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Analysis of antibiotics in urine and wipe samples from environmental and biological monitoring—Comparison of HPLC with UV-, single MS- and tandem MS-detection

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Abstract

Results of the simultaneous determination of the structurally different antibiotics cefazoline, cefotiame, cefuroxime, chloramphenicol, ciprofloxacin, ofloxacin, sulfamethoxazole and trimethoprim from environmental and biological monitoring using high-performance liquid chromatography with UV, single mass and tandem mass spectrometry were compared. For sample enrichment and clean-up a SPE method using bakerbond C18 cartridges was developed. Mean recovery rates were above 70%. Because of the complex urine matrix, only the wipe samples could be analyzed by UV-detection. However, UV-detection and single MS-detection are useful for control measurements after spillage, e.g. (LOD = 1–2 ng/cm^2). Samples from biological monitoring of occupational uptake should be analyzed by LC–MS/MS. The limits of detection (LOD) in urine ranged from 0.4 to 70 μg/L for LC–MS and 0.01 to 0.9 μg/L for LC–MS/MS detection. The limits of detection in wipe samples ranged from 0.003 to 0.13 ng/cm^2.

Keywords: Liquid chromatography-mass spectrometry; Antibiotics; Sulfonamides; β-Lactames; Fluoroquinolones; Wipe Samples; Urine; Environmental and biological monitoring; Occupational exposure

1. Introduction

The occurrence of pharmaceuticals – especially antibiotics and hormones – in the environment and in food as well as the occupational exposure of farm workers caused increasing attention [1–4]. Moreover, occupational exposure of health care personnel against cytotoxic drugs has been studied intensively [5–7] and this resulted in new guidelines for handling these substances in many countries [8–12]. In contrast to the carcinogenic and teratogenic effects of these drugs, long-term exposure to antimicrobial agents has been associated with an increased risk of development and spread of antibiotic resistance [13]. The World Health Report 1998 of the World Health Organisation (WHO) described the increasing occurrence of resistant bacteria and their quick spreading in the world population as one of the biggest health problems of the 21st century [14,15]. Smith and Coast pointed out the requirement for global research and characterized strategies for emergence and transmission of resistance [16].

The aim of this study was to develop an analytical procedure to be applied in the biological and environmental monitoring of antibiotics in health care facilities which has not been investigated to our knowledge so far.

In order to enhance performance of larger monitoring studies, a method for simultaneous analysis (sample pre-treatment and determination) of as many agents as possible is necessary. The analyzed compounds have been selected with special attention to the quantities consumed [17,18]. We have chosen the structurally very different antibiotics cefazoline, cefotiame, cefuroxime, chloramphenicol, ciprofloxacin, ofloxacine, sulfamethoxazole and trimethoprim for method development (Fig. 1).

Several articles and reviews describe the analysis of the selected compounds by HPLC-UV and LC–MS from

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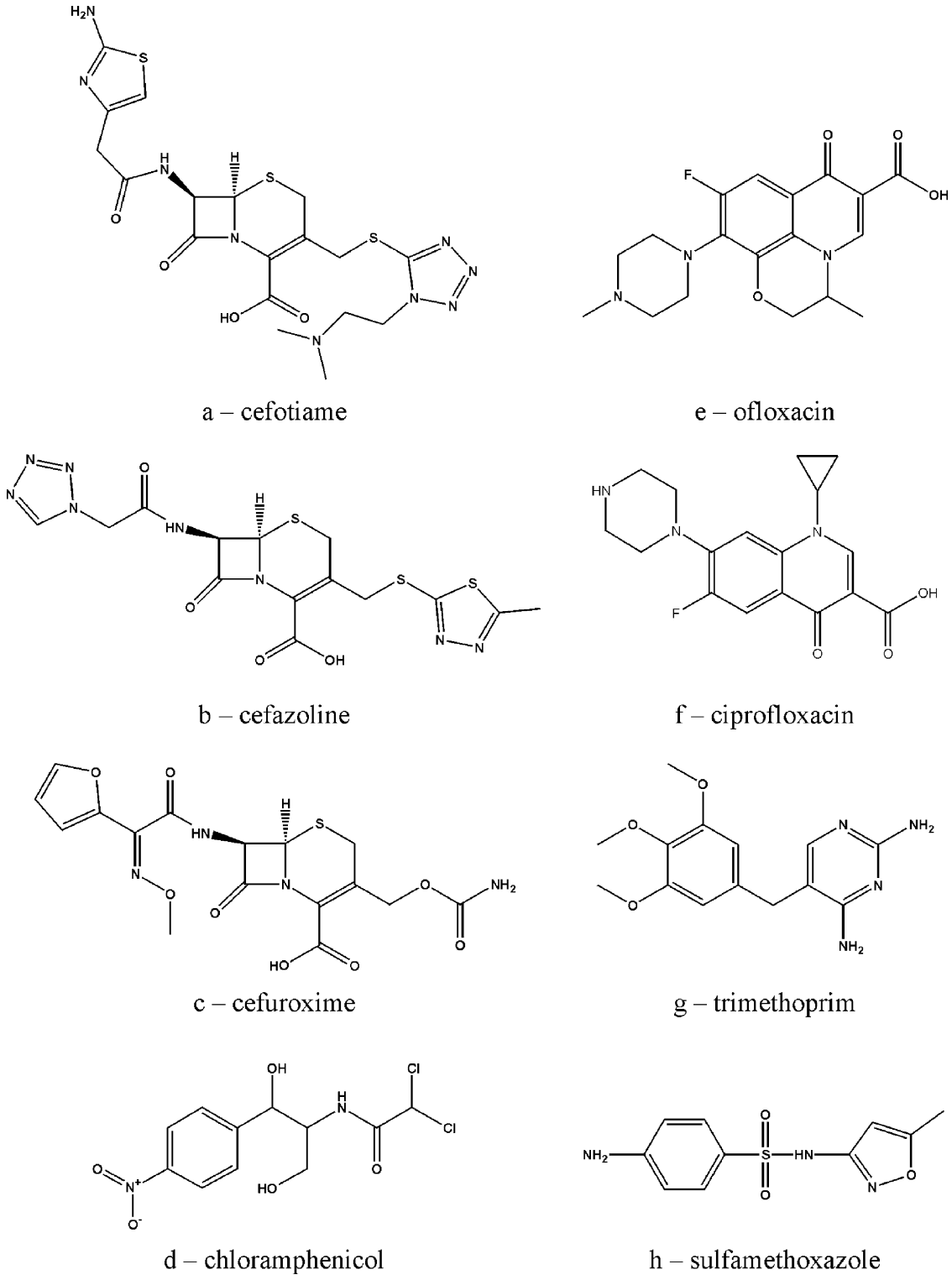

Fig. 1. Chemical structures of selected antibiotics.

pharmacokinetic studies or determination of drug residues in food products [19–23]. Recently several methods have been developed for the simultaneous determination of pharmaceuticals in the aqueous environment using solid-phase extraction (SPE) and detection by liquid chromatography–tandem mass spectrometry [24–26].

Because of the structural and chemical differences of the analysed pharmaceuticals, especially sample enrichment and clean-up by SPE is difficult and requires a separation of the compounds in groups of similar polarity. Hirsch et al. [27] solved this problem by lyophilisation of water samples. Due to the complex urine matrix, this is not possible for samples

from biological monitoring. The commonly utilized techniques for the extraction and clean-up of antibiotics from biomatrices involve liquid–liquid extraction (LLE) or solid-phase extraction [28]. Mostly polymeric adsorbents like styrene divinylbenzene (SDB), styrene divinylbenzene-copolymer (ENV+) or hydrophilic–lipophilic balance (HLB) are used for the enrichment of surface water samples. The instrumental detection limits (IDL) range from 0.1 to 10 ng on column [22–27,29]. Due to the instable β-lactam ring, cephalosporines and penicillines are not included in multi-methods for environmental monitoring of waste and surface waters. β-Lactams from food samples (milk and tissue) were mostly extracted by SPE on C18-, diol- or anion-exchange-cartridges whereas use of LLE is limited because of the instability of β-lactams in methanol and in aqueous acids and bases. These methods are optimized to control the multiple residue limits (MRL) between 5 μg/L for milk up to 300 μg/kg for kidney. Sample clean-up by C18 SPE for food control applications of chloramphenicol is also well described. Gantverg et al. [30] describe a clean-up method for muscle and urine using LPE/C18-SPE and LC–MS/MS detection with a detection limit of 0.02 μg/kg.

Sulfonamides as well as the synergist trimethoprim could be extracted with several different adsorption materials. More difficult is the integration of flouroquinolone antibiotics to a multi-method. The main problem is strong adsorption to the SPE materials. But also separation problems were observed. For example Miao et al. [31] extracted quinolones together with sulfonamides, trimethoprim and tetracyclines, but every group is measured separately by LC–MS/MS. To avoid the adsorption of fluoroquinolones on C18 material, the addition of strong elution solvents like tetrahydrofuran is often described [32–35]. The IDL's for ciprofloxacin range from 0.02 ng on column measured by triple quadrupole mass spectrometry [31], and 0.1 ng on column with fluorescence to 4 ng on column for UV-detection [36].

2. Experimental

2.1. Chemicals

Acetonitrile (HPLC-grade) and tetrahydrofuran (picograde) were purchased from LGC Promochem (Wesel, Germany). High-purity water was prepared by a Millipore Elix 10 water purification system (Millipore, Eschborn, Germany). Reference compounds and formic acid were delivered from Sigma–Aldrich (Taufkirchen, Germany). Stock solutions (0.5 g/L) were prepared in acetonitrile–water (1:1, v/v) and stored at 4 °C up to three months. The calibration standards were dissolved in deionized water or blank urine for matrix calibration.

2.2. Environmental monitoring

The contamination of working surfaces was analysed using wipe samples. These samples were obtained by wiping spiked test surfaces with three 20 cm × 21 cm KIMWIPES® Lite 100 tissues (Kimberly-Clark, Mainz, Germany), each wetted with 1 mL deionized water. The three tissues were extracted with 25 mL deionized water in a 50 mL PE-Tube (Greiner bio-one, Solingen, Germany) for 15 min by sonification. Prior to injection the extracts were filtered through a 0.45 μm cellulose acetate syringe filter (Schleicher & Schuell, Dassel, Germany).

2.3. Solid-phase extraction of urine

Bakerbond C18 SPE cartridges from Baker (1000 mg/6 mL, Deventer, The Netherlands) were conditioned with 6 mL methanol and 6 mL deionized water. A solid-phase extraction manifold (Baker, Deventer, The Netherlands) with a PTFE stopcock and needles was used. The urine samples (5 mL) were passed through the cartridge with a flow rate of approximately 5 mL/min and dried for 5 min by sucking air through the column. The antibiotics were eluted with 5 mL of a mixture of methanol–tetrahydrofuran (1:1, v/v). These extracts were dried in a gentle nitrogen stream and redissolved in 1 mL deionized water. Prior to injection, the extracts were filtered through a 0.45 μm cellulose acetate syringe filter (Schleicher & Schuell, Dassel, Germany).

2.4. High-performance liquid chromatography

The first experiments were carried out with a Rheos 2000 HPLC pump (Flux Instruments, Basel, Switzerland) equipped with a HTS-PAL autosampler (CTC Analytics, Zwingen, Switzerland) and a UV 6000 LP diode array detector (Finnigan MAT, Bremen, Germany). This system was additionally coupled with a single mass spectrometer. An Agilent 1100 binary pump with a second HTS-PAL autosampler equipped with a stack cooler for sample storage at 4 °C until injection was used for the triple quadrupole mass spectrometry. For UV-detection we used a 250 mm × 3 mm Nucleosil 100-5 C18 HD (Fig. 2), for single MS-detection a 125 mm × 3 mm Nucleodur 100-5 C18 EC (Figs. 3 and 4) and for tandem mass spectrometry a 125 mm × 2 mm Nucleodur 100-5 C18 EC column (Fig. 5) with binary gradients of 0.1% formic acid in water (v/v, solvent A) and 0.1% formic acid in acetonitrile (v/v, solvent B). The detailed gradients are described in the figure legends. All HPLC columns were purchased from Macherey-Nagel (Düren, Germany).

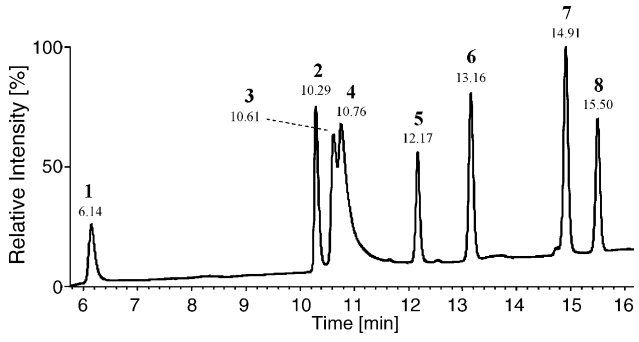

Fig. 2. HPLC-DAD chromatogram of a 5 mg/L standard solution on the 250 mm × 3 mm Nucleosil 100-5 C18 HD column, $\vartheta = 30\,^{\circ}C$, flow rate: 300 μL/min, gradient: 0–1 min 95% A, 15 min 50% A, 17 min 50% A, 18 min 95% A, 22 min 95% A, mobile phase A: 0,1% formic acid in deionized water, mobile phase B: acetonitrile. (**1**) Cefotiame, (**2**) trimethoprim, (**3**) ofloxacin, (**4**) ciprofloxacin, (**5**) cefazoline, (**6**) cefuroxime, (**7**) sulfamethoxazole and (**8**) chloramphenicol.

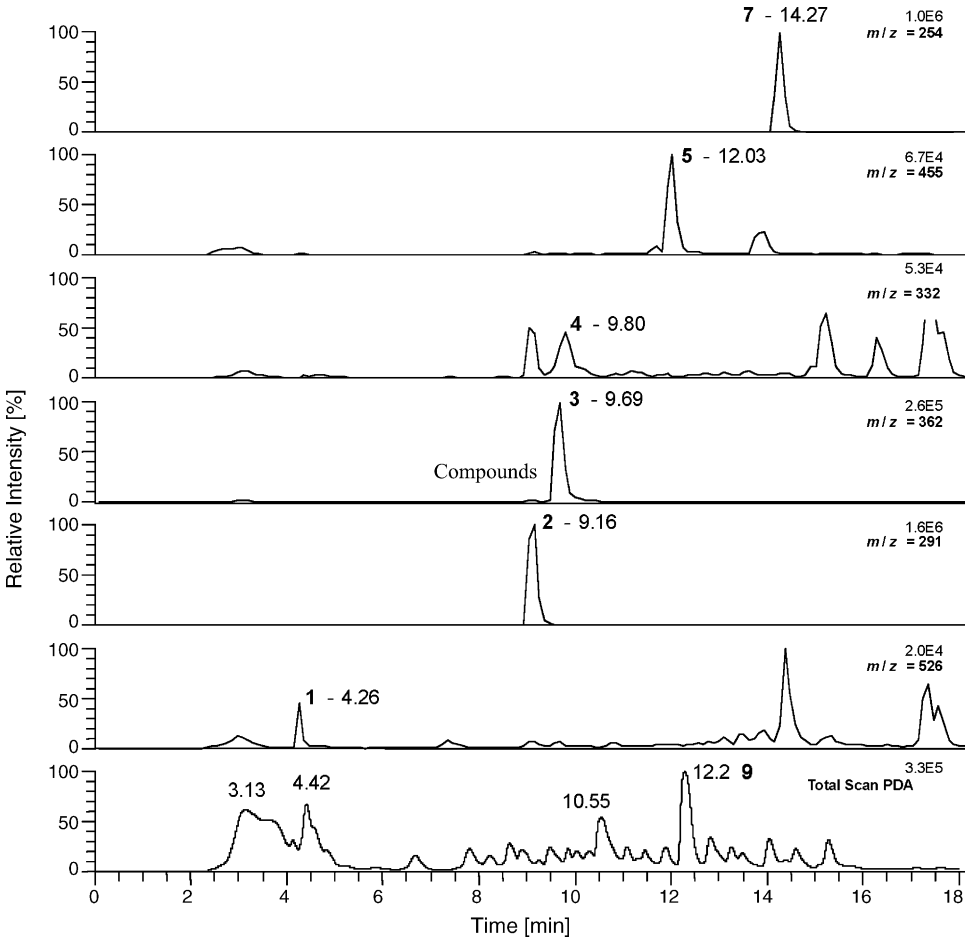

Fig. 3. HPLC-DAD (λ = 200–450 nm) and positive mode LC–MS chromatograms (SIM of six ions) of a spiked urine sample (5 mg/L) on a 125 mm × 3 mm Nucleodur 100-5 C18 EC column. Gradient: 0–1 min 95% A, 14 min 50% A, 16 min 50% A, 17 min 95% A, 20 min 95% A. (**1**) Cefotiame, (**2**) trimethoprim, (**3**) ofloxacin, (**4**) ciprofloxacin, (**5**) cefazoline and (**7**) sulfamethoxazole.

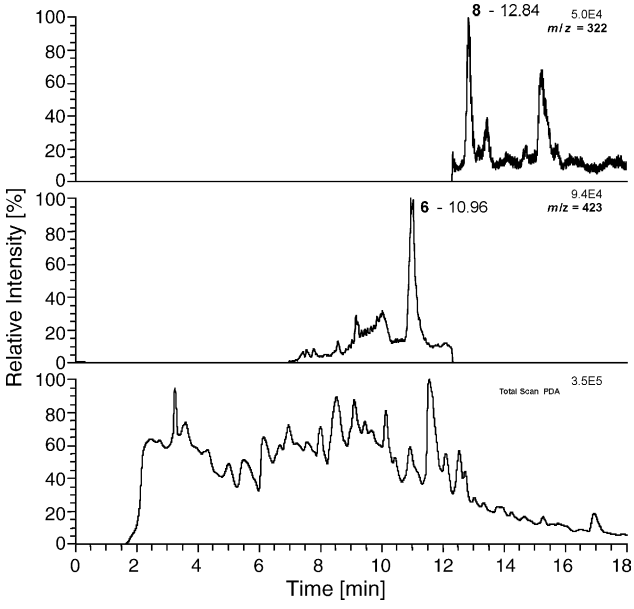

Fig. 4. HPLC-DAD and negative mode LC–MS chromatograms of a spiked urine sample (5 mg/L) on a 125 mm × 3 mm Nucleodur 100-5 C18 EC column. Period 1: 0–12 min SIM of m/z = 423 (**6**, cefuroxime), period 2: 12–20 min SIM of m/z = 322 (**8**, chloramphenicol). Gradient: 0–1 min 95% A, 14 min 50% A, 16 min 50% A, 17 min 95% A, 20 min 95% A.

2.5. Single mass spectrometry

An Automass Multi-mass spectrometer (ThermoQuest Finnigan, Egelsbach, Germany) equipped with an electrospray source operating in positive and negative modes with selected ion monitoring (SIM) was used. The optimised corona and cone voltages are shown together with the precursor ions and the quantification wavelengths of DA-detection in Table 1. The samples were injected twice, first for the positive and second time for the negative mode single MS measurement. The two negative SIM masses were separated in two periods (Fig. 4).

Table 1
UV-detection wavelengths and MS-parameters with optimized corona (CV) and cone voltages

	λ (nm)	m/z (U)	CV (V)	Cone (V)
Cefotiame (**1**)	258	526	+2670	+34
Trimethoprim (**2**)	220	291	+2670	+34
Ofloxacin (**3**)	296	362	+2670	+34
Ciprofloxacin (**4**)	282	332	+2670	+34
Cefazoline (**5**)	272	455	+2670	+34
Cefuroxime (**6**)	278	423	−2670	−7
Sulfamethoxazole (**7**)	270	254	+2670	+34
Chloramphenicol (**8**)	278	322	−2920	−21

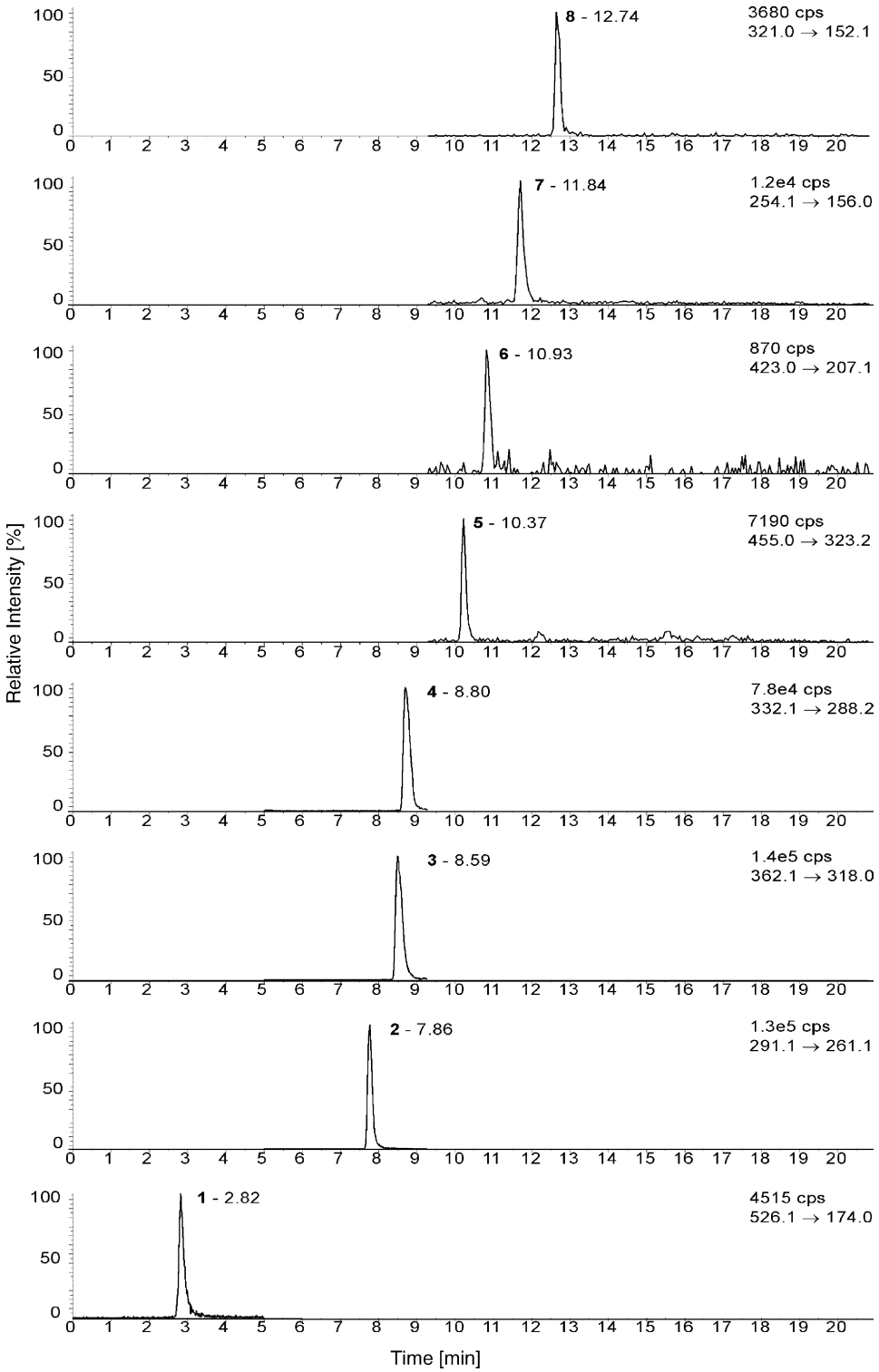

Fig. 5. LC–MS/MS chromatograms of a spiked urine sample (100 μg/L) on a 125 mm × 2 mm Nucleodur 100-5 C18 EC column. $\vartheta = 30\,^{\circ}C$, flow rate: 300 μL/min. Period 1: 0–5 min, period 2: 5–9.2 min, period 3: 9.3–21 min. Gradient: 0–1 min 95% A, 15 min 30% A, 17 min 30% A, 18 min 95% A, 24 min 95% A mobile phase A: 0.1% formic acid in deionized water, mobile phase B: 0.1% formic acid in acetonitrile. (**1**) Cefotiame, (**2**) trimethoprim, (**3**) ofloxacin, (**4**) ciprofloxacin, (**5**) cefazoline, (**6**) cefuroxime, (**7**) sulfamethoxazole and (**8**) chloramphenicol.

2.6. Tandem mass spectrometry

An API 3000 triple quadrupole mass spectrometer (Applied Biosystems, Darmstadt, Germany) equipped with TurboIonSpray™ interface operating at 450 °C in positive and negative modes with ion spray probe voltages of 5000 and −4500 V was used. For measurements in positive and negative mode in one experiment, a settling time of 700 ms

Table 2
Optimized MS/MS-detection parameters

	Orifice voltage (V)	Ring voltage (V)	Precursor ion (U)	Product ion I (U)	Product ion II (U)
Cefotiame (**1**)	31	+240	526.1	174.1	113.0
Trimethoprim (**2**)	56	+320	291.1	261.1	123.1
Ofloxacin (**3**)	61	+340	362.1	318.1	261.1
Ciprofloxacin (**4**)	56	+340	332.1	288.1	245.1
Cefazoline (**5**)	41	+270	455.0	323.1	167.0
Cefuroxime (**6**)	−51	−320	423.1	207.0	318.0
Sulfamethoxazole (**7**)	71	+350	254.1	156.0	92.1
Chloramphenicol (**8**)	−76	−330	322.0	152.0	257.0

was adjusted. The parameter settings for nebulizer, curtain and collision gases (nitrogen each) were 12, 14 and 6 arbitrary units, respectively. Orifice and focusing ring voltage were optimized by continuous flow experiments (Table 2). The analytes were detected by multiple reaction monitoring (MRM). The pause time was set at 5 ms and the dwell time at 150 ms.

2.7. Method validation

The precision and accuracy of the complete method was based on analysis of spiked urine samples. For the single quadrupole LC–MS system only a duplicate analysis was done on one day. The intra- and inter-day means, standard deviations and coefficients of variation (CV) were calculated by standard

Table 3
Intra-day accuracy and precision of the SPE sample clean-up of each analyte in spiked urine samples

	Target concentration (μg/L)	Detected concentration mean ± S.D. (μg/L)	Precision CV (%)	Accuracy	
				Recovery (%)	R.E. (%)
Cefotiame (**1**)	20	19 ± 4	20	95	22
	100	93 ± 3	3.7	93	−4.2
	200	179 ± 4	2.5	89	2.7
	5000	3703 ± 958	26	74	n.d.[a]
Trimethoprim (**2**)	20	18 ± 1	5.1	92	6.0
	100	92 ± 5	5.8	92	−5.8
	200	180 ± 1	0.2	90	0.3
	5000	4920 ± 99	2.0	98	n.d.[a]
Ofloxacin (**3**)	20	16 ± 1	6.4	81	−7.4
	100	97 ± 3	3.4	97	−3.2
	200	192 ± 2	1.1	96	1.2
	5000	4535 ± 113	2.5	91	n.d.[a]
Ciprofloxacin (**4**)	20	14 ± 1	3.2	69	3.6
	100	62 ± 2	2.9	62	3.3
	200	167 ± 4	2.6	83	2.7
	5000	2238 ± 18	0.8	45	n.d.[a]
Cefazoline (**5**)	20	14 ± 1	1.9	68	−2.2
	100	85 ± 4	4.8	85	−5.5
	200	232 ± 12	5.2	116	−5.2
	5000	2213 ± 25	1.1	44	n.d.[a]
Cefuroxime (**6**)	20	18 ± 2	8.2	92	8.7
	100	85 ± 4	5.2	85	−5.5
	200	178 ± 9	4.8	89	5.3
	5000	2790 ± 14	0.5	56	n.d.[a]
Sulfamethoxazole (**7**)	20	15 ± 1	7.2	76	−7.9
	100	93 ± 5	5.4	93	5.1
	200	160 ± 7	4.2	80	4.3
	5000	3583 ± 53	1.5	72	n.d.[a]
Chloramphenicol (**8**)	20	14 ± 1	5.7	71	−5.6
	100	79 ± 11	14	79	−15.4
	200	124 ± 2	1.6	62	−1.8
	5000	2440 ± 21	0.9	49	n.d.[a]

The highest level (5000 μg/L) was measured by single LC–MS ($n = 2$), the others by tandem mass spectrometry ($n = 3$).
[a] n.d.: not determined.

Table 4
Inter-day accuracy and precision of the SPE sample clean-up of each analyte in spiked urine samples measured by LC–MS/MS ($n = 9$)

	Target concentration (μg/L)	Detected concentration (mean ± S.D.) (μg/L)	Precision CV (%)	Accuracy	
				Recovery (%)	R.E. (%)
	20	18 ± 3	16	91	22
Cefotiame (**1**)	100	87 ± 8	8.7	87	20
	200	189 ± 17	9.2	95	−16
	20	19 ± 2	9.3	94	−16
Trimethoprim (**2**)	100	93 ± 14	15	93	30
	200	201 ± 33	16	101	−31
	20	16 ± 2	9.5	82	−18
Ofloxacin (**3**)	100	85 ± 4	5.1	85	9.4
	200	190 ± 14	7.2	95	12
	20	12 ± 2	16	62	24
Ciprofloxacin (**4**)	100	56 ± 8	14	56	25
	200	168 ± 28	16	84	−23
	20	12 ± 1	11	61	−16
Cefazoline (**5**)	100	94 ± 10	11	94	−22
	200	188 ± 39	21	94	35
	20	20 ± 3	13	102	20
Cefuroxime (**6**)	100	82 ± 11	14	82	31
	200	188 ± 32	17	94	36
	20	15 ± 2	15	77	−19
Sulfamethoxazole (**7**)	100	94 ± 7	7.6	94	12
	200	182 ± 22	12	91	−25
	20	13 ± 1	10	66	15
Chloramphenicol (**8**)	100	73 ± 8	11	73	−26
	200	122 ± 14	11	61	−19

methods of triplicate experiments for three days using the triple quadrupole mass spectrometer. The accuracy is expressed as recovery rate and the relative error (R.E.) as maximum relative error from the calculated mean concentration.

3. Results and discussion

Extracts from wipe samples could be analyzed directly without prior clean-up. Sample enrichment and clean-up for urine was carried out with solid-phase extraction on baker-bond C18 cartridges. Our results confirm those of Mizuno et al. [33]; that the addition of tetrahydrofuran for the elution of fluoroquinolones from C18-cartridges is necessary. The other pharmaceuticals could be eluted by methanol. Breakthrough experiments with spiked urine showed that 5 mL of the sample could be sucked through the cartridge without the loss of one or more of the eight compounds. Recoveries and standard deviation are shown in Tables 3 and 4. The recovery experiments were carried out in triplicate on different days with the LC–MS/MS system. In comparison to other solid-phase extraction applications, recoveries range for these compounds from 70 to 100%. Except at some points, our recoveries are in the same area. The highest level ($c = 5000$ μg/L) was measured on the single mass spectrometer only on one day. The used single mass spectrometer was not stable enough for triplicates on different days. This was mainly a stability problem of the instrument. Additional problems caused the usage of the system as a LC–MS and as a GC–MS instrument. After switching of the interface it was not possible to reach the same performance as before. Because of these experiences we would recommend to use a mass spectrometer either as GC–MS or LC–MS system.

The limits of detection (LOD) for extracts of the wipe samples and urine samples are shown in Table 5. Enrichment factors and recovery rates are not considered. Therefore, the LOD of the biological monitoring are approximately better by a factor of 5. Under consideration of the mean recovery

Table 5
Limits of detection (signal-to-noise ratio = 3:1) for wipe sample extracts and urine sample extracts using UV-, MS- and MS/MS-detection

	Wipe sample extracts (μg/L)			Urine sample extracts (μg/L)	
	UV	MS	MS/MS	MS	MS/MS
Cefotiame (**1**)	60	100	1	160	2
Trimethoprim (**2**)	75	0.3	0.05	2	0.06
Ofloxacin (**3**)	30	25	0.2	100	0.3
Ciprofloxacin (**4**)	30	45	0.4	200	0.4
Cefazoline (**5**)	60	45	0.8	160	2
Cefuroxime (**6**)	50	25	0.3	75	4
Sulfamethoxazole (**7**)	75	1.2	0.1	9	0.2
Chloramphenicol (**8**)	30	100	2	250	3

Table 6
Intercept, slope and correlation coefficient (r^2) of weighted ($1/x$) matrix-matched and solvent-based standard calibration with TurboIonSpray Ionisation in MRM

Compound	Calibration	Intercept	Slope	r^2
Cefotiame	Matrix	−1.01E2	2.82E2	0.9974
	Standard	−5.05E2	3.24E2	0.9988
Trimethoprim	Matrix	3.74E4	1.94E3	0.9906
	Standard	1.27E5	2.81E4	0.9975
Ofloxacin	Matrix	2.21E4	8.45E2	0.9998
	Standard	−1.53E4	1.36E4	0.9981
Ciprofloxacin	Matrix	1.53E4	1.07E3	0.9988
	Standard	−2.07E4	8.12E3	0.9968
Cefazoline	Matrix	1.38E3	9.52E1	0.9973
	Standard	6.65E2	2.38E3	0.9997
Cefuroxime	Matrix	2.66E3	2.62E1	0.9985
	Standard	2.86E3	1.09E3	0.9992
Sulfamethoxazole	Matrix	2.54E3	1.12E1	0.9900
	Standard	−1.16E3	1.55E3	0.9991
Chloramphenicol	Matrix	−1.12E2	1.27E2	0.9964
	Standard	1.42E4	2.53E3	0.9970

rates and a surface area of 400 cm^2 the limits of detection for environmental monitoring range from 0.003 to 0.13 ng/cm^2.

3.1. Quantification

The analytical performance of the developed methods was evaluated. The limits of detection defined as signal-to-noise ratio (S/N) of 3:1 are presented in Table 5. The instrumental limits of detection (ILD) for LC–MS/MS range from 1 to 80 pg on column. This is the same range as for comparable triple quadrupole mass spectrometers [22–27,29]. The calibration was performed as an external standard calibration with at least five, for the final analysis with LC–MS/MS even with seven, calibration levels from 5 to 1000 μg/L. A matrix effect for urine samples was noticed for all compounds. Therefore, the calibration of urine samples was carried out by matrix calibration using blank urine extracts. Correlation coefficients (r^2) calculated by weighted ($1/x$) regression analysis were better than 0.99 for both matrix and standard calibration (Table 6). The slopes of the matrix-matched calibration curves were less than those of the solvent-based standard calibration curves. This matrix suppression effect was noticed for all compounds. In contrast to this, for wipe samples no matrix effects were observed.

3.2. Accuracy and precision

Precision and accuracy are shown in Tables 3 and 4. Except for cefotiame the CV at 20, 100, 200 and 5000 μg/L ranged from 0.2 to 8.2% and the R.E. from 0.3 to −15.4% for intra-day. The worse CV of 26% for cefotiame at 5000 μg/L is probably a result of instrumental problems caused by the single mass spectrometer. The CV at 20, 100 and 200 μg/L ranged from 5.1 to 21% and the R.E. ranged from −26 to 36% for inter-day.

4. Conclusions

We developed a sensitive multi-method for eight structurally very different antibiotics by HPLC-UV, –MS and –MS/MS. Only wipe samples could be analyzed by UV-detection. Due to the complex urine matrix and the expected concentrations in the lower μg/L range samples from biological monitoring of occupational uptake should be measured by tandem mass spectrometry.

We also developed a well-suited SPE method on C18 cartridges with recovery rates above 70% and an enrichment factor of five. Extension of the method to further antibiotics is also possible. UV-detection and single MS-detection are useful for control measurements of wipe samples after spillage; samples from biological monitoring should be analyzed by LC–MS/MS. The precision and accuracy developed in this method are suitable and sensitive to determine antibiotics in environmental and biological samples.

This new method will be applied to further investigations to quantify contamination of workplaces and uptake by exposed personnel in order to assess and reduce possible health risks. First results showed the occurrence of one or more of the analyzed antibiotics in 93% of the wipe samples ($n = 81$) and 7.5% of the urine samples ($n = 40$).

The detailed results will be published after the release of the study results in a forthcoming publication.

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