

Ciprofloxacin, enrofloxacin, lincomycin, penicillin G, and penicillin V – Determination of selected antibiotics in urine by LC-MS/MS

Biomonitoring Method – Translation of the German version from 2022

Keywords

antibiotics; ciprofloxacin; enrofloxacin; lincomycin; penicillin G; penicillin V; biomonitoring; urine; LC-MS/MS

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Abstract

The working group “Analyses in Biological Materials” of the Permanent Senate Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area developed and verified the presented biomonitoring method. This method allows for the sensitive and precise determination of selected antibiotics (ciprofloxacin, enrofloxacin, lincomycin, penicillin G, and penicillin V) in human urine. Sample preparation includes extracting the analytes by solid-phase extraction on Oasis HLB cartridges, followed by concentrating the eluates under a stream of nitrogen. The analytes are separated from matrix compounds by liquid chromatography and subsequently detected with tandem mass spectrometry using electrospray ionisation. Quantitative evaluation is carried out via external calibration in urine.

The good precision data and accuracy data show that the method provides reliable and accurate measurement values. Any matrix effects are effectively compensated for by the use of isotope-labelled internal standards. This finding holds similarly true for ciprofloxacin, for which isotope-labelled enrofloxacin was used as internal standard (ISTD). With quantitation limits of 0.1 µg/l for ciprofloxacin, enrofloxacin, and lincomycin as well as 0.3 µg/l for penicillin G and penicillin V, this method is very sensitive and enables the reliable quantitation of occupational exposure to the selected antibiotics.

1 Characteristics of the method

| | |
|-----------------------------|--|
| Matrix | Urine |
| Analytical principle | Liquid chromatography with tandem mass spectrometry (LC-MS/MS) |

Parameters and corresponding hazardous substances

| Hazardous substance | CAS No. | Parameter | CAS No. |
|---------------------|------------|---------------|------------|
| Ciprofloxacin | 85721-33-1 | Ciprofloxacin | 85721-33-1 |
| Enrofloxacin | 93106-60-6 | Enrofloxacin | 93106-60-6 |
| | | Ciprofloxacin | 85721-33-1 |
| Lincomycin | 154-21-2 | Lincomycin | 154-21-2 |
| Penicillin G | 61-33-6 | Penicillin G | 61-33-6 |
| Penicillin V | 87-08-1 | Penicillin V | 87-08-1 |

Reliability data

Ciprofloxacin

| | | |
|-----------------------------------|---|--|
| Within-day precision: | Standard deviation (rel.) | $s_w = 5.43\text{--}6.98\%$ or $3.25\text{--}15.8\%$ |
| | Prognostic range | $u = 15.1\text{--}19.4\%$ or $9.02\text{--}43.9\%$ |
| | at a spiked concentration of 0.15 µg or 0.55 µg ciprofloxacin per litre of urine and n = 5 determinations | |
| Day-to-day precision: | Standard deviation (rel.) | $s_w = 7.62\%$ or 14.3% |
| | Prognostic range | $u = 32.8\%$ or 61.5% |
| | at a spiked concentration of 0.15 µg or 0.55 µg ciprofloxacin per litre of urine and n = 3 determinations | |
| Accuracy: | Mean accuracy (rel.) | $r = 90.8\text{--}106\%$ or $88.9\text{--}116\%$ |
| | at a spiked concentration of 0.15 µg or 0.55 µg ciprofloxacin per litre of urine and n = 5 determinations | |
| Detection limit: | 0.038 µg ciprofloxacin per litre of urine | |
| Quantitation limit (theoretical): | 0.064 µg ciprofloxacin per litre of urine | |
| Quantitation limit (in practice): | 0.1 µg ciprofloxacin per litre of urine | |

Enrofloxacin

| | | |
|-----------------------|--|--|
| Within-day precision: | Standard deviation (rel.) | $s_w = 10.8\text{--}20.4\%$ or $3.67\text{--}10.1\%$ |
| | Prognostic range | $u = 30.0\text{--}56.6\%$ or $10.2\text{--}28.0\%$ |
| | at a spiked concentration of 0.15 µg or 0.55 µg enrofloxacin per litre of urine and n = 5 determinations | |
| Day-to-day precision: | Standard deviation (rel.) | $s_w = 9.87\%$ or 3.33% |
| | Prognostic range | $u = 31.4\%$ or 10.6% |
| | at a spiked concentration of 0.15 µg or 0.55 µg enrofloxacin per litre of urine and n = 4 determinations | |

| | | |
|-----------------------------------|---|---|
| Accuracy: | Mean accuracy (rel.) | $r = 76.0\text{--}99.1\%$ or $98.0\text{--}106\%$ at a spiked concentration of $0.15\ \mu\text{g}$ or $0.55\ \mu\text{g}$ enrofloxacin per litre of urine and $n = 5$ determinations |
| Detection limit: | 0.043 μg enrofloxacin per litre of urine | |
| Quantitation limit (theoretical): | 0.072 μg enrofloxacin per litre of urine | |
| Quantitation limit (in practice): | 0.1 μg enrofloxacin per litre of urine | |

Lincomycin

| | | |
|-----------------------------------|--|--|
| Within-day precision: | Standard deviation (rel.) | $s_w = 5.44\text{--}9.65\%$ or $1.89\text{--}15.0\%$ |
| | Prognostic range | $u = 15.1\text{--}26.8\%$ or $5.25\text{--}41.6\%$ at a spiked concentration of $0.15\ \mu\text{g}$ or $0.55\ \mu\text{g}$ lincomycin per litre of urine and $n = 5$ determinations |
| Day-to-day precision: | Standard deviation (rel.) | $s_w = 11.8\%$ or 4.24% |
| | Prognostic range | $u = 50.8\%$ or 18.2% at a spiked concentration of $0.15\ \mu\text{g}$ or $0.55\ \mu\text{g}$ lincomycin per litre of urine and $n = 3$ determinations |
| Accuracy: | Mean accuracy (rel.) | $r = 90.3\text{--}113\%$ or $96.3\text{--}105\%$ at a spiked concentration of $0.15\ \mu\text{g}$ or $0.55\ \mu\text{g}$ lincomycin per litre of urine and $n = 5$ determinations |
| Detection limit: | 0.0015 μg lincomycin per litre of urine | |
| Quantitation limit (theoretical): | 0.0035 μg lincomycin per litre of urine | |
| Quantitation limit (in practice): | 0.1 μg lincomycin per litre of urine | |

Penicillin G

| | | |
|-----------------------------------|--|--|
| Within-day precision: | Standard deviation (rel.) | $s_w = 2.40\text{--}4.15\%$ or $1.00\text{--}6.27\%$ |
| | Prognostic range | $u = 6.66\text{--}11.5\%$ or $2.78\text{--}17.4\%$ at a spiked concentration of $0.25\ \mu\text{g}$ or $1.00\ \mu\text{g}$ penicillin G per litre of urine and $n = 5$ determinations |
| Day-to-day precision: | Standard deviation (rel.) | $s_w = 4.19\%$ or 6.50% |
| | Prognostic range | $u = 18.0\%$ or 28.0% at a spiked concentration of $0.25\ \mu\text{g}$ or $1.00\ \mu\text{g}$ penicillin G per litre of urine and $n = 3$ determinations |
| Accuracy: | Mean accuracy (rel.) | $r = 99.4\text{--}108\%$ or $90.0\text{--}102\%$ at a spiked concentration of $0.25\ \mu\text{g}$ or $1.00\ \mu\text{g}$ penicillin G per litre of urine and $n = 5$ determinations |
| Detection limit: | 0.0036 μg penicillin G per litre of urine | |
| Quantitation limit (theoretical): | 0.0081 μg penicillin G per litre of urine | |
| Quantitation limit (in practice): | 0.1 μg penicillin G per litre of urine | |

Penicillin V

| | | |
|-----------------------------------|--|--|
| Within-day precision: | Standard deviation (rel.) | $s_w = 1.99\text{--}5.46\%$ or $2.52\text{--}7.31\%$ |
| | Prognostic range | $u = 5.52\text{--}15.2\%$ or $7.00\text{--}20.3\%$ |
| | at a spiked concentration of 0.50 µg or 2.50 µg penicillin V per litre of urine and n = 5 determinations | |
| Day-to-day precision: | Standard deviation (rel.) | $s_w = 7.59\%$ or 7.76% |
| | Prognostic range | $u = 32.7\%$ or 33.4% |
| | at a spiked concentration of 0.50 µg or 2.50 µg penicillin V per litre of urine and n = 3 determinations | |
| Accuracy: | Mean accuracy (rel.) | $r = 87.0\text{--}99.7\%$ or $90.5\text{--}106\%$ |
| | at a spiked concentration of 0.50 µg or 2.50 µg penicillin V per litre of urine and n = 5 determinations | |
| Detection limit: | 0.031 µg penicillin V per litre of urine | |
| Quantitation limit (theoretical): | 0.071 µg penicillin V per litre of urine | |
| Quantitation limit (in practice): | 0.3 µg penicillin V per litre of urine | |

2 General information on the selected antibiotics

Enrofloxacin, lincomycin, penicillin G, and penicillin V are applied as common antibiotics on poultry farms (Paul et al. 2019), whereby the administration of these antibiotics commonly takes place via the drinking water. The structural formulas of enrofloxacin (including its main metabolite ciprofloxacin), lincomycin, penicillin G, and penicillin V are shown in Figure 1. Occupational exposure may occur during isolation or production of the active substances, during the preparation and packaging of the medicines, and during their medical use in humans and animals. On poultry farms, occupational exposure can occur during preparation of drinking water for antibiotic treatment or by inhaling contaminated poultry-stall dusts. Biomonitoring can be used to measure employees' systemic exposure to antibiotics.

Enrofloxacin is the first synthetic broad-spectrum antibiotic of the fluoroquinolone class that was used in veterinary medicine. Fluoroquinolones inhibit DNA replication in bacteria via interaction with the bacterial DNA topoisomerases II and IV (Trouchon and Lefebvre 2016; Wolfson and Hooper 1989). In 1991, Bayer AG first introduced enrofloxacin into the market under the name Baytril® for oral dosage in poultry. Enrofloxacin is currently administered either orally or via injection for the treatment of house pets and livestock.

Depending on dosage method and recipient, enrofloxacin is largely metabolised to its main metabolite ciprofloxacin, which also functions as an antibacterial. Both substances are, among other elimination pathways, excreted with the urine. Ciprofloxacin itself, which also belongs to the fluoroquinolone class, is used as an antibiotic in human medicine.

Based on the antibacterial activity and the accompanying risk to human health by the intake of enrofloxacin, for example via food products, an acceptable daily intake (ADI) of 0–2 µg/kg body weight per day (WHO 1998) or 6.2 µg/kg body weight per day (EMA 2002) was established for enrofloxacin. The elimination half-life of enrofloxacin is approximately 2–4 h (Paul et al. 2019).

Lincomycin is an antibiotic of the lincosamide class and is used, in Germany, exclusively in veterinary medicine. Lincomycin is primarily applied for the treatment of anaerobic gram-positive and gram-negative bacteria. Its effects rely on the inhibition of bacterial protein synthesis (Spížek and Řezanka 2004). For lincomycin, which may be ingested via food products, an ADI of 0–30 µg/kg body weight has been established (WHO 2004). The elimination half-life of lincomycin is approximately 2–4 h (Paul et al. 2019).

Penicillin G was the first penicillin to be produced and used on a large scale in 1943. While penicillin G was obtained by fermentation, the first synthetically produced penicillin—penicillin V—could be manufactured shortly thereafter in 1957 (Arnaud 2005; Gaynes 2017).

Penicillin V and penicillin G are particularly used in the treatment of gram-positive bacteria. Their action is based on disrupting the formation of the bacterial cell wall. It is recommended to limit the daily intake of both penicillin G and penicillin V to less than 30 μg (EMEA 1999). The elimination half-life of penicillin G and penicillin V is less than 1 h (Brodt and Smollich 2013).

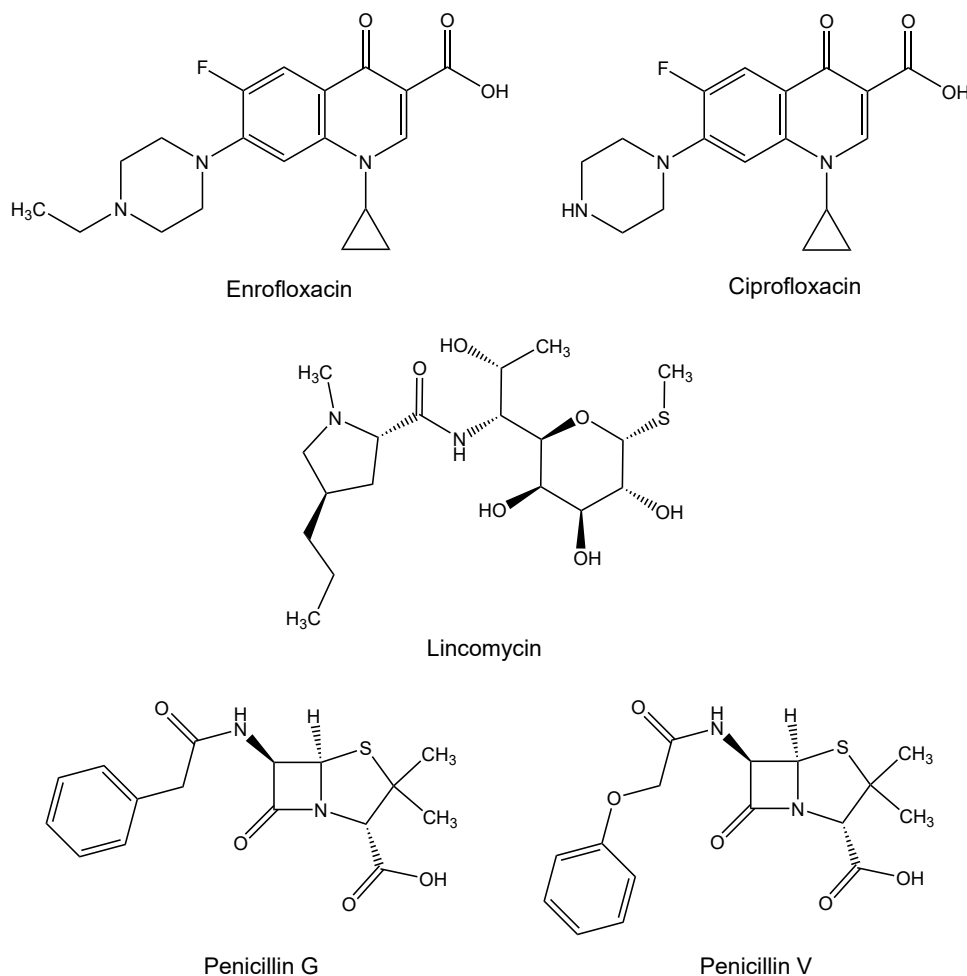


Fig. 1 Structural formulas of ciprofloxacin, enrofloxacin, lincomycin, penicillin G, and penicillin V

The method described herein was used to detect occupational exposure to veterinary antibiotics on poultry farms. The urinary excretion of each antibiotic within 24 h was 0.23 to 18.4 $\mu\text{g}/24\text{ h}$ (sum of enrofloxacin and ciprofloxacin; $n = 4$), 0.80 to 1.9 $\mu\text{g}/24\text{ h}$ (lincomycin; $n = 2$), and 0.44 to 14.4 $\mu\text{g}/24\text{ h}$ (penicillin V; $n = 2$) (Paul et al. 2019).

3 General principles

Selected antibiotics (ciprofloxacin, enrofloxacin, lincomycin, penicillin G, and penicillin V) are determined in human urine. Sample preparation includes extracting the analytes by solid-phase extraction, followed by concentrating the eluates under a stream of nitrogen. The analytes are separated from matrix compounds by liquid chromatography

and subsequently detected with tandem mass spectrometry using electrospray ionisation. Quantitative evaluation is carried out via external calibration in urine.

4 Equipment, chemicals, and solutions

4.1 Equipment

- HPLC system (e.g. Agilent 1260) comprised of a degasser, a binary pump, an autosampler, a thermostat, and a column oven (TCC) (e.g. Agilent Technologies Germany GmbH & Co. KG, Waldbronn, Germany)
- Tandem mass spectrometer (e.g. QTRAP 5500, AB SCIEX Germany GmbH, Darmstadt, Germany)
- HPLC column: Kinetex C18 (2.6 μm ; 50 \times 2.1 mm) (e.g. Phenomenex Ltd. Deutschland, Aschaffenburg, Germany)
- Vacuum unit (e.g. VisiPrepTM SPE Vacuum Manifold, Merck KGaA, Darmstadt, Germany)
- Blow-off station (e.g. TurboVap[®] LV, Biotage Sweden AB, Uppsala, Sweden)
- Analytical balance (e.g. Sartorius AG, Göttingen, Germany)
- pH meter (e.g. Mettler-Toledo GmbH, Gießen, Germany)
- Vortex mixer (e.g. Multi Reax, Heidolph Instruments GmbH + Co. KG, Schwabach, Germany)
- SPE cartridges (Oasis HLB, 6 ml, 150 mg) (e.g. Waters GmbH, Eschborn, Germany)
- Syringe filter with a regenerated cellulose membrane (\varnothing 13 mm, 0.45 μm) (e.g. MULTOCLEAR-13, CS-Chromatographie Service GmbH, Langerwehe, Germany)
- 60-ml polypropylene SPE cartridges with 20- μm polyethylene frits (e.g. Supelco, Merck KGaA, Darmstadt, Germany)
- 15-ml polypropylene tubes (e.g. Kuhnle GmbH, Karlsruhe, Germany)
- 1.5-ml polypropylene vials (e.g. CS-Chromatographie Service GmbH, Langerwehe, Germany)
- Screw caps (e.g. CS-Chromatographie Service GmbH, Langerwehe, Germany)
- Various volumetric flasks (e.g. witeg Labortechnik GmbH, Wertheim, Germany)
- Various beakers (e.g. BRAND GMBH + CO KG, Wertheim, Germany)
- Various pipettes and Multipettes[®] with matching pipette tips (e.g. Eppendorf AG, Hamburg, Germany)
- Urine cups (z.B. Sarstedt AG & Co. KG, Nümbrecht, Germany)

4.2 Chemicals

- Acetonitrile, LC-MS purity (e.g. No. 9821, Th. Geyer GmbH & Co. KG, Renningen, Germany)
- Formic acid, 98–100% (e.g. No. 5.33002, Merck KGaA, Darmstadt, Germany)
- Ciprofloxacin (e.g. No. 17850, Merck KGaA, Darmstadt, Germany)
- Dichloromethane for HPLC (e.g. No. 34856, Merck KGaA, Darmstadt, Germany)
- Enrofloxacin (e.g. No. 17849, Merck KGaA, Darmstadt, Germany)
- Enrofloxacin-d₅ hydrochloride (e.g. No. CH005, WITEGA Laboratorien Berlin-Adlershof GmbH, Berlin, Germany)
- Ethylenediaminetetraacetic acid (EDTA) disodium salt dihydrate (e.g. No. E4884, Merck KGaA, Darmstadt, Germany)
- Ultra-pure water, LC-MS purity (e.g. No. 9825, Th. Geyer GmbH & Co. KG, Renningen, Germany)
- Isopropanol for liquid chromatography (e.g. No. R34965-1L, Honeywell International Inc., Morristown, USA)

- Lincomycin (e.g. No. L0650000, Merck KGaA, Darmstadt, Germany)
- Lincomycin-d₃ (e.g. No. L466202, Toronto Research Chemicals, Toronto, Canada)
- Methanol, LC-MS purity (e.g. No. 34860, Merck KGaA, Darmstadt, Germany)
- Penicillin G potassium salt (e.g. No. 46609, Merck KGaA, Darmstadt, Germany)
- Penicillin G-d₇ N-ethylpiperidinium salt (e.g. No. 32985, Merck KGaA, Darmstadt, Germany)
- Penicillin V potassium salt (e.g. No. 46616, Merck KGaA, Darmstadt, Germany)
- Penicillin V-d₅ (e.g. No. P223502, Toronto Research Chemicals, Toronto, Canada)
- 1 mol/l hydrochloric acid (e.g. No. 109970, Merck KGaA, Darmstadt, Germany)

4.3 Solutions

- EDTA solution (0.05 g/l)
50 mg of EDTA is weighed into a 1000-ml volumetric flask and dissolved in 900 ml of ultra-pure water. The volumetric flask is made up to the mark with ultra-pure water.
- Water (pH = 4.0)
In a 1000-ml beaker, one litre of ultra-pure water is adjusted to pH = 4.0 using 1 mol/l hydrochloric acid. The acidified water is stored in a laboratory reagent bottle.
- 2% Methanol (v/v)
2 ml of methanol are placed in a 100-ml volumetric flask. The volumetric flask is then made up to the mark with ultra-pure water.
- Methanol : water (10 : 90, v/v)
50 ml of methanol are placed in a 500-ml volumetric flask. The volumetric flask is then made up to the mark with ultra-pure water.
- Methanol : water (50 : 50, v/v)
250 ml of methanol are placed in a 500-ml volumetric flask. The volumetric flask is then made up to the mark with ultra-pure water.
- Acetonitrile : water (10 : 90, v/v)
50 ml of acetonitrile are placed in a 500-ml volumetric flask. The volumetric flask is then made up to the mark with ultra-pure water.
- Mobile phase A (0.1% formic acid in water)
2 ml of formic acid are pipetted into a 2-l volumetric flask. The volumetric flask is then made up to the mark with ultra-pure water.
- Mobile phase B (0.1% formic acid in methanol)
2 ml of formic acid are placed in a 2-l volumetric flask. The volumetric flask is then made up to the mark with methanol.
- Needle-wash solution (acetonitrile : methanol : isopropanol : 0.1% formic acid in water (1 : 1 : 1 : 1; v/v/v/v))
250 ml of acetonitrile, 250 ml of methanol, and 250 ml of isopropanol are placed in a 1000-ml volumetric flask. The volumetric flask is then made up to the mark with 0.1% formic acid in water.

4.4 Internal standards (ISTDs)

- ISTD stock solutions (1000 mg/l)

The ISTD stock solutions are prepared as single-analyte standards. For this purpose, 10 mg of each deuterated standard are weighed into 10-ml volumetric flasks and dissolved in methanol:water (50:50; v/v). The volumetric flasks are each made up to the mark with methanol:water (50:50; v/v) and the solutions thoroughly mixed.

To improve the solubility of the deuterated standard of enrofloxacin, 100 µl of 1 mol/l HCl may be added if necessary. The stock solutions are transferred to 15-ml polypropylene tubes and stored at -20 °C.

- ISTD working solutions I (10 mg/l)

The ISTD working solutions I are prepared as single-analyte standards. For this purpose, 100 µl of each stock solution are pipetted into 10-ml volumetric flasks and the volumetric flasks are made up to the mark with methanol:water (10:90; v/v).

The ISTD working solutions I are transferred into 15-ml polypropylene tubes and stored at -20 °C.

- ISTD working solution II (enrofloxacin-d₅ and lincomycin-d₃; 1 mg/l)

For the ISTD working solution II, 100 µl of each of the ISTD working solutions I for enrofloxacin-d₅ and lincomycin-d₃ are mixed with 800 µl of methanol:water (10:90; v/v) in a 1.5-ml vial.

The ISTD working solution II for enrofloxacin-d₅ and lincomycin-d₃ is stored in the refrigerator at 4 °C.

4.5 Calibration standards

- Stock solutions (1000 mg/l)

The stock solutions are prepared as single-substance standards. For this purpose, 10 mg of the pure substance of each analyte are weighed into a 10-ml volumetric flask and dissolved in methanol:water (50:50; v/v). The volumetric flasks are each made up to the mark with methanol:water (50:50; v/v) and the solutions are thoroughly mixed.

The solubility of the pure substances for enrofloxacin and ciprofloxacin can be improved by the addition of 100 µl of 1 mol/l HCl.

The stock solutions are transferred into 15-ml polypropylene tubes and stored at -20 °C.

- Working solutions I (10 mg/l)

The working solutions I are prepared as single-substance standards. For this purpose, 100 µl of each stock solution are pipetted into 10-ml volumetric flasks; the flasks are then made up to the mark with methanol:water (10:90; v/v) for ciprofloxacin, enrofloxacin, and lincomycin or with acetonitrile:water (10:90; v/v) for penicillin G and penicillin V.

The working solutions I are transferred into 15-ml polypropylene tubes and stored at -20 °C.

- Working solution II for ciprofloxacin, enrofloxacin, and lincomycin (1 mg/l)

For the working solution II (multisubstance standard for ciprofloxacin, enrofloxacin, and lincomycin), 100 µl of each working solution I for ciprofloxacin, enrofloxacin, and lincomycin are mixed with 700 µl methanol:water (10:90; v/v) in a 1.5-ml vial.

The working solution II for ciprofloxacin, enrofloxacin, and lincomycin is stored in the refrigerator at 4 °C.

- Working solutions II for penicillin G or penicillin V (1 mg/l)

For the working solutions II (single-substance standards for penicillin G or penicillin V), 100 µl of each working solution I for penicillin G or penicillin V are mixed with 900 µl acetonitrile:water (10:90; v/v) in a 1.5-ml vial.

The working solutions II for penicillin G or penicillin V are stored in the refrigerator at 4 °C.

Calibration standards are prepared according to the pipetting schemes given in Tables 1–3 by spiking pooled urine with the corresponding volumes of the working solutions. The calibration standards thus prepared are processed as described in Section 5. The tables also contain the pipetting schemes for the individual quality-control samples (see Section 10).

Tab. 1 Pipetting scheme for the preparation of calibration standards for the determination of ciprofloxacin, enrofloxacin, and lincomycin in urine

| Calibration standard | Working solution I [μl] | Working solution II [μl] | ISTD working solution II [μg] | Pooled urine [ml] | Analyte concentration [μg/l] |
|----------------------|-------------------------|--------------------------|-------------------------------|-------------------|------------------------------|
| 00 | – | – | – | | 0.00 |
| 0 | – | – | 50 | | 0.00 |
| 1 | – | 10 | 50 | | 0.10 |
| 2 | – | 20 | 50 | | 0.20 |
| 3 | – | 30 | 50 | | 0.30 |
| 4 | – | 40 | 50 | ad 100 | 0.40 |
| 5 | – | 50 | 50 | | 0.50 |
| 6 | – | 60 | 50 | | 0.60 |
| 7 | 10 | – | 50 | | 1.00 |
| Q _{low} | – | 15 | 50 | | 0.15 |
| Q _{high} | – | 55 | 50 | | 0.55 |

Tab. 2 Pipetting scheme for the preparation of calibration standards for the determination of penicillin G in urine

| Calibration standard | Working solution I [μl] | Working solution II [μl] | ISTD working solution I [μg] | Pooled urine [ml] | Analyte concentration [μg/l] |
|----------------------|-------------------------|--------------------------|------------------------------|-------------------|------------------------------|
| 00 | – | – | – | | 0.00 |
| 0 | – | – | 10 | | 0.00 |
| 1 | – | 10 | 10 | | 0.10 |
| 2 | – | 25 | 10 | | 0.25 |
| 3 | – | 50 | 10 | | 0.50 |
| 4 | 10 | – | 10 | ad 100 | 1.00 |
| 5 | 20 | – | 10 | | 2.00 |
| 6 | 30 | – | 10 | | 3.00 |
| Q _{low} | – | 25 | 10 | | 0.25 |
| Q _{high} | 10 | – | 10 | | 1.00 |

Tab. 3 Pipetting scheme for the preparation of calibration standards for the determination of penicillin V in urine

| Calibration standard | Working solution I [μl] | Working solution II [μl] | ISTD working solution I [μg] | Pooled urine [ml] | Analyte concentration [μg/l] |
|----------------------|-------------------------|--------------------------|------------------------------|-------------------|------------------------------|
| 00 | – | – | – | | 0.00 |
| 0 | – | – | 10 | | 0.00 |
| 1 | – | 30 | 10 | | 0.30 |
| 2 | – | 50 | 10 | | 0.50 |
| 3 | 10 | – | 10 | ad 100 | 1.00 |
| 4 | 20 | – | 10 | | 2.00 |
| 5 | 30 | – | 10 | | 3.00 |
| 6 | 40 | – | 10 | | 4.00 |
| Q _{low} | – | 50 | 10 | | 0.50 |
| Q _{high} | 25 | – | 10 | | 2.50 |

5 Specimen collection and sample preparation

5.1 Specimen collection

Urine samples are collected in sealable plastic containers and stored at -20°C until analysis. Prior to analysis, the samples are brought to room temperature and thoroughly mixed.

5.2 Sample preparation

100 ml of the urine samples are mixed with 2 ml of the EDTA solution. Subsequently, 50 μl of the ISTD working solution II (enrofloxacin-d₅ and lincomycin-d₃) as well as 10 μl each of the ISTD working solutions I (penicillin G-d₇ and penicillin V-d₃) are added.

Purification of the urine sample is performed by solid-phase extraction (SPE) using Oasis HLB cartridges. The cartridges are first conditioned with 5 ml of methanol and then with 5 ml of water (pH = 4.0). The 60-ml empty cartridges with 20-μm frits are placed on the SPE cartridges; the samples are then transferred through the 60-ml cartridges onto the SPE cartridges applying a vacuum (about 4 ml/min). After the sample has passed through, the SPE cartridges are washed with 2 ml of 2% methanol and dried for 5 min under a vacuum. The analytes and ISTDs are eluted from the SPE cartridges twice, each time with 5 ml methanol; the eluates are collected in 15-ml polypropylene vials.

200 μl of ultra-pure water are added to each of the eluates. The eluates are then reduced to 200 μl under a stream of nitrogen at 50°C .

For the determination of ciprofloxacin, enrofloxacin, and lincomycin, 100 μl of the concentrated eluates are pipetted off and mixed with 400 μl of methanol: water (10 : 90; v/v). For the determination of penicillin V, the remaining 100 μl of the concentrated eluate are mixed with 400 μl of acetonitrile: water (10 : 90; v/v). If penicillin G is to be determined instead, 400 μl of ultra-pure water are added to the remaining 100 μl of the concentrated eluate.

The measurement solutions thus prepared are mixed for 20 seconds on the vortex mixer and subsequently filtered into a polypropylene HPLC vial using a 0.2-μm syringe filter.

The verifier of the method added 400 μl of acetonitrile: water (10 : 90; v/v) to the remaining 100 μl of the concentrated eluate and successfully determined both penicillins in parallel in this solution.

6 Operational parameters

Analytical determination was performed using a device configuration comprised of an LC system with a tandem mass spectrometer (LC-MS/MS).

6.1 Liquid chromatography

| | |
|--------------------------|--|
| Analytical column: | Kinetex Core Shell C18; 2.6 µm; 50 × 2.1 mm |
| Column temperature: | 40 °C, isothermal |
| Injection volume: | 10 µl |
| Autosampler temperature: | 7 °C, cooled |
| Needle-wash: | Acetonitrile : methanol : isopropanol : 0.1% formic acid in water (1 : 1 : 1 : 1; v/v/v/v) |
| Mobile phase: | A: 0.1% formic acid in water B: 0.1% formic acid in methanol |
| Flow rate: | 500 µl/min |
| Gradient program: | See Table 4 |

All other parameters must be optimised according to manufacturer specifications.

Tab. 4 Gradient program for the determination of ciprofloxacin, enrofloxacin, lincomycin, penicillin G, and penicillin V in urine

| Time [min] | Mobile phase A [%] | Mobile phase B [%] |
|------------|--------------------|--------------------|
| 0.00 | 98 | 2 |
| 0.30 | 98 | 2 |
| 7.27 | 20 | 80 |
| 7.37 | 1 | 99 |
| 8.28 | 1 | 99 |
| 9.00 | 98 | 2 |
| 13.00 | 98 | 2 |

6.2 Tandem mass spectrometry

| | |
|------------------------------|-------------------------------|
| Source: | Turbo Spray |
| Ionisation: | Electrospray ionisation (ESI) |
| Mode: | Positive |
| Source temperature: | 450 °C |
| Detection mode: | Scheduled MRM |
| Target scan time: | 0.4 s |
| Parameter-specific settings: | see Table 5 |

The instrument-specific parameters must be ascertained and adjusted by the user for the individual MS/MS system used. The instrument-specific parameters mentioned in this section were determined and optimised for the system used for method development.

Tab. 5 Parameter-specific settings and retention times for the determination of ciprofloxacin, enrofloxacin, lincomycin, penicillin G, and penicillin V in urine

| Analyte/ISTD | Q1 [m/z] | Q3 [m/z] | Retention time [min] | DP [V] | CE [eV] | CXP [V] |
|-----------------------------|-------------|-------------|-------------------------|-----------|------------|------------|
| Ciprofloxacin | 332.0 | 314.0 | 4.98 | 70 | 23 | 13 |
| | 332.0 | 288.0 | 4.98 | 70 | 20 | 13 |
| Enrofloxacin | 360.0 | 342.0 | 5.04 | 70 | 30 | 30 |
| | 360.0 | 316.0 | 5.04 | 70 | 30 | 30 |
| Enrofloxacin-d ₅ | 365.0 | 347.0 | 5.04 | 70 | 30 | 30 |
| | 365.0 | 321.0 | 5.04 | 70 | 30 | 30 |
| Lincomycin | 407.5 | 125.5 | 4.06 | 90 | 25 | 25 |
| | 407.5 | 359.0 | 4.06 | 90 | 25 | 25 |
| Lincomycin-d ₃ | 410.0 | 128.6 | 4.06 | 90 | 32 | 25 |
| | 410.0 | 362.0 | 4.06 | 90 | 32 | 25 |
| Penicillin G | 333.0 | 192.0 | 6.93 | -13 | -14 | -15 |
| | 333.0 | 289.0 | 6.93 | -19 | -10 | -7.5 |
| Penicillin G-d ₇ | 340.0 | 199.0 | 6.93 | -25 | -17 | -15 |
| | 340.0 | 296.0 | 6.93 | -25 | -12 | -28 |
| Penicillin V | 350.9 | 159.8 | 7.41 | 15 | 20 | 20 |
| | 350.9 | 113.8 | 7.41 | 15 | 20 | 20 |
| Penicillin V-d ₅ | 355.9 | 159.8 | 7.41 | 15 | 20 | 20 |
| | 355.9 | 113.8 | 7.41 | 15 | 20 | 20 |

7 Analytical determination

Of each of the samples processed according to [Section 5](#), 10 µl are injected into the LC-MS/MS system. The analytes are identified by their specific ions or ion transitions as well as their retention times (see [Table 5](#)). The retention times given in [Table 5](#) can only serve as a point of reference. The user must ensure the separation performance of the column used and the resulting retention behaviour of the analytes. Chromatograms of the lowest respective calibration standards and of a blank urine are shown in [Figure 2](#).

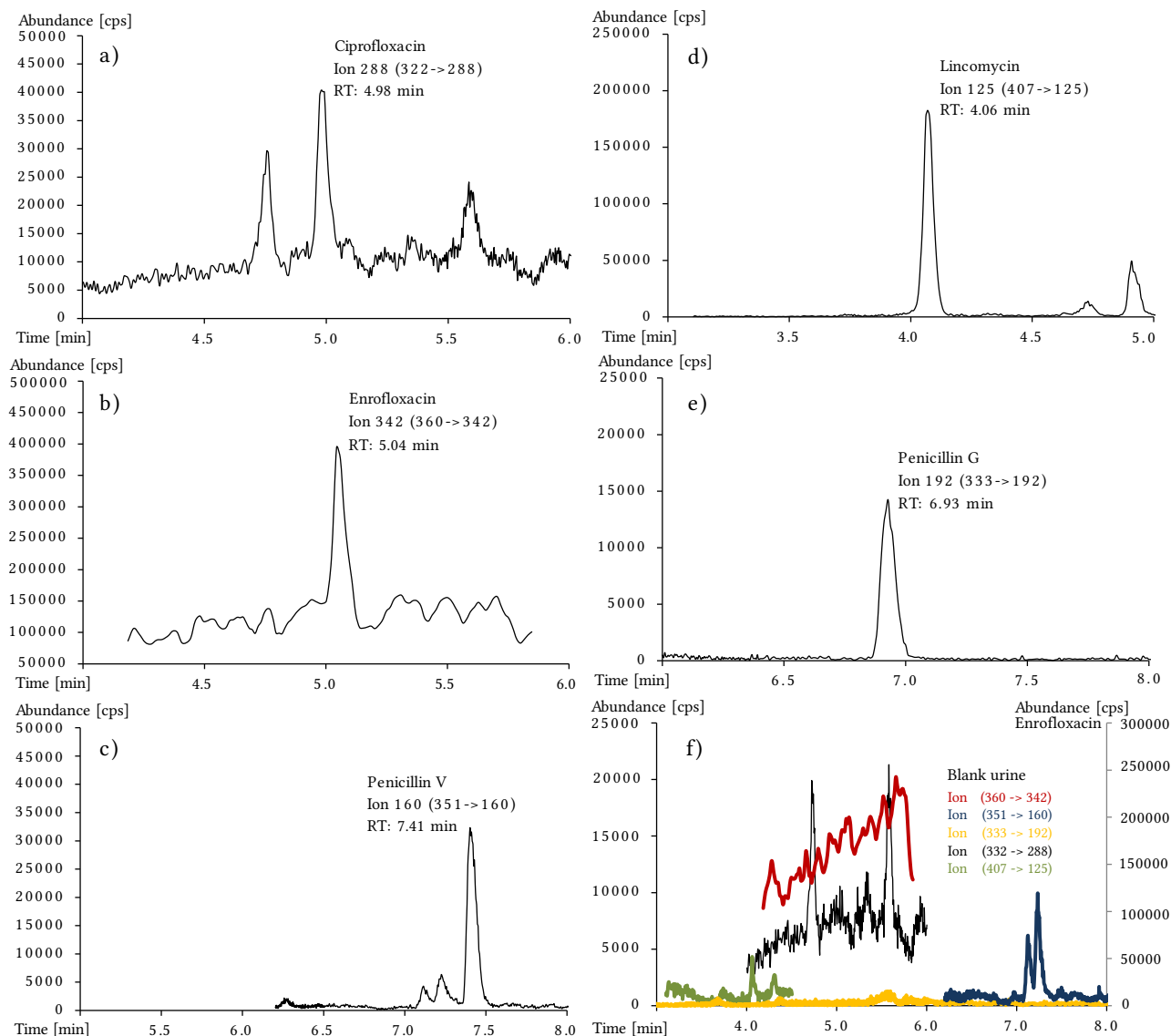


Fig. 2 Chromatograms of the lowest respective calibration standards: urine spiked with a) ciprofloxacin (0.1 µg/l), b) enrofloxacin (0.1 µg/l), c) penicillin V (0.3 µg/l), d) lincomycin (0.1 µg/l), and e) penicillin G (0.3 µg/l); Chromatogram f) shows the respective MRM traces (quantifier) in an unspiked urine sample (blank urine)

8 Calibration

The calibration standards are prepared as described in [Section 4.5](#), processed analogously to the urine samples (see [Section 5.2](#)), and analysed. The calibration curve is generated by plotting the peak-area ratio of the analyte to the deuterated ISTD against the spiked concentration. Enrofloxacin- d_5 is used as ISTD for ciprofloxacin. The calibration curves were linear in the concentration ranges given in [Table 6](#). Representative calibration curves are depicted in [Figure 3.1](#) and [3.2](#).

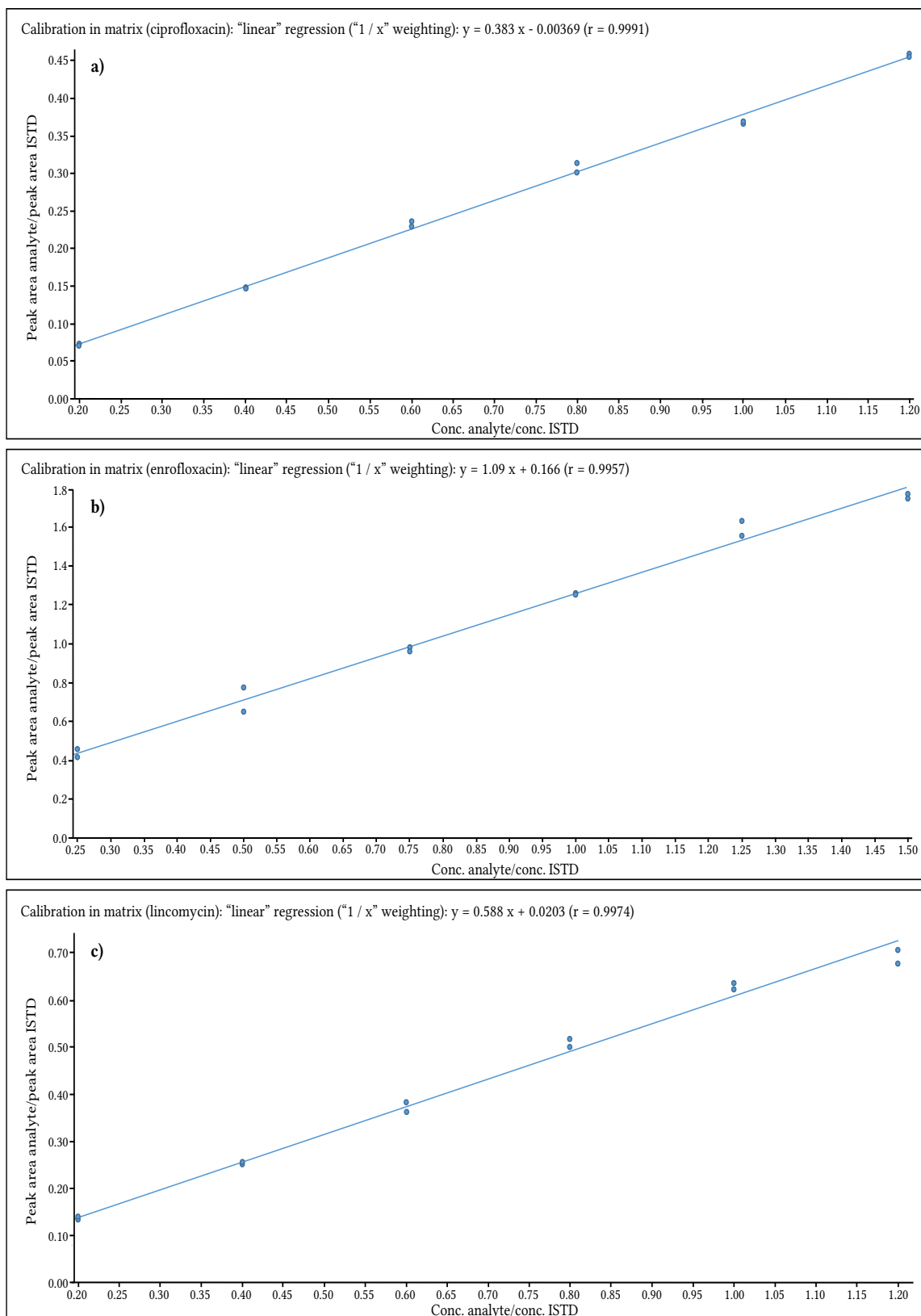


Fig. 3.1 Calibration curves for the determination of a) ciprofloxacin, b) enrofloxacin, c) lincomycin in urine

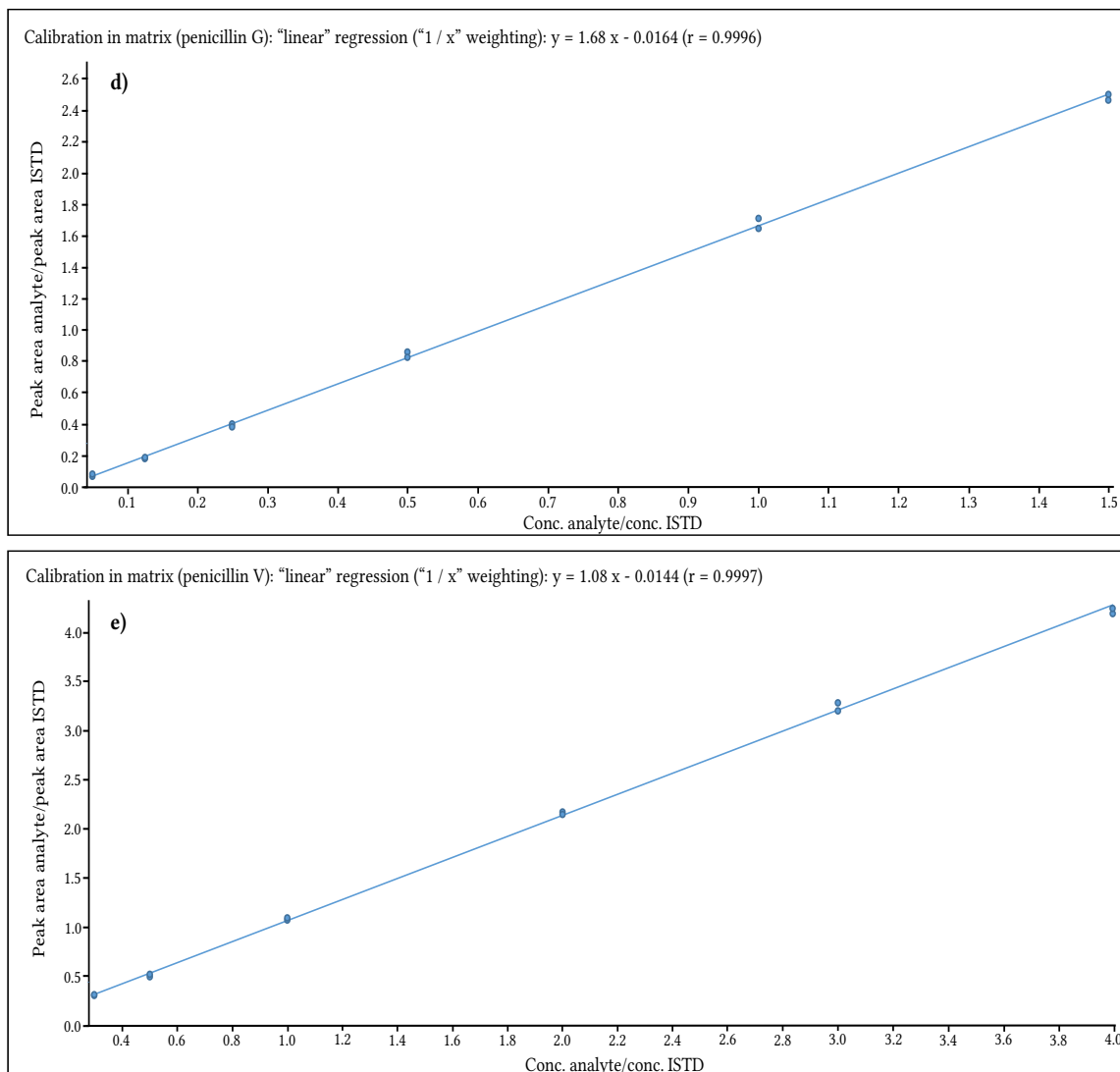


Fig. 3.2 Calibration curves for the determination of d) penicillin G and e) penicillin V in urine

Tab. 6 Calibration ranges for the determination of ciprofloxacin, enrofloxacin, lincomycin, penicillin G, and penicillin V in urine

| Analyte | Calibration range [$\mu\text{g/l}$] |
|---------------|---------------------------------------|
| Ciprofloxacin | 0.10–0.60 |
| Enrofloxacin | 0.10–1.00 |
| Lincomycin | 0.10–1.00 |
| Penicillin G | 0.10–3.00 |
| Penicillin V | 0.30–4.00 |

9 Calculation of the analytical results

The analyte concentration of a urine sample is calculated by dividing the peak area of the analyte by the peak area of the ISTD. Using the calibration function of the respective analytical run, the quotient thus calculated can be used to calculate the analyte concentration in µg/l urine.

If the measured result lies above the calibration range, the sample is diluted with ultra-pure water, reprocessed, and newly analysed.

10 Standardisation and quality control

Quality control of the analytical results is carried out as stipulated in the guidelines of the *Bundesärztekammer* (German Medical Association) and in a general chapter published by the Commission (Bader et al. 2010; Bundesärztekammer 2014).

In order to ensure precision, two quality-control samples with constant analyte concentrations are investigated as part of each analytical run. Since commercial material is not available, the control material must be prepared in the in-house laboratory by spiking urine with standard analyte solutions. The analyte concentrations of the quality-control materials should lie within the relevant concentration range (see Tables 1–3). The nominal values and tolerance ranges of the quality-control materials are determined within a pre-analytical period (one analysis of each quality-control material on ten different days) (Bader et al. 2010).

Moreover, as part of quality control, double-blank and blank values are analysed in each analytical run. The samples of a run are preceded and succeeded by quality-control samples, and at the end of each analytical run, two calibration standards are measured a second time for quality-control purposes.

11 Evaluation of the method

The reliability of this method was confirmed by comprehensive validation as well as by replication and verification in a second, independent laboratory.

11.1 Precision

Within-day precision

For the determination of within-day precision, five quality-control samples of each spiking level (Q_{low} and Q_{high}) were processed and analysed on three days. The within-day precision data thus obtained are presented in Table 7, giving the range of the means of all three runs.

Tab. 7 Within-day precision for the determination of ciprofloxacin, enrofloxacin, lincomycin, penicillin G, and penicillin V in urine (n=5)

| Analyte | Spiked concentration [µg/l] | Standard deviation (rel.) s_w [%] | Prognostic range u [%] |
|---------------|-----------------------------|-------------------------------------|--------------------------|
| Ciprofloxacin | 0.15 | 5.43–6.98 | 15.1–19.4 |
| | 0.55 | 3.25–15.8 | 9.02–43.9 |
| Enrofloxacin | 0.15 | 10.8–20.4 | 30.0–56.6 |
| | 0.55 | 3.67–10.1 | 10.2–28.0 |
| Lincomycin | 0.15 | 5.44–9.65 | 15.1–26.8 |
| | 0.55 | 1.89–15.0 | 5.25–41.6 |

Tab. 7 (continued)

| Analyte | Spiked concentration [µg/l] | Standard deviation (rel.) s_w [%] | Prognostic range u [%] |
|--------------|--------------------------------|--|-----------------------------|
| Penicillin G | 0.25 | 2.40–4.15 | 6.66–11.5 |
| | 1.00 | 1.00–6.27 | 2.78–17.4 |
| Penicillin V | 0.50 | 1.99–5.46 | 5.52–15.2 |
| | 2.50 | 2.52–7.31 | 7.00–20.3 |

Day-to-day precision

For the determination of day-to-day precision, five quality-control samples of each spiking level (Q_{low} and Q_{high}) were processed and analysed on three different days (enrofloxacin: four different days; ciprofloxacin: 1 × 3 technical replicates, 2 × 5 technical replicates). The day-to-day precision data thus obtained are presented in [Table 8](#).

Tab. 8 Day-to-day precision for the determination of ciprofloxacin, lincomycin, penicillin G, and penicillin V in urine (n=3) as well as enrofloxacin in urine (n=4)

| Analyte | Spiked concentration [µg/l] | Standard deviation (rel.) s_w [%] | Prognostic range u [%] |
|---------------|--------------------------------|--|-----------------------------|
| Ciprofloxacin | 0.15 | 7.62 | 32.8 |
| | 0.55 | 14.3 | 61.5 |
| Enrofloxacin | 0.15 | 9.87 | 31.4 |
| | 0.55 | 3.33 | 10.6 |
| Lincomycin | 0.15 | 11.8 | 50.8 |
| | 0.55 | 4.24 | 18.2 |
| Penicillin G | 0.25 | 4.19 | 18.0 |
| | 1.00 | 6.50 | 28.0 |
| Penicillin V | 0.50 | 7.59 | 32.7 |
| | 2.50 | 7.76 | 33.4 |

11.2 Accuracy

The accuracy of the method was ascertained from the data on within-day and day-to-day precision. The data thus obtained are presented in [Table 9](#).

Tab. 9 Mean accuracy for the determination of ciprofloxacin, enrofloxacin, lincomycin, penicillin G, and penicillin V in urine

| Analyte | Spiked concentration [µg/l] | Mean accuracy r (Within-day precision) [%] | Mean accuracy r (Day-to-day precision) [%] |
|---------------|--------------------------------|---|---|
| Ciprofloxacin | 0.15 | 90.8–106 | 98.7 |
| | 0.55 | 88.9–116 | 100 |
| Enrofloxacin | 0.15 | 76.0–99.1 | 91.4 |
| | 0.55 | 98.0–106 | 101 |
| Lincomycin | 0.15 | 90.3–113 | 104 |
| | 0.55 | 96.3–105 | 100 |
| Penicillin G | 0.25 | 99.4–108 | 103 |
| | 1.00 | 90.0–102 | 96.8 |

Tab. 9 (continued)

| Analyte | Spiked concentration [µg/l] | Mean accuracy <i>r</i> (Within-day precision) [%] | Mean accuracy <i>r</i> (Day-to-day precision) [%] |
|--------------|--------------------------------|--|--|
| Penicillin V | 0.50 | 87.0–99.7 | 91.8 |
| | 2.50 | 90.5–106 | 97.9 |

11.3 Limits of detection and quantitation

The limits of detection and quantitation presented in Table 10 were determined using the MRM trace of the quantifier. The limit of detection was determined on the basis of a signal-to-noise ratio of 3:1. The limit of quantitation was similarly ascertained from the tenfold signal-to-noise ratio.

Due to the complexity of the urine matrix, which may be subject to substantial variations, the theoretical quantitation limits were raised. The practical quantitation limits correspond to the analyte concentration thus calculated in the lowest respective calibration standard.

Tab. 10 Limits of detection and quantitation for the determination of ciprofloxacin, enrofloxacin, lincomycin, penicillin G, and penicillin V in urine

| Analyte | Detection limit [µg/l] | Quantitation limit (theoretical) [µg/l] | Quantitation limit (in practice) [µg/l] |
|---------------|---------------------------|--|--|
| Ciprofloxacin | 0.038 | 0.064 | 0.1 |
| Enrofloxacin | 0.043 | 0.072 | 0.1 |
| Lincomycin | 0.0015 | 0.0035 | 0.1 |
| Penicillin G | 0.0036 | 0.0081 | 0.1 |
| Penicillin V | 0.031 | 0.071 | 0.3 |

11.4 Specificity

The specificity of the MRM transitions was investigated using the corresponding chromatographic traces of the double-blank values, blank values, and the respective lowest standard. The selected MRM transitions showed sufficient specificity for all analytes.

11.5 Analyte stability

Stability in urine matrix

The stability of the analytes in the urine matrix was investigated at room temperature and at –20 °C. Stability at room temperature is relevant for sample preparation and was investigated over a period of 24 h. Stability at –20 °C is relevant for sample storage and was investigated over a period of 8 to 12 weeks. These investigations were performed for each analyte at two spiking levels (Q_{low} and Q_{high}) with five replicates each.

After 24 h at room temperature, enrofloxacin and lincomycin exhibited the worst recovery for the Q_{low} standard with 68% and 65%, respectively. For all other analytes, mean recoveries between 84% and 114% were determined. The data on mean recoveries are presented in Table 11.

The investigations on storage stability at –20 °C showed that the enrofloxacin concentrations decrease over time. As such, the mean recovery after twelve weeks was about 77% for both the Q_{low} and the Q_{high} samples. Mean relative recoveries of 90–106% were determined for all other analytes.

Tab. 11 Analyte stability in the urine matrix

| Analyte | Spiked concentration [µg/l] | Storage at room temperature | | Storage at -20 °C | |
|---------------|-----------------------------|-----------------------------|-----------------------------|--------------------------|-----------------------------|
| | | Storage duration [h] | Rel. recovery Mean ± SD [%] | Storage duration [weeks] | Rel. recovery Mean ± SD [%] |
| Ciprofloxacin | 0.15 | 24 | 84.1 ± 4.5 | 12 | 106 ± 8.1 |
| | 0.55 | 24 | 114 ± 4.4 | 12 | 106 ± 11.0 |
| Enrofloxacin | 0.15 | 24 | 68.1 ± 7.3 | 12 | 77.1 ± 7.3 |
| | 0.55 | 24 | 95.2 ± 2.6 | 12 | 76.7 ± 7.3 |
| Lincomycin | 0.15 | 24 | 64.5 ± 6.3 | 12 | 96.4 ± 10.0 |
| | 0.55 | 24 | 107 ± 2.3 | 12 | 105 ± 13.2 |
| Penicillin G | 0.50 | 24 | 99.1 ± 5.1 | 8 | 95.0 ± 1.6 |
| | 1.00 | 24 | 105 ± 2.9 | 8 | NA |
| Penicillin V | 0.50 | 24 | 84.2 ± 3.3 | 8 | 89.6 ± 4.2 |
| | 2.50 | 24 | 96.6 ± 4.2 | 8 | 92.7 ± 2.1 |

NA: Not analysed due to lack of spiking

Analyte stability in the sample extracts

Analyte stability in the sample extracts at -20 °C was measured over a period of three to six weeks. These investigations were performed five times for each analyte at two spiking levels (Q_{low} and Q_{high}).

After 42 days, the mean recoveries for penicillin G were 62% and 58%; shortening the storage period to 14 days resulted in mean recoveries of 91% and 125%. Regarding enrofloxacin, it should be noted that two outliers occurred per spiking concentration; thus storage periods of less than 22 days are recommended for extracts containing this antibiotic.

Data on analyte recovery after storage of sample extracts are presented in [Table 12](#).

Tab. 12 Analyte stability in the sample extracts

| Analyte | Spiked concentration [µg/l] | Rel. recovery after storage at -20 °C | |
|---------------|-----------------------------|---------------------------------------|-------------------------|
| | | Storage duration [d] | Mean ± SD [%] |
| Ciprofloxacin | 0.15 | 27 | 96.3 ± 8.4 |
| | 0.55 | 27 | 99.5 ± 7.4 |
| Enrofloxacin | 0.15 | 22 | 101 ± 26 ^{a)} |
| | 0.55 | 22 | 99.6 ± 34 ^{a)} |
| Lincomycin | 0.15 | 27 | 125 ± 12 |
| | 0.55 | 27 | 118 ± 8.0 |
| Penicillin G | 0.50 | 42 | 61.5 ± 2.3 |
| | 1.00 | 42 | 58.4 ± 5.4 |
| | 0.50 | 14 | 90.5 ± 1.8 |
| | 1.00 | 14 | 78.6 ± 5.6 |
| Penicillin V | 0.50 | 27 | 110 ± 5.1 |
| | 2.50 | 27 | 111 ± 8.2 |

^{a)} two outliers in the five replicates

Analyte stability in the extracts at -20 °C was tested by the verifying laboratory over the entire calibration range of each analyte. These tests showed that ciprofloxacin, enrofloxacin, and lincomycin were stable in the extracts for at least 63 days and both penicillins for at least 28 days.

11.6 Sources of error

Interference-free chromatograms were obtained for all of the analytes with the method. Even in the blank and the double-blank samples, the ion traces of the analytes were not disturbed. During external method verification, however, there were overlaps with interfering peaks at ion trace $m/z=314$ of ciprofloxacin in samples with a high matrix load, which influenced the quantifier/qualifier ratio. In these cases, the ion traces $m/z=288$ (quantifier) and $m/z=231$ (qualifier) were used.

Lincomycin and enrofloxacin as well as its main metabolite ciprofloxacin show a linear working range of 0.10–1.0 µg per litre urine and 0.10–0.60 µg (ciprofloxacin) per litre urine, respectively. During external method verification, a linear working range of 0.10–1.00 µg per litre urine was also found for ciprofloxacin. For penicillin G and penicillin V, the calibration curves were linear over concentration ranges of 0.10–3.00 µg/l urine and 0.30–4.00 µg/l urine, respectively.

During external method verification, the negative ionisation mode was used for the quantification of the penicillins, which proved to be more sensitive with the instrument configuration applied (Shimadzu Nexera XR (HPLC system) with SCIEX QTRAP 5500 (triple-quadrupole mass spectrometer)).

The robustness of the method when using smaller sample volumes was investigated for the antibiotics enrofloxacin and lincomycin. It was shown that sample volumes between 20 ml and 100 ml can be used without affecting the validity of the method. This should similarly apply to ciprofloxacin, penicillin G, and penicillin V.

In general, depending on the antibiotics to be quantified, the calibration standards can also be prepared as multi-element standards containing all five analytes.

12 Discussion

The LC-MS/MS method presented here allows for the sensitive and precise determination of ciprofloxacin, enrofloxacin, lincomycin, penicillin G, and penicillin V in human urine. The good precision data and accuracy data show that the method provides reliable and accurate measurement values. Any matrix effects are effectively compensated for by the use of isotope-labelled internal standards. This also holds true for ciprofloxacin, for which the isotope-labelled enrofloxacin was used as ISTD. With quantitation limits of 0.1 µg/l for ciprofloxacin, enrofloxacin, and lincomycin as well as 0.3 µg/l for penicillin G and penicillin V, this method is very sensitive and allows for the reliable quantitation of occupational exposure to the selected antibiotics. The method has already been used successfully in a field study (Paul et al. 2019).

Instruments used HPLC system (Agilent 1260) comprised of a degasser, a binary pump, an autosampler, a thermostat, and a column oven (TCC) (Agilent Technologies Germany GmbH & Co. KG, Waldbronn, Germany); HPLC column: Kinetex C18 (2.6 µm; 50 × 2.1 mm) (Phenomenex Ltd. Deutschland, Aschaffenburg, Germany); Tandem mass spectrometer (QTRAP 5500, AB SCIEX Germany GmbH, Darmstadt, Germany).

Notes

Competing interests

The established rules and measures of the Commission to avoid conflicts of interest (www.dfg.de/mak/conflicts_interest) ensure that the content and conclusions of the publication are strictly science-based.

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