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Short communication

Determination of amikacin in cerebrospinal fluid by high-performance liquid chromatography with pulsed electrochemical detection

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ABSTRACT

A highly sensitive and fast reversed-phase liquid chromatographic (LC) method combined with pulsed electrochemical detection (PED) was developed for the direct quantification of the aminoglycoside antibiotic amikacin in cerebrospinal fluid (CSF). The limit of quantification obtained was 0.06 μg/ml and linearity was established over the concentration range 0.06–4.00 μg/ml. The recovery was found to be close to 100%. This method was developed in order to study CSF pharmacokinetics of amikacin in neonates. The narrow therapeutic range calls for monitoring to ensure optimal therapy and to minimize the risk of toxic side effects such as nephro- and ototoxicity, especially in populations like preterm neonates at birth, where the predictability of amikacin clearance is limited. Typical problems to be solved were the low amikacin concentrations and the limited sample volume of CSF.

1. Introduction

Amikacin is a semisynthetic, water soluble, broad spectrum aminoglycoside antibiotic derived from kanamycin A by acylation of the 1-amino group in the 2-deoxystreptamine moiety with L-(−)-γ-amino-α-hydroxybutyric acid (L-HABA) (Fig. 1).

It is commonly administered parenterally for the treatment of Gram-negative infections resistant to gentamacin, kanamycin and tobramycin because the amikacin molecule has fewer points susceptible to enzymatic attack than the other aminoglycosides. The administration of amikacin based on extended dose interval and in combination with ampicillin is a well established antibiotic treatment regime, also in neonates [1,2].

The narrow therapeutic range, i.e. small differences between the effective and toxic concentrations, calls for monitoring to ensure optimal therapy and to minimize the risk of toxic side effects such as renal and ototoxicity especially in populations like preterm neonates at birth, where the predictability of amikacin clearance is limited [3].

Traditionally microbiological assays have been used to determine amikacin and other aminoglycosides in biological samples. Although microbiological assays used to be popular for monitoring aminoglycosides during therapy, there has been a massive decline in their use due to their non-specificity and poor sensitivity. More convenient techniques for quantitative analysis of aminoglycosides in body fluids were introduced. Automated immunoassays were shown to be more appropriate for aminoglycoside determination in body fluids [4]. In 1981, Abbott (Abbott Park, IL, USA) introduced the TDx® system which uses the fluorescence polarization immunoassay (FPIA) methodology and rapidly has become the market leader in therapeutic drug monitoring. The Abbott FPIA system has been shown to be suitable for the analysis of aminoglycosides in serum and other biological fluids. In the study of Jandreski and Garbincius, the FPIA TDx® system was used for the measurement of aminoglycosides in CSF and sensitivity obtained for amikacin was 0.18 μg/ml [5]. However, the disadvantage of immunoassays is the possibility of cross-reactivity between different aminoglycosides and the requirement for the suitable antibody and tracer, as well as expertise, facilities and time required to produce them.

In order to perform analyses in less demanding conditions and to achieve adequate selectivity for specific determination of aminoglycosides, LC methods, often developed originally for drug purity control were also introduced. Most LC methods for the determination of amikacin in various samples required the use of different derivatization reactions to provide adequate detection. First LC analysis of aminoglycosides was involving either pre- or post-column derivatization with *o*-phthalaldehyde (OPA) [6–8] or pre-column derivatization with 1-fluoro-2,4-dinitrobenzene [9], 2,4,6-trinitrobenzene sulphonic acid [10], 1-naphtoyl chloride [11], 1-naphtyl isothiocyanate [12] or 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate [13] prior to UV detection.

The main disadvantage in the use of derivatization is that it is time-consuming and gives problems with quantification. To overcome the derivatization step, different alternative detection

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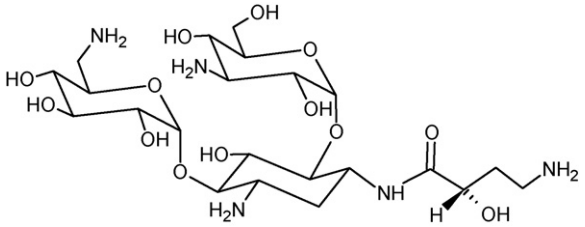

Fig. 1. Chemical structure of amikacin.

methods following LC have been proposed like pulsed electrochemical detection (PED) [14,15], fluorescence detection based on a ligand displacement reaction [16], mass spectrometry [17] and evaporative light scattering detection [18]. Recently, a LC method using chemiluminescence detection was reported for the determination of amikacin in body fluids [19].

From the above mentioned, LC methods using mass spectrometry and chemiluminescence detection have found their application for the determination of amikacin in biological fluids. The LC method combined with mass spectrometry for the determination of amikacin in serum showed a limit of quantification (LOQ) of 0.1 μg/ml [17]. The method involves solid phase extraction as sample pre-treatment, followed by hydrophilic interaction chromatography. The determination of amikacin in body fluids such as plasma and urine by LC with chemiluminescence (CL) detection has been described by Serrano and Silva [19]. This method involves centrifugation of the sample after dilution with an ethanol/sodium carbonate mixture, separation on a reversed-phase C18 column and indirect detection based on the inhibitory effect of the amikacin on the CL reaction between luminol and hydrogen peroxide catalysed by Cu(II). The limit of detection obtained was 0.05 μg/ml and relative recoveries were found to be over 92%.

The intention of this study was to develop a relatively simple, specific and highly sensitive technique for direct quantification of very low amikacin concentrations in CSF in order to estimate serum-CSF compartmental pharmacokinetics of amikacin. Based on one single study on amikacin CSF concentrations in 10 neonates, it was hereby anticipated that the concentrations will be very low, necessitating a highly sensitive method [20]. This study was the analytical part of research on CSF kinetics of amikacin in neonates [21]. Amikacin was administrated as part of the empiric treatment for infection in (pre)term neonates at the neonatal intensive care unit [22]. The aim of the research was to describe the relationship between plasma and CSF concentrations and to explore the impact of CSF inflammatory markers on that relationship.

Based on the good results obtained for purity control of amikacin using LC combined with PED [14,15], the performance of this technique was to determine low concentrations of amikacin in CSF samples was investigated here.

2. Experimental

2.1. Reagents

A Milli-Q water purification system (Millipore, Bedford, MA, USA) was used to purify demineralized water. Tetrahydrofuran (THF), stabilized with 2,6-di-*tert*-butyl-4-methylphenol and anhydrous sodium sulphate extra pure was obtained from Merck (Darmstadt, Germany). Sodium 1-octanesulphonate was obtained from Acros (Geel, Belgium) and helium was from Messer (Machelen, Belgium). The buffer was prepared by mixing a 0.2 M phosphoric acid solution and a 0.2 M potassium dihydrogen phosphate solution till a pH of 3.0 was obtained. Concentrated phosphoric acid (85% m/m) used for preparation of a 0.2 M phosphoric acid solution and potassium dihydrogen phosphate were obtained from Acros. The 0.5 M sodium hydroxide solution was prepared by adding 25 ml of a 50% (m/m) sodium hydroxide solution (J.T. Baker, Deventer, the Netherlands) to 975 ml of purified water previously degassed with helium (10 min). The mixture was degassed again for 10 min.

Table 1
Final chromatographic conditions

Stationary phase	Hypurity Elite (150 mm × 4.6 mm, 5 μm)	
Guard column	Gemini C18 (4 mm × 2 mm)	
Mobile phase composition per liter	Sodium-1-octanesulphonate, 1.5 g Anhydrous sodium sulphate, 20 g Tetrahydrofuran, 15 ml 0.2 M phosphate buffer pH 3, 250 ml Milli-Q^{50} water, up to 1000 ml	
Flow rate of mobile phase	1.0 ml/min	
Injection volume	100 μl	
Column temperature	40 °C	
Post-column addition of 0.5 M NaOH	0.5 ml/min	
Pulsed electrochemical detector		
Working electrode	Gold	
Reference electrode	HyREF	
Counter electrode	Carbon filled polytetrafluoroethylene (PTFE)	
Detector settings	*t* (s)	*E* (V)
	0.0–0.40	+0.05
	0.41–0.60	+0.70
	0.61–1.00	−0.15
Integration period	0.20–0.40 s	
Sensitivity	5 μA	

2.2. HPLC analysis

The mobile phase was delivered by a LC pump (L-6200 Intelligent pump, Merck–Hitachi, Darmstadt, Germany) at a flow rate of 1.0 ml/min. An automatic injector (Elite LaChrom L-2200) was used to inject the samples. The column was immersed in a water bath with a heating immersion circulator (Julabo EC, Germany). 0.5 M sodium hydroxide was added post-column using a helium-pressurized reservoir to increase the pH of the column effluent above 12. This alkaline pH is necessary to enhance the detection sensitivity. Addition of the post-column solution should be pulse-free to avoid disturbances in the baseline. The post-column solution was added at a flow rate of 0.5 ml/min. After mixing both solutions in a mixing coil (1.2 m, 500 μl) (Dionex, Sunnyvale, CA, USA) the resulting solution entered into the electrochemical cell of the PED (Antec Decade II), which was equipped with a gold working electrode, a HyRef reference electrode and a carbon filled polytetrafluoroethylene (PTFE) counter electrode. The measuring cell was kept in a hot air oven to keep the temperature constant at 35 °C. The detector settings were as follows: E_1, E_2 and E_3 were respectively +0.05 V, +0.70 V and −0.15 V with the assigned pulse durations t_1: 0.00–0.40 s, t_2: 0.41–0.60 s and t_3: 0.61–1.00 s. Integration of the signal occurred between 0.20 s and 0.40 s. An overview of the settings is also given in Table 1. Data acquisition software (Chromeleon, Dionex) was used to record the signal.

2.3. Sample preparation

As reference substance Amukin® (Bristol-Myers Squibb, Brussels, Belgium) was used. Each vial contained 100 mg of amikacin base. The pediatric dosage form of the same medicine was administrated to the patients.

The content of a vial was dissolved in mobile phase and diluted to 100.0 ml with the same solvent. Reference solutions in mobile phase were prepared at concentrations of 0.06 μg/ml, 0.08 μg/ml,

0.1 μg/ml, 0.2 μg/ml, 0.5 μg/ml, 1 μg/ml, 2 μg/ml, 3 μg/ml and 4 μg/ml (expressed as amikacin base).

From June 2005 until May 2007 CSF samples were prospectively collected from neonates in whom amikacin had been administered before a diagnostic lumbar puncture was performed. Samples were kept in the freezer till analysis was performed. Blank samples from three neonates were also available.

A volume of 250 μl of CSF sample was pipetted into a 1.5 ml Eppendorf vial and centrifuged 3 min at a speed of 10,000 rpm using centrifuge mini Spin plus (Eppendorf, Hamburg, Germany). The supernatant was transferred into a sample vial containing a special 250 μl insert and 100 μl was directly injected.

3. Results and discussion

3.1. Method development

The method described by Zawilla et al. was used as starting point for further method development [15]. It included the use of a two-step gradient LC system using mobile phases composed of an aqueous solution containing sodium 1-octanesulphonate, tetrahydrofuran, phosphate buffer pH 3.0 and sodium sulphate. The gradient was performed by increasing the amount of sodium sulphate, which has been used in ion-pairing mobile phases to shorten retention times as the sulphate anions are more hydrophilic than the anions of the ion-pairing agent [23]. A reversed-phase C_{18} column was used and maintained at 40 °C.

Intention of this study was to optimize this method in order to analyse amikacin in CSF samples. Most important are sensitivity and absence of interference from the matrix. To check the latter, blank samples were available.

The following reversed-phase C_{18} columns were investigated: Hypersil BDS (Thermoquest, Runcorn, England, 100 mm × 4.6 mm, 3 μm), Symmetry (Waters, Milford, MA, Ireland, 150 mm × 4.6 mm, 5 μm) and Hypurity Elite (Thermoquest, Runcorn, England, 150 mm × 4.6 mm, 5 μm). The best condition for further analysis was achieved with the Hypurity Elite column. Since biological samples were injected directly, the use of a guard column during analysis was necessary. As guard column, a Gemini C18 (Phenomenex, Torrance, USA, 4 mm × 2 mm) was used.

Since only one compound had to be determined, an isocratic LC system was preferred for simplicity. Thus, the composition of the mobile phase previously described [15] was adapted in order to obtain good chromatographic conditions for the determination of amikacin in CSF. The main concern was to provide conditions which would assure absence of interference from the blank sample. Different amounts of sodium sulphate in the mobile phase were tested and no interference of the blank sample was achieved when 20 g/l was used. Amounts of other components in the mobile phase were also slightly adapted, 1.5 g/l of sodium 1-octanesulphonate as ion-pairing agent and 15 ml/l of THF as organic modifier were used in order to decrease as much as possible the retention time and to improve the sensitivity of the method.

The pH of the buffer is not very critical, but an acidic environment is necessary so that the analyte molecules are positively charged and capable to interact with the negatively charged ion-pairing agent. So, 50 ml/l of phosphate buffer pH 3 was used, similar as in the already published method. During analysis it was noticed that the amikacin peak obtained after injecting a blank sample spiked with amikacin was eluted faster than the amikacin peak from the reference solution dissolved in mobile phase. This indicated that the necessary acidic environment for the analysis of amikacin in the CSF sample was not achieved. Knowing that the normal pH value for CSF could change after removal from the body [24], the pH of the samples was experimentally checked and found to be around 9. Increasing the buffer concentration in the mobile phase to 250 ml/l solved the problem and the amikacin peak in the CSF sample was now eluted at the same retention time as the reference.

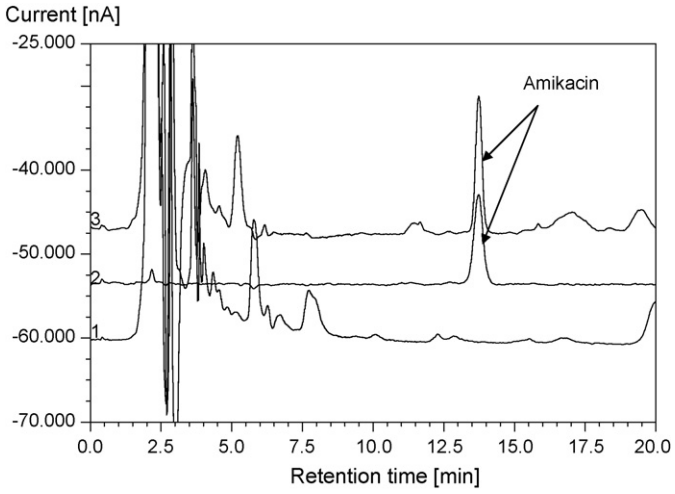

Fig. 2. Chromatograms obtained with: (1) CSF blank sample, (2) 1 μg/ml amikacin base reference solution and (3) CSF sample.

The final chromatographic conditions are shown in Table 1.

3.2. Quantitative aspects

3.2.1. Limit of quantification

A signal-to-noise ratio (S/N) of 10 is generally accepted for estimating the limit of quantification (LOQ), which is the lowest concentration of a substance that can be quantified with acceptable precision. The LOQ value for amikacin base at a S/N = 10 was found to be 0.06 μg/ml (100 μl injected), which is slightly better than other, already described methods.

3.2.2. Linearity and repeatability

The linearity of amikacin base was examined in the concentration range from 0.06 μg/ml to 4.0 μg/ml. Good linearity was obtained with a correlation coefficient greater than 0.99.

The repeatability was checked by analyzing a 1.0 μg/ml amikacin base reference solution three times. The precision (RSD) on the peak area was 0.9%.

Data are shown in Table 2.

Table 2
Linearity and repeatability data

	Repeatability (n = 3) RSD (%)	Linearity			
		Range x (μg/ml) (n = 3)	R^2	y	$S_{y,x}$
Amikacin base reference solution (1.0 μg/ml)	0.9	0.06–4.00	0.99	3575x+303	447

RSD: relative standard deviation, range: concentration range studied, n: number of injections per concentration, R^2: coefficient of determination, y: peak area, x: concentration (μg/ml), $S_{y,x}$: standard error of estimate.

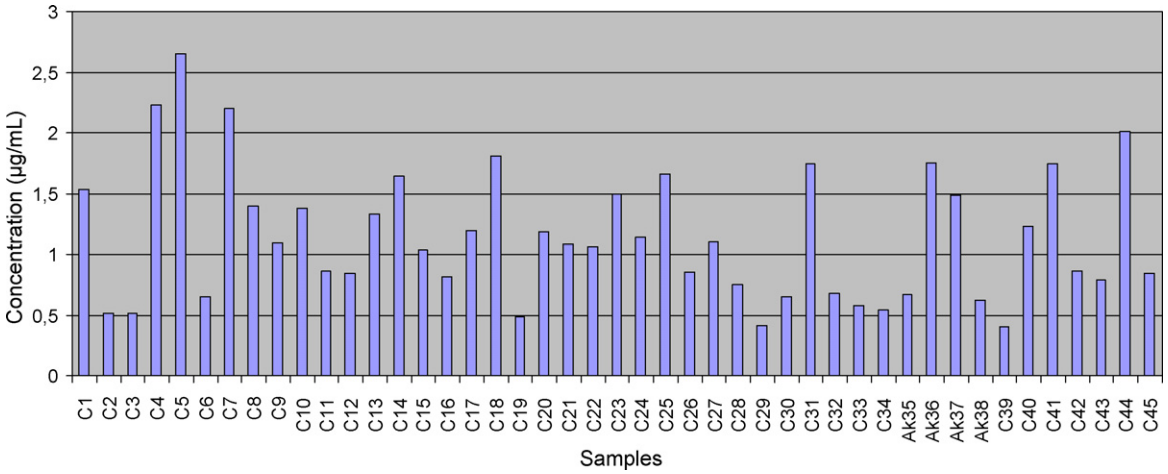

Fig. 3. Concentrations of amikacin in cerebrospinal fluid samples.

3.2.3. Recovery

Two hundred microlitres of a drug-free CSF sample were pipetted into a 1.5 ml Eppendorf vials and 50 μl of 1.0 μg/ml or 10.0 μg/ml amikacin base reference solutions were added to obtain final concentrations of 0.2 μg/ml and 2.0 μg/ml, respectively. Samples were centrifuged 3 min at the speed of 10,000 rpm, supernatant was taken and directly injected. Reference solutions were prepared in the same way using mobile phase instead of blank CSF samples. The recovery was found to be 96.6% and 99.8% respectively.

3.3. Application to cerebrospinal fluid samples

3.3.1. Blank sample

Comparing the chromatograms obtained from the blank and reference solution, it was shown that there was no interference from the blank sample (Fig. 2). The three blank samples were investigated and no interference was found. Since no blank samples of all neonates were available, it was assumed that all samples are giving the same matrix peaks.

3.3.2. Concentrations of amikacin in cerebrospinal fluid samples

CSF samples for amikacin assay were taken by a lumbar puncture. Samples were kept in the freezer till analysis was performed. Altogether, 45 samples were analyzed.

Concentrations were calculated using a 1.0 μg/ml amikacin base reference solution. The median amikacin concentration in the CSF was found to be 1.08 (0.40–2.65) μg/ml. Concentrations obtained are shown in Fig. 3.

Based on the differences obtained in amikacin concentrations and in combination with plasma concentrations collected for therapeutic drug monitoring in the same patients, it was documented that the permeability of the blood–brain barrier for amikacin in neonates displayed major interindividual variability. Two distinct groups of neonates were found. The one with higher amikacin concentrations indicated rapid movement of drug across the blood–brain barrier which suggested higher permeability of the barrier, while the second group, with lower amikacin concentrations, indicated limited permeability. The white blood cells (WBC) count (>21/mm^3) of the CSF, reflecting inflammation, was the best indicator of increased amikacin permeability.

4. Conclusion

A rapid and highly sensitive LC method with pulsed electrochemical detection was developed for the direct quantification of amikacin in cerebrospinal fluid. Detection is based on the measurement of the current resulting from the oxidation reaction of the amikacin molecules at the gold electrode. The limit of quantification obtained was 0.06 μg/ml and linearity was established over the concentration range 0.06–4.00 μg/ml with a correlation coefficient greater than 0.99.

Comparing with available immunoassay methods and alternative chromatographic determinations of amikacin in biological fluids, this method is a suitable choice for therapeutic drug monitoring and clinical and pharmacokinetic research on amikacin.

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