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Short communication

Assay of amikacin in the skin by high-performance liquid chromatography

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Abstract

Amikacin is used in the systemic treatment of serious infections, but also locally for the treatment of skin infections. The aim of this work was to develop and validate a simple procedure for amikacin determination inside the epidermal tissue: this implies a simple method for an efficient drug extraction from the skin and a clean and easy HPLC analysis. Amikacin was extracted from epidermis samples with 500 μl of a mixture methanol–water–0.05 M NaOH (5:5:2 v/v/v) at 60 °C for 1 h. After filtration, the obtained solution was derivatized (1-fluoro-2,4-dinitrobenzene at 90 °C for 10 min) and analyzed by HPLC, on a C18 μBondapack 300 mm × 4.6 mm column thermostatted at 45 °C. The mobile phase was a mixture of acetonitrile–water–acetic acid (47:53:0.1 v/v/v) at a flow rate of 1.5 ml/min and the UV detector was set at 365 nm. The derivatization and HPLC analysis were validated in the concentration interval 1.64–49.21 μg/ml. The linearity resulted very good ($R = 0.9995$); the R.S.D.% varied between 0.20% and 3.89% depending on the concentration and the ER% was included between 5.4 and 0.9. The extraction method used demonstrated to be specific and the recovery resulted about 93%. The extraction, derivatization and HPLC assay has good reproducibility, sensitivity and specificity resulting in a reliable method for biopharmaceutical studies of AK distribution in the epidermis.

Keywords: Amikacin; Skin extraction; Derivatization; Reversed-phase chromatography

1. Introduction

Amikacin (AK) a semi synthetic analogue of kanamycin, is an aminoglycosidic antibiotic active against most of gram-negative bacteria including gentamycin- and tobramycin-resistant strains. Amikacin is typically used for the treatment of serious general infections, but also for the treatment of skin infections [1] and more recently of keratitis [2,3]. Amikacin is administered parenterally also in the treatment of generalized skin infections. When the affected area is limited, or when poor circulation creates uncertainty as to whether an efficacious dose would be provided by systemic administration [4], local application of AK can be advisable, because it reduces the systemic side effects. Bacterial skin infections can be located in the viable epidermis, the stratum corneum or the hair follicle (e.g. Pyodermas, impetigo, folliculitis, erythrasma). Published studies [1] have demonstrated that topical amikacin is therapeutically effective, suggesting that it accumulates in the site of action in therapeutic concentrations. Nevertheless no literature data are available on the quantification of amikacin or other aminoglycosidic antibiotics in the epidermis.

The aim of this work was to develop and validate a simple procedure for amikacin determination inside the epidermal tissue: this implies a simple method for an efficient drug extraction from the skin and a clean and easy HPLC analysis. The applicability of the procedure was checked by studying the epidermal accumulation of AK after in vitro permeation experiments.

At present, amikacin analytical determination in biological samples is made using microbiological, immunological (RIA, radiochemical, EIA) and chromatographic methods [5]. Due to lack of volatility and chromophore of aminoglycosides, most chromatographic methods use derivatization to produce volatile, UV/vis absorbing [6] or fluorescent derivatives [7]; a cleanup procedure is often required prior to or after derivatization to eliminate interfering products. Pre-column derivatization is usually preferred, over post-column, because it does not require special equipment [8].

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2. Materials and methods

2.1. Materials

Amikacin bisulphate (AKSS) (chemically: D-Streptamine, *O*-3-amino-3-deoxy-alpha-D-glucopyranosyl-(1-6)-*O*-(6-amino-6-deoxy-alpha-D-glucopyranosyl-(1-4))-*N*1-(4-amino-2-hydroxy-1-oxobutyl)-2-deoxy-, (*S*)-sulfate (1:2) MW = 781.8) (USP 29, Eur Pharm 5) was a gift from Lisapharma (Erba, Como, Italy). The derivatizing agent 1-fluoro-2,4-dinitrobenzene (FDNB) was purchased from Sigma (St. Louis, USA). For HPLC analysis, acetonitrile (HPLC grade) and distilled water were used. All other chemicals used were of analytical grade.

2.1.1. Derivatization and HPLC conditions

Amikacin was derivatized by mixing 100 μl of aqueous solution of the drug with 300 μl of methanol, 40 μl of NaOH 0.05 M and 50 μl of a methanolic solution of the derivatizing agent (FDNB) 180 mg/ml (modified from Refs. [6,9]). The presence of a high proportion of methanol is necessary to maintain FDNB in solution, while NaOH serves to have un-protoned AK amino groups, thus allowing the derivatization reaction to take place. When AK was dissolved in vehicles different from water, the derivatization volumes were changed according to Table 1 in order to maintain the same solvents proportion and to avoid unnecessary dilution. The obtained mixture was heated at 90 °C in an air-circulating oven for 10 min, then cooled and injected in HPLC. Each solution was separately derivatized prior to injection.

The HPLC apparatus consisted of an isocratic pump (Series 200, Perkin-Elmer instrument, Norwalk, CT, USA) equipped with a μBondapack C_{18} 300 mm × 4.6 mm column (particle size: 10 μm; pore size: 125 Å; endcapped) (Waters, Milford, MA, USA) thermostatted at 45 °C. The mobile phase was a mixture of acetonitrile–water–acetic acid (47:53:0.1 v/v/v) pumped at 1.5 ml/min. An autosampler (Prostar 410, Varian, Leinì (To), I) with a loop of 100 μl and a spectrophotometric detector (LC 290, Perkin-Elmer) working at 365 nm were used.

2.1.2. Calibration

A calibration series of aqueous AK bisulphate solutions (Table 2, concentration range expressed as AK free base: 0.16–49.21 μg/ml) was derivatized and analyzed in order to estimate if the derivatization conditions (time, temperature and amount of FDNB added) were sufficient to achieve reproducible results. For each concentration 3–9 different samples were derivatized and analyzed individually. The linearity of the calibration curve was tested and the correlation coefficient (*r*) was calculated. Accuracy was determined through the relative error (ER% = (fitted value − nominal value)/nominal value), and the precision of the method, expressed as the relative standard deviation (R.S.D.% = 100 S.D./mean), was also evaluated. The limit of quantification (LOQ) was calculated as the concentration corresponding to a signal-to-noise ratio of 10, while the limit of detection (LOD) to a signal-to-noise ratio of 3. The LOQ was further confirmed by analyzing an AK solution having the computed concentration. Number of theoretical plates and tailing factor were also calculated according to USP 27 and EP 4 (suppl. 4.6).

Table 2
Nominal value, fitted value ($y = 63{,}737x - 35{,}784$), R.S.D.% and ER% calculated for each concentration tested

Nominal AK concentration (μg/ml)	Fitted value	S.D.	*n*	R.S.D.%	ER%
1.64	1.60	0.02	6	2.2	2.70
4.10	3.99	0.07	3	2.15	2.68
8.20	7.76	0.28	6	3.89	5.41
16.40	16.23	0.06	8	0.4	1.04
32.81	32.74	0.42	9	1.31	1.11
49.21	49.65	0.10	3	0.2	0.90

2.1.3. Drug extraction from skin samples

At the end of each permeation experiment, the skin was washed and a disc of tissue was cut, fitting the area covered by donor compartment (0.6 cm^2 area).

Then, the skin sample was heated with a hair dryer for about 20 s and the epidermis was separated from dermis. The two skin layers were placed in individual plastic test tubes, weighted and then AK was extracted following different experimental conditions (volume and composition of the extracting solution and temperature). The conditions tested are reported in Table 3. After extraction, the supernatant was filtered through a 0.45 μm filter, derivatized (see Table 1) and analyzed by HPLC.

2.1.4. Validation of AK extraction

For the validation of AK extraction, epidermis and dermis samples (which had not previously been in contact with the drug) were separated and used in specificity and recovery determination. Some of the blank skin tissues were submitted to the extraction procedure and the retention time of extracted, and derivatized, endogenous compounds was compared with that of AK. For recovery determination, a known amount of AK solution (5 μl of AK 150 μg/ml) was added to blank epidermis and dermis specimens. After 1 h of contact, the tissues were

Table 1
Details on derivatization volumes

AK vehicle	Volume derivatized (μl)	MeOH added (μl)	NaOH 0.05 M added (μl)	FDNB[a] added (μl)
H_2O	100	300	40	50
MeOH:H_2O (50:50)	200	200	40	50
MeOH:H_2O:NaOH 0.05 M (5:5:2)	240	200	–	50

[a] 80 mg/ml solution in MeOH.

Table 3
Extraction conditions and recovery of amikacin from rabbit epidermis and dermis (extraction time: 1 h, mean ± S.E.M.)

Extraction solvent	Volume (μl)	Temperature (°C)	*n*	Recovery %
Epidermis				
MeOH:H_2O (50:50)	250	45	4	77.6 ± 28.3
MeOH:H_2O (50:50)	500	45	3	66.4 ± 14.6
MeOH:H_2O:NaOH 0.05M (5:5:2)	500	45	3	67.3 ± 7.2
MeOH:H_2O:NaOH 0.05M (5:5:2)	500	60	3	92.9 ± 1.1
Dermis				
MeOH:H_2O (50:50)	250	45	4	15.5 ± 9.6
MeOH:H_2O (50:50)	500	45	3	26.6 ± 24.9
MeOH:H_2O:NaOH 0.05 M (5:5:2)	500	45	3	53.9 ± 16.3
MeOH:H_2O:NaOH 0.05 M (5:5:2)	500	60	3	nd

nd: not determined because of precipitation during heating.

submitted to the above-described assay of extraction, derivatization and analysis. The recovery was determined by computing the ratio of the amount of AK extracted from spiked skin to the amount of AK added (determined by direct injection of spiked solutions in the absence of skin). By modifying the volume of extraction, pH and temperature, a recovery of 93% was obtained for epidermis. In this case, 500 μl of the mixture MeOH–H_2O–0.05 M NaOH (5:5:2 v/v/v) was added to epidermis. The test tube was heated in a water bath at 60 °C for 1 h. After filtration, 240 μl of extraction solution was added to 200 μl of methanol and 50 μl of FDNB solution. The derivatization was performed as before.

2.1.5. Application of the method to permeation experiments

Permeation experiments were conducted in vertical Franz-type diffusion cells (Disa, Milan, I), using rabbit ear skin as barrier. The donor compartment was filled with 1 ml of AK 50 mg/ml aqueous solution (as bisulphate) at pH 7.4 or pH 4.0. The receptor phase was 0.9% sodium chloride solution, thermostatted at 37 °C and magnetically stirred. At the end of the experiment (2 h) the cells were dismantled, AK was extracted from the skin as previously described and the receptor solution was sampled. The solutions were derivatized and analyzed by HPLC for the determination of AK permeated and accumulated. Blank permeation experiments (where the donor was represented by 1 ml of saline solution) were performed in order to check the possible presence of interfering compounds. Each permeation experiment was replicated at least three times.

3. Results and discussion

In this paper, a specific and simple method of extraction and HPLC-UV/vis analysis for AK is described. This method enabled us to determine amikacin in samples of receptor solution and of skin, obtained from in vitro permeation experiments. The method used for this purpose needs to be highly specific, since this kind of samples usually contain endogenous compounds released from the skin. Aminoglycosides can be determined by HPLC with UV/vis detection after derivatization. We have adapted the methods of Barends [6] and Wong [9], in which the reaction between all four primary amino groups of AK and FDNB produces a stable UV/vis absorbing 2,4-dinitrophenyl derivative. The adapted method resulted considerably simpler and quicker, probably because of the different biological matrix and HPLC column used. In fact, the method of Barends [6] required an extensive pre-derivatization treatment of the sample and the one of Wong [9] necessitated an extensive post-derivatization cleanup to eliminate interfering substances and the excess of derivatizing agent.

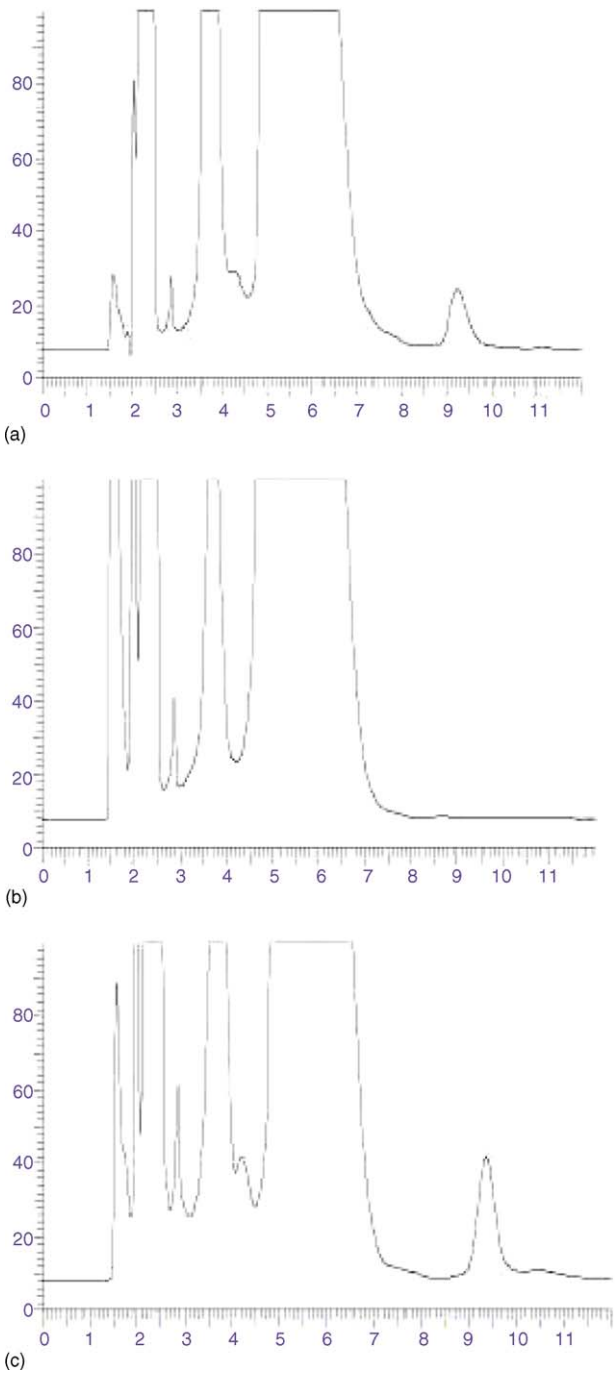

Fig. 1. Typical chromatograms of: AK standard solution (concentration: 5 μg/ml) (panel a), extraction solution of "blank" epidermis (panel b) and extraction solution of epidermis after a permeation experiment (panel c).

Table 4
AK accumulated and permeated through rabbit ear skin after 2 h of application of a 50 mg/ml AKSS solution (average ± S.E.M.)

Donor solution pH	*n*	AK permeated (μg/cm^2)	AK accumulated in the epidermis (μg/mg)
4.0	3	0.77 ± 0.03	0.93 ± 0.09
7.4	4	9.06 ± 3.18	2.01 ± 0.38

3.1. Derivatization and HPLC analysis

The chromatogram of a standard solution of amikacin 5 μg/ml, derivatized in the conditions described in Table 1, is illustrated in Fig. 1 (panel a). The retention time of AK is about 9.5 min. The limit of quantification resulted 1.64 μg/ml and the limit of detection was 0.63 μg/ml (expressed as free base). The efficiency of the column, expressed as the number of theoretical plates, was about 1150. The tailing factor resulted very close to 1 and the linearity resulted good ($R = 0.9994$). The R.S.D.% varied between 0.20 and 3.89 depending on the concentration. Nominal value, fitted value ($y = 63{,}737x - 35{,}784$), R.S.D.% and ER% are illustrated in Table 2 for each concentration. The inter-day variability was also checked on an AK solution 4 μg/ml and resulted 4.3%. The good analytical results obtained demonstrate also that the derivatization procedure is reproducible and overall the analytical method produces reliable results.

3.2. Validation of drug extraction from skin samples

The recovery of AK from the skin was tested in different experimental conditions, the results being illustrated in Table 3. By modifying the volume of extraction, pH and temperature, a satisfactory recovery of 93% was obtained for epidermis. When blank epidermis samples were derivatized and analyzed, no peaks with the same retention time as AK were found (Fig. 1, panel b), indicating the specificity of the extraction and analysis procedure.

In the conditions tested, the recovered from dermis was always very variable and reached a maximum value of 54% (see Table 3). The extraction temperature could not be further increased, because a precipitate formed during the derivatization step. No further attempts were made to improve the extraction from dermis, because dermis concentration is not predictable of what takes place in vivo: blood circulation, responsible for drug clearance from the skin, is located in the upper dermis and then in vivo dermis concentration will be generally lower than in vitro. On the contrary, epidermis concentration of drugs obtained in in vitro permeation experiments is considered a good reference of the in vivo situation [10].

3.2.1. Application of the method to permeation experiments

The extraction and derivatization method was then applied to actual samples. Permeation experiments were carried out at two different pH values (4.0 and 7.4) to evaluate the effect of drug charge on its transport and skin accumulation. The results obtained (Table 4) show a higher accumulation and a higher permeation from the solution at pH 7.4 compared to pH 4.0. This can be explained considering the ionization characteristics of the drug: AK, with four amino groups whose pK_a are in the range between 6.7 and 9.7 [11,12], can cross the skin more easily at pH 7.4 where it is less charged. Finally, Fig. 1 (panel c) reports an illustrative chromatogram of AK extracted from an epidermis sample. These results indicate the ability of the analytical method set up in this work to determine small amounts of AK, without any interference from the biological matrix. The extraction method was validated only on rabbit ear skin, but the same method can probably be adjusted for human skin, by simply changing extraction time and temperature, in analogy with former data on nicotinamide [13].

4. Conclusion

A simple procedure has been set up and validated for the extraction, derivatization and HPLC-UV/vis quantification of amikacin in the epidermis. The procedure has good reproducibility, sensitivity and specificity resulting in a reliable method for biopharmaceutical studies of AK distribution in the epidermis. This can allow for the determination of drug concentration in the target tissue and then for the development of effective topical formulations. The same method was used for the analysis of both AK permeated and AK accumulated, with a simple variation of the solvent proportions in the derivatization step. The procedure was validated only on rabbit ear skin, but the same method can probably be adjusted for human skin, by simply changing the extraction conditions.

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