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Analytical method for the determination of the aminoglycoside gentamicin in hospital wastewater via liquid chromatography–electrospray-tandem mass spectrometry

Dirk Löffler, Thomas A. Ternes*
ESWE-Institute for Water Research and Water Technology, Soehnleinstrasse 158, D-65201 Wiesbaden, Germany

Abstract

A method for the determination of gentamicin residues in hospital wastewater has been developed using kanamycin as a surrogate standard. The method consists of solid-phase extraction (SPE) and detection by ion-pair chromatography with electrospray tandem mass spectrometry (LC–ES-tandem MS). The SPE was performed on a weak cation exchanger. Filtration should be avoided in the sample preparation, otherwise a significant loss of gentamicin occurs. Chromatographic separation on a C_{18}-column was achieved using a ternary eluent containing methanol, water and 20 mmol l^{-1} heptafluorobutyric acid solution. Mean relative recoveries of the analytes in hospital wastewater varied between 107 and 111%. The limit of quantification (LOQ) was 0.20 μg l^{-1} in hospital wastewater. Gentamicin was found in native hospital wastewater in a concentration range between 0.4 and 7.6 μg l^{-1}.

Keywords: Hospital wastewater; Solid-phase extraction; Water analysis; Gentamicin; Antibiotics

1. Introduction

The aminoglycoside antibiotic gentamicin is widely used in hospitals for the treatment of serious infections caused by Gram-negative and Gram-positive bacteria. In general, gentamicin is mostly administered by intramuscular injection because of its low oral absorption. Since it is excreted almost entirely nonmetabolised with urine, it is therefore expected to be present in hospital wastewater. Despite an application quantity of about 1 ton per year for human medical purposes in Germany [1], little is known about its occurrence in wastewater and the aqueous environment. The presence of antibiotics in wastewater and the environment is of particular interest because of the potential formation of antibiotic-resistant strains in pathogenic bacteria. Numerous methods for the determination of aminoglycosides in a variety of biological matrices such as blood, urine, tissue and milk are reported in literature [2–9]. They are based on separations by liquid chromatography (LC), gas chromatography (GC), capillary electrophoresis (CE) and thin-layer chromatography (TLC). Mass spectrometry is the detection method of choice for aminoglycosides, due to the lack of chromophores and fluorophores in the molecule [10,11]. Using other detection systems than mass spectrometry require intensive derivatizations [2,12]. In the last years several analytical methods have been described for the determination of various antibiotics in aqueous matrices such as wastewater and river water [13–20]. However, to date no

*Corresponding author. Tel.: +49-611-780-4343; fax: +49-611-780-4375.
E-mail address: thomas.ternes@eswe.com (T.A. Ternes).

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appropriate analytical method is published allowing for the determination of gentamicin in aqueous environmental samples.

Since gentamicin is mainly used in hospitals, the aim of the current work was to develop an analytical method primarily for its determination in hospital wastewater. The development was based on a method reported by Heller et al. [11] which allows for the determination of gentamicin in milk.

2. Experimental

2.1. Chemicals and materials

Gentamicin was purchased from Sigma (Deisenhofen, Germany), kanamycin sulfate and heptafluorobutyric acid from Fluka (Buchs, Switzerland), acetic acid and methanol (Suprasolv) from Merck (Darmstadt, Germany). Standard solutions were prepared in water–methanol (50:50, v/v) and stored at −20 °C.

Because of the high sorption affinity of the aminoglycosides to polar surfaces, only laboratory equipment made of PTFE and polypropylene were used during the sample preparation. In order to prevent losses by adsorption all contacts with glass should be avoided as much as possible.

2.2. Solid-phase extraction

Aminoglycosides are extremely hydrophilic, due to the high number of amino- and hydroxyl moieties. Therefore, a weak cation exchanger was used for a solid-phase enrichment based on a method reported for the analysis of gentamicin in milk [11]. The water quantity which can be used for the enrichment of the cation exchanger is limited by salinity of the samples. The latter was very high in hospital wastewater in comparison to the used groundwater, with conductivities of 1313 and 83 μS cm^{-1}, respectively. SPE-cartridges (Widepore CBX, 500 mg/6 ml, ANSYS Technologies, Germany) were pre-conditioned using 5 ml of methanol and 5 ml of water successively (M. Carson, personal communication, 2001). Hospital wastewater (20–50 ml) at neutral pH (7–8) was spiked with 1 μg of kanamycin as a surrogate standard. The water samples were then passed through the pre-conditioned SPE cartridges with a flow-rate of 1–2 droplets per minute (approximately 50 ml h^{-1}). The flow-rate decreased significantly during sample enrichment due to the deposition of solid particles in the cartridges. The cartridges were then eluted three times with 1 ml methanol–acetic acid (10:1, v/v) into conical polypropylene vials (Labcon, Heppenheim, Germany). The extracts were reduced to dryness in a gentle nitrogen stream and were filled up to 500 μl with a solution of 5 mmol l^{-1} aqueous HFBA. Sample extracts were stored at −20 °C until measurement.

2.3. HPLC conditions

The HPLC system consisted of a Perkin-Elmer Series Autosampler Series 400 connected to a Perkin-Elmer quaternary pump Series 400. The ion-pair chromatographic separation was achieved using a 50×3-mm Chrompack Omnispher C_{18} (3 μm) stainless steel column and a ternary eluent containing water, methanol and heptaflourobutyric acid solution (20 mmol l^{-1}). Sample aliquots of 50 μl were injected onto the HPLC column. The flow-rate of the eluent was 300 μl min^{-1} and the gradient is shown in Table 1.

2.4. MS–MS parameters

For detection, the tandem MS detector API 365 (PE Sciex) was run in the positive mode with the turbo-electrospray ionisation (ESI). Nitrogen was used as curtain gas with a flow-rate of 1.0 l min^{-1} and synthetic air with a pressure of 250 kPa as a nebulizer gas with an approximate flow-rate of

Table 1
Eluent gradient for HPLC separation

Time (min)	Duration (min)	Water (%)	Methanol (%)	HFBA 20 mmol l^{-1} (%)
0	5	18	42	40
5	1	3	57	40
6	0.1	3	57	40
6.1	3	5	95	0
9.1	0.1	5	90	5
9.2	3	18	42	40
12.2	7.8	18	42	40

Table 2
Retention time, precursor ions and product ions for gentamicin and kanamycin

Substance	Retention time (min)	Precursor ion (m/z) $[M+H]^+$	Product ion (m/z)
Gentamicin C_1 (dimethyl)	16.1	478.0	322.0
Gentamicin C_2 (methyl)	16.1	464.0	322.0
Kanamycin A, C	15.0	485.0	324.0

1.0 l min^{-1}. The ESI interface was heated to 400 °C and the LC effluent was split 1:10 in the interface resulting in an approximate spray flux of 40 ml min^{-1}. Orifice voltages varied from 30 to 44 V depending on the individual analytes. Tandem MS parameters were optimised as follows. After determination of the best conditions for the isolation of the target ion, the quadrupole and lens conditions for the argon induced fragmentation were optimised (precursor scan: 1-Da steps, 4 ms dwell time; product scan: 0.1-Da steps, 2 ms dwell time; multiple reaction monitoring (MRM): dwell time >200 ms depending on number of recorded mass traces). Precursor and product ions of the compounds are given in Table 2.

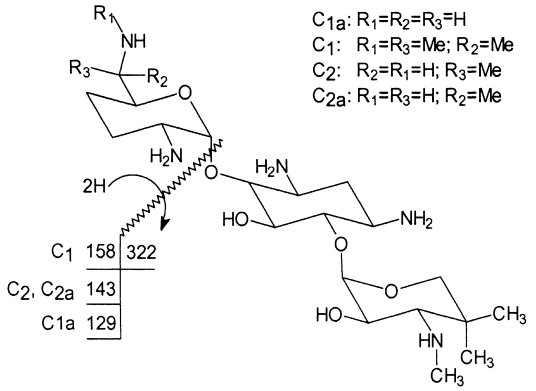
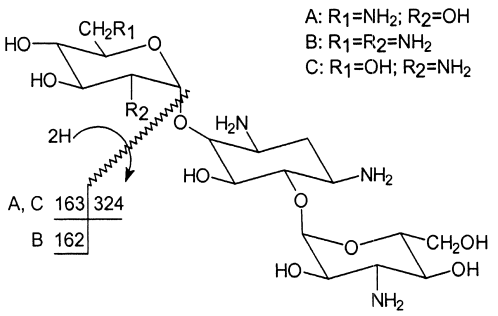
Gentamicin consists of the major components referred to as gentamicin C_1, $C_{2(a)}$ and a minor component gentamicin C_{1a} (Table 3). The latter was not found in the reference compound purchased from Sigma. For the major components (C_1, $C_{2(a)}$) the MS–MS parameters were optimised. Thus, gentamicin was quantified always as a sum of the major compounds gentamicin C_1 and $C_{2(a)}$. The most abundant fragments (m/z=322) of all gentamicin components are formed by the cleavage of the purpurosamine group (Table 3).

The surrogate standard kanamycin, consists of the main component kanamycin A and the minor components kanamycin B and C. The most abundant fragments of the kanamycin components are caused by the cleavage of the kanosamine group. The resulting fragment m/z=324 was found for all kanamycin components as shown in Table 3. The fragmentation patterns for gentamicin and kanamycin are in accordance to those found by other authors [11,21].

2.5. Recoveries and calibration

Filtered hospital wastewater was prepared by

Table 3
Main fragmentation of gentamicin and kanamycin in tandem detection

Gentamicin	Kanamycin
	

passing raw hospital wastewater through 0.45-μm polystyrene filters. Recoveries of gentamicin and kanamycin were determined for the matrices groundwater, raw (unfiltered) hospital wastewater and filtered hospital wastewater. Groundwater and the hospital wastewater (each 50 ml) were spiked at a level of 10 μg l^{-1} with both compounds in three replicates and were then analysed as described above. The recoveries were calculated in comparison to a non-enriched standard solution. However, for gentamicin its native concentration had to be subtracted prior to the calculation. The calibration curve for the determination of gentamicin in native hospital wastewater samples was prepared with groundwater, which was known to be free of anthropogenic organic contamination. For this, groundwater (100 ml) was spiked with at least 10 concentrations of gentamicin ranging from 0.1 to 100 μg l^{-1} and were analysed as described above.

3. Results and discussion

3.1. Chromatographic separation

When using HPLC for the chromatographic separation of aminoglycosides several challenges arise caused by the sorption properties and the polarity of the compounds. Since the aminoglycosides themselves are not retained by reversed-phase columns, the HPLC separation was achieved by ion-pair chromatography. Perfluorinated ion pair agents are most appropriate for MS detection due to their elevated volatility [22]. The ion pair agent trifluoroacetic acid (TFA) causes a dramatic ion suppression in the ESI, whereas with heptafluorobutyric acid (HFBA) the ion suppression is limited [11,23,24]. It is known that the presence of matrix can influence retention times, peak shapes and the analyte responses [10]. A matrix-induced peak splitting of the kanamycin peak was observed in the hospital wastewater extracts showing presumably the separated components kanamycin A and C (Fig. 1). This effect did not occur in standard solutions and the calibration samples, nor was it found for the gentamicin components which were never resolved. Interferences of matrix compounds with kanamycin can be excluded, since unspiked hospital wastewater exhibited no significant signals within the retention time of kanamycin. Therefore, both kanamycin peaks were considered for quantification. It should be noted that memory effects were found for gentamicin due to its sorption properties. Up to 1% of the gentamicin injected was found in the consecutive analysis. Therefore, after each analysis of a sample containing gentamicin, a blank analysis was performed with an injection of 5 mmol l^{-1} aqueous HBFA.

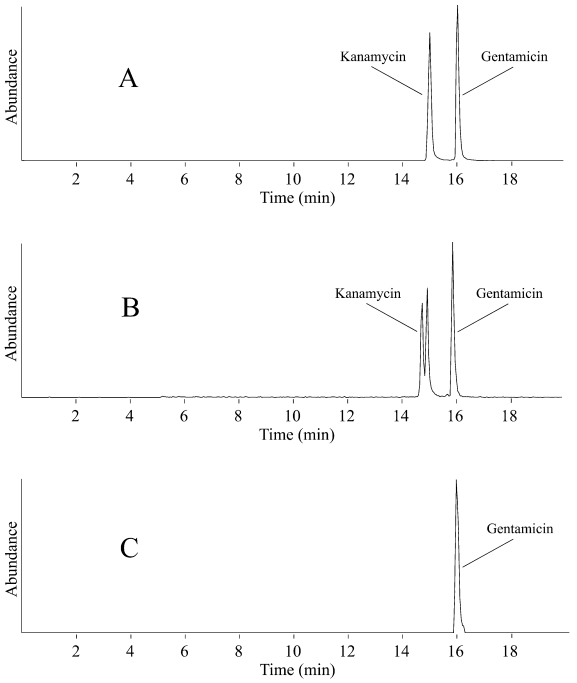


Fig. 1. LC–ES-tandem MS total ion chromatogram of a standard mixture (A), hospital wastewater with kanamycin addition (B) and hospital wastewater without kanamycin addition (C).

3.2. Recoveries and limit of quantification

Absolute recoveries of gentamicin were 93% for ground water, 68% for filtered and 49% for unfiltered hospital wastewater. The corresponding recoveries of the surrogate standard kanamycin did not differ significantly from those of gentamycin. As consequence, the mean relative recoveries of gentamicin ranged between 105 and 111% in all three matrices (Table 4). Presumably, ion suppression in the electrospray interface caused analyte losses which could be compensated by the surrogate stan-

Table 4
Recoveries of gentamicin and kanamycin in hospital wastewater ($n=3$)

	Groundwater		Filtered wastewater		Unfiltered wastewater	
	Gentamicin	Kanamycin	Gentamicin	Kanamycin	Gentamicin	Kanamycin
Recovery (%)	93	89	68	53	49	53
RSD (%)	9	12	6	1	3	1
Relative recovery (%)	105	–	111	–	107	–
RSD (%)	7	–	6	–	8	–

dard kanamycin. Further, it can also be expected that analyte losses occurred by sorption onto all kind of surfaces and particles. For instance, it has to be noted that it is rather unlikely that the elution process of the dried SPE cartridges led to a complete desorption of the sorbed gentamicin from the deposed wastewater particles. Since the relative recoveries were comparable from groundwater to unfiltered hospital wastewater, groundwater was used for calibration which is known to be free of anthropogenic organic pollutions. The LOQ for gentamicin in hospital wastewater was 0.2 $\mu g\ l^{-1}$ which represents the second lowest calibration point within the linear correlation. Further it was confirmed that for gentamicin the signal-to-noise ratio (S/N) was >10 in all calibration points and in the hospital wastewater analysed. In general, the sufficient relative recoveries and the low RSDs exhibited the high accuracy of the developed method for hospital wastewater.

3.3. Occurrence of gentamicin in hospital wastewater

Wastewater from a German hospital was collected through the course of a day by a time-related automatic sampler which took samples in 10-min intervals. The antibiotic gentamicin was found in concentrations between 0.4 and 7.6 $\mu g\ l^{-1}$ in the unfiltered hospital wastewater (Table 5). The concentrations observed in filtered hospital wastewater varied between 0.5 and 1.3 $\mu g\ l^{-1}$ and, thus, were much lower than those found in the unfiltered samples (Table 5). Due to its extremely high sorption properties, most of the gentamicin present in wastewater should be sorbed onto solid particles and colloidal organic matter (micro particles). Filtration (<0.45 μm) removes most of the solid particles, while micro particles frequently pass through [25]. Further, it can be expected that during filtration dissolved gentamicin is sorbed onto the filter or onto the deposition on the filter. Therefore, filtration should be avoided in any case. Without filtration a deposition of the solid wastewater particles occurs on the top of the SPE cartridges. Since the gentamicin recoveries of unfiltered wastewater are lower than those for groundwater and filtered wastewater, the desorption of gentamicin from these particles during the SPE elution with methanol–acetic acid (10:1, v/v) seems to be incomplete. However, the excellent relative recoveries after using the surrogate standard kanamycin indicate that the incomplete desorption can be compensated, due to the very similar sorption–desorption behaviour of gentamicin and kanamyin.

Table 5
Gentamicin ($\mu g\ l^{-1}$) in hospital wastewater samples ($n=4$)

	Minimum	Median	90-Percentile	Maximum
Filtered	0.2	0.5	1.1	1.3
Unfiltered	0.4	3.1	6.4	7.6

4. Conclusion

The analytical method described allows for the determination of gentamicin in hospital wastewater down to the ng l^{-1} range. Since the hospital wastewater used had an extremely high salt content, it can be assumed that also municipal wastewater and other less contaminated waters such as river water can be analysed for gentamicin with the current method.

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