DEVELOPMENT OF AN ANALYTICAL METHOD FOR PHARMACEUTICAL RESIDUES IN WATER ON A NANO-UPLC-Q-TOF-MS SYSTEM

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Introduction

The development of analytical sciences during the last decades made it possible to observe a wide spectrum of organics of anthropogenic origin in the environment. In recent years, highly water soluble pharmaceutical residues came in the scope of research due to elevated prescription numbers and consumption of pharmaceuticals in industrial countries. In contrast to rather lipophilic persistent organic pollutants, these substances behave difficult in terms of separation from matrix components such ashumic acids. For multi-residue-analysis of water samples, mass spectrometry coupled with electrospray ionization to high-performance and ultra-performance liquid chromatography (HPLC/UPLC) has evolved as a common approach[1,2]. The analytical systems allow the researcher to monitor organic residues in water samples down to concentrations in the low ppb range or lower without enrichment step. Nevertheless, the sensitive instruments struggle in practice with response issues regarding linearity as well as signal suppression and enhancement[3]. Method development concentrates on compensation of these adverse effects as well as gathering of system specific parameters and configurations for the specific analytical task.

In this context the aim of this work is to develop a novel analytical method for the determination of highly water soluble substances. The method development will cover all aspects needed to carry out an analysis for the selected compounds including sampling, extraction, clean-up and instrumental measurement.As a first step in that direction, the establishment of an instrumental method on the existing analytical equipment for the selected analytes is presented.

Materials and methods

Acetonitrile, methanol and formic acid (all UHPLC/MS grade) used for chromatography and preparation of standard solutions have been purchased from LGC Promochem (Wesel, Germany). Ultrapure water was obtained from a water purification system consisting of a RiOs reverse osmosis unit with Progard 1 Silver Cartridge and a Milli-Q Gradient unit with Quantum EX Ultrapure Organex Cartridge + Q-Guard 1, both from Merck Millipore (Darmstadt, Germany). Ammonium formatewas purchased from Sigma-Aldrich (Steinheim, Germany). Reference standards of Atenolol, Diclofenac, Ibuprofen, Naproxene,Paracetamol and Sulfamethoxazole were obtained from Dr. Ehrenstorfer (Augsburg, Germany), Simazin, Atrazine and Linuron fromRiedl-de Haën (Seelze, Germany) and Carbamazepine from Sigma-Aldrich (Steinheim, Germany).

For the preparation of a 100 ppm multi-standard stock solution of all analytes in acetonitrile, a precision balance SBC 21 purchased from SCALTEC Instruments GmbH (Goettingen, Germany) was used. The graduated cylinder with the stock solution was stored in the fridge at 4 degree Celsius protected from any light sources. It was only used to produce a 10 ppm working solution in acetonitrile by 1:10 dilution. For measuring purposes, unless otherwise stated, further dilutions have been prepared in a mixture of 80:20 of the mobile phase solvents A and B used for chromatography.

Chromatographic analysis and method development was carried out on a Waters nanoAcquity UPLC system (Milford, USA) characterized by a very low analytical flow rate of 0.3 µL/min. The system is equipped with two pumps, one for gradient elution (nano binary solvent manager) and one for sample preconcentration (auxiliary solvent manager). Sample injection was carried out by an autosampler (sample manager) which carries two trays with sample vials in a sampling compartment which is held at a constant temperature of 8 degree Celsius. Chromatographic separation was run on a Waters C-18 HSS-T3 column with the dimensions 75 µm x 150 mm and a particle size of 1.8 µm. Due to the low volume of the analytical column, a trapping column (Waters

Symmetry C-18 180 µm x 20 mm, 5 µm particle size) pre-concentrates the analytes in a injected sample volume of 2 µL. The temperature of the analytical column was held constant at 40 degree Celsius at all time. Solvent A as well as trapping solvent was a mixture of 0.1 % formic acid and 1 % acetonitrile in ultrapure water, solvent B was 0.1 % formic acid in acetonitrile. Washing agents for the autosampler needle were 0.1 % formic acid and 10 % acetonitrile in ultrapure water as weak wash and 0.1 % formic acid in acetonitrile as strong wash. Additionally, the pumps had to be supplied with seal wash which consisted of 10 % acetonitrile in ultrapure water. Solvent A was prepared daily or at least every 72 hours according to the advice of the manufacturerwhich led to a reduction in background in LC-MS. The final chromatographic method started with 2 min of isocratic trapping with a flow rate of 3 µL/min trapping solvent, resulting in rinsing the 2 µL sample loop with 3 loop volumes of trapping solvent in total. During the trapping phase, the outflow of the trapping column was directed into waste. In the meantime, the analytical column was flushed at the analytical flow rate of 0.3 µL/min with the starting gradient of 40 % solvent A. Subsequently, elution startedisocratically by connecting the trapping column with the gradient pump and directing the outlet to the analytical column. The starting gradient of 40 % solvent A was held for 3 min, followed by a gradient of 2 min down to 0 % A whichwas held isocratically for 5 min. After elution of all compounds,the system was set to switch directly back to the starting gradient of 40 % A and to hold that for 15 min (about 10 analytical column volumes) till system equilibration. In total a complete analytical run lasted 28 min.

The chromatographic system was coupled via a nano-electrospray interface to a quadrupol time-of-flight mass spectrometer (Q-TOF-MS) Q-TOF2 from Waters-Micromass. The MS features a quadrupoleanalyser followed by a collision cell and a time-of-flight tube. To gain a stable spray,capillary voltage was adjusted between 1.8 and 2.3 kV. In TOF-MS-mode, which was used for most of the experiments, maximum sensitivity for the analytes could be reached with a cone voltage of 28 V. Collision energy was held constant at 10 V which minimized fractionation of analytes and enabled detection of molecular ion masses. Scanning of the first quadrupole was minimized and adjusted to the narrow mass range needed for small molecule detection. The TOF was operated with a scan time of 0.5 s to achieve enough data points for narrow chromatographic peaks. Ionization behavior and fragmentation spectra of the analytes were gained by directly infusing individual standard solutions with a concentration of 1 ppm via syringe pump into the MS.

Results and discussion

The compounds chosen for method development are listed in table 1. The development presented here incorporates work in positive ionization mode only. For the applied chromatography in nano-scale dimension no other separation technology than C-18 was available.Consequently, the chromatographic method development was concentrated in the optimization of gradient elution. At the beginning of each analytical run, the sample volume of 2 µL had to be pre-concentrated on a trapping column which is also of C-18 chemistry. In several pre-studies, the conditions of this trapping step have been optimized. The trapping was carried out with pure solvent A for two minutes at a flow rate of 3 µL/min. The sample loop is thereby flushed with 3 times of its volume ensuring the whole sample to be transferred onto the trapping column. At the same time, the composition of solvent A appeared to be critical to avoid elution of highly polar compounds off the short trapping column.

Table 1 Properties of target compounds for method development

Compound	Description	logP	ESI Ionization mode [pos/neg]
Atenolol	Beta-blocker	0.335	pos
Atrazine	Herbicide	2.64	pos
Carbamazepine	Anti-epileptic	1.90	pos
Linuron	Herbicide	3.13	pos
Diclofenac	nonsteroidal anti-inflammatory drug	4.55	neg
Ibuprofen	nonsteroidal anti-inflammatory drug	3.50	neg
Naproxen	nonsteroidal anti-inflammatory drug	2.88	pos/neg
Paracetamol	Analgesic	0.475	pos/neg
Simazine	Herbicide	2.28	pos
Sulfamethoxazole	Anti-infective	0.659	pos

The chromatographic system provided two possibilities for sample elution from the trapping column to the analytical column. In forward flush mode, sample was eluted from the trapping column in the same direction than trapping flow. In reverse flush configuration, however, the elution was carried out in opposite direction of trapping flow. Comparative measurements with both configurations showed benefits for reverse flushing of the trapping column. By that means it was possible to significantly reduce the peak width especially for compounds with a logP value above 2 which led in the same time to an increase in sensitivity. At last, the analytical gradient was optimized for best peak shape and sensitivity. At the beginning of this process, a simple linear gradient was used, starting with 100 % A for 1 min, then decreasing gradually to 0 % within 9 min and holding that isocratically for 10 min till all compounds have been eluted. After another 15 min of equilibration time with 100 % A the system was ready for the next analytical run. Unfortunately, this gradient led to unsatisfactory peak shape of Simazine. Moreover, the sensitivity for compounds with a logP-value below 1 was low, too. For method optimization, isocratic elution at different concentrations of A was carried out. Promising results have been achieved using 40 % A which was adopted as starting gradient for the final method. Fig. 1 shows the BPI chromatogram of an 80 ppb calibration standard in positive ionization mode. Except of Atrazine and Naproxene, co-elution could not be observed (the signal of Atenolol is not visible due to its weak ionization with ESI).
One important aspect of conducting quantitative measurements with a TOF-MS is dead time. During one MS-

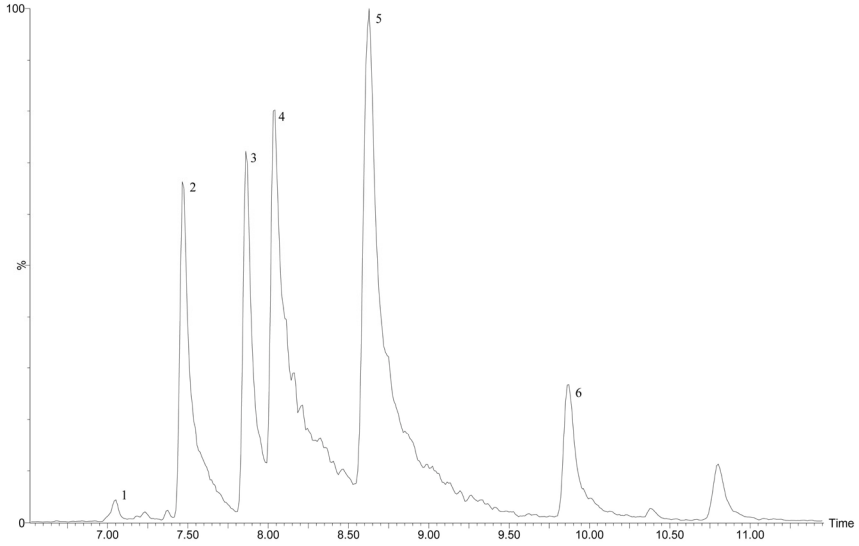

Fig. 1: BPI chromatogram of a 80 ppb standard solution; Compounds in elution order: Atenolol/Paracetamol (1), Sulfamethoxazole (2), Carbamazepine (3), Simazine (4), Atrazine/Naproxene (5), Linuron (6)

scan, a certain amount of ions from the constant ion beam is directed into the time of flight tube. Dependent on their mass, the ions reach the detector after a certain flight time. In the case of the Q-TOF2, data acquisition is accomplished by a time to digital converter (TDC). After each registration of an ion, there is a minimum time interval before a subsequent ion arrival can be registered. This time interval is called dead time and is of the order of 5 nanoseconds. According to the time-of-flight, masses can be assigned to the ions with high accuracy. The main drawback of this kind of mass acquisition is the dead time in which the analyser is not available for further ions. First of all, this fact reduces the sensitivity of the TOF instrument. Furthermore, the range of linear response is significantly reduced. Above ion currents of approximately 400 cps, the instrument applies dead time correction in order to avoid influence on the calculated chromatographic peak areas. Nevertheless, the model is limited till ion currents of about 10,000 cps of one centered mass peak. Above this limit, no dead time correction is applied resulting in a quite narrow concentration range in which linear response of the MScould be confirmed. Forquantitative analysis, however, a linear response upon different analyte concentrations is mandatory.Fig. 2 andfig. 3 show exemplary the calibration curves of Sulfamethoxazole and Naproxene gathered by injection of 10, 20, 40, 60 and 80 ppb concentrated multi-analyte standard in positive ESI mode.Sulfmethoxazole as a good ionizer already exceeds dead time correction limits at the maximum injected concentration of 80 ppb. Consequently, the data points for the 80 ppb solution have not been included in calculation of a linear fit. Within

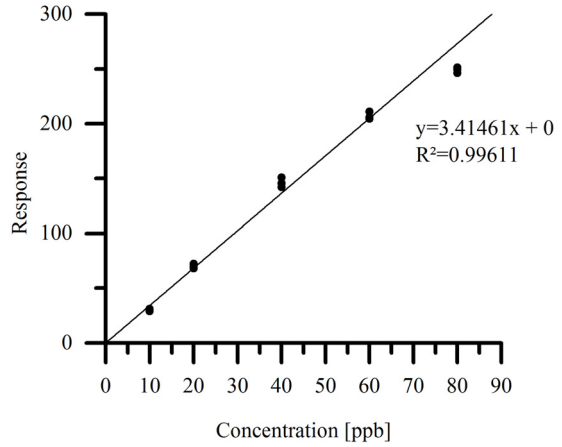

Fig. 2: Calibration curve of Sulfamethoxazole; each concentration was measured three times (n=3); signal for 80 ppb standard not included in linear fit.

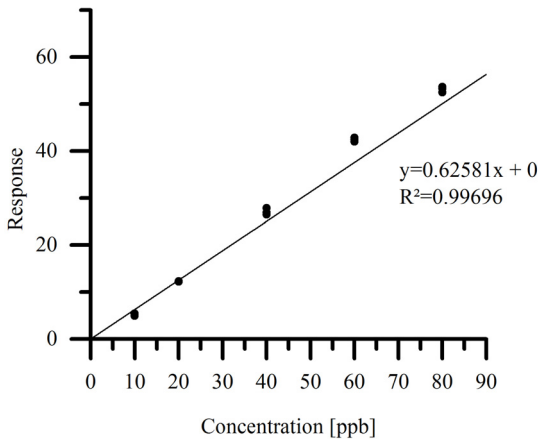

Fig. 3: Calibration curve of Naproxene; samples; each concentration was measured three times (n=3).

the concentration range of 10-60 ppb, however, good linear response could be achieved with a correlation coefficient near 1. Due to the fact that Naproxene shows less good sensitivity than Sulfamethoxazole in positive ion mode, there is no exceeding of the dead time correction seen in the calibration curve shown infig. 2and all recorded data points could be used to calculate a linear fit. Like in the case of Sulfamethoxazole, the corresponding correlation coefficient is close to 1.

This finding has to be regarded for future quantification experiments involving the Q-TOF2-MS as detector. Quantification can only be done within the narrow linear working area of the instrument. In combination with standard addition experiments, it may happen easily that the concentration exceeds the linear limits of response at least for good ionizing analytes. For future analysis of environmental samples, sample dilution has to be considered whenever the measurement results are suspicious of exceeding the linear response range.

Table 2 shows the calibration range as well as the calculated instrumental detection limit (IDL). Due to the low noise in the extracted ion chromatograms of each analyte, the IDL was calculated based on the standard deviation of the analyte signal during 9 injections of a 10 ppb standard solution and the corresponding t-value of the one-sided Student t-distribution at a confidence level of 99 %. The limit of quantification (LOD) also shown in table 2, was regarded as 5 times the IDL.

Table 2:system calibration parameters

Compound	calibration range [ppb]	R^2	IDL [ppb]	LOD [ppb]
Atenolol	20-80	0.9900	3.6	18.1
Atrazine	10-60	0.9881	2.0	10.1
Carbamazepine	10-60	0.9940	1.8	8.9
Linuron	10-80	0.9919	4.5	22.3
Naproxene	10-80	0.9911	5.7	28.7
Paracetamol	10-80	0.9943	4.4	21.9
Simazin	10-80	0.9928	1.9	9.7
Sulfamethoxazole	10-60	0.9932	3.7	18.3

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