (IS = Internal standard).

derivatives of these components are structurally closely related. In the initial investigation on the influence of pH of the background electrolyte on selectivity of gentamicin components, it was found that pH has a considerable influence, and a pH between 9.75 and 10.25 showed improved selectivity.

The influence of cyclodextrins, inclusion complex forming agents, which are well known to improve selectivity of some closely related compounds, was investigated. Neutral cyclodextrins such as α-cyclodextrin, β-cyclodextrin and γ-cyclodextrin, were investigated. It was found that only β-cyclodextrin improved the selectivity of C_2 and C_{1a} to achieve baseline separation at a concentration of 5–10 m*M* but could not separate C_{1a} and C_{2a}.

Organic modifiers are frequently employed to achieve desired selectivity, probably due to changes in pK_a values, solvation effects, decreasing conductivity and changes in partition coefficient which help to dissolve hydrophobic compounds [38]. In this work the effect of methanol in improving the selectivity and the resolution of the critical peak pair C_{2a} and C_{1a}, was investigated. Methanol at 10–15% gave good selectivity and resolution.

4.2. Optimization of selectivity

As a first step in the optimization process, it is beneficial to perform screening experiments where a relatively large number of variables are examined concerning their significance [36]. Screening experiments, which involved a full factorial design, were performed to study the influence of several electrophoretic parameters on the selectivity between critical peak pairs. It was established from these screening experiments that only the concentration of β-cyclodextrin, methanol and pH have a significant effect while the change of tetraborate concentration was non-significant.

These were used for further optimization by a central composite response–surface modelling experiment. The RSM experiment included three factors, and as response variables, selectivities $S_{C2a-C1a}$

Table 2
Factorial analysis nominal values, corresponding to (−), (0) and (+) levels of separation conditions

Electrophoretic parameter	Low value (−)	Central value (0)	High value (+)
β-Cyclodextrin (m*M*)	5.0	7.5	10.0
Methanol (%, v/v)	10.0	12.5	15.0
pH	9.8	10.0	10.2

Table 3
Central composite face-centered (CCF) design used in the multivariate analysis of selectivity between gentamicin components

Exp. No.	β-CD (m*M*)	Methanol (%, v/v)	pH	$S_{C2a-C1a}$	S_{C1a-C2}
1	5	10	9.8	1.032	1.028
2	10	10	9.8	1.014	1.048
3	5	15	9.8	1.048	1.019
4	10	15	9.8	1.025	1.048
5	5	10	10.2	1.029	1.028
6	10	10	10.2	1.012	1.046
7	5	15	10.2	1.046	1.031
8	10	15	10.2	1.024	1.058
9	5	12.5	10	1.041	1.030
10	10	12.5	10	1.022	1.051
11	7.5	10	10	1.018	1.040
12	7.5	15	10	1.035	1.041
13	7.5	12.5	9.8	1.029	1.042
14	7.5	12.5	10.2	1.027	1.045
15	7.5	12.5	10	1.025	1.042
16	7.5	12.5	10	1.024	1.039
17	7.5	12.5	10	1.024	1.037

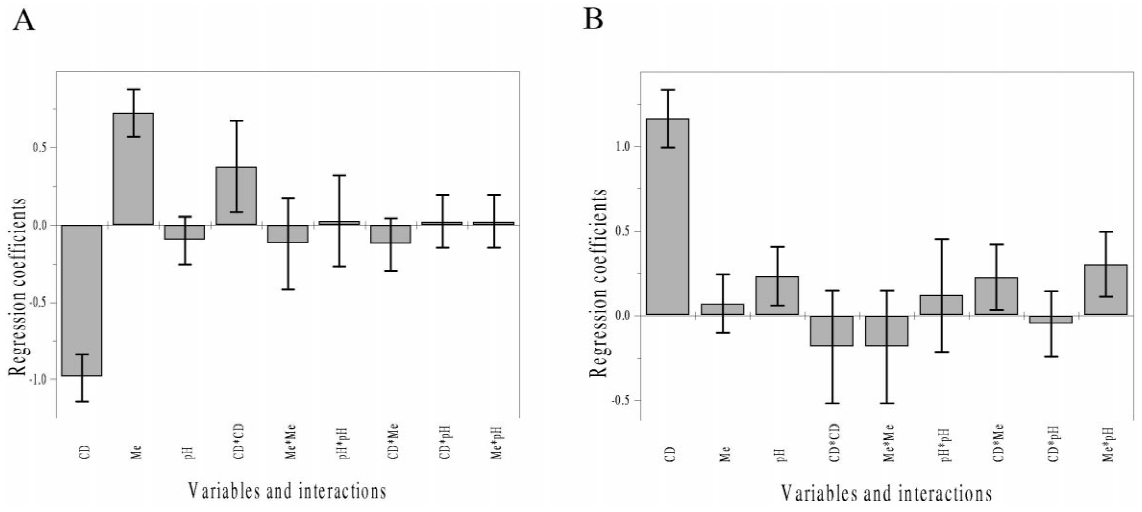

Fig. 3. Regression coefficients plots for the separation selectivity. (A) $S_{C2a-C1a}$ = selectivity between critical peak pair C_{2a} and C_{1a}, (B) S_{C1a-C2} = selectivity between critical peak pair C_{1a} and C_2. (Me = methanol, BC = β-cyclodextrin).

and S_{C1a-C2} corresponding to critical peak pairs C_{2a}–C_{1a} and C_{1a}–C_2 were chosen. Full factorial analysis nominal values, applied for optimization of selectivity are shown in Table 2 and a summarized work sheet is shown in Table 3. The tetraborate concentration was kept constant at 30 m*M*.

A full factorial multiple linear regression (MLR) analysis showed a significant effect on a number of peak selectivities from the addition of β-CD and methanol in the background electrolyte. The statistical analysis of a model containing the important main factors i.e. β-CD, methanol and pH yielded a R^2 (fraction of variation of response that can be explained by the model) greater than 0.97 and Q^2 (fraction of variation of response that can be predicted by this model) greater than 0.93 both for selectivity $S_{C2a-C1a}$ and S_{C1a-C2}. This shows that our experimental data were well fitted with the model. R^2 and Q^2 values were close to unity, which indicates the suitability of this model in predicting the optimum conditions.

4.2.1. The influence of β-CD on responses

In the concentration range investigated, it has been observed that β-CD has a significant influence both on $S_{C2a-C1a}$ and $S_{C1a-C2.}$ The effect is negative for the former and positive for the latter as illustrated in the regression coefficients plots (Fig. 3A and B) and in the response surface plots (Fig. 4A and B).

These observations can be explained as follows: the enhancement of selectivity by the use of β-CD in CE can be attributed to its ability to selectively include a variety of molecules into its hydrophobic cavities. When gentamicin–OPA derivative forms a complex with β-CD, its mobility is greatly reduced owing to change in apparent molecular mass. The complex stability is governed by factors such as Van der Waals interaction, solvation effect, and hydrogen bonding. As a result of this complexation, the charge density and electrophoretic mobility are finally reduced which imparts a differential mobility and hence improved selectivity.

4.2.2. The influence of methanol on responses

The effect of methanol on the two selectivities has been investigated. It was found that an increase in methanol concentration greatly increases the selectivity $S_{C2a-C1a}$, with a relatively small influence on selectivity S_{C1a-C2} as illustrated in the regression coefficient plots in Fig. 3A and B response surface plots in Fig. 4A and B, respectively. The addition of methanol in this separation buffer has proved to be useful in modifying selectivity especially between C_{2a} and C_{1a}, which in the first instance presented some difficulty. Methanol alters the electrophoretic properties that depend on physicochemical nature such as viscosity, zeta potential, pK_a and partition coefficient. The electroosmotic flow (EOF) de-

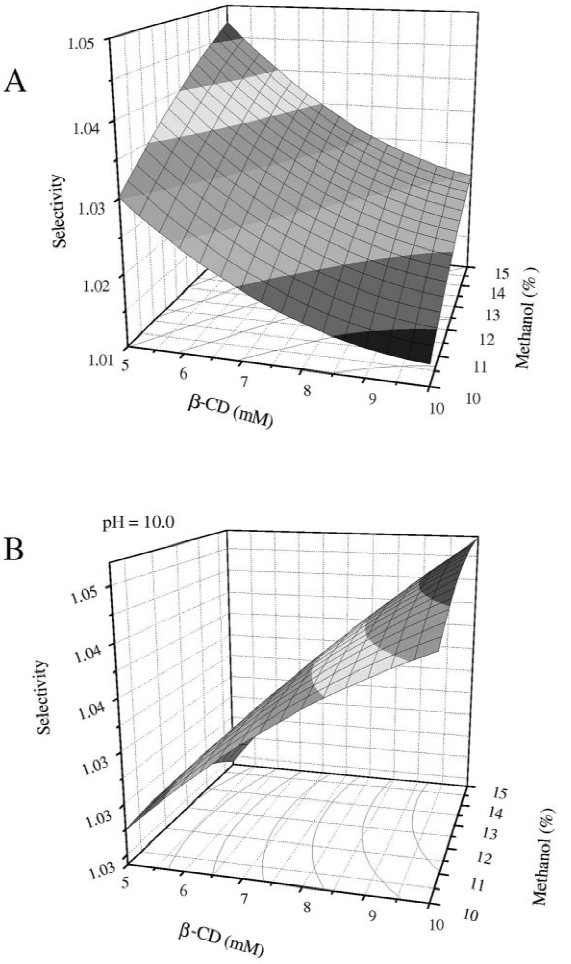

Fig. 4. Response surface plots of selectivity as a function of significant separation parameters. (A)$S_{C2a-C1a}$ = selectivity between critical peak pair C_{2a} and C_{1a}, (B)S_{C1a-C2} = selectivity between critical peak pair C_{1a} and $C_{2.}$.

Table 4
Factorial analysis nominal values, corresponding to (−), (0) and (+) levels of derivatization conditions

Reaction parameter	Low value (−)	Central value (0)	High value (+)
Molar ratio	3.2	9.6	16
Temperature (°C)	40	70	100
Time (min)	5	15	25

creases, prolonging migration time and hence improving overall selectivity. Solvation effects and alteration of pK_a could also result in further improvement in the solubility of hydrophobic analytes. The effect of methanol and β-CD on selectivity $S_{C2a-C1a}$ is opposite. The influence of methanol on the partition coefficient of the C_{1a} and C_{2a} derivatives between the hydrophobic β-cyclodextrin core and hydrophilic background electrolyte might be a possible explanation for this antagonism.

4.2.3. The influence of pH on the response

Separation in CZE is mainly governed by the ratio of the charge to mass of each analyte. The pH and the ionic strength of the background electrolyte are main factors that determine dissociation of the analyte into ions. This investigation was done at pH range 9.8 to 10.2, in which case the analytes were fully ionized and carried negative charge. This is the possible reason as to why pH did not have much influence on the selectivity.

4.2.4. Robustness and optimum selectivity prediction

Robustness testing is an important aspect of method validation. Appropriate experimental design can be used [39]. The central composite experiment design above was used to evaluate the response surface plot for concentration of β-CD, methanol and pH on selectivity (Fig. 4). Each selectivity investigated had its own optimum separation conditions. The selection of the overall optimum point took in consideration the balancing of effects (Table 1). The method under evaluation was found robust enough

Table 5
Central composite face-centered (CCF) design used in the multivariate analysis of derivatization conditions

Exp. No.	Temp. (°C)	Molar ratio	Time (min)	C_1/IS	C_{1a}/IS	C_2/IS
1	40	3.2	5	0.405	0.114	0.107
2	100	3.2	5	0.387	0.122	0.114
3	40	16	5	1.178	0.918	0.886
4	100	16	5	1.104	1.102	0.94
5	40	3.2	25	0.392	0.11	0.106
6	100	3.2	25	0.347	0.108	0.099
7	40	16	25	1.076	1.079	0.944
8	100	16	25	1.095	1.099	0.935
9	40	9.6	15	1.095	0.983	0.911
10	100	9.6	15	1.057	1.104	0.943
11	70	3.2	15	0.399	0.121	0.122
12	70	16	15	1.186	1.18	1.006
13	70	9.6	5	1.131	1.098	0.979
14	70	9.6	25	1.059	1.105	0.949
15	70	9.6	15	1.102	1.124	0.948
16	70	9.6	15	1.079	1.133	0.962
17	70	9.6	15	1.049	1.086	0.921

within the operating range for parameters examined in this study.

4.3. Optimization of derivatization conditions

A central composite design experiment and a multilinear regression analysis using Modde 4.0 statistical software enabled optimization of derivatization reaction. $2^k + 2k + n = 17$ experiments were performed where $k = 3$ is number variables and $n = 3$ is the number of centre points which were included to evaluate repeatability around this area. As variables the molar ratio (gentamicin base:1,2-phthalic dicarboxaldehyde), the derivatization time and the temperature were chosen, and as a response the ratios of corrected peak area of gentamicin to picric acid (an internal standard used to improve precision). Full factorial analysis nominal values applied for optimization of derivatization are shown in Table 4 and a summarized work sheet is shown in Table 5.

The statistical analysis of a model containing the important main factors yielded $R^2 > 0.99$ and $Q^2 > 0.98$ for all major components of gentamicin. This shows that our experimental data fitted well the model with reliable optimum condition prediction. Experimental results show that only the molar ratio (MR) between gentamicin and 1,2-phthalic dicarboxaldehyde strongly influences the derivatization yield (see Fig. 5A). Temperature and reaction time have no significant influence within the experimental limits investigated. In the presence of large excess of reagent the reaction kinetics are fast such that the reaction completion is well below the limits of time investigated. The response increases with increased molar ratio up to an optimum value at 12.8, with further increase, the response decreases possibly due to instability of *N*-substituted 1-alkylthioisoindole derivatives in presence of excess OPA reagent [40–44] see Fig. 5B. To make this method valuable and effective a molar ratio of 12.8, lower reaction temperature of 40°C and short reaction time of 5 min were chosen and established to be the optimum derivatization condition for this assay.

4.4. Quantitative analysis

Quantitative features of this analytical CE method for gentamicin C_1, C_{1a}, C_2, C_{2a}, and C_{2b} were investigated (using the ratio of corrected peak area of gentamicin to internal standard). For this study an amount of 38 ng was used by injecting 19 nl of a 2 mg/ml solution. The results for the limit of quantification (LOQ, at a signal-to-noise ratio, $S/N = 10$) and the limit of detection (LOD, $S/N = 3$) are summarized in Table 6 while the results found for linearity of gentamicin C_1, C_{1a}, C_2, C_{2a}, and C_{2b} are shown in Table 7. A commercial sample of gentamicin was used for linearity because pure reference substances were not available in sufficient quantities.

Analyzing six times a 2 mg/ml solution of gentamicin sulphate allowed to calculate the repeatability. The RSD values of the corrected peak areas for both intra-day and inter-day repeatability

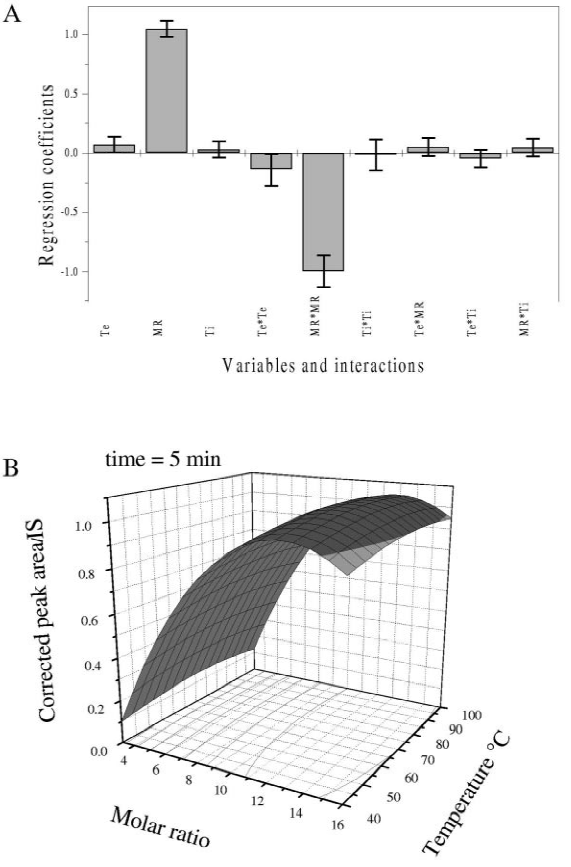

Fig. 5. Regression coefficients plots for the OPA–gentamicin derivative yield. (B) response surface plot of OPA–gentamicin derivative yield as assessed from the ratio of corrected peak area of gentamicin to that of internal standard (IS) (Ti = time, MR = molar ratio, Te = temperature).

Table 6
Limits of quantitation (LOQs) and limits of detection (LODs) for the four major gentamicin components

	Gentamicin	C_1	C_{2a}	C_{1a}	C_2
LOQ[a]	Concentration (μg/ml)	1.0	0.42	0.30	1.00
$S/N=10$	Absolute mass (pg).	19.00	7.98	5.70	19.00
	Relative (%)[b]	0.0500	0.0210	0.0150	0.0500
	RSD (%) ($n=6$)	10.6	3.4	7.9	8.9
LOD[a]	Concentration (μg/ml)	0.50	0.13	0.10	0.50
$S/N=3$[c]	Absolute mass (pg).	9.50	2.34	1.90	9.50
	Relative (%)[a]	0.0250	0.0063	0.0050	0.0250

[a] Injection volume was 19 nl for 8 s.
[b] relative to 2 mg/ml.
[c] S/N=signal-to-noise ratio.

Table 7
Linearity [a] of gentamicin components C_1, C_{2a} C_{1a} and C_2

Gentamicin	Regression equation	r	$S_{y,x}$	n_c	n_i
C_1	$y=5719.1x-274$	0.9963	325	8	3
C_{2a}	$y=2163.4x-193$	0.9924	178	8	3
C_{1a}	$y=5909.7x-358$	0.9926	480	8	3
C_2	$y=5124.2x-257$	0.9955	322	8	3

[a] The linearity range was from 1% to 120% relative to 2 mg/ml. y=corrected peak area/internal standard, x is the total concentration of gentamicin, r is the coefficient of correlation, $S_{y,x}$=standard error of y estimate, n_c number of experimental concentrations studied and n_i number of injection/concentration.

are summarized in Table 8. In each experiment a freshly derivatized sample was used. The R.S.D values contain information on the repeatability of analysis and derivatization yield.

4.5. Stability of gentamicin-OPA derivative

The stability of gentamicin–OPA derivative was investigated within 12 h for a derivatized solution and stored at room temperature throughout the study time. The results were evaluated using the ratio of the corrected peak area of the peaks due to gentamicin components to that of internal standard. Over the time range investigated the ratio remained stable (see Fig. 6) with intermediate R.S.D values between intra-day and inter-day repeatability. It can be concluded from these results that the derivative is stable at room temperature within this period of time.

5. Conclusion

The method developed using pre-capillary derivatization of gentamicin with OPA/MAA allows fast, selective and sensitive separation of the components C_1, C_{1a}, C_2, C_{2a}, C_{2b}, sisomicin and several other minor unknown components, which were not identified because the reference standards were not available. The method offers the advantage of being fast, compared to previous LC methods, which needed about 60 min for complete analysis. The use of higher ratio of reagent to gentamicin allowed the use of lower reaction temperature and short reaction time which are the excellent features of this method over the methods prescribed in the United States Pharmacopeia and European Pharmacopoeia.

The quantitative analysis established that the method is sensitive enough for the analysis of gentamicin in commercial preparations.

Table 8
Repeatability of the corrected peak areas[a]

Gentamicin	C_1	C_{2b}	C_{2a}	C_{1a}	C_2	Number of experiments (n)
Intra-day RSD (%)	0.7	4.0	2.0	1.9	2.3	6
Inter-day RSD (%)	2.1	12.1	3.8	3.0	3.0	18 (6 days)

[a] RSD values were calculated based on ratio of gentamicin corrected peak areas to internal standard.

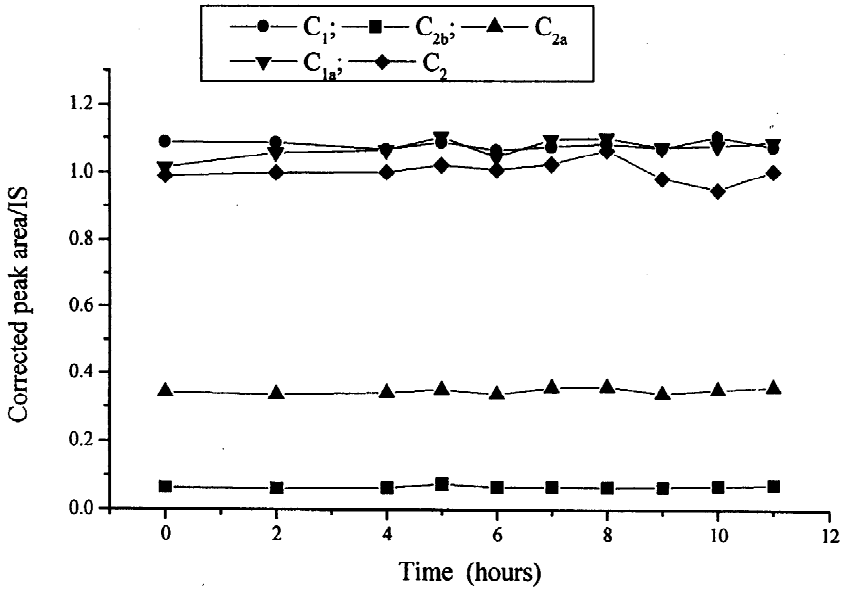

Fig. 6. Stability of OPA–gentamicin derivative over a period of 12 h at room temperature.

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