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Determination of amikacin in body fluid by high-performance liquid-chromatography with chemiluminescence detection

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Abstract

A simple and sensitive method was developed for the quantification of amikacin in human plasma and urine samples. The method involves centrifugation of body fluid plasma after dilution with an ethanol/sodium carbonate mixture, and then an aliquot of the supernatant is directly injected into the chromatograph. After separation on a reversed-phase C18 column (runtime 20 min), aminoglycoside is detected on the basis of its complex formation reaction with Cu(II), the catalyst of the luminol/hydrogen peroxide chemiluminescence system. Using a volume of 500 μl biological sample, linearity is established over the concentration range 0.15–2.0 μg/ml and the limit of detection (LOD) is ca. 50 μg/l in plasma or urine. The intra-day and inter-day precision (measured by relative standard deviation, R.S.D.%) are always less than 9%, and relative recoveries are found to be over 92%.

Keywords: Amikacin; Chemiluminescence; Reversed-phase chromatography; Human plasma and urine samples

1. Introduction

Amikacin is a semisynthetic, water soluble, broad spectrum aminoglycoside antibiotic. It is commonly administered parenterally for the treatment of Gram-negative infections resistant to gentamicin, kanamycin or tobramycin because the amikacin molecule has fewer points susceptible to enzymatic attack than most other aminoglycosides [1]. Since clinical chemotherapy with aminocyclitol antibiotics is frequently associated with oto- and nephrotoxicity, careful monitoring of blood levels is required especially when therapy is of long duration. To assure therapeutic serum concentration and to minimize these toxicities, frequent and careful monitoring of amikacin levels in blood or urine is essential. Therefore, it is necessary to develop a simple, efficient, and sensitive method for the determination of amikacin in both biological fluids.

Microbiological assays have been used traditionally to determine amikacin and other aminoglycosides in biological fluids; however, these methods determine the total antibiotic activity in a sample, that is, neither identifying nor quantitating specific aminoglycoside while also being tedious and time-consuming [2]. On the other hand, high-performance liquid-chromatography (HPLC) appears to be the prevailing technique for the determination of amikacin in various matrices [2–4]. However, the HPLC of amikacin is not straightforward because the absence of chromogenic or fluorogenic groups in its molecule necessitates the use of different derivatization reactions to provide adequate detection. Thus, pre- and post-column derivatization with *o*-phthalaldehyde (OPA) [5–7] and pre-column derivatization with 1-fluoro-2,4-dinitrobenzene [8], 2,4,6-trinitrobenzenesulfonic acid [9], 1-naphthoyl chloride [10], 1-naphthyl isothiocyanate [11], 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate [12], etc., have also been described. However, these techniques are time-consuming and give problems with quantitation. It has been reported that pre-column derivatization with OPA or 1-fluoro-2,4-dinitrobenzene resulted in unstable derivatives [6,8]. To overcome the derivatization step, direct HPLC methods based on tandem-mass spectrometric (MS–MS) [13], pulsed electrochemical (PED) [14], evaporative light scattering (ELSD) [15] and indirect fluorimetric detection [16] have been proposed.

This work reports a rapid, straightforward method for the direct trace analysis of amikacin in human plasma and urine samples with a liquid chromatograph equipped with a chemi-

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luminescence (CL) detector, which avoids the use of derivatization and provides LOD at the microgram-per-litre level. It is based on the inhibitory effect of the aminoglycoside on the CL reaction between luminol and hydrogen peroxide catalysed by Cu(II). We have previously used this CL detector for the multiresidue analysis of aminoglycoside antibiotics in environmental waters following strong cation-exchange chromatographic separation [17]. In this work, the chromatographic separation is carried out using a classical reversed-phase C18 column, which allows the selective determination of amikacin in the presence of other aminoglycoside antibiotics. The special features of the CL detector provide lower LOD for amikacin than do existing chromatographic alternatives, which allow the determination of this aminoglycoside antibiotic in human plasma and urine in and below its therapeutic level without the use of derivatization.

2. Experimental

2.1. Chemicals

All chemicals employed were of analytical-reagent grade and milli-Q water was used throughout. Amikacin sulfate (≥99% purity) was purchased from Fluka (Sigma–Aldrich Química, Madrid, Spain). A standard solution (100 μg/ml) was prepared in milli-Q water and stored at 4 °C in a refrigerator. The reagent/catalyst solution was made by mixing the following chemicals at a final concentration of 6.0×10^{-4} mol/l luminol (Aldrich, Sigma–Aldrich Química), 1.0 μg/ml Cu(II) (Aldrich), 8×10^{-2} mol/l sodium hydroxide (Merck), 1.0 mol/l sodium chloride (Merck) and adjusting the pH at 12.4. The oxidant/micellar solution was prepared by mixing concentrated hydrogen peroxide (Merck) and 0.25 mol/l Triton X-100 (polyoxyethylene octyl phenyl ether, Fluka) solutions, and making up to 100 ml with milli-Q water so that the final concentrations were 7.0×10^{-3} and 2.5×10^{-2} mol/l for hydrogen peroxide and surfactant, respectively.

2.2. HPLC analysis

The HPLC system consisted of a Phenomenex C_{18} Synergi RP 80A 250 mm × 4.6 mm (4 μm) column (Phenomenex, Torrance, CA, USA), a Waters W-600E multisolvent pump and a Rheodyne Model 7161 injector (Cotati, CA, USA) fitted with a 20-μl injection loop. The mobile phase was 10^{-2} mol/l potassium hydrogen phthalate at pH 3.35, adjusted with diluted sodium hydroxide, and acetonitrile (90:10, v/v), which was filtered and degassed on a 0.45 μm nylon membrane filter before use. A flow-rate of 1.0 ml/min provided a retention time for amikacin of 9.5 min, whereas the runtime was ca. 20 min for the chromatographic analysis of urine or plasma samples (see Section 3.4). The analytical signal was monitored with a post-column CL detector [17] consisting of a Gilson Minipuls-3 peristaltic pump (Middleton, WI, USA) used to deliver the reagent/catalyst (0.7 ml/min) and the oxidant/micellar (0.5 ml/min) solutions, which were mixed with the mobile phase via mixing tees, and a Jasco luminescence detector Model CL-2027 (Jasco Corporation, Tokyo, Japan) with a flow cell made from a PTFE tube.

2.3. Preparation of plasma and urine samples

A volume of 500 μl of plasma or urine sample was mixed to 2.5 ml of ethanol and 2.0 ml of 10^{-2} mol/l sodium carbonate (pH 11). After vortex mixing, the sample was centrifuged at 2500 rpm for 15 min, and then the supernatant passed through a 0.45 μm nylon membrane syringe filter (e.d. 2.5 cm) prior injection of a 20-μl aliquot into the HPLC–CL system.

3. Results and discussion

3.1. CL detection system

Although the ability of aminoglycosides to form stable complexes with the Cu(II) ion [18–20] is well-known, this reaction has scarcely ever been used for analytical purposes and especially as the basis for designing a detection system in HPLC. Thus, to our knowledge, only two detection approaches have been reported. One is based on the displacement reaction between aminoglycosides and the Cu(II)–L-tryptophan complex, in which the resulting increase in L-tryptophan fluorescence is indicative of the presence of the aminoglycoside [16], and the other is the CL detection system used in this study, which is supported on the amikacin inhibition of the CL emission generated from the oxidation of luminol in alkaline medium by hydrogen peroxide catalyzed by Cu(II).

The optimisation of the CL detection approach used in this work is not necessary because it has been reported elsewhere [17]. In this context, it is important to point out that the inhibitory effect of amikacin on the luminol/hydrogen peroxide/Cu(II) system has formerly been used by Bosque-Sendra and co-workers [21] for the determination of this antibiotic in pharmaceutical preparations by using a flow injection (FI) system; however, significant differences in sensitivity are found between the two methods. In fact, the FI method provides a linear range of 9.89–20 mg/l with a LOD of 2.97 mg/l, whereas the method reported herein (see below) is linear over the range 15–150 μg/l with a LOD of 5 μg/l, in both cases for amikacin aqueous standard solutions. This behaviour can be basically ascribed to the use of higher concentrations of luminol as well as the enhanced effect of the micellar medium (Triton X-100) and the ionic strength (sodium chloride) used in the present paper on the formation of the amikacin–Cu(II) complex.

3.2. Conditions of chromatography

The selection of mobile phase components was a critical factor in achieving good chromatographic peak shape and CL efficiency. A solvent system of potassium hydrogen phthalate and acetonitrile (90:10, v/v) was selected as a mobile phase for its good sensitivity. As can be seen in Fig. 1, the pH and the concentration of the buffer show a noteworthy effect on the peak height and the retention time for amikacin. A 10^{-2} mol/l potassium hydrogen phthalate (pH 3.35) was selected as opti-

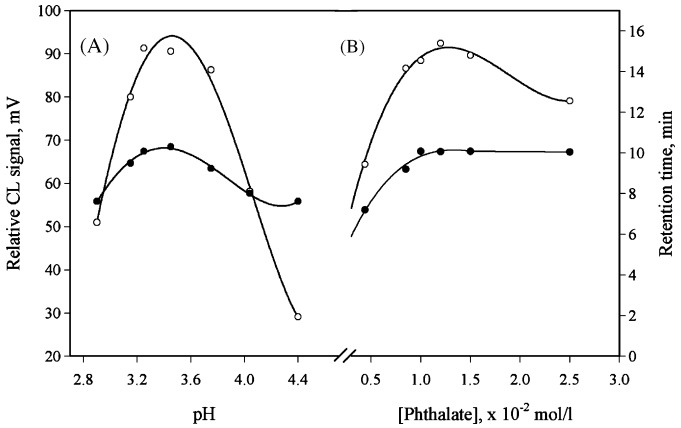

Fig. 1. Effect of the (A) pH and (B) concentration of the buffer of the mobile phase on the peak height (◯) and retention time (●) provided by 350 μg/l of amikacin. All other conditions as in Section 2.

mum as a compromise between the two variables. Although with this mobile phase amikacin can be assumed to form an ion-pair with the phthalate acid ion (the pK_a values for phthalic acid are 2.95 and 5.41), it does not offer sufficient hydrophobicity to be retained on the C_{18} column because only amikacin is retained and other aminoglycosides are not retained and eluted with the mobile phase. The singular chromatographic behaviour of the amikacin can be ascribed to the presence of the aliphatic chain, which provides the additional hydrophobicity to the corresponding ion-pair to be separated by reversed-phase chromatography.

3.3. Analytical calibration and precision

The calibration graph was constructed using least-squares regression of amounts of antibiotic standard in milli-Q water versus peak height under selected experimental conditions (see Section 2). Table 1 gives the least-squares parameters of the working curve, its LOD, calculated as the concentrations of amikacin providing chromatographic signals equal to three times peak-to-peak noise.

The precision was calculated for three concentration levels: 25, 50 and 100 μg/l of amikacin. Precision was measured using six antibiotic standards in milli-Q water for each concentration level and each day. As can be seen in Table 2, the evaluation of method precision was carried out in a day (intra-day precision) and in 3 different days (inter-day precision) and evaluated by means of the relative standard deviation (R.S.D.). In summary, the proposed method allows the determination of amikacin at levels that can permit its drug monitoring in clinical studies with quite good precision.

Table 1
Characteristic parameters of the calibration graphs for the determination of amikacin in biological fluids

Sample	Linear range[a] (μg/l)	Regression equation[b]	r	LOD (μg/l)
Milli-Q water	15–150	$H = 0.6 + 2.68 \times 10^{-1} C$	0.9987	5
Urine	150–2000	$H = -0.8 + 2.51 \times 10^{-2} C$	0.9977	50
Plasma	150–2000	$H = -1.5 + 2.46 \times 10^{-2} C$	0.9954	55

[a] Urine and plasma samples diluted 10 times.
[b] H, peak height (in mV); C, analyte concentration (in μg/l).

Table 2
Precision obtained for three different amikacin concentration levels ($n = 6$)

Sample	Concentration level					
	250 μg/l		500 μg/l		1000 μg/l	
	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day
Milli-Q water[a]	5.3	6.2	3.8	4.5	3.7	4.3
Urine[b]	6.2	8.3	4.5	5.7	4.1	5.3
Plasma[b]	6.4	8.9	5.2	7.1	5.3	6.9

[a] For comparison, the concentration levels of amikacin in milli-Q water were 25, 50 and 100 μg/l, because the urine and plasma samples were diluted 10 times.
[b] Samples spiked at these three concentrations were analysed on each day of the 3-day validation ($n = 6$ at each concentration).

3.4. Determination of amikacin in biological fluid samples

Several assays were carried out to evaluate the capability of the present HPLC–CL method for the determination of amikacin contents in urine and plasma samples. Initially, spiked urine samples were directly analysed under different dilution factors and only the 1:100 dilution afforded accuracy results (recoveries higher than 90%). This option provides a limit of quantification (LOQ) for amikacin (ca. 2.0 μg/ml) that allows its therapeutic monitoring in urine samples. In fact, amikacin is eliminated in unchanged form by glomerular filtration, and about 40–90% of an administered dose (0.5 g usually) appears in the urine within 24 h. So, if amikacin was injected intramuscularly, the amount that was excreted in urine was far above the present LOQ [22,23]. Nevertheless, additional experiments were carried out to increase the sensitivity of the proposed method by using smaller dilution factors per urine sample, which requires an additional sample treatment. Thus, accuracy results can be achieved when the urine sample is diluted 10 times with a mixture of ethanol and 10^{-2} mol/l sodium carbonate at pH 11 as stated in Section 2.3. As can be seen in Table 1 and Fig. 2B, no matrix effect was observed in the determination of amikacin in urine samples under these experimental conditions: the recovery, measured as the sensitivity ratio in urine and milli-Q water, was ca. 93.7%. In addition, the method showed a good linearity and intra-day and inter-day precision (see Table 2) with a LOD at microgram-per-litre level.

The method proposed for urine samples (1:10-fold dilution with a mixture of ethanol and 10^{-2} mol/l sodium carbonate at pH 11) also provided accuracy and precise results for the analysis of plasma samples (see Tables 1 and 2 and Fig. 2C). In this case, the recovery was about 92%, and, as in the urine samples, the method was highly sensitive and reliable for the determination of amikacin in this biological fluid.

At this point, it may be interesting to compare the proposed chromatographic method with existing alternatives for the chromatographic determination of amikacin in human urine and plasma samples. The following conclusions can be drawn. First, reversed-phase ion-pairing HPLC has been widely used for this

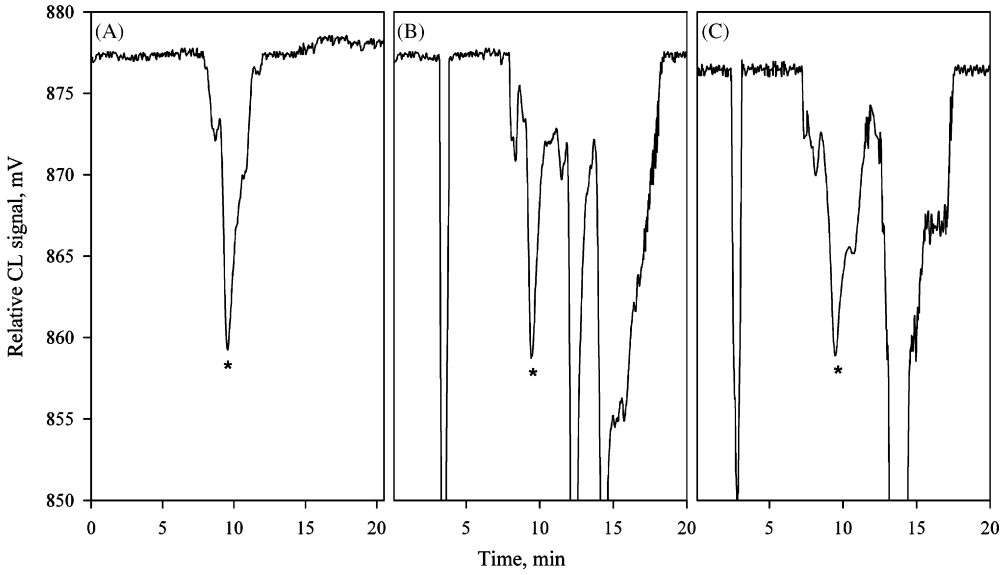

Fig. 2. Representative chromatograms of (A) standard solution of 50 μg amikacin/l; (B) urine and (C) plasma samples spiked with 500 μg/l of aminoglycoside. *Amikacin. Other conditions as described in Section 2.

purpose with UV [8,24] and fluorescence [25] detection, the latter by using post-column derivatization with OPA. As expected, the OPA method provides higher sensitivity with a LOD of 125 μg/l. Second, poorer LODs are obtained by using reversed-phase HPLC with UV detection [11], in which amikacin was pre-column derivatized with 1-naphthyl isothiocyanate, capillary electrophoresis with amperometric detection [26,27] and micellar electrokinetic chromatography with fluorescence detection after derivatization of amikacin with 1-methoxycarbonyl-indolizine-3,5-dicarbaldehyde [28]. In all cases, LODs ranged from 0.5 to 0.7 μg/ml. Finally, the third conclusion is that recently hydrophilic interaction chromatography combined with tandem-mass spectrometry has been used for the determination of this aminoglycoside in biological samples with LOQ at 100 μg/l [29]. From these results, it can be seen that the HPLC–CL method described compares favourably in terms of sensitivity and cost with existing chromatographic alternatives and is a suitable choice for therapeutic drug monitoring and for clinical and pharmacokinetic research on amikacin.

4. Conclusions

A rapid and sensitive method was developed for the direct determination of amikacin in human urine and plasma. This HPLC method used the inhibitory effect of aminoglycoside on the CL signal provided by the luminol–hydrogen peroxide system catalysed by Cu(II). This detection system avoided limitations of those approaches involving derivatization, such as time-consuming and labor-intensive sample preparation and the instability of the derivatives. The method is a powerful and robust alternative for quantification of amikacin with an excellent LOD, accuracy and precision and is capable of detecting this aminoglycoside in urine and plasma samples at levels as low as microgram-per-litre.

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