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Sample preparation for residue determination of gentamicin and neomycin by liquid chromatography

Andrzej Posyniak*, Jan Zmudzki, Jolanta Niedzielska

Department of Pharmacology and Toxicology, National Veterinary Research Institute, Al. Partyzantow 57, 24-100 Pulawy, Poland

Abstract

The effect of sample preparation on the determination of gentamicin and neomycin residues in animal tissues was investigated. The extract was mixed with an ion-pair reagent and applied to an octadecyl cartridge. The cartridges were washed with buffer followed by water, and analytes were eluted with ion-pair buffer–acetonitrile mixture. The aminoglycosides were derivatized with 9-fluorenylmethyl chloroformate prior to liquid chromatography using a reversed-phase column and fluorescence detection. Under the conditions applied neomycin was fully separated from all the gentamicin compounds. The highest recoveries of gentamicin and neomycin from spiked tissues were obtained using trichloroacetic acid after initial extraction with phosphate-buffered saline. No interfering peaks from endogenous compounds of matrix were noted at the elution position of the analytes. An intra-laboratory validation of the whole procedure was performed. The calibration graphs were linear from 0.1 to 1.0 mg/kg for gentamicin, and from 0.2 to 1.0 mg/kg for neomycin. Limits of detection were 0.05 mg/kg and 0.10 mg/kg for gentamicin and neomycin, respectively. Limits of quantitation for gentamicin and neomycin were 0.1 and 0.20 mg/kg muscle, liver or kidney tissue, respectively. Recoveries of gentamicin spiked at levels of 0.1 mg/kg porcine tissues ranged from 76 to 86%. Recoveries of neomycin spiked at levels of 0.2 mg/kg porcine tissues ranged from 77 to 83%. The validated procedure was used to determine gentamicin concentrations in porcine tissue after dosing with gentamicin at a level of 5 mg/kg body mass.

Keywords: Food analysis; Gentamicin; Neomycin; Aminoglycosides; Antibiotics; Glycosides

1. Introduction

Gentamicin and neomycin are aminoglycoside antibiotics used to treat bacterial infections in animals because of their good spectrum of activity against gram-negative bacteria. Aminoglycosides are rapidly absorbed from an injection site but poorly absorbed after oral administration. They are not inactivated in the intestine and are eliminated quantitatively in feces. Gentamicin and neomycin, systemic compounds, are excreted almost entirely as parent compound. Aminoglycosides bind to tissue proteins and macromolecules via ionic bonds, but binding to plasma proteins is low (<25%). Gentamicin and neomycin in tissues are usually found in low concentrations, except in the renal cortex where they tend to concentrate. There is a human health risk associated with the use of gentamicin and neomycin, since they have both been found to cause damage to the cranial nerves, resulting in hearing loss [1,2]. To keep harmful aminoglycoside residues out of the human food chain, international authorities require long withdrawal periods before dosed animals may be slaughtered. The maximum residue levels (MRLs) recommended by the Joint FAO/WHO Expert Committee on Food Additives

*Corresponding author. Tel.: +48-81-8863-051 ext. 159; fax: +48-81-8862-595.

E-mail address: aposyn@piwet.pulawy.pl (A. Posyniak).

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(JECFA) are 0.50–10.0 mg neomycin/kg, and 0.10–5.0 mg gentamicin/kg, depending on the different tissues [2]. The European Union [3] has fixed the MRLs in edible animal products for gentamicin from 0.1 to 1.0 mg/kg. In the case of neomycin the MRLs were set from 0.5 to 5.0 mg/kg. Regulatory methods for gentamicin and neomycin that could perform at these concentrations were needed, but development of such a method required overcoming challenges in sample preparation and analysis of drug content.

Chromatographic methods are capable of simultaneous determination of gentamicin and neomycin in animal tissues. However, separations are difficult to achieve because of the structural similarity of aminoglycoside moieties and some endogenous compounds. Additionally, gentamicin and neomycin have no UV or visible absorption and most procedures have to use pre- or post-column derivatization. The derivative reaction with *o*-phthaldialdehyde (OPA) [1,4,5], dansyl chloride [6], fluorescamine [7] or 9-fluorenylmethyl chloroformate (FMOC-Cl) [8–10] is the most desirable because fluorescence detection generally provides better sensitivity and selectivity than UV absorption.

The purpose of this study was to develop a simple and rapid method for the extraction of gentamicin and neomycin from tissue samples and a solid-phase extraction (SPE) as a clean-up procedure, which would yield a very high rate of analyte recovery and clean sample extracts for analysis by high-performance liquid chromatography (HPLC).

2. Experimental

2.1. Materials and reagents

All reagents were analytical or HPLC grade. Acetonitrile and Bakerbond octadecyl SPE cartridges were obtained from J.T. Baker (Deventer, The Netherlands). The cartridges were filled with 100, 500 or 1000 mg of packing material. FMOC-Cl, sodium hexane-1-sulfonic acid (HSA), gentamicin sulfate and neomycin sulfate were obtained from Sigma (Poole, UK).

2.2. Solutions

Phosphate-buffered saline (PBS) solution was made by dissolving 8.5 g sodium chloride, 1.1 g dibasic sodium phosphate and 0.27 g monobasic sodium phosphate in 1000 ml water. The solution was adjusted to pH 7.4 using 2 *M* sodium hydroxide or 3 *M* phosphoric acid. Sample extraction 0.2 *M* ion-pair reagent was prepared by dissolving 1.88 g of HSA in water and diluting to 50 ml. The SPE conditioning buffer was prepared by dissolving 1.88 g of HSA in water, adding 1 ml of acetic acid and diluting with water to 500 ml (pH 3.3). Borate buffers were prepared by dissolving 24.7 g boric acid in 1000 ml water. The buffer was adjusted to pH 10 with potassium hydroxide (45%). Trichloroacetic acid (TCA) 50% solution in water was also prepared.

2.3. Tissue samples

Muscle, liver and kidney samples were obtained from pigs that had not been exposed to any aminoglycosides within the previous 6 months. Tissue samples were stored at −20°C until the time of the in vitro study. A sample of kidney, liver and muscle (2 g wet mass) was accurately weighed.

For recovery, precision and linearity studies, gentamicin sulfate and neomycin sulfate diluted in PBS to concentrations of 0.10 to 1.00 mg analyte/kg tissue were added.

To study removal of matrix interference, tissue samples without any addition were used.

2.4. Instrumentation and methods

2.4.1. Extraction

The aminoglycosides were isolated from the spiked tissue samples after using extraction procedures according to the literature [1,11,12]. Some modifications were introduced to the procedures.

2.4.1.1. Procedure I

A portion of tissue sample was transferred to a 50-ml polypropylene centrifuge tube. Then 5 ml PBS was added and the mixture was incubated at room temperature for 1 h. The mixture was homogenized using a homogenizer (Polytron 3000; Kinematica,

Germany) for 30 s at medium speed. The suspension was centrifuged at 10 000 *g* for 10 min at room temperature. The supernatant liquid was separated, the pH was adjusted to 3.5±0.2 with 5% acetic acid and 2 ml of 0.2 *M* ion-pair reagent was added.

2.4.1.2. Procedure II

To a 50-ml polypropylene centrifuge tube with spiked tissue sample, 5 ml PBS was added and incubated as above; then 10 ml of 50% TCA was added. The mixture was homogenized and the suspension was centrifuged at 3500 *g*, for 15 min at 4°C. The supernatant liquid was transferred to another tube and 2 ml of 0.2 *M* ion-pair reagent was added.

2.4.1.3. Procedure III

To the previously spiked sample 5 ml PBS was added and incubated as above; the content was homogenized. An equal volume of 2 *M* NaOH was added to the homogenate, and the mixture was incubated for 20 min at 70°C, and then cooled to room temperature. The suspension was centrifuged at 3500 *g*, for 15 min at 4°C, and the supernatant liquid was transferred to another tube. The pH was adjusted to 3.5±0.2 with 10% acetic acid, and 2 ml of 0.2 *M* ion-pair reagent was added.

2.4.2. Solid-phase extraction

A SPE 12G vacuum manifold (J.T. Baker) was used for testing all the cartridges. The samples were applied to SPE columns that were conditioned previously with 3 ml of methanol and 3 ml of HSA buffer. The cartridge was then washed with 3 ml of buffer and twice with 3 ml of water.

Recoveries of test analytes from SPE phases were determined following elution with acetonitrile–0.02 *M* HSA buffer mixtures of increasing eluotropic strength (from 10 to 100% acetonitrile).

In order to investigate the effect of matrix compounds on extraction efficiency, extracts of tissue samples containing 5 μg of each of the analytes, and extracts from non-spiked tissues, were applied onto SPE cartridges using the conditions described above.

2.5. High-performance liquid chromatography

2.5.1. Pre-column derivatization

Derivatization of aminoglycosides was performed as described previously [8,9]. Eluate from the SPE column was adjusted to pH 8.0 with borate buffer. The mixture (1.0 ml) was added to 0.5 ml of FMOC solution in a 2-ml autosampler vial and reacted. After 15 min, reaction was stopped by adding 50 μl glycine (0.1 *M*).

2.5.2. Analysis

Analyses of the samples were performed on a Shimadzu VP Series HPLC (Duisburg, Germany) modular system consisting of a pump LC-10AT, autosampler SIL-10AP and system controller SCL-10A utilized RS-232C interface for communication with CLASS-VP chromatography workstation. A fluorescence detector FR-10AXL was used to measure signals at excitation and emission wavelengths of 260 and 315 nm, respectively.

The separation was performed on a 5 μm Hypersil BDS C_{18} column (100×4.6 mm) (Runcorn, UK) with acetonitrile–water (85:15, v/v) as the mobile phase at a flow-rate of 1.0 ml/min at room temperature (20–25°C).

2.6. Recovery and precision studies

The spiked tissue samples were used for validation of the PBS–TCA procedure. At each concentration, five analyses were performed and repeated on 5 days. The peak areas of spiked tissue samples were compared with those of the standards to determine the recovery. A linear regression equation was prepared by plotting the peak area (corrected by the recovery) against concentrations.

2.7. In vivo studies

Gentamicin, in a dose 5 mg/kg, was given orally to four piglets. Tissue samples (kidney, liver and muscle) were taken 24 h after administration. Immediately after sampling, all tissue samples were stored at −21°C until assay.

Table 1
Separation factors (relative retention) of gentamicin components in relation to neomycin B

Compounds	Separation factor, α
Neomycin B/gentamicin C_1	1.2
Neomycin B/gentamicin C_{1a}	0.8
Neomycin B/gentamicin C_{2a}	0.6
Neomycin B/gentamicin C_2	0.4

3. Results and discussion

3.1. Chromatographic conditions

Both gentamicin and neomycin showed strong fluorescence after derivatization with FMOC-Cl. The pre-column procedure was very simple, fast and occurred at room temperature. Reactions ensued quantitatively at pH 8 and derivatives were stable for 12 h. Optimal reactions of neomycin and gentamicin with FMOC-Cl occurred similarly to those reported previously [8,9]. The structural differences between these aminoglycosides and the ion-pair reagent did not show important dependence on the reaction efficiency.

The chromatographic conditions usually described for the elution of aminoglycosides are based on the separation of the major components of gentamicin (C_1, C_{1a}, C_2 and C_{2a}) and neomycin A and neomycin B [13–15]. The gentamycin-FMOC and neomycin-FMOC derivatives were eluted as separate peaks on a C_{18} column with an acetonitrile–water mobile phase [8,9]. However, the best peak shapes were obtained from a column packing consisting of a C_4 adsorbent bonded to a wire-pore spherical base [10].

Our examinations indicated that the BDS C_{18} stationary phase resulted in a symmetry factor, calculated at 10% above baseline, of less than 1.2 for all separated gentamicin and neomycin components. Under the conditions LC used neomycin-FMOC and gentamicin-FMOC derivatives are eluted as separated peaks. Table 1 shows the separation factors (α) of the gentamicin components in relation to neomycin B.

3.2. Extraction

Previous experiments [10–12,16,17] showed that gentamicin and neomycin could be extracted from the biological matrix at pH 6–8. Both alkaline and acid deproteination was used to separate the analytes from co-extractive compounds.

The results of the recovery of analytes from spiked pig kidney tissues are shown in Table 2. With PBS solution as the extraction solvent, the recoveries of analytes were about 80%. The recoveries were improved when the TCA precipitation procedure or NaOH digestion were additionally applied. Table 2 further shows that the recoveries of analytes by TCA precipitation and NaOH digestion were similar when spiked tissue samples were used. The TCA precipitation procedure seems to be more practical because it is less laborious and time consuming. Additionally, obtained extracts were more clear and applicable to SPE clean-up. Therefore, only PBS extraction cou-

Table 2
Comparison of recovery of gentamicin and neomycin extracted from porcine kidney by various methods of extraction[a]

Aminoglycoside component	Recovery (%)		
	BSA extraction	TCA precipitation	NaOH digestion
Gentamicin C_1	64.2	85.5	82.7
Gentamicin C_{1a}	57.4	83.3	86.6
Gentamicin C_{2a}	55.8	81.8	84.2
Gentamicin C_2	49.3	78.5	76.2
Neomycin B	63.2	81.5	83.7

[a] Samples were spiked with 0.50 μg/g of each drug. Mean results of six replicates.

pled with TCA precipitation was used in subsequent experiments.

3.3. SPE clean-up

Most of the reported SPE of aminoglycosides have applied ion-exchange cartridges at high or low pH [14,18,19]. However, the use of ion-exchange SPE cartridges have the same minor drawbacks. The value of the adjusted pH is the critical step in the whole procedure and usually the extract would be diluted before being loaded on an ion exchanger for further clean up. The ion exchange is a very slow reaction and the loading of a high volume of extracts is time consuming. Additionally, the recoveries are strongly influenced by the manufacturer/batch of SPE column used.

In our studies, octadecyl SPE columns were used. As it was found in a previous study [20], the analytes were completely retained on octadecyl cartridges when extracted from buffer at pH between 2 and 4. Since the separations using C_{18} cartridges and reversed-phase (RP) columns are based on the same principles, we supposed that analytes could be eluted from C_{18} cartridges with mobile phase, in the ion-pair mode. The ion-pairing protocol generally requires an equilibration period for the RP column. Therefore, HSA was added to the extracts and HSA buffer was used for the conditioning of the C_{18} cartridges. The results of elution studies with acetonitrile–HSA buffer are shown in Fig. 1. When more than 50% acetonitrile was used in the eluent mixture, good recoveries were obtained.

The combined effects of the amount of C_{18} sorbent, matrix interference and volume of elution phase were determined to study the recoveries of gentamicin and neomycin. Elution curves for applied phases were relatively sharp, indicating good mass transfer properties and the likelihood that the analyte could be recovered in a small volume of solvent. In our studies, we did not find any adverse effects of the biological matrix on analyte recoveries from C_{18} cartridges. Elution with 2 ml of acetonitrile–HSA buffer (55:45, v/v) from 500 mg C_{18} cartridges was sufficient for high recoveries of analytes and separation from endogenous components, and therefore it was used in subsequent experiments. As Fig. 2 indicates, there is no interference from muscle and kidney extracts.

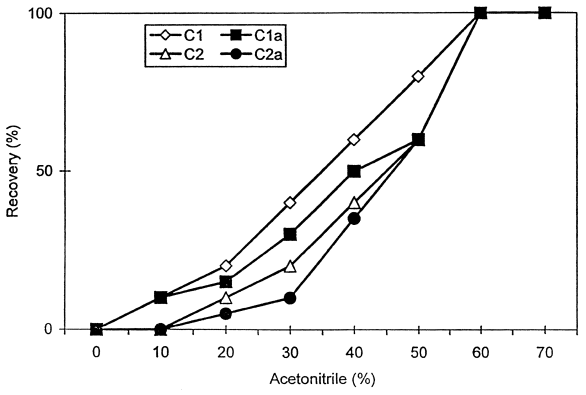

Fig. 1. Cumulative elution curves for gentamicin components from 500 mg Bakerbond C_{18} SPE cartridge: C1, gentamicin C_1; C1a, gentamicin C_{1a}; C2, gentamicin C_2; C2a, gentamicin C_{2a}.

3.4. Validation

The recoveries of gentamicin and neomycin components extracted from spiked muscle, liver or kidney, calculated by comparison with a solution of analyte that had been derivatised directly are shown in Table 3. All aminoglycoside compounds were extracted with a high efficiency (75–86%) from biological matrices. As found in previous papers [10,14], the highest recoveries were obtained from kidney.

Calibration lines of gentamicin and neomycin components were obtained by analysis of spiked kidney samples at concentrations of 0.10, 0.20, 0.40, 0.80 and 1.00 μg/g. The results are shown in Table 4. The assay showed good linearity over the whole of the procedure. Correlation coefficients (r) were almost identical and all >0.99. Detection limits were established as the amount of analyte producing a response at five times the noise level. Limits of detection (LODs) for gentamicin and neomycin compounds were 0.05 and 0.1 μg/g porcine tissue samples, respectively. The practical limit of quantification (LOQ) was estimated as the concentration equal to twice the LOD.

Table 5 shows results of intra-assay (precision),

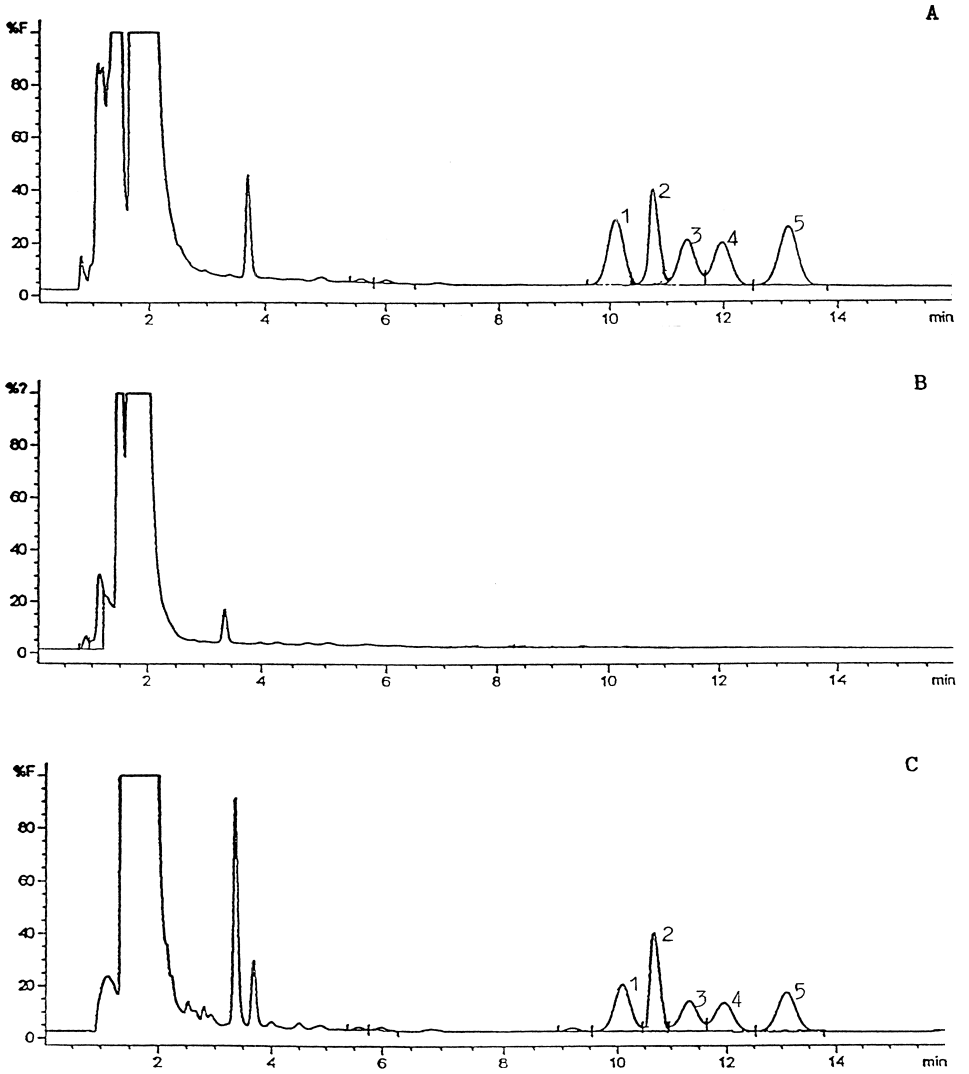

Fig. 2. Typical HPLC examples of standard gentamicin and neomycin, control and spiked kidney. All 20-μl injections with HPLC conditions specified in Experimental. (A) 0.2 μg/ml gentamicin and neomycin HPLC working standards; (B) control kidney; (C) spiked kidney at level of 0.2 μg/g: 1, gentamicin C_1; 2, neomycin B; 3, gentamicin C_{1a}; 4, gentamicin C_{2a}; 5, gentamicin C_2.

and inter-assay (day-to-day variation) of the method. Average variabilities for all types of assays were calculated at less than 10%.

Concentrations of gentamicin obtained from tissue samples of treated piglets are shown in Table 6. The results of the study involving depletion of gentamicin residues in piglets will be published elsewhere.

The above procedure is less time consuming than procedures using ion-exchange cartridges [10,18]. The whole procedure takes about 2 h to complete the sample preparation for HPLC analysis.

4. Conclusions

The described sample preparation procedure for determination of gentamicin and neomycin is rapid, sensitive, reproducible and simple. Following an

Table 3
Recovery of gentamicin and neomycin extracted from porcine tissues[a]

Aminoglycoside component	Recovery[b] (%)		
	Liver	Kidney	Muscle
Gentamicin C_1	76.8	83.3	77.9
Gentamicin C_{1a}	78.2	86.5	75.9
Gentamicin C_{2a}	76.1	80.2	81.4
Gentamicin C_2	78.6	78.9	80.9
Neomycin B	77.5	81.5	83.1

[a] Samples were spiked with 0.1 μg/g of gentamicin, and 0.2 μg/g of neomycin.
[b] Mean results of six replicates.

extraction and deproteination the extracts were cleaned up by SPE. Analytes were separated and detected by HPLC with fluorescence detection followed by pre-column derivatization with FMOC. No internal standards were used. The method described allowed determination of gentamicin and neomycin at concentrations of 0.1 and 0.2 μg/g, respectively. Applicability of the method has been demonstrated by the analysis of gentamicin concentrations in piglet tissues.

Table 6
Concentrations of gentamicin components in piglet tissue samples (24 h post administration of 5 mg/kg body mass)

Component	Concentration[a] (μg/g)		
	Muscle	Kidney	Liver
Gentamicin C_1	0.2 (±0.06)	1.1 (±0.09)	0.2 (±0.05)
Gentamicin C_{1a}	0.3 (±0.09)	1.2 (±0.04)	0.4 (±0.09)
Gentamicin C_{2a}	0.2 (±0.07)	1.8 (±0.11)	0.5 (±0.12)
Gentamicin C_2	0.1 (±0.04)	1.4 (±0.06)	0.2 (±00.4)

[a] Mean results of four different samples (±standard deviation).

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Table 4
Calibration lines for the HPLC assay of gentamicin and neomycin in kidney tissue[a]

Aminoglycoside component[b]	Linear regression equation ($y=a+bx$)		
	Slope, b (SD)	Intercept, a (SD)	Correlation, r^2
Gentamicin C_1	128.2 (3.2)	−2.4 (2.6)	0.9998
Gentamicin C_{1a}	117.9 (2.4)	−3.8 (3.2)	0.9997
Gentamicin C_{2a}	95.6 (1.6)	−2.9 (3.9)	0.9995
Gentamicin C_2	79.3 (3.2)	−3.4 (3.3)	0.9996
Neomycin B	85.4 (3.7)	−4.5 (3.6)	0.9998

[a] Results are expressed as peak areas (mm^2) with detector settings: gain ×1, sensitivity: low, response: 3.
[b] Mean results of four replicates.

Table 5
Reproducibility of the HPLC assay of gentamicin and neomycin in kidney tissue

Aminoglycoside component	Precision (RSD, %)[a]					
	Intra-day			Inter-day		
	0.1 μg/g	0.2 μg/g	1.0 μg/g	0.1 μg/g	0.2 μg/g	1.0 μg/g
Gentamicin C_1	7.9	9.2	8.3	8.2	8.4	7.5
Gentamicin C_{1a}	8.6	7.6	9.4	7.8	9.8	6.9
Gentamicin C_{2a}	9.3	8.5	7.5	8.5	7.4	6.5
Gentamicin C_2	8.4	9.4	8.6	8.6	7.9	8.3
Neomycin B	n.d.[b]	7.5	7.9	n.d.	8.2	6.7

[a] Results of five replicates.
[b] n.d., Not determined.

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