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Determination of neomycin and bacitracin in human or rabbit serum by HPLC–MS/MS

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Abstract

The method for the simultaneous determination of neomycin and bacitracin in human or rabbit serum was developed by using ion pairing reversed phase chromatography and tandem mass spectrometry (MS/MS) detection with electrospray (ESI) in positive mode. Both substances elute under these conditions at the same time and also kanamycin as internal standard elutes almost at the same time. The sample preparation was simple—only using 0.1 mL serum by protein precipitation with acetonitrile. Neomycin and bacitracin were detected as two-fold charged ions as well as the internal standard. The calibration range of these quite difficult detectable substances was 0.2–50 μg/mL of serum. The method was validated for both human or rabbit serum. The inter batch precision of quality control samples in human serum for neomycin ranged from 4.46% to 8.99% and for bacitracin from 6.85% to 11.17%. The inter batch accuracy for neomycin ranged from 98.7% to 100.7% and for bacitracin from 99.2% to 103.0%. At lower limit of quantitation (LLOQ) level of 0.2 μg/mL inter batch precision in human serum for neomycin was 12.05% and for bacitracin 11.91%, whereas accuracies were 99.9% for neomycin and 102.7% for bacitracin. Bench top stability in human or rabbit serum was given over three freeze thaw cycles and 4 h at room temperature.

The method can be considered to be specific and recoveries for sample preparation were high.
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Keywords: Neomycin; Bacitracin; Liquid chromatography mass spectrometry; Human or rabbit serum; Antibiotics

1. Introduction

Determination of neomycin and bacitracin – both old antibiotics – is necessary because of checking systemic absorption for the two antibiotics which was not methodically feasible at that time when the antibiotics first entered into the market and law-requirements were not as strict as they are nowadays. Neomycin is ototoxic and nephrotoxic therefore oral absorption via intestinal tract or via damaged skin should be avoided. Bacitracin is nephrotoxic and therefore systemic absorption should be avoided. Even methods for determining only one of the two substances described in literature in biological samples are rare. There is no method described in literature for the simultaneous determination of both analytes together. For neomycin an aminoglycoside high-performance liquid chromatography (HPLC)–fluorescence after post column derivatization [1] with an LLOQ of 0.3 μg/mL of serum, TLC after derivatization with fluram [2] with a quantitation limit of 0.05 μg/mL of plasma or HPLC–tandem-MS [3] with a quantitation limit of 0.1 μg/mL, and an HILIC–tandem-MS method with a quantitation limit of 0.1 μg/mL serum [4] is described. For bacitracin only an ELISA [5] for the determination in biological samples and a review on identification of polypeptide antibiotics including bacitracin [6] are described. Both substances lack a usable chromophore but derivatization of the primary amino groups which both analytes possess is possible. As neomycin has six of them reproducibility of derivatization is quite a problem. On the other hand bacitracin as it is used for application consists of about nine different similar substances—all polypeptides. Simultaneous determination of both substances sometimes is necessary because of checking of oral or dermal application of both substances in one galenic formulation. The difficulty to determine bacitracin after derivatization (at least nine different polypeptides) and neomycin (six primary amino groups which cannot be derivatized equally precolumn, therefore post column derivatization only would work) should be avoided when using a HPLC–tandem-MS method. During method development the discovery was made that both analytes tend to strongly tail

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chromatographically and severe carryover was discovered for both analytes. Furthermore neomycin is hardly retained on a reversed phase column material. For both analytes ion yields in MS are low and a charge distribution occurs from one to multiple charges per molecule which decreases sensitivity, too. Furthermore charge distribution varies with analyte concentration which results in non-linear calibration curves for each charged ion. With ion pairing chromatography (with nonafluoropentanoic acid and formic acid) neomycin could be retained well on the chosen column as well as carryover could be eliminated and peak shape could be improved. Stabilisation of the charge could be gained from that (as well as by optimising the MS parameters) preferably to the doubly charged ion of the respective substance.

2. Experimental preface

2.1. Neomycin

In a first approach HPLC–MS in single ion monitoring (SIM) with atmospheric pressure chemical ionization (APCI) in positive ion mode was used for neomycin (Fig. 1, taken from Merck Index, 13th ed., 2001) and with ion pairing reagent enough retention was gained. Although different mobile phases were used in this phase with any of these injections of a dilution series (increments of factor of 10) of just the reference item resulted in non-linear response (30–60 times less signal for every 10th part of concentration). At that stage the distribution of charge was not checked and singly charged ions were monitored only. Of course similar results were obtained when monitoring in MRM mode. Similar results were gained by Heller et al. [7]. Since neomycin possesses six primary amino groups post column derivatization with NDA and OPA were checked. Both derivatization reagents resulted in adducts with neomycin which were not sensitive enough (about 5 ng amount detection limit). Later on charge distribution with HPLC–MS/MS for different multiply charged ions were checked and finally the decision was made to use the doubly charged ion and optimised all MS parameters.

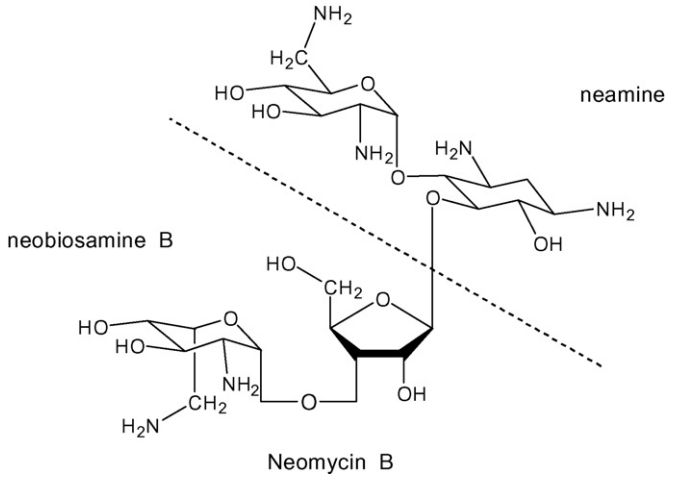

Fig. 1. Structure of Neomycin B.

2.2. Bacitracin

According to the Merck Index this molecule does not possess a definite molecular weight. Several components when first analysing this substance with HPLC–MS were indeed discovered (Fig. 2, taken from Merck Index, 13th ed., 2001). After post column derivatization with OPA there were two main peaks and at least nine components in total. Finally the decision was made to determine both neomycin and bacitracin simultaneously with HPLC–MS/MS with optimised ionization (doubly charged ions) and ion pairing chromatography in serum of humans and rabbits.

3. Experimental

3.1. Chemicals, reference items and matrices

Chemicals used were acetonitrile (gradient grade), formic acid (p.A.), and methanol (gradient grade) by Merck, Germany. Furthermore nonafluoropentanoic acid (NFPA, p.A.) provided by Sigma–Aldrich, USA; trifluoroacetic acid (TFA, purum) provided by Fluka, Switzerland, and water (ASTM-1 grade) by pharm-analyt, Austria.

Reference items were neomycin and bacitracin provided by Altana Pharma AG, Germany and the internal standard kanamycin provided by Sigma–Aldrich, USA.

Human blank serum used for spiking of calibration standards and quality control samples was pooled by Pharm-Analyt, Austria; rabbit blank serum used for spiking of quality control samples was provided by Kraeber, Germany.

Study samples derived from a dermal study with humans and from a rabbit parenteral study.

3.2. Sample preparation (human or rabbit serum)

After thawing at approximately 20–25 °C in a water bath or taking from ambient conditions (Standards, QC-samples), all samples used in a batch or analytical sequence were prepared as follows:

- One hundred microliters of the samples used were aliquot transferred into a test tube each. Thereafter 20 μL of IS work-

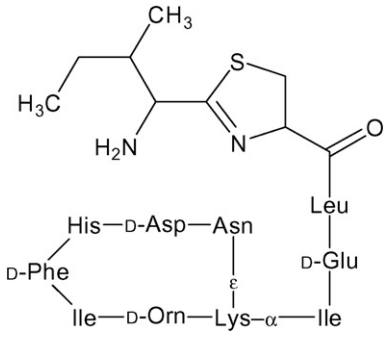

Fig. 2. Structure of bacitracin.

ing solution were added to each aliquot and 150 μL ACN were added later on. Each test tube was vortexed for approx. 10 s, to allow full protein precipitation. Thereafter each sample was stored for approx. 10 min at 2–8 °C.
- Subsequently each sample was centrifuged at about 3040 × *g* for approx. 3 min.
- Auto-sampler vials were charged with 20 μL of 50 mM NFPA. Thereafter 200 μL of the supernatant of the liquid phase of each sample were transferred into an auto-sampler vial (containing 50 mM NFPA already) each. Finally, previous to analysis, the vials were sealed with an aluminium crimp cap and vortexed for approx. 5 s.

3.3. High-performance liquid chromatography (human or rabbit serum)

A gradient HPLC Method on a reversed phase column was used for the determination of both analytes in human or rabbit serum. The 2 HPLC pumps PE Series 200 were provided by Perkin Elmer, USA. The column oven Jetstream 2 Plus was provided by W.O. Electronics, Austria:

Mobile phase	Solvent A: 20 mM formic acid and 10 mM NFPA in water Solvent B: 20 mM formic acid and 10 mM NFPA in methanol
Gradient	0.0–3.0 min linear: 50% B → 95% B 3.0–3.5 min isocratic: 95% B 3.5–4.0 min isocratic: 50% B[a]
Column	Luna C18(2) 3 μm, 50 × 2 mm i.d. (Phenomenex)
Flow	0.5 mL/min
Temperature	25 ± 2 °C
Injection volume	10 μL
Retention time	Approx. 2.6 min: neomycin ($k' \sim 6$) Approx. 2.6 min: bacitracin Approx. 2.2 min: kanamycin (IS; $k' \sim 5$)

[a] Auto-sampler cycle time is >0.5 min; therefore, full equilibration was given (total of >1.0 min).

3.4. Mass spectrometer parameters (human or rabbit serum)

Mass spectrometer	API 3000 (PE Sciex, Canada)
Operational mode	Turbo Spray (ESI), positive ion mode
Vaporizer temperature	500 ± 10 °C
Ion spray voltage	5000 V
Horizontal position	Approx. 3 mm
Lateral position	Approx. 15 mm
Detection mode	MRM
Transitions	308.5 → 454.0 *m/z*: neomycin 712.0 → 119.0 *m/z*: bacitracin 243.4 → 163.2 *m/z*: kanamycin

3.5. Method validation

The analytical method was validated in three batches (including demonstration of linearity, accuracy, precision, specificity, recovery and LLOQ). A minimum of one set of calibration standards and five sets of quality control samples were analysed within these three different batches as well as a carry-over, a blank, and a zero sample. "Blank sample" refers to Standard 0 without analyte and internal standard and "zero sample" refers to Standard 0 with internal standard. Carryover refers to analyte-free injection solution injected after the highest calibration standard. Calibration standards were made at eight concentration levels by adding defined volumes of aqueous solutions containing neomycin and bacitracin or a higher concentrated calibration standard to analyte-free human serum. Quality control samples were prepared alike but spiked in a different batch of human or rabbit serum with different solutions of the analytes deriving from a second weighing. Since not enough rabbit serum from the sponsor was available a cross-validation rabbit serum to human serum only could be performed. Concentrations of calibration standards were between 0.20 and 50 μg/mL for both analytes in human serum and at about 0.50/3.50/42.5 μg/mL for quality control samples (serum of both species).

To determine the assay's linearity, precisions (CVs) and accuracies at least three batches should be analysed, each consisting of at least one set of calibration standards, a zero sample (or Standard 0), a blank sample and five QC-samples at each of three concentration levels.

Intra- (for QC-samples only) and inter-batch (for calibration standards and QC-samples) precisions (as CVs in [%]) and accuracies (in [%]) of the assay should be derived from the results of the validation batches mentioned above.

Concentration values of both the calibration standards and the QC-samples should be back-calculated from the appropriate calibration curve. Thereof inter-batch mean values, precisions (as CVs) and accuracies should be calculated.

3.6. Specificity

Acceptable specificity was defined as an area of possible interferences in serum in blank and zero samples. Blank and zero samples had to be below 1/3 of the area of calibration standard 1 (at level of LLOQ) or not detectable. The specificity of the method was determined by analysing six sample pairs consisting of one zero and one blank human and blank rabbit serum sample per volunteer.

3.7. Recovery

Recovery was determined by comparing the areas of quality control samples with areas of aqueous solutions (without sample preparation but appropriate dilution in accordance to sample preparation of QC-samples) at three concentration levels. Each peak area of the QC-samples was divided by the mean peak area of those direct aqueous solutions. Aqueous DIR-samples had to be analysed in triplicate at each of these concentration levels. This is the usual procedure at pharm-analyt of checking recovery (including possible matrix effects). Only in case of recovery <50% or greater >150% matrix or other effects are additionally checked. As in this case adsorption took place for lower concentrated samples in aqueous/methanolic solution recovery was determined differently (see Section 4.3.).

3.8. Method linearity

The calibration range was from 0.20 to 50 μg/mL for both analytes in human serum. The inter batch coefficient of variation (CV) had to be <15% (20% at LLOQ level) for precision, and for accuracy the mean value had to be within ±15% of the actual value (20% at LLOQ level). However, at LLOQ level, 20% was acceptable for both inter batch precision and accuracy. If the calibration curve was rejected, the batch had to be rejected also. A linear (neomycin, weighting factor $1/x^2$) and a quadratic regression (bacitracin, weighting factor $1/x$) should be used (after pre-study tests). The coefficient of correlation (R) has to achieve a degree of certainty of $R = 0.99$.

Accuracy of individual calculated values must not deviate more than ±15% (20% at LLOQ level) from their expected ones.

Seventy-five percent of all individual values, but at least six concentration levels have to match the specifications mentioned above.

If any values failed ($\langle o\, o\, s \rangle$), the respective calibration curve will be calculated anew without them, retaining the upper conditions unchanged. At least 50% of all individual values at a certain concentration level have to be valid, else this concentration level fails ($\langle o\, o\, s \rangle$).

$\langle o\, o\, s \rangle$ concentration levels are allowed unless those being adjacent ones. These criteria were used for all cited tables.

3.9. Precision and accuracy for QC-samples

Quality control samples were prepared in human or rabbit serum whereas rabbit serum was only used in one sequence for cross-validation purposes.

Five replicates of quality control samples (three for cross-validation) at three concentration levels each had to be analysed. Quality control samples were prepared at three concentrations (≤3× LLOQ, mid-range and at least 80% of the highest calibration concentration) and were, at least in triplicate, incorporated into each sequence. According to the results of the QC-samples a sequence was accepted or rejected. At least six of the nine QC-samples had to be within ±15% of their respective nominal values; three of the nine QC-samples (but not at the same concentration) may have been outside the ±15% of their respective nominal values.

If a batch did not adhere to these criteria, the batch was rejected. QC-samples outside ±15% (±20% at LLOQ level) are called "out of specifications", whereas QC-samples outside ±30% (±40% at LLOQ level) are called "outlier". These criteria were used for all cited tables.

4. Results and discussion

As both substances – neomycin and bacitracin – are sometimes applied together and both have a severe toxicity in higher concentrations in human plasma measurement of both together is a big issue. Inconsistency of bacitracin (a mixture of different polypeptides) and many primary amino groups without a usable UV absorption of neomycin make it necessary to use mass spectrometry as detector.

4.1. Carryover

Preliminary tests have shown carryover values higher than 5% for neomycin when flushing twice with 250 μL 50% MeOH, a solution which is normally used as flushing solution, however, no significant carryover was observed for bacitracin. As it was careful examined the carryover of neomycin can be reduced by addition of TFA into the flushing solution (50% MeOH without TFA: carryover >5%, with 10 mM TFA in 50% MeOH: carryover >1%; 50 mM TFA in 50% MeOH: carryover approx. 0.7%).

Within validation the auto-sampler was flushed five times with 250 μL of 50 mM TFA in 50% MeOH after any injection. The carryover resulted in 0.18% to 0.56% (maximum equivalent to 0.28 μg/mL serum) for neomycin (Table 1) and in 0.00% to 0.09% for bacitracin (Table 2).

Table 1
Linear regression parameters and carryover of neomycin in human serum

Sequence	Intercept	Slope	R	Calibration range (μg/mL)	No. of standards	w.F.	Carryover (%)
Neo_1804_A7	0.000732	0.0376	0.9964	0.20–50.14	8	$1/x^2$	0.56
Neo_1804_A8	0.000228	0.0385	0.9961	0.20–50.14	8	$1/x^2$	0.45
Neo_1804_A9	0.000540	0.0403	0.9982	0.20–50.14	15	$1/x^2$	0.41
Neo_1804_B4	0.000649	0.0303	0.9959	0.20–50.14	8	$1/x^2$	0.18

Table 2
Regression parameters and carryover of bacitracin in human serum

Sequence	A	b	c	R	Calibration range (μg/mL)	No. of standards	w.F.	Carryover (%)
Neo_1804_A7	−2.45E−05	0.01070	−1.32E−04	0.9998	0.20–50.02	8	$1/x$	0.09
Neo_1804_A8	−2.15E−05	0.00869	−3.73E−04	0.9995	0.20–50.02	8	$1/x$	0.00
Neo_1804_A9	−1.78E−05	0.00727	−2.02E−04	0.9991	0.20–50.02	15	$1/x$	0.00
Neo_1804_B4	9.40E−07	0.00460	1.26E−04	0.9987	0.20–50.02	8	$1/x$	0.05

$y = ax^2 + bx + c$.

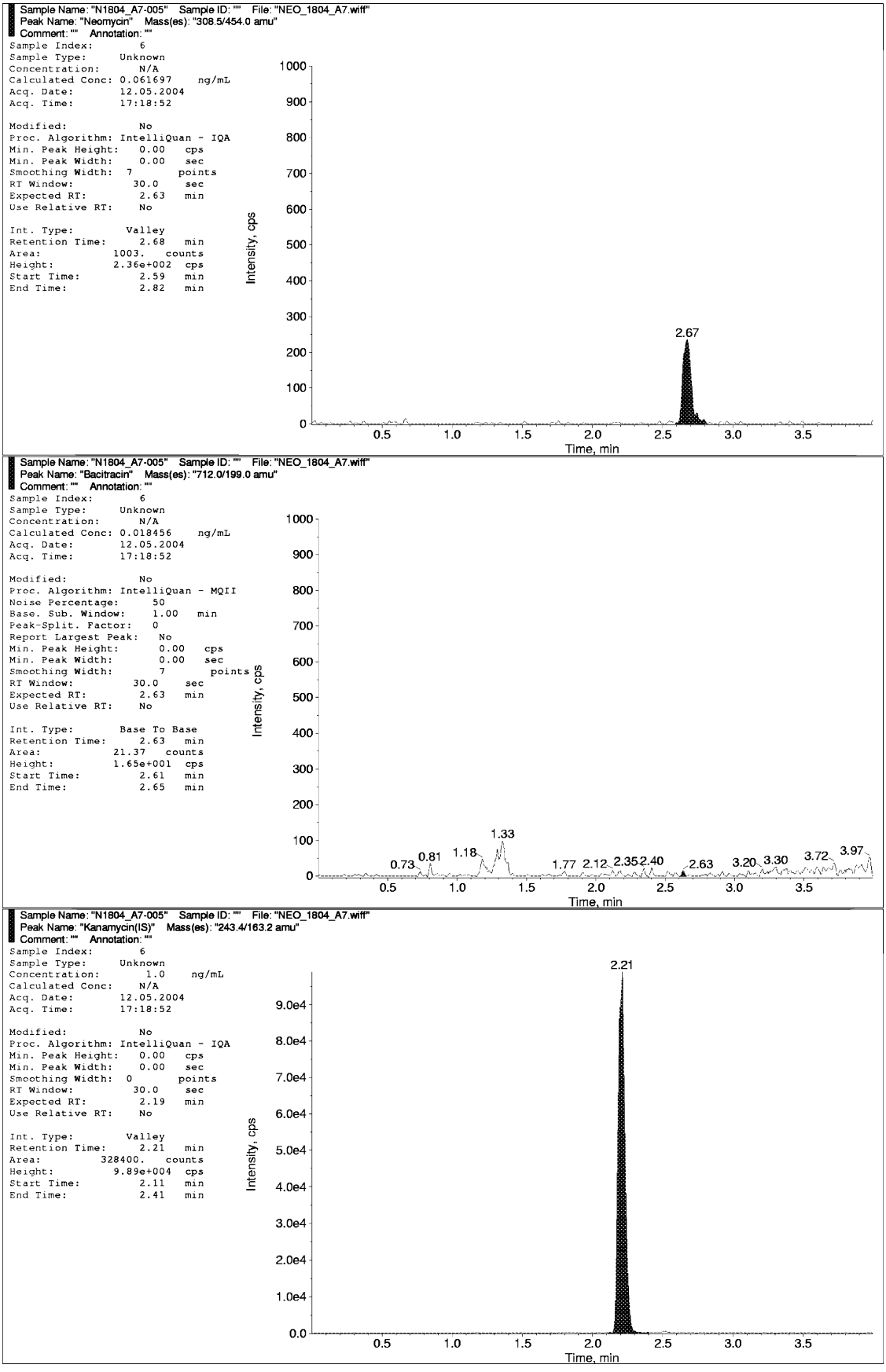

Fig. 3. Chromatogram of zero sample (Std0A) in human serum. *Note:* In the following figures the chromatograms of neomycin, bacitracin and the internal standard are shown top down. Erroneously the concentration units are specified as ng/mL instead of the correct unit μg/mL in all chromatograms.

Table 3
Linearity, precision (CV) and accuracy of neomycin in human serum (Stds)

Sequence	Unit	Calculated concentration (μg/mL)							
		Std 1A	Std 2A	Std 3A	Std 4A	Std 5A	Std 6A	Std 7A	Std 8A
NEO_1804_A7	μg/mL	0.21	0.38	0.92	2.20	5.67	10.96	18.22	52.17
NEO_1804_A8	μg/mL	0.21	0.39	0.89	2.30	5.75	9.37	19.55	54.27
NEO_1804_A9	μg/mL	0.19	0.40	0.90	2.27	5.68	10.34	19.57	48.39
		0.21	0.41	[0.86]	2.02	5.65	10.31	20.10	49.89
NEO_1804_B4	μg/mL	0.21	0.37	0.89	2.07	5.55	10.65	20.19	54.51
Mean	μg/mL	0.21	0.39	0.90	2.17	5.66	10.33	19.53	51.85
S.D.	μg/mL	0.01	0.01	0.02	0.12	0.07	0.59	0.79	2.69
CV	%	3.10	3.80	1.78	5.70	1.26	5.76	4.04	5.18
Number		5	5	4	5	5	5	5	5
Experimental concentration	μg/mL	0.20	0.40	1.04	2.16	5.37	10.03	19.98	50.14
Accuracy	%	102.9	97.7	86.6	100.7	105.3	103.0	97.7	103.4

Note: If outliers of QC-samples occurred, they were excluded from calculation and are displayed in brackets (e.g. [1 1 1]). Whereas QC-samples out of specification were included into calculation and are displayed as underlined values (e.g. 2 2 2).

Table 4
Linearity, precision (CV) and accuracy of bacitracin in human serum (Stds)

Sequence	Unit	Calculated concentration (μg/mL)							
		Std 1A	Std 2A	Std 3A	Std 4A	Std 5A	Std 6A	Std 7A	Std 8A
NEO_1804_A7	μg/mL	0.22	0.39	0.90	2.26	5.26	10.12	19.98	49.98
NEO_1804_A8	μg/mL	0.21	0.41	0.92	2.11	5.78	10.20	19.25	50.25
NEO_1804_A9	μg/mL	[0.28]	0.35	0.92	2.30	5.29	10.32	18.67	49.11
		0.22	0.42	0.97	2.24	5.66	10.75	19.31	51.53
NEO_1804_B4	μg/mL	0.19	0.41	0.95	2.23	5.84	10.62	18.44	50.44
Mean	μg/mL	0.21	0.40	0.93	2.23	5.56	10.40	19.13	50.26
S.D.	μg/mL	0.02	0.03	0.03	0.07	0.27	0.27	0.60	0.87
CV	%	7.51	7.01	2.87	3.19	4.94	2.60	3.15	1.73
Number		4	5	5	5	5	5	5	5
Experimental concentration	μg/mL	0.20	0.40	1.04	2.15	5.36	10.00	19.93	50.02
Accuracy	%	104.9	99.0	89.5	103.4	103.8	104.0	96.0	100.5

Table 5
Assay performance for analysis of neomycin and bacitracin in human serum

	Neomycin			Bacitracin		
	QC-A1	QC-B1	QC-C1	QC-A1	QC-B1	QC-C1
Expected concentration (μg/mL)	0.50	3.49	42.45	0.50	3.49	42.50
Experimental mean (μg/mL)	0.50	3.44	42.66	0.52	3.47	42.57
Inter-batch CV (%)	8.99	7.67	4.46	11.17	8.38	6.85
Inter-batch accuracy (%)	100.7	98.7	100.5	103.0	99.2	100.2
Number of records	17	18	18	18	18	18

Table 6
Summary inter-batch results of LLOQ samples in human serum

Analyte	Experimental concentration (μg/mL)	Mean calculated concentration (μg/mL)	CV (%)	Mean accuracy (%)	*n*
Neomycin	0.20	0.20	12.04	101.7	18
Bacitracin	0.20	0.20	12.66	103.2	18

As neomycin has the tendency of higher carryover Fig. 3 shows a chromatogram of a zero human serum sample with internal standard (bottom).

To exclude an appreciable carryover as far as possible, a 50% ACN solution was injected subsequent to each high-level QC-sample and highly concentrated study sample, respectively. These requirements were implemented into SOPs for analyzing study samples.

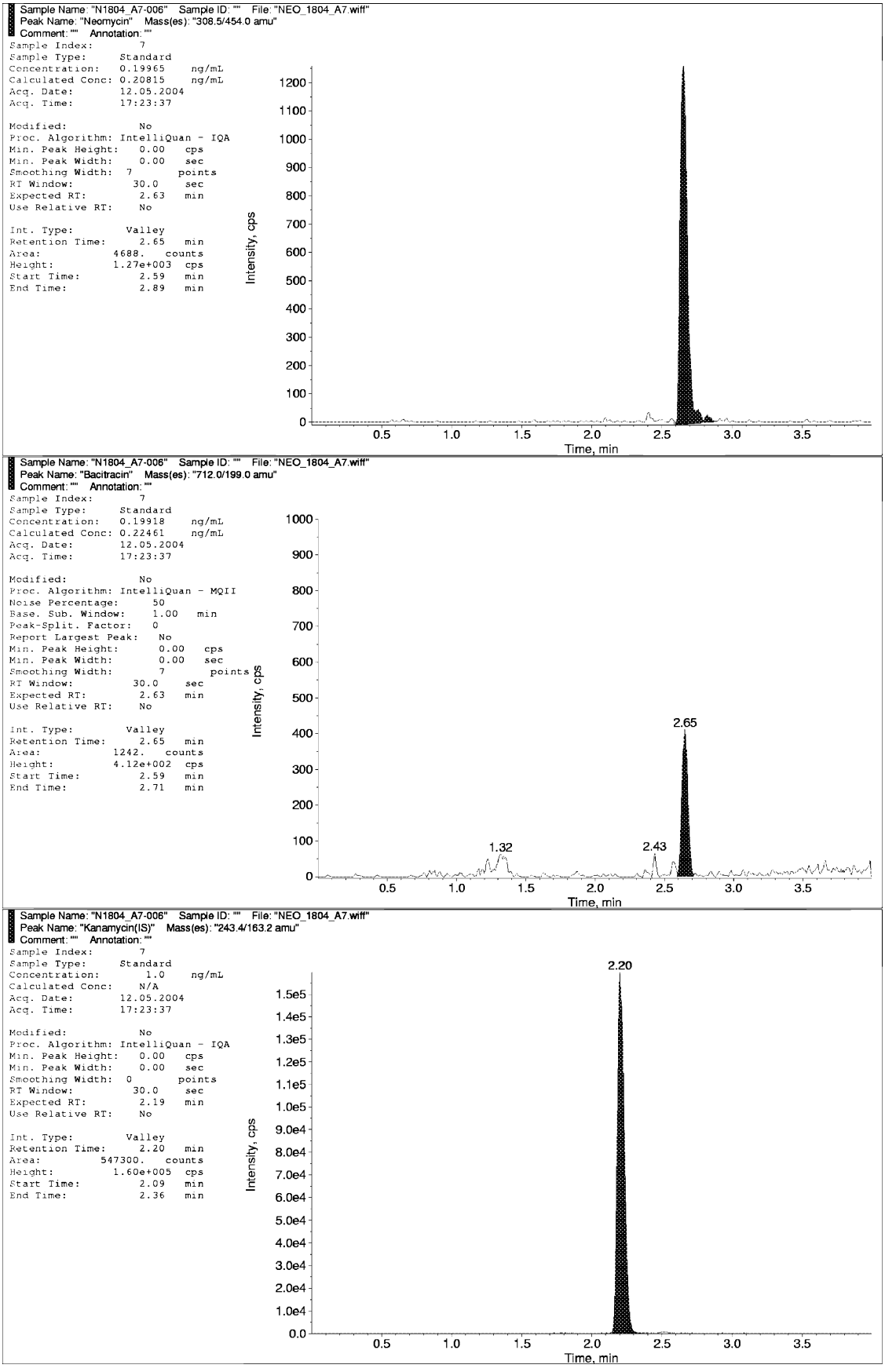

Fig. 4. Calibration standard Std1A (at level of LLOQ) in human serum.

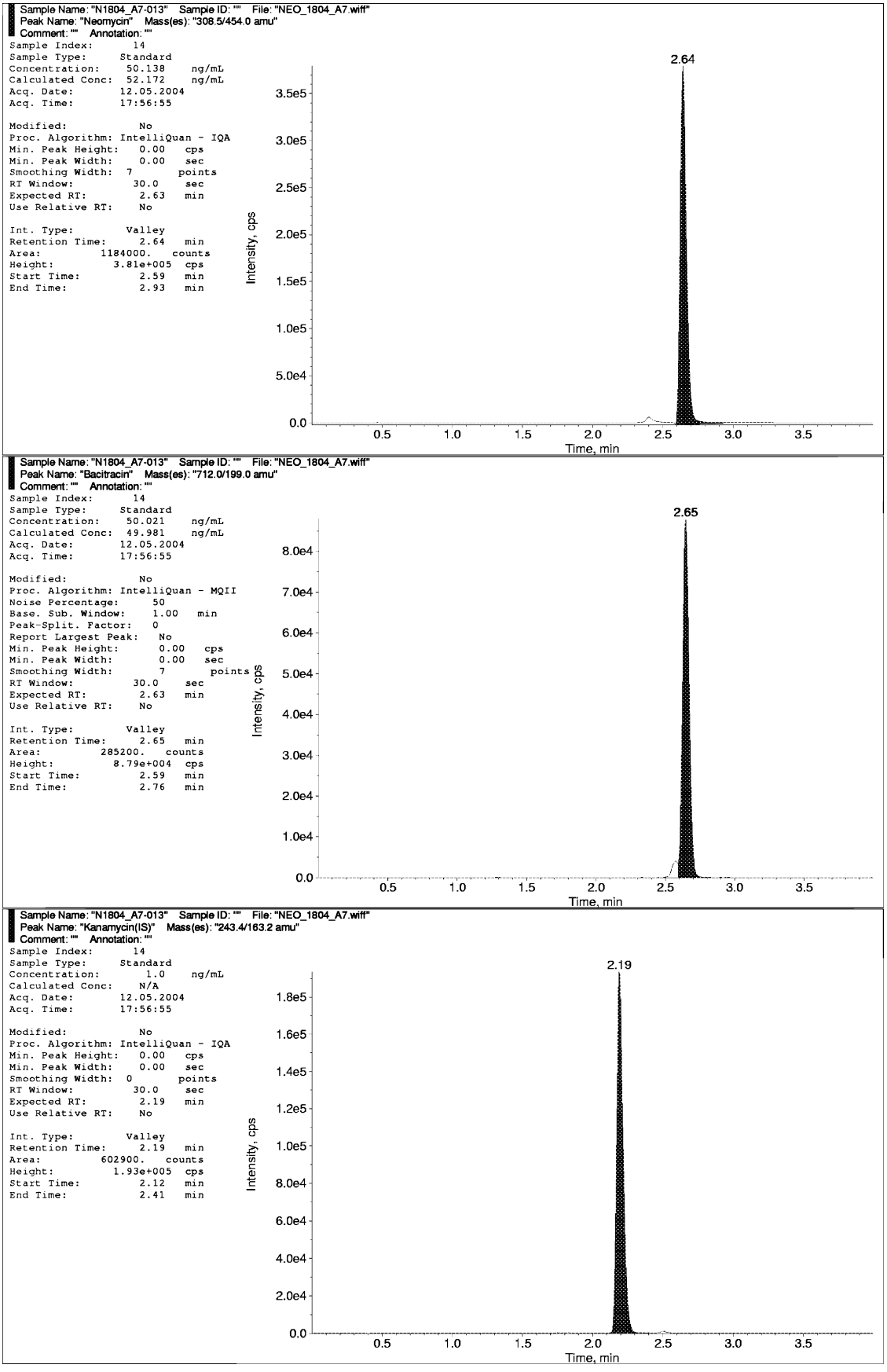

Fig. 5. Calibration standard Std8A (at level of ULOQ) in human serum.

Table 7
Cross-validation of neomycin in rabbit serum

Sample	Filename	Calculated concentration (μg/mL)	Mean (μg/mL)	CV (%)	Experimental concentration (μg/mL)	Accuracy (%)	Accuracy reference mean
QC-A1-R	N1804_B4-020	0.47	0.44	10.70	0.50	94.6	88.6
	N1804_B4-021	0.47			0.50	93.6	
	N1804_B4-022	*0.39*			0.50	77.7	
QC-B1-R	N1804_B4-029	3.50	3.47	4.84	3.50	99.9	99.1
	N1804_B4-030	3.29			3.50	94.0	
	N1804_B4-031	3.62			3.50	103.5	
QC-C1-R	N1804_B4-038	43.59	43.30	3.41	42.50	102.6	101.9
	N1804_B4-039	44.62			42.50	105.0	
	N1804_B4-040	41.70			42.50	98.1	

Table 8
Cross-validation of bacitracin in rabbit serum

Sample	Filename	Calculated concentration (μg/mL)	Mean (μg/mL)	CV (%)	Experimental concentration (μg/mL)	Accuracy (%)	Accuracy reference mean
QC-A1-R	N1804_B4-020	0.52	0.49	10.17	0.50	103.6	98.4
	N1804_B4-021	0.52			0.50	104.7	
	N1804_B4-022	0.43			0.50	86.8	
QC-B1-R	N1804_B4-029	3.53	3.70	4.92	3.51	100.5	105.5
	N1804_B4-030	3.89			3.51	110.9	
	N1804_B4-031	3.68			3.51	105.0	
QC-C1-R	N1804_B4-038	42.78	42.32	2.93	42.55	100.5	99.5
	N1804_B4-039	40.92			42.55	96.2	
	N1804_B4-040	43.27			42.55	101.7	

4.2. Linearity, precision, and accuracy

By using the described method the following calibration curves for neomycin (Table 3) and bacitracin (Table 4) in human serum resulted for the concentration range of 0.2 to 50 μg/mL of serum. There is no peak at the retention time of bacitracin but a small peak at the retention time of neomycin (Fig. 3). Otherwise the LLOQ could have been lowered to about 0.05 μg/mL in regards to sensitivity. Table 1 shows the regression parameters and the carryover for neomycin in four validation batches. Table 2 shows this for bacitracin. Fig. 4 shows a chromatogram of the LLOQ for neomycin (top) and bacitracin (middle trace) and Fig. 5 of the highest calibration standard of 50 μg/mL of human serum each. Table 5 shows the inter-batch precision and accuracy of neomycin for the QC-samples at 0.50/3.49/42.45 μg/mL in human serum and the same parameters for bacitracin in human serum. Table 6 shows the LLOQ of both substances in three validation batches in human serum. As it was necessary to measure also both substances in rabbit serum a cross-validation from human to rabbit serum was done (Tables 7 and 8, spiked rabbit serum quality control samples versus spiked human serum quality control samples and calibration standards; following the same sample preparation method). Table 9 shows the results of the LLOQ samples of both substances in rabbit serum.

4.3. Specificity and recovery

Specificity criteria were met for both matrices (six samples in each matrix with/without Internal Standard). Therefore, the method can be considered specific.

As in this case adsorption took place for lower concentrated samples in aqueous/methanolic solution recovery was determined differently: blank samples were extracted and the analytes as well as the internal standard were spiked after extraction procedure into the blank samples. Therefore the recovery without matrix effects was determined in this case and no adsorption effects occurred in serum or precipitated serum. Recovery was found to be 66.6% for neomycin in human serum and 104.3% for bacitracin. For the internal standard kanamycin recovery was found to be 67.3%.

Table 9
Summary inter-batch results of LLOQ samples in rabbit serum

Analyte	Experimental concentration (μg/mL)	Mean calculated concentration (μg/mL)	CV (%)	Mean accuracy (%)	*n*
Neomycin	0.20	0.18	12.59	91.8	6
Bacitracin	0.20	0.18	12.92	89.4	6

4.4. Acceptance criteria

All criteria concerning specificity, recovery, linearity and precision and accuracy of calibration and quality control samples were fully met. However, carryover of neomycin was unusually high and this fact was taken into consideration when analyzing real samples (see Section 4.1).

4.5. Ruggedness of the HPLC–MS/MS system

Although protein precipitation only was used for sample preparation no problems in regards of system stability due to dirty samples and visible contamination of the MS system was encountered while analyzing hundreds of serum samples. This has a lot to do with the rugged Turbo Ion Spray and interface system of the API 3000 system.

4.6. Charge distribution

During prevalidation experiments mainly the doubly and partly the singly charged (and very little of the triply charged) ions of the analytes were seen. The most influencial and therefore critical parameters on this distribution were found to be the LC flow and the spray position of the electrospray. After having optimised the HPLC conditions the spray position was optimised on the doubly charged ions.

5. Conclusions

The described method for the determination of neomycin and bacitracin in human or rabbit serum fulfils all acceptance criteria in regards to GLP validation guidelines [8]. Except for carryover criteria of neomycin were not fully met. Therefore, after injection of highly concentrated known samples (e.g. Standard 8 or QC-C1) an injection of a blank sample was necessary before an injection of a low level sample (e.g. QC-A1 or LLOQ). The method can be used in the range of 0.2–50 μg/mL of human serum for neomycin and bacitracin. This method is for the first time capable of determining both substances together from serum. With a simple sample preparation (only protein precipitation with acetonitrile) only 0.1 mL of serum for the determination of both substances is required.

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