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Improved reversed-phase liquid chromatographic method combined with pulsed electrochemical detection for the analysis of amikacin

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Abstract

A two-step gradient liquid chromatographic method combined with pulsed electrochemical detection is described for the determination of amikacin and its impurities. The mobile phase is composed of an aqueous solution containing 1.8 g/l sodium 1-octanesulphonate, 14 ml/l tetrahydrofuran, 50 ml/l of phosphate buffer pH 3.0 and sodium sulphate, which was 20 g/l in mobile phase A and 28 g/l in mobile phase B. 0.5 M sodium hydroxide was added post-column to enhance the detection. An investigation of different reversed-phase columns indicated that the Discovery (C_{18}, 5 μm, 250 mm × 4.6 mm i.d.) column was the most suitable. Compared to previously published investigations, the proposed method showed higher sensitivity and efficiency, allowing the separation of the main component amikacin from 16 impurities, 7 of which were of unknown identity. A central composite experimental design was used to assess the robustness. The method showed good repeatability and linearity in the assay range. The method was further applied to analyze some commercial samples.

Keywords: Pulsed electrochemical detection; Reversed-phase liquid chromatography; Amikacin; Impurity profile

1. Introduction

Amikacin or BB-K8 is a semisynthetic, water-soluble, broad spectrum aminoglycoside antibiotic derived from kanamycin A [1]. It is commonly used for treating severe, hospital-acquired infections caused by Gram-negative bacteria resistant to other aminoglycosides [2]. The blood levels however, have to be monitored carefully because of its ototoxicity and nephrotoxicity [3,4].

Amikacin is synthesised by acylation of the amino group in position 1 of kanamycin A with L-(−)-γ-amino-α-hydroxybutyric acid (L-HABA) (Fig. 1). Therefore, kanamycin A and L-HABA can be expected to be possible impurities in commercial samples. As the molecule of kanamycin A has four primary amino groups, it is possible during the synthesis to obtain side products that differ only in the position of the acyl group. BB-K11 (acylation at position 3″), BB-K29 (acylation at position 3) and 1,3-di-HABA kanamycin A (acylation at positions 1 and 3) are reported as potential impurities in the European Pharmacopoeia (Ph.Eur.) [5]. Other potential impurities are BB-K6 (acylation at position 6′), 1,6′-di-HABA kanamycin A (acylation at positions 1 and 6′), BB-K9 (acylation with D-(+)-HABA instead of L-(−)-HABA) and BB-K26 (acylation of kanamycin B).

Direct UV detection of amikacin and its impurities cannot be performed because of the lack of a strong UV-absorbing chromophore. Traditionally, HPLC analysis of amikacin involves either pre-column [5–8] or post-column [9] derivatization prior to UV detection. In the actual Ph.Eur. method, amikacin and its impurities are determined by LC-UV following pre-column derivatization with 2,4,6-trinitrobenzene sulphonic acid [5]. Only four impurities are described to be separated. The drawback in the use of derivatization is that it is time-consuming and difficult for quantification. Amikacin can also be analyzed using pulsed electrochemical detection (PED) because it contains functional groups that can be oxidized to give an efficient signal at the detector [10]. Other non-derivatization methods reported are: LC with fluorescent detection based on a ligand displacement reaction [11], fluorimetric determination using lanthanide probe ion spectroscopy [12] and hydrophilic interac-

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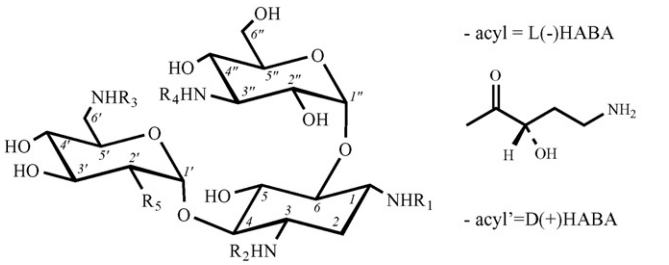

	R_1	R_2	R_3	R_4	R_5
Amikacin (BB-K8)	acyl	H	H	H	OH
Kanamycin A	H	H	H	H	OH
BB-K29	H	acyl	H	H	OH
BB-K6	H	H	acyl	H	OH
BB-K11	H	H	H	acyl	OH
1,3-*di*-HABA kanamycin	acyl	acyl	H	H	OH
1,6'-*di*-HABA kanamycin	acyl	H	acyl	H	OH
BB-K9	acyl'	H	H	H	OH
BB-K26	acyl	H	H	H	NH_2

Fig. 1. Structures of amikacin and its impurities.

tion chromatography combined with tandem-mass spectrometry [13]. Recently, a LC method was reported for the determination of amikacin in drug products, using evaporative light scattering detection [14]. Among these methods, the most interesting combination for the analysis of amikacin, including purity testing, was found to be LC-PED. For the ion-pair method described in reference [10] a poly(styrene-divinylbenzene) column was used. Although this type of stationary phase has proved to be more stable than conventional C_8 or C_{18} columns, it shows poor efficiency. More recently, several new reversed-phases, like a C_{18} derivatised polymer, came on the market while the general quality of columns improved significantly.

In this study, the performance of different C_{18} columns towards the analysis of amikacin will be examined in order to separate more impurities. The composition of the mobile phase used in this study was based on that previously developed in our laboratory [10]. The method has been used to analyze commercial samples.

2. Experimental

2.1. Reagents and samples

A milli-Q water purification system (Millipore, Bedford, MA, USA) was used to purify glass-distilled water. The buffer was prepared by mixing a 0.2 M phosphoric acid solution and a 0.2 M potassium dihydrogen phosphate solution to obtain pH 3.0. These solutions were prepared using 85% (mass/mass) phosphoric acid from Acros (Geel, Belgium) and potassium dihydrogen phosphate from Merck (Darmstadt, Germany). Sodium 1-octanesulphonate, HPLC grade, was also from Acros. Tetrahydrofuran (THF) (stabilised with 2,6-di-tert-butyl-4-methyl phenol) and sodium sulphate (anhydrous, extra pure fine powder) were obtained from Merck. The 0.5 M sodium hydroxide solution was prepared using 50% (mass/mass) sodium hydroxide aqueous solution (Baker, Deventer, The Netherlands) and was added post-column to the effluent of the column by a pneumatic device pressurised with helium from Air Liquide (Machelen, Belgium).

Reference substances to identify the peaks as shown in Fig. 1 originated from Gist-Brocades (Capua, Italy). Bulk samples were from Bristol-Myers Squibb (Sermoneta, Italy and Paris, France). Sample solutions were prepared at 0.5 mg/ml in mobile phase.

2.2. Instrumentation

The chromatographic procedure was carried out using a L-6200 Intelligent pump (Merck, Hitachi, Darmstadt, Germany), an autosampler AS100 Spectra Series (San Jose, CA, USA) equipped with a 20 μl loop, a laboratory made pneumatic device, allowing pulse-free post-column addition of the sodium hydroxide solution and Chromeleon 6.50 software (Dionex, Sunnyvale, CA, USA) for data acquisition. The pulsed electrochemical detector was a Decade II from Antec (Leyden, The Netherlands). The electrochemical cell consisted of a gold working electrode, a hydrogen reference electrode and a carbon filled polytetrafluoroethylene counter electrode. The electrochemical cell was kept at 35 °C in the detector oven. The following columns were investigated (unless indicated otherwise, all the column dimensions were 250 mm × 4.6 mm i.d., 5 μm): Astec C_{18} polymer (Agilent, Wilmington, DE, USA), Brava BDS (Alltech, Deerfield, IL, USA), Discovery C_{18} (Supelco, Bellefonte, PA, USA), Gemini C_{18} (Phenomenex, Macclesfield, UK), Hypersil BDS C_{18} (Thermo, Bellefonte, PA, USA), HyPurity Elite C_{18} (ThermoQuest, Runcorn, UK), Intersil ODS-2 (Alltech), Symmetry C_{18} (Waters, Milford, MA, USA), Supelcosil C_{18} (Supelco), Supelcosil LC-C_{18}-DB (Supelco), Supelcosil LC-C_{18}-DB, 3 μm, 150 mm × 4.6 mm (Supelco) and XTerra RP 18 (Waters). The columns were maintained at 40 °C in a water bath heated by means of a Julabo EM thermostat (Julabo, Seelbach, Germany).

2.3. Chromatography

The mobile phase consisted of 1.8 g/l of sodium 1-octanesulphonate, 14 ml/l of tetrahydrofuran, 50 ml/l of phosphate buffer pH 3.0 and sodium sulphate, which was present at a concentration of 20 g/l in mobile phase A and 28 g/l in mobile phase B. The mobile phases were degassed with helium before use. The LC flow rate was 1 ml/min. A two-step gradient elution was necessary to obtain a good separation together with a reasonable analysis time: 0–3.0 min, 100% A; 3.1–38.0 min, 100% → 30% A, while 0% → 70% B; 38.1–68.0 min, 100% B; 68.1–78.0 min, 100% A. The LC flow rate was 1.0 ml/min. All substances were dissolved in mobile phase and 20 μl was injected onto the column.

Through a mixing tee, 0.5 M sodium hydroxide was added post-column at 0.3 ml/min from a helium-pressurized reservoir

and mixed in a packed reaction coil (1.2 m, 500 μl) from Dionex. The pH of the mobile phase has to be raised to 13 to enhance the electrochemical reaction and so to improve the sensitivity of the detection. The 0.5 M NaOH solution was prepared starting from a 50% (mass/mass) aqueous solution which was pipetted in helium degassed water. The solution was degassed in order to avoid the formation of carbonates, which foul the electrodes of the electrochemical cell and hamper further reaction.

The time period and voltage parameters for the detector were set as follows: t_1 (0–0.40 s), E_1 (+0.05 V); t_2 (0.41–0.60 s), E_2 (+0.75 V); t_3 (0.61–1.00 s), E_3 (−0.15 V). Integration of the signal occurred between 0.20 and 0.40 s.

2.4. Experimental design

A robustness study was performed by means of an experimental design and multivariate analysis with Modde 5.0 software (Umetrics, Umea, Sweden). A central composite design was applied, which is composed of a full or fractional factorial design, star points and replicated centre points. The star points enable the model to estimate the curvature response. These star points are located at the centre and both extreme levels of the experimental domain. For a complete central composite design, which includes the points of a two level full factorial design, the number of runs is equal to $2^k + 2k + n$, where k is the number of parameters and n is the number of centre points. In this study, five parameters (amount of sodium sulphate, amount of sodium 1-octanesulphonate, volume of THF, temperature of the column, pH of the phosphate buffer) were investigated. With this number of parameters and three centre points, a complete central composite design would result in a number of runs equal to 45. In order to reduce the number of runs, a central composite design which includes the points of a two level half fractional factorial design was chosen, with a number of runs equal to $2^{k-1} + 2k + n = 29$. The statistical relationship between a response Y and the experimental variables X_i, X_j, ... is of the following form:

$$Y = \beta_0 + \beta_i X_i + \beta_j X_j + \beta_{ij} X_i X_j + \beta_{ii} X_i^2 + \beta_{jj} X_j^2 + \cdots + E \quad (1)$$

where the βs are the regression coefficients and E is the overall experimental error.

The linear coefficients β_i and β_j describe the quantitative effects of the respective variables. The cross coefficient β_{ij} measures the interaction effect between the variables and the square terms $\beta_{ii} X_i^2$ and $\beta_{jj} X_j^2$ describe the non-linear effects on the response.

3. Results and discussion

3.1. Method development

A previously described method, using a polymer column, was taken as a starting point [10]. It was tried to improve this method in order to separate as many impurities as possible within a reasonable analysis time. Different C_{18} columns, which possess much higher efficiency compared to polymer stationary phases, were investigated. The selection of different columns was based on a column classification system previously developed in our laboratory [15]. The columns investigated are mentioned in Section 2.2. Astec, a C_{18} derivatised polyvinyl alcohol material, was included in this study since this stationary phase combines the separation efficiency of traditional reversed-phase column with the stability of polymer columns. It showed a selectivity similar to that of the PLRP-S column used previously in [10]. The normal silica-based columns and the hybrid Gemini column showed a better selectivity overall. The selectivity and peak shape obtained with the Discovery column was superior to that of the other columns and this end-capped stationary phase was chosen for further investigation.

In further method development it was tried to reduce the amount of salt (up to 60 g/l) in the mobile phase since this can cause stability problems for silica-based stationary phases. It was adapted to 20 g/l in mobile phase A and 28 g/l in mobile phase B. However, decreasing the amount of salt leads to an increase in analysis time. To reduce the analysis time, THF was added. This organic modifier also improved the selectivity. An amount of 14 ml/l was found to be a good compromise between the resolution of the different peak pairs and the analysis time. The most optimal concentration of sodium 1-octanesulphonate was 1.8 g/l, since a lower amount showed peak shape distortion and higher amounts gave too long retention times. An acidic environment (pH 3) is required since the analyte molecules are then positively charged and can efficiently interact with the negatively charged ion-pairing agent. The temperature was investigated between 38 °C and 42 °C and 40 °C was chosen as a good compromise, a higher temperature resulted in reduction of retention and resolution. The analysis time of the new method is longer than that of the previous one (78 min versus 60 min). This is due to the late elution of BB-K26, an impurity that was not described to be separated by the previous method. A typical chromatogram of a commercial sample is shown in Fig. 2. Amikacin was com-

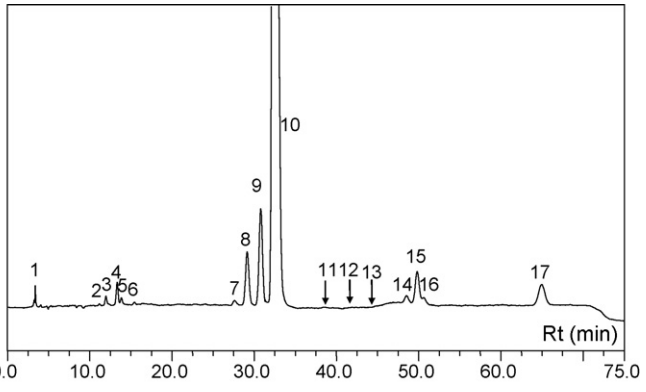

Fig. 2. A typical chromatogram of a commercial amikacin sample under the LC conditions mentioned in Section 2.3. Column: discovery C_{18}, 5 μm, 250 mm × 4.6 mm i.d. Elution order: (1) L-HABA; (2) unknown 1; (3) unknown 2; (4) unknown 3; (5) unknown 4; (6) unknown 5; (7) unknown 6; (8) 1,6′-di-HABA–kanamycin A; (9) 1,3-di-HABA–kanamycin A; (10) amikacin; (11) BB-K9; (12) BB-K11; (13) BB-K6; (14) kanamycin A; (15) BB-K29; (16) unknown 7; (17) BB-K26.

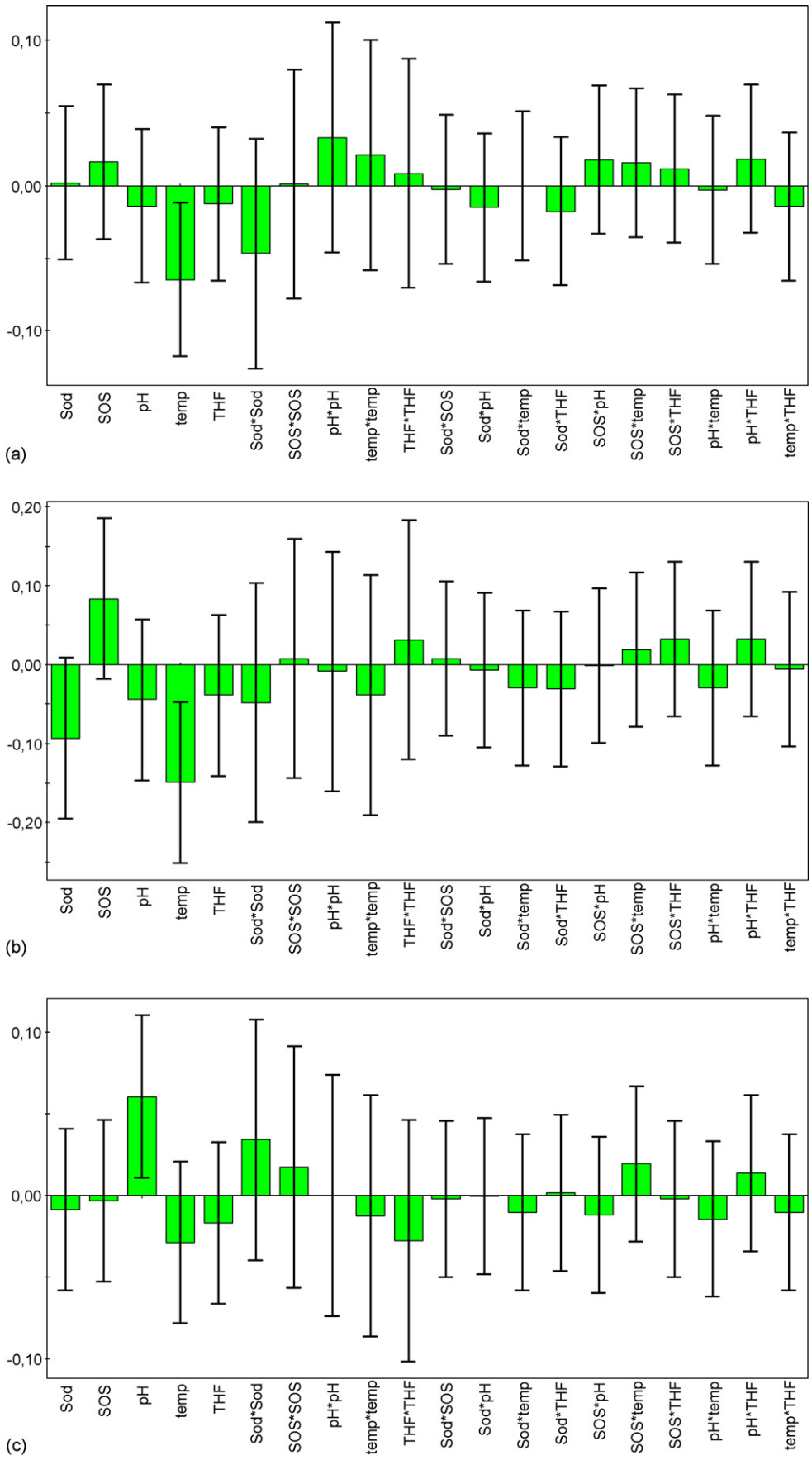

Fig. 3. Regression coefficient plots for the separation of the pairs (a) 1,3-di-HABA–kanamycin A–amikacin (Rs_1), (b) BB-K6–kanamycin A (Rs_2) and (c) kanamycin A–BB-K29 (Rs_3). Sod: sodium sulphate, SOS: sodium 1-octanesulphonate and Temp: temperature.

Table 1
Factorial analysis: nominal values corresponding to −1, 0 and +1 level

Chromatographic parameter	−1	0	+1
Sodium sulphate (g/l)	27	28	29
Sodium octanesulphonate (g/l)	1.7	1.8	1.9
pH of the phosphate buffer	2.5	3.0	3.5
Volume of THF (ml/l)	13	14	15
Column temperature (°C)	38	40	42

pletely separated from nine known related substances as well as several unknown impurities. The nine known substances were also well separated from each other, but unknown 7 could only be partly separated.

3.2. Robustness study

The robustness study was performed by means of an experimental design as mentioned in Section 2.4. The different chromatographic parameters of the design are listed in Table 1. The values indicated for sodium sulphate, sodium 1-octanesulphonate and THF were only applied to mobile phase B while the pH was adapted in both mobile phases A and B. The individual, interaction and quadratic effects on the resolution for the pairs 1,3-di-HABA–amikacin (Rs_1), BB-K6–kanamycin A (Rs_2) and kanamycin A–BB-K29 (Rs_3) are summarized in Fig. 3.

The plots consist of bars, which correspond to the regression coefficients and which are proportional to the magnitude of the variable effects. The 95% confidence interval limits are expressed by using error lines. A regression coefficient smaller than the error line interval shows that the variation of the response caused by that variable change is smaller than the experimental error and, in this case, the effect is considered not to be significant. The magnitude of the effect is proportional to the regression coefficient (see Eq. (1)). It is observed that temperature had a negative effect on the resolution of Rs_1 and Rs_2, which means that an increase in temperature decreases the resolution of the considered peak pairs. The pH had a positive effect on Rs_3. The other factors had no significant effect on the resolutions studied and no important interactions between different parameters were found. It can be concluded that the method is robust.

3.3. Quantitative aspects

3.3.1. Limits of detection (LOD) and quantification (LOQ)

A signal-to-noise ratio (S/N) of 3 is generally accepted for estimating the LOD, which is the lowest concentration that can be detected. The LOQ ($S/N \geq 10$) is the lowest concentration of a substance that can be quantified with acceptable precision. The LOD and LOQ values for amikacin were 0.2 and 0.5 μg/ml (0.1 %, $n = 6$), respectively.

3.3.2. Linearity and repeatability

Linearity was investigated for amikacin with solutions prepared at 11 levels ranging from 1 to 625 μg/ml (100% corresponds to 500 μg/ml). However, the response of the detector in this wide range was not linear. Overloading of the detector was clearly noticed for concentrations above 100 μg/ml. This implies that a lower concentration is required for the assay of amikacin. By further study, good linearity was obtained from LOQ to 25 μg/ml, with a correlation coefficient greater than 0.99. The precision (R.S.D.) for the measurements of amikacin at 25 μg/ml was 0.53% for six injections.

Table 2
Composition of commercial amikacin sulphate samples (% (mass/mass)), expressed as amikacin

	Sample 1	Sample 2	Sample 3
L-HABA	0.18	0.17	0.13
Unknown 1	<LOQ	<LOQ	<LOQ
Unknown 2	<LOQ	0.12	0.12
Unknown 3	0.30	0.31	0.34
Unknown 4	<LOQ	0.12	<LOQ
Unknown 5	<LOQ	<LOQ	<LOQ
Unknown 6	0.16	0.11	0.12
1,6′-Di-HABA-kanamycin A	0.98	1.35	1.40
1,3-Di-HABA-kanamycin A	2.81	2.55	2.55
BB-K9	<LOD	<LOD	<LOD
BB-K11	<LOD	<LOD	<LOD
BB-K6	<LOD	<LOD	<LOD
Kanamycin A	0.19	0.23	0.16
BB-K29	1.01	0.96	0.93
Unknown 8	0.14	0.18	0.20
BB-K26	0.99	0.97	0.95
Total	6.76	7.07	6.89

3.4. Application to commercial samples

The proposed LC-PED method was applied to commercial samples of amikacin. Samples from three different batches were dissolved and diluted with the mobile phase to the appropriate concentrations. The impurity profile was investigated at a concentration of 0.5 mg/ml. Data obtained are summarized in Table 2. All related components are expressed as amikacin, using chromatograms obtained with a 5% dilution (25 μg/ml) of the sample.

4. Conclusion

The proposed method, using a reversed-phase C_{18} column, allows the separation of amikacin from 16 impurities, 9 of which were known related substances and 7 were of unknown identity. The method was robust, sensitive and repeatable. The method has been used for the analysis of commercial samples.

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