Talanta 83 (2011) 795–803

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Simultaneous determination of 15 top-prescribed pharmaceuticals and their metabolites in influent wastewater by reversed-phase liquid chromatography coupled to tandem mass spectrometry

Isabela Tarcomnicu[a], Alexander L.N. van Nuijs[a,*], Ward Simons[a], Lieven Bervoets[b], Ronny Blust[b], Philippe G. Jorens[c], Hugo Neels[a], Adrian Covaci[a,b]

[a] Toxicological Centre, University of Antwerp, Universiteitsplein 1, 2610 Antwerp, Belgium
[b] Laboratory for Ecophysiology, Biochemistry and Toxicology, Department of Biology, University of Antwerp, Groenenborgerlaan 171, 2020 Antwerp, Belgium
[c] Department of Clinical Pharmacology/Clinical Toxicology, University of Antwerp, Antwerp University Hospital, Universiteitsplein 1, 2610 Antwerp, Belgium

ARTICLE INFO

Article history:
Received 15 July 2010
Received in revised form 18 October 2010
Accepted 26 October 2010
Available online 4 November 2010

Keywords:
Pharmaceuticals
Metabolites
Influent wastewater
Reversed-phase liquid chromatography–tandem mass spectrometry
Solid-phase extraction
Validation

ABSTRACT

A fast and sensitive high performance reversed-phase liquid chromatography–tandem mass spectrometry method was developed and validated for the analysis of 15 prescription pharmaceuticals and four of their metabolites in influent wastewater. The selected pharmaceuticals belonged to various classes, such as angiotensin converting enzyme inhibitors, angiotensin receptor antagonists, calcium antagonists, β-blockers, antidepressants, analgetics, anticonvulsants, platelet antiaggregants, and cholesterol lowering agents. They were selected from the list of top-sold prescription pharmaceuticals in Belgium. The chromatographic separation was optimized in order to achieve suitable retention times, good resolution for analytes susceptible of mass spectrometric cross-talk and high sensitivity in one single run. All compounds eluted within 9 min on a Phenomenex Kinetex C18 column, based on a newly developed technology that allows a very narrow distribution of the core–shell particles, providing high separation efficiency. Sample preparation was executed with solid-phase extraction on Oasis MCX cartridges. The method was validated by assessing specificity, selectivity, lower limit of quantification (LLOQ), linearity, accuracy, precision, extraction recovery, and matrix effects following Food and Drug Administration guidelines. The method LLOQs ranged from 0.5 to 25 ng/L. Calibration curves and LLOQs were designed to provide a good analytical performance at concentrations expected in real influent wastewater samples for each target compound. Eight deuterated analogues were used as internal standards for quantification. The method was applied to influent wastewater samples collected from 17 different wastewater treatment plants throughout Belgium. Most of the analytes were measured in the samples at concentrations above LLOQ. Seven of the compounds were for the first time reported in influent wastewater. The newly developed analytical method is currently used to assess relationships between sales figures of pharmaceuticals and their corresponding concentrations in influent wastewater.

1. Introduction

The widespread occurrence of pharmaceutical compounds in the environment is nowadays well established and as a result they are classified as emerging environmental contaminants [1]. The environmental impact of pharmaceuticals is believed to be primarily the consequence of their presence in the aquatic environment. Because of their immense worldwide consumption, pharmaceutically active compounds are continuously released in wastewater due to excretion via urine and/or faeces or due to direct disposal of leftovers. An incomplete removal of these pharmaceutical compounds in wastewater treatment plants (WWTPs) and drinking water treatment plants (DWTPs) is often observed, and as a result they can be detected in surface water and drinking water [2]. Because the acute and long-term effects of these compounds on the environment need to be studied more intensively, it is absolutely necessary that sensitive and specific analytical methods are developed, validated and applied to monitor the presence of pharmaceuticals in wastewater, surface water and drinking water.

Analytical methods to measure low concentrations (in the ng/L range or even lower) of pharmaceuticals in water samples of different sources should allow the simultaneous determination of a wide

* Corresponding author at: Toxicological Centre, University of Antwerp, Universiteitsplein 1, 2610 Wilrijk-Antwerpen, Belgium.
Tel.: +32 3 265 2743; fax: +32 3 265 2722.
E-mail address: alexander.vannuijs@ua.ac.be (A.L.N. van Nuijs).

0039-9140/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.talanta.2010.10.045

range of pharmaceutically-active compounds in a single analytical run with high sensitivity and specificity. Therefore, techniques based on liquid chromatographic (LC) separation coupled to mass spectrometry (MS) detection are the most appropriate. Due to the often polar and non-volatile character of the studied pharmaceuticals, LC is favorable above gas chromatography (GC), which often needs a time-consuming derivatization step and is not compatible with thermolabile compounds. Several LC–MS and LC–MS/MS methods for the analysis of a wide range of pharmaceuticals in wastewater have been published up to date, in most cases based on reversed-phase LC (RPLC) [3–8]. Sample preparation, to remove interferences from the complex matrix and to concentrate the analytes of interest, is usually done by off-line solid-phase extraction (SPE), but also on-line SPE and solid-phase micro extraction (SPME) have been reported [3,8,9].

In this work, a sensitive and specific method based on RPLC–MS/MS was developed and fully validated for the analysis of 15 top-prescribed pharmaceuticals in Belgium and four of their important metabolites in influent wastewater. The pharmaceutical compounds selected in this study were chosen based on earlier published concentrations, the possibility to detect them in influent wastewater (depending on the pharmacokinetics of the compounds), as well as on the available Belgian sales statistics [10]. The studied pharmaceuticals were: angiotensin II receptor antagonists (losartan – LOS and its major carboxylic metabolite – LOS MTB; telmisartan – TEL; valsartan – VAL), angiotensin converting enzyme inhibitors (enalapril – ENA and its major metabolite enalaprilate – ENT; lisinopril – LIS; perindopril – PER and its major metabolite perindoprilate – PET), calcium antagonists (diltiazem – DIL), antidepressants (venlafaxine – VEN; fluoxetine – FLU), a statin (atorvastatin – ATO), an anticonvulsant (carbamazepine – CAR), a benzodiazepine (diazepam – DIA), an antiplatelet agent (clopdigrel – CLO and its major carboxylic metabolite – CLO MTB), a β-blocker (bisoprolol – BIS) and an analgetic (tramadol – TRA).

The research presented here is part of a larger project, aiming at measuring the concentrations of several classes of prescription pharmaceuticals in influent wastewater obtained from different Belgian wastewater treatment plants (WWTPs). Further, these concentrations will be correlated with the detailed official sales figures, taking into account the metabolism and the excretion pattern of the pharmaceuticals. The first stage of this project was thus to develop sensitive analytical methods to detect and quantify the selected pharmaceuticals in influent wastewater samples.

2. Material and methods

2.1. Reagents and materials

The analytes of interest (ATO, BIS, CAR, CLO, CLO MTB, DIA, DIL, ENA, ENT, FLU, LIS, LOS, LOS MTB, PER, PET, TEL, TRA, VAL, VEN) and deuterated internal standards (ATO-d_5, BIS-d_5, CAR-d_2, ENA-d_5, FLU-d_5, nordiazepam-d_5 – NOR-d_5, TRA-d_6, VAL-d_3) were purchased from Cerilliant (Round Rock, TX, USA) or Toronto Research Chemicals (North York, Ontario, Canada) as chemical powders or as solutions at concentrations of 1 mg/mL or 100 μg/mL in methanol (MeOH) or acetonitrile (AcN). From the powder, concentrations of 1 mg/mL in MeOH were prepared. Further dilutions and working mixtures with concentrations between 100 μg/mL and 10 ng/mL were prepared in MeOH starting from the stock solutions. LC-grade AcN and MeOH, as well as hexane, analytical grade hydrochloric acid (HCl), ammonium hydroxide (NH_4OH), formic acid and ammonium acetate were obtained from Merck (Darmstadt, Germany). Milli-Q water was prepared by purifying demineralised water in a Milli-Q system (Millipore, Bedford, MA, USA). Oasis HLB (3 mL, 60 mg), Oasis MCX (3 mL, 60 mg) and Oasis MAX (3 mL, 60 mg) solid-phase extraction (SPE) cartridges were acquired from Waters (New Bedford, MA, USA) and a Supelco Visprep SPE Vacuum Manifold with 12 ports and a self-cleaning dry vacuum system Welch 2023 was used for the conditioning, loading, washing and drying of the SPE cartridges.

2.2. Samples

Influent wastewater samples were collected from 17 WWTPs in Belgium. All WWTPs served more than 20,000 inhabitants. The WWTP in Brussels was sampled on 5 different dates (four samples in 2009; one sample in 2007). All samples were collected in a volume-proportional manner, meaning that a fixed amount of wastewater is automatically sampled when a certain volume of wastewater has passed through the sampling device. This procedure was repeated for 24 h, so that a 24-h composite sample, representative for a whole day, was obtained. After collection, the samples were immediately stored in glass bottles in the dark at −20 °C and at pH 2. The samples were collected in the frame of another project in which they were analyzed for illicit drugs and where adjustment to pH 2 was necessary to prevent degradation during storage.

2.3. Sample preparation

In this study, we compared three common SPE cartridges for the isolation of the selected pharmaceuticals and metabolites from influent wastewater. Because the samples were stored at pH 2, the pH was adjusted, when necessary, as described below before loading onto SPE cartridges.

Oasis HLB cartridges contain a copolymeric sorbent with hydrophilic and lipophilic properties and can be used for a wide range of target compounds. The conditioning of the Oasis HLB cartridges was done with consecutively 6 mL MeOH and 6 mL Milli-Q water. After loading of the samples (at pH 7), the cartridges were washed with 6 mL of Milli-Q water, dried under vacuum and eluted with 8 mL of MeOH.

Oasis MCX cartridges consist of a sorbent with strong cation-exchange sulfonic acid groups and are thus mostly suitable for the sample preparation of basic compounds. For Oasis MCX, conditioning was executed with consecutively 6 mL MeOH, 4 mL Milli-Q water and 4 mL Milli-Q water brought at pH 2. After loading of the samples (at pH 2), the cartridges were washed with 6 mL Milli-Q water, dried under vacuum and eluted with 4 mL MeOH and 4 mL MeOH with 5% NH_4OH.

Oasis MAX cartridges, containing a sorbent with quaternary amine groups, are mostly applied in sample preparation for compounds with acidic functional groups. For Oasis MAX, conditioning was done with 6 mL MeOH, 4 mL Milli-Q water and 4 mL Milli-Q water brought at pH 12. After loading of the samples (at pH 11), the cartridges were washed with 6 mL Milli-Q water, dried under vacuum and eluted with 4 mL MeOH and 4 mL MeOH with 5% formic acid.

To evaluate the extraction recovery for each analyte, the areas of each compound in the chromatograms resulting from the spiking of 50 mL of blank surface water before or after the SPE procedure with 100 ng/L analyte were compared.

Before applying the SPE procedures, samples were first passed through a microfibre glass filter to remove solid particles. After SPE, the methanolic eluates were evaporated to dryness under a nitrogen stream at 40 °C and the residue was redissolved in 50 μL AcN and 150 μL 5 mM ammonium acetate in Milli-Q water under thorough vortexing and after filtration, the extract was transferred into a glass vial for injection in the LC–MS/MS system.

2.4. HPLC–MS/MS

The LC system consisted of an Agilent 1200 series binary pump and autosampler. Chromatographic separation of the target compounds was achieved with a Kinetex C18 column (100 mm × 2.1 mm, 2.6 μm) maintained at 40 °C in an Agilent thermostat. The mobile phase was composed of (A) ammonium acetate 5 mM in Milli-Q water with 0.1% formic acid and (B) AcN, using a gradient as follows: 0–0.5 min: 5% B; 0.5–2.0 min: from 5% B to 70% B; 2.0–6.5 min: 70% B; 6.5–6.6 min: from 70% B to 100% B; 6.6–9.5 min: 100% B; 9.5–9.8 min: from 100% B to 5% B and then 5.7 min in these conditions for column equilibration. The flow rate was 0.2 mL/min and the injection volume was optimized and set at 5 μL. All compounds eluted within 9 min (Fig. 1) and the total run-time with column equilibration was 15.5 min. The MS system consisted of an Agilent 6410 triple quadrupole mass spectrometer equipped with an electrospray interface operating in positive ionization mode. The capillary voltage was 4500 V, the drying gas (nitrogen) temperature was 350 °C and the nebulizer pressure was set at 40 psi. Quantitative analyses were performed in multiple reaction monitoring (MRM) mode and the two most abundant fragmentation products (selected as quantifier and qualifier) were recorded for each compound. The LC flow was diverted to the waste in the first 3.5 min of the analytical run and after 10.5 min of the acquisition to avoid excessive contamination of the mass spectrometer interface. Table 1 gives an overview of the MS parameters and the retention times for all analytes and internal standards.

2.5. Quantification and method validation

For each compound, the most abundant MRM transition was used for quantification (quantifier), while the other transition was used for confirmation (qualifier). Only one transition was used for the deuterated internal standards. The analytes were considered confirmed in unknown samples if the retention time did not differ by more than ±0.4 min than that of the reference standard [11] and if the ratio quantifier/qualifier in the extracted samples were within the range of ±20% of the ratio in the reference standards [12]. Surface water originating from a small creek near the laboratory was used as the matrix for the method validation. We have chosen this approach because influent wastewater always contained measurable concentrations of the investigated compounds and thus could not be used as matrix for the validation. The matrix effects and extraction recovery in influent wastewater were evaluated using the standard addition method. These experiments justified the execution of validation in surface water.

The method was validated in at least 3 separate analysis days, following the Food and Drug Administration (FDA) guidelines, by assessing linearity, low limit of quantification (LLOQ), specificity, selectivity, accuracy and precision [13].

For each analyte, multi-level calibration curves (7 points) were prepared by spiking 50 mL of surface water with different working standard solutions and a fixed amount of the deuterated internal standards. The range of the calibration curves was based on the published concentrations in wastewater samples. When such data was not available (CLO MTB, ENT, LOS MTB, PER, PET and TEL), the calibration range was based on roughly estimated concentrations in preliminary analyses of real samples. Linearity was considered acceptable when three requirements were fulfilled: (a) the coefficient of determination of the calibration curves was at least 0.99, (b) calibrators had accuracies between 85% and 115% and (c) precision was <15% relative standard deviation (RSD). At the lowest calibration point (being the LLOQ), limits for accuracy were set between 80% and 120% and the limit for precision was <20% RSD [13].

The LLOQ was defined as the lowest concentration producing peaks with a signal to noise ratio of at least 10 and with an accuracy and precision within ±20% of the target concentration. The LLOQ was further evaluated by comparing the analyte peak areas in the LLOQ samples with the areas in blank samples. The peak areas in zero blank samples cannot exceed 20% of the mean analyte LLOQ peak areas [13].

To asses the method's precision and accuracy, quality control (QC) samples at three concentration levels (QC low, between point 1 and 2 of the calibration curve; QC medium, between point 4 and 5; QC high, between point 6 and 7 of the calibration curve) were prepared by spiking 50 mL surface water with different working standard solutions and a fixed amount of the deuterated internal standards. A minimum of five replicates per concentration level was analyzed in one validation day and this was repeated for minimum three days. In this way, it was possible to assess accuracy and precision both within-run and between-run. The limits for precision and accuracy for replicated quality controls at the three concentration levels were set as follows: within 15% RSD for precision and between 85% and 115% for accuracy [13].

Specificity was evaluated by comparing blank surface water samples ($n = 3$) and surface water samples spiked at the QC high concentration ($n = 3$). For each analyte, this was done separately. No peaks should be present in the blank samples at the retention time of the analyte.

Extraction efficiencies of the optimized method were assessed by comparing the peak areas in the chromatograms of the analyses of 50 mL surface water samples spiked at QC low, QC medium and QC high concentration before or after SPE. The extraction recovery was calculated for two replicate samples at each concentration level and the total mean was generated ($n = 6$). To assess the comparability between the recoveries in influent wastewater and surface water, the extraction recovery in influent wastewater was evaluated by standard addition tests. Six different influent wastewater samples were spiked before and after SPE with QC high concentration and the peak areas were compared, after subtraction of the area of the native analyte in the sample (without spiking).

Matrix effects were evaluated according to the recommendations of Matuszewski et al. [14] and Kelly et al. [15]. Analyte peak areas in blank surface water samples (50 mL) spiked at QC high concentration after SPE were compared with peak areas in mobile phase solutions containing QC high concentration of the analytes. Because calibration curves were prepared in surface water instead of influent wastewater (blank influent wastewater is not available), the matrix effects in influent wastewater were further evaluated by standard addition tests. Three influent wastewater samples were processed following the normal protocol, and in parallel the same samples were spiked with calibrator 7 concentration. The matrix effect between surface and wastewater was then given by the following formula:

$$\text{matrix effect (\%)} = \left(\left(\frac{\text{area spiked wastewater} - \text{area non spiked wastewater}}{\text{area cal 7 from curve}}\right) - 1\right) \times 100\%$$

The matrix effects were evaluated in the same way for the internal standards. The assessment of these matrix effects is extremely important, since one of the principal criteria to assign an internal standard to an analyte is that the internal standard has to compensate for the occurring matrix effects. If not, calculations of concentrations in real samples are not correct.

3. Results and discussion

3.1. Sample preparation

The sample preparation step should deliver consistent, precise and reproducible extraction efficiencies [13]. This means that the

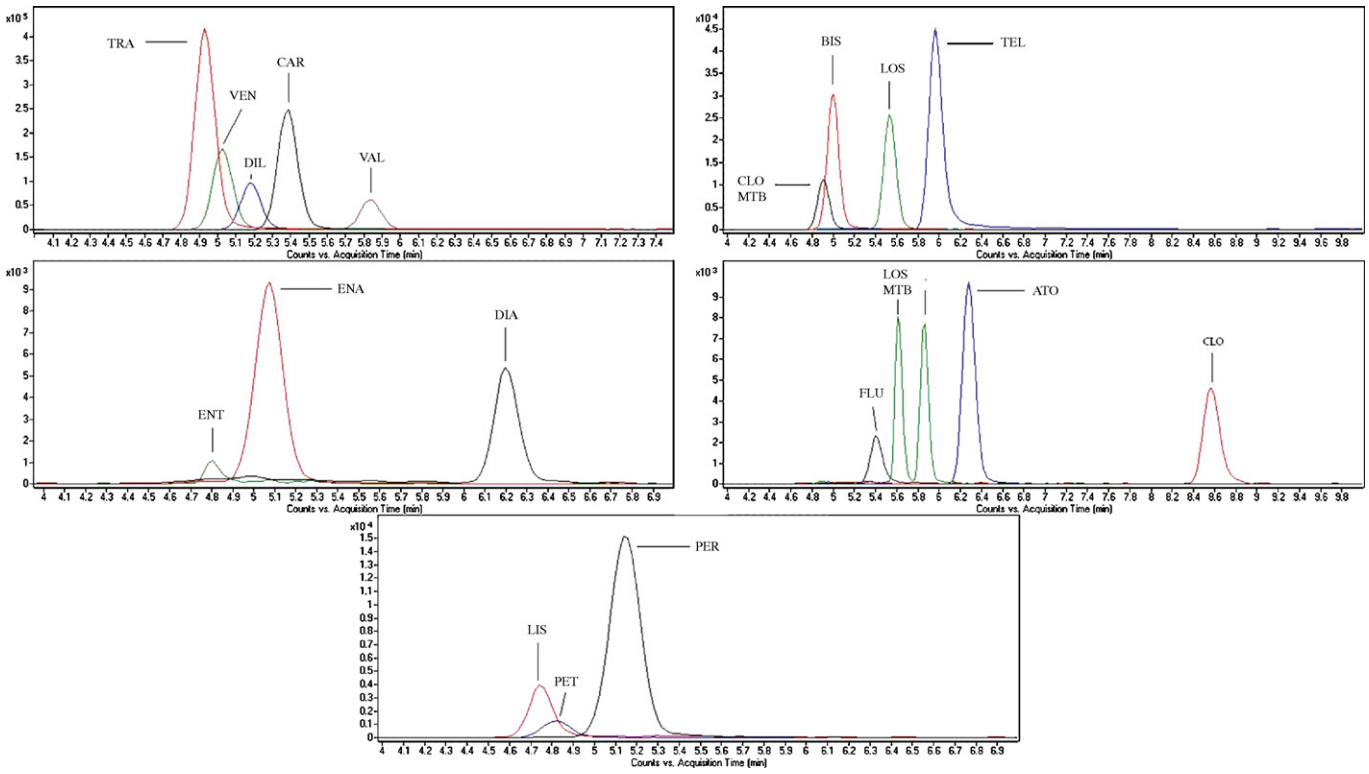

Fig. 1. Chromatogram of the quantifier MRM transition for blank surface water spiked with compounds at QC medium concentration. ATO: atorvastatin; BIS: bisoprolol; CAR: carbamazepine; CLO: clopidogrel; CLO MTB; clopidogrel metabolite; DIA: diazepam; DIL: diltiazem; ENA: analapril; ENT: enalaprilate; FLU: fluoxetine; LIS: lisinopril; LOS: losartan; LOS MTB: losartan metabolite; PER: perindopril; PET: perindoprilate; TEL: telmisartan; TRA: tramadol; VAL: valsartan; VEN: venlafaxine. *: cross talk LOS MTB and VAL. Column: Kinetex C18 (100 mm × 2.1 mm, 2.6 μm); mobile phase: (A) 5 mM aqueous ammonium acetate with 0.1% formic acid and (B) acetonitrile; flow: 0.2 mL/min; gradient conditions.

Table 1
LC–MS/MS parameters of the selected analytes. ATO: atorvastatin; BIS: bisoprolol; CAR: carbamazepine; CLO: clopidogrel; CLO MTB; clopidogrel metabolite; DIA: diazepam; DIL: diltiazem; ENA: analapril; ENT: enalaprilate; FLU: fluoxetine; LIS: lisinopril; LOS: losartan; LOS MTB: losartan metabolite; NOR: nordiazepam; PER: perindopril; PET: perindoprilate; TEL: telmisartan; TRA: tramadol; VAL: valsartan; VEN: venlafaxine. NA = not applicable.

Pharmaceutical	Retention time (min)	Internal Standard	Precursor ion (m/z)	Fragmentor voltage (V)	Quantifier		Qualifier	
					Product ion (m/z)	Collision energy (V)	Product ion (m/z)	Collision energy (V)
ATO	6.2	ATO-d_5	559.4	190	440.3	20	NA	NA
BIS	5.0	BIS-d_5	326.2	125	116.2	20	74.1	20
CAR	5.4	CAR-d_2	237.1	125	194.1	20	178.9	30
CLO	8.6	NOR-d_5	322.1	110	212.1	15	184.0	20
CLO MTB	4.9	ENA-d_5	308.0	120	198.1	15	151.9	20
DIA	6.2	CAR-d_2	285.0	125	193.0	35	154.0	93
DIL	5.2	FLU-d_5	415.2	125	178.0	25	150	30
ENA	5.1	ENA-d_5	377.2	130	234.1	15	130.2	25
ENT	4.8	ENA-d_5	349.1	120	206.0	15	101.9	30
FLU	5.4	FLU-d_5	310.2	90	44.2	10	148.1	3
LIS	4.7	ENA-d_5	406.2	110	84.0	30	246.2	25
LOS	5.5	VAL-d_3	423.2	100	207.0	20	192.0	25
LOS MTB	5.6	VAL-d_3	437.1	120	235.0	15	207.0	25
PER	5.1	ENA-d_5	369.3	125	172.1	20	72.2	30
PET	4.8	ENA-d_5	341.1	120	144.1	20	170.0	15
TEL	6.0	FLU-d_5	515.3	110	276.1	50	305.2	30
TRA	4.9	TRA-d_6	264.1	90	58.1	20	NA	NA
VAL	5.8	VAL-d_3	436.3	125	207.0	25	291.1	15
VEN	5.0	TRA-d_6	278.2	90	58.1	20	121.0	15
ATO-d_5	6.2		564.2	190	445.3	20		
BIS-d_5	5.0		331.2	125	121.1	20		
CAR-d_2	5.3		239.0	125	196.1	20		
ENA-d_5	5.0		382.1	120	239.2	20		
FLU-d_5	5.3		315.1	90	44.1	10		
NOR-d_5	5.7		276.0	100	140.0	35		
TRA-d_6	4.9		270.2	90	64.2	15		
VAL-d_3	5.8		439.2	120	207.1	15		

Table 2
Linearity, quantification limit (LLOQ), accuracy, precision and extraction recovery results for the developed LC–MS/MS method. ATO: atorvastatin; BIS: bisoprolol; CAR: carbamazepine; CLO: clopidogrel; CLO MTB: clopidogrel metabolite; DIA: diazepam; DIL: diltiazem; ENA: analapril; ENT: enalaprilate; FLU: fluoxetine; LIS: lisinopril; LOS: losartan; LOS MTB: losartan metabolite; PER: perindopril; PET: perindoprilate; TEL: telmisartan; TRA: tramadol; VAL: valsartan; VEN: venlafaxine. SD = standard deviation; RSD = relative standard deviation; QC = quality control; SW = surface water; WW = wastewater.

Pharmaceutical	Range (ng/L)	r^2	LLOQ (ng/L)	Within-run accuracy (% (SD))			Between-run accuracy (% (SD))			Within-run precision (% RSD)			Between-run precision (% RSD)			Extraction recovery (% (SD))	
				QC low (n = 6)	QC medium (n = 6)	QC high (n = 6)	QC low (n = 18)	QC medium (n = 18)	QC high (n = 18)	QC low (n = 6)	QC medium (n = 6)	QC high (n = 6)	QC low (n = 18)	QC medium (n = 18)	QC high (n = 18)	SW (n = 6)	WW (n = 6)
ATO	4–360	0.9959	4	95 (12)	93 (4)	103 (3)	90 (11)	95 (5)	104 (5)	12	5	3	12	5	4	40 (9)	47 (3)
BIS	1–360	0.9991	1	104 (5)	94 (4)	99 (3)	96 (7)	98 (5)	101 (4)	5	4	3	7	5	4	101 (8)	96 (5)
CAR	25–1500	0.9998	25	102 (2)	103 (2)	102 (1)	101 (3)	104 (2)	102 (1)	2	2	1	3	2	1	110 (3)	101 (6)
CLO	0.5–20	0.9969	0.5	107 (10)	99 (10)	94 (6)	98 (12)	95 (9)	93 (9)	9	10	7	12	3	10	97 (5)	91 (5)
CLO MTB	5–500	0.9978	5	107 (9)	111 (1)	112 (4)	101 (11)	111 (6)	109 (8)	8	1	4	10	5	7	108 (9)	98 (9)
DIA	1–360	0.9949	1	108 (12)	98 (2)	99 (4)	100 (16)	96 (7)	94 (6)	12	2	4	16	7	7	98 (4)	93 (4)
DIL	5–500	0.9972	5	107 (8)	113 (7)	112 (7)	101 (10)	110 (10)	103 (11)	8	6	6	10	9	11	71 (5)	77 (6)
ENA	1–60	0.9964	1	108 (5)	101 (3)	108 (5)	104 (10)	100 (6)	103 (6)	5	3	5	9	6	6	98 (6)	100 (7)
ENT	1–60	0.9909	1	108 (10)	106 (5)	106 (4)	103 (13)	104 (9)	102 (9)	10	5	4	12	9	9	97 (8)	91 (4)
FLU	1–60	0.9938	1	95 (4)	101 (5)	98 (4)	94 (7)	97 (6)	98 (5)	4	5	4	8	6	5	91 (2)	87 (2)
LIS	25–1500	0.9998	25	110 (13)	101 (7)	114 (7)	100 (16)	106 (10)	110 (7)	12	7	6	16	10	6	72 (3)	80 (3)
LOS	5–500	0.9992	5	92 (6)	111 (6)	111 (4)	95 (9)	106 (7)	106 (8)	7	5	3	9	6	8	92 (6)	96 (5)
LOS MTB	1–360	0.9967	1	101 (7)	94 (9)	98 (5)	94 (14)	94 (8)	102 (8)	7	10	5	13	8	6	96 (7)	96 (7)
PER	1–90	0.9985	1	99 (11)	95 (2)	108 (4)	105 (9)	96 (5)	104 (5)	11	3	4	9	5	5	99 (10)	106 (7)
PET	1–90	0.9981	1	96 (12)	104 (11)	110 (7)	100 (11)	100 (9)	104 (7)	12	10	6	11	9	7	102 (9)	97 (5)
TEL	1–360	0.9990	1	94 (9)	89 (4)	97 (5)	93 (7)	94 (10)	97 (10)	9	4	6	7	10	10	85 (1)	91 (5)
TRA	20–1500	0.9998	20	100 (2)	101 (2)	103 (2)	106 (6)	103 (3)	103 (2)	2	2	2	6	3	3	108 (2)	103 (2)
VAL	25–1500	0.9987	25	94 (9)	100 (4)	104 (5)	96 (7)	99 (3)	104 (5)	9	4	4	7	3	5	98 (3)	98 (4)
VEN	5–1000	0.9992	5	99 (2)	100 (3)	108 (1)	103 (6)	102 (3)	103 (9)	2	3	1	6	3	1	108 (1)	100 (1)

Table 3
Matrix effects for compounds and internal standards in surface and wastewater. Positive values represent ion enhancement, negative values represent ion suppression. ATO: atorvastatin; BIS: bisoprolol; CAR: carbamazepine; CLO: clopidogrel; CLO MTB; clopidogrel metabolite; DIA: diazepam; DIL: diltiazem; ENA: analapril; ENT: enalaprilate; FLU: fluoxetine; LIS: lisinopril; LOS: losartan; LOS MTB: losartan metabolite; NOR: nordiazepam; PER: perindopril; PET: perindoprilate; TEL: telmisartan; TRA: tramadol; VAL: valsartan; VEN: venlafaxine.

Pharmaceutical or internal standard	Matrix effect between mobile phase and surface water (%)	Matrix effect between surface- and wastewater (%)	Total matrix effect (%)[a]
ATO	−38	53	−5
BIS	−56	−45	−76
CAR	−57	−35	−72
CLO	−13	61	40
CLO MTB	−20	−49	−59
DIA	−19	−48	−58
DIL	−41	−57	−75
ENA	21	−51	−41
ENT	7	−77	−75
FLU	−66	−48	−82
LIS	−50	143	22
LOS	−29	−24	−46
LOS MTB	0	−43	−43
PER	8	−52	−48
PET	26	−71	−63
TEL	−14	−66	−71
TRA	−73	−67	−91
VAL	13	−30	−21
VEN	−59	−58	−83
ATO-d_5	−36	52	−5
BIS-d_5	−59	−47	−78
CAR-d_2	−59	−38	−75
ENA-d_5	16	−50	−42
FLU-d_5	−70	−52	−86
NOR-d_5	−9	−7	−15
TRA-d_6	−72	−66	−90
VAL-d_3	5	−35	−32

[a] Matrix effect between mobile phase and wastewater calculated from column 2 and 3.

extraction recovery does not have to be 100% for all 19 compounds, but it should be sufficient to allow the detection of low concentrations in real wastewater samples. The sorbent which offered the best combination of highest extraction efficiencies together with best reproducibility was further used for the method validation.

Reproducible and high recoveries were obtained on Oasis HLB cartridges, but for three compounds (ENT, LIS, and PET), the extraction recovery was lower than 30%. Since ENT and PET were not reported until now in water samples, we decided to select the SPE procedure with the highest extraction recovery for these two compounds. On Oasis MAX and MCX, recoveries of PET and ENT were higher than 95% and therefore, these two adsorbents were selected for further tests in the early stages of optimisation. Oasis MAX and MCX cartridges showed reproducible recoveries for all compounds. For the procedure using Oasis MAX cartridges, an extraction recovery lower than 70% was observed for six analytes (AML, DIL, LOS MTB, FLU, LIS, and ATO). With Oasis MCX, an extraction recovery lower than 70% was obtained only for ATO, but it was enough to ensure the selected LOQ (4 ng/L). Based on these results, the protocol using Oasis MCX cartridges was chosen for sample preparation in the optimized analytical method.

3.2. HPLC–MS/MS

The 19 selected compounds were suitable for a chromatographic separation in reversed phase conditions. Due to their very different physico-chemical properties, a gradient approach was preferred. Various stationary phases were tested: classical octadecylsilica (Phenomenex Gemini NX C18; 150 mm × 2 mm, 5 μm),

Table 4
Concentrations (ng/L) of the selected pharmaceuticals in influent wastewater samples. ATO: atorvastatin; BIS: bisoprolol; CAR: carbamazepine; CLO: clopidogrel; CLO MTB: clopidogrel metabolite; DIA: diazepam; DIL: diltiazem; ENA: analapril; ENT: enalaprilate; FLU: fluoxetine; LIS: lisinopril; LOS: losartan; LOS MTB: losartan metabolite; PER: perindopril; PET: perindoprilate; TEL: telmisartan; TRA: tramadol; VAL: valsartan; VEN: venlafaxine.

WWTP	Date	ATO	BIS	CAR	CLO[a]	CLO MTB	DIA	DIL	ENA	ENT	FLU	LIS[a]	LOS MTB	LOS	PER	PET	TEL	TRA	VAL	VEN
LLOQ		4	1	25	0.5	5	1	5	1	1	1	25	5	1	1	1	1	20	25	5
Antwerp-North	20/8/07	<LLOQ	6	437	1	200	<LLOQ	<LLOQ	8	6	9	287	94	32	12	5	73	385	84	121
Antwerp-South	17/12/07	4	2	550	2	268	<LLOQ	<LLOQ	15	16	14	174	49	25	22	8	45	402	108	209
Bruges	24/12/07	7	7	586	4	346	<LLOQ	<LLOQ	13	23	15	293	169	44	28	13	32	333	289	308
Brussels	18/12/07	29	179	771	3	92	1	31	22	25	13	64	697	360	20	13	424	666	1809	408
Brussels	30/3/09	11	177	440	6	306	9	30	16	32	15	84	73	37	65	68	17	918	545	409
Brussels	16/4/09	16	83	513	2	234	6	38	17	23	23	54	68	17	47	16	12	784	251	430
Brussels	16/6/09	11	182	555	5	310	5	89	28	38	22	35	111	69	71	68	135	1018	892	432
Brussels	17/7/09	19	72	603	1	239	4	157	18	24	30	249	358	84	46	36	75	794	719	529
Destelbergen	15/10/07	5	19	647	1	201	2	<LLOQ	6	9	10	170	74	36	23	4	19	347	150	249
Deurne	30/8/07	10	<LLOQ	841	4	381	1	<LLOQ	18	15	17	383	67	26	28	13	57	671	140	386
Genk	18/10/07	5	3	316	4	412	<LLOQ	<LLOQ	5	13	11	356	54	29	31	19	203	370	162	126
Ghent	16/8/07	4	5	811	2	213	1	<LLOQ	10	9	13	209	150	58	18	5	33	479	205	322
Harelbeke	24/12/07	6	3	427	4	276	2	<LLOQ	13	29	16	231	284	94	32	15	79	435	446	312
Hasselt	18/10/07	23	5	786	2	389	1	<LLOQ	11	19	26	336	181	36	35	36	70	625	200	406
Lokeren	16/8/07	6	16	402	2	202	2	<LLOQ	9	8	8	330	182	36	14	2	17	326	183	387
Mechelen	22/10/07	8	4	581	2	518	2	<LLOQ	8	34	14	419	118	46	27	14	83	673	243	377
Mol	12/12/07	15	4	593	2	386	2	<LLOQ	16	22	11	277	121	45	23	10	69	429	372	308
Mons	18/2/08	5	29	680	2	197	3	9	10	6	16	131	119	34	41	14	61	789	156	312
Morkhoven	13/12/07	7	4	227	1	107	<LLOQ	<LLOQ	4	3	9	158	124	31	12	11	34	267	205	175
Nijvel	20/12/07	58	15	553	<LLOQ	224	1	<LLOQ	7	22	14	351	197	38	21	2	7	442	220	403
Ostend	21/2/08	28	119	1028	4	561	<LLOQ	10	25	30	18	648	195	68	91	84	51	830	862	519

[a] Original calculated concentrations corrected for different matrix effects between analyte and internal standard. LLOQ: limit of quantification.

amide (Supelco Ascentis RP-Amide; 150 mm × 2.1 mm, 5 μm) and pentafluoropropionyl (Supelco Discovery HSF5; 100 mm × 2.1 mm, 5 μm). Experiments have been carried out with mobile phases containing as aqueous solvent ammonium acetate 5 mM or 20 mM, formic acid 0.05%, 0.1%, or 0.2% in water, or a mixture containing both ammonium acetate (5 mM) and formic acid 0.1% in water, and as organic modifier AcN, MeOH or a mixture of both (75/25 or 87.5/12.5, v/v).

For some analytes, specific chromatographic issues were encountered during method development. For the final method, a compromise between sensitivity, selectivity and peak shape was needed. First, the possibility of cross-talk due to close molecular masses was evaluated for the susceptible groups: angiotensin II receptor antagonists (VAL, LOS, LOS MTB, TEL and VAL-d_3) and angiotensin converting enzyme inhibitors (ENA, ENT, PER, PET and ENA-d_5). Cross-talk was observed for VAL, LOS MTB and VAL-d_3, and therefore it was mandatory to obtain a good chromatographic selectivity (valley < 10%) for the respective analytes. The best separation was achieved on the amide column, with a mobile phase consisting of formic acid 0.1% in water and acetonitrile, with a linear gradient starting from 5% up to 70% AcN. In these conditions, although not showing cross-talk, peak splitting occurred for ENT and PET due to isomeric separation. This fact was reported in literature and the usual solution to avoid peak splitting is to maintain the column at a higher temperature (accelerating the conformational changes between isomers) [16]. Initially, the amide column was operated at 60 °C. Further, ammonium acetate was added to the aqueous mobile phase to improve the retention and peak shape for LIS. ATO and CLO, being the most apolar structures, were strongly retained on the stationary phase and a step gradient up to 100% AcN was necessary to elute these two compounds within 10 min (Fig. 2).

During method development, Phenomenex Kinetex C18 columns became commercially available. Such columns are based on a new developed technology and allow an extremely narrow distribution of core–shell particles, which reduces the effects of Eddy diffusion, enhancing column efficiency. Since the particles are not fully porous, the mass-transfer is faster, resulting in less band broadening. The more ordered particle distribution also leads to reasonable column pressures, making possible to achieve high speed analyses and high separation efficiency of sub-2 micron columns without the need for ultra-high performance LC systems (UHPLC) [17]. Testing the Kinetex C18 column (100 mm × 2.1 mm, 2.6 μm) with a gradient adapted from the previously optimized method, a better resolution for the problematic "cross-talk" analytes was obtained. The sensitivity also improved due to peak narrowing (Fig. 1) and therefore, this column was further used for method validation. A gradient was applied, at a flow of 0.2 mL/min, and the mobile phase consisted of (A) 5 mM aqueous ammonium acetate with 0.1% formic acid and (B) acetonitrile. The gradient was as follows: 0–0.5 min: 5% B; 0.5–2.0 min: 5% B–70% B; 2.0–6.5 min: 70% B; 6.5–6.6 min: 720% B–100% B; 6.6–9.5 min: 100% B; 9.5–15.5 min: 5% B for column equilibration. The injection volume was 5 μL and the column was kept at 40 °C.

Specific MS parameters, such as fragmentor voltage, collision energy and ionization mode, were optimized for each compound separately by injecting standard solutions at a concentration of 400 pg/μL without an LC column. Electrospray ionization (ESI) as well as atmospheric pressure chemical ionization (APCI) were tested (with column attached), but the overall performance for the selected analytes was better with ESI, which was preferred. To monitor two MRM traces (quantifier and qualifier) for 19 analytes and one for each of the eight internal standards, a short dwell time was used to gather at least 12 data points per peak for a good quantification. The dwell time was set at 15 or 25 ms, depending on the sensitivity and required LLOQ for each compound. Only one MRM

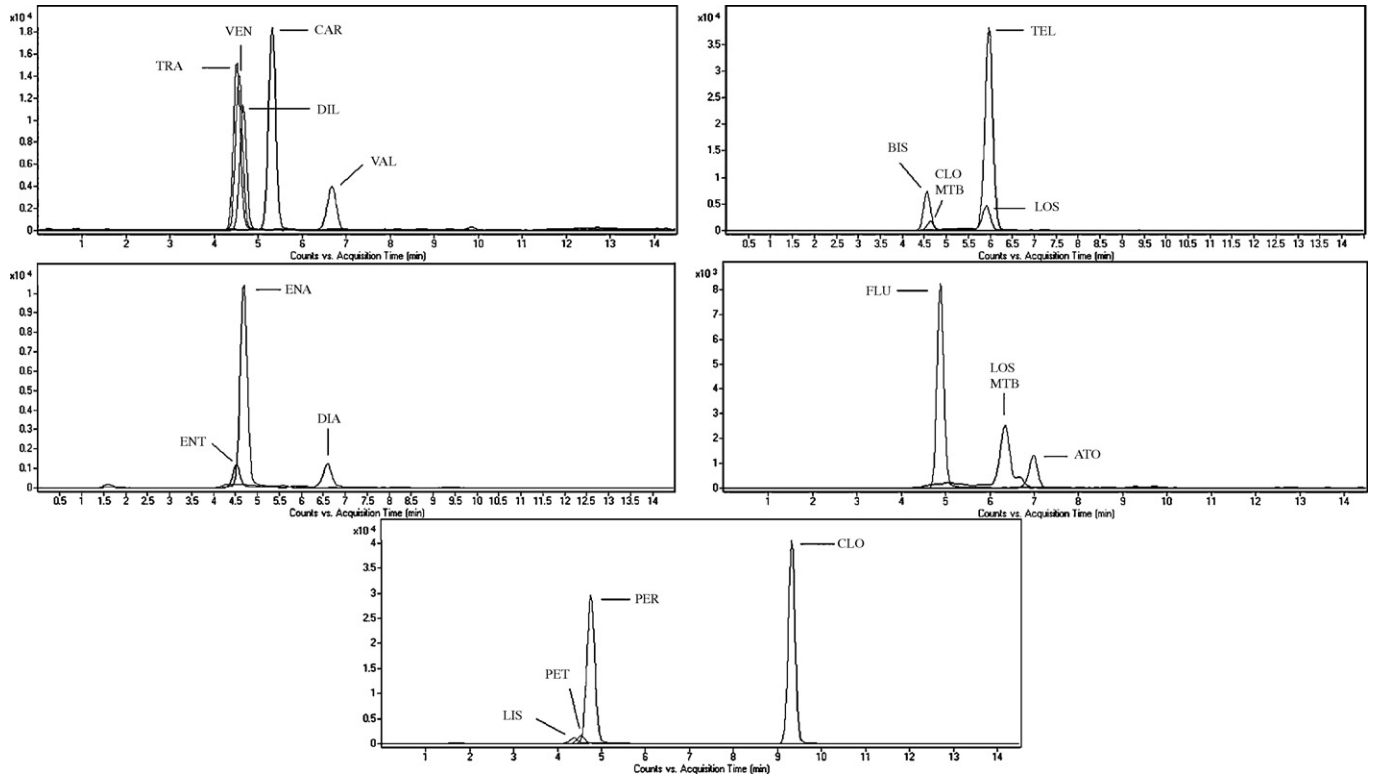

Fig. 2. Chromatogram of the quantifier MRM transition for blank surface water spiked with the analytes at 100 ng/L. ATO: atorvastatin; BIS: bisoprolol; CAR: carbamazepine; CLO: clopidogrel; CLO MTB; clopidogrel metabolite; DIA: diazepam; DIL: diltiazem; ENA: analapril; ENT: enalaprilate; FLU: fluoxetine; LIS: lisinopril; LOS: losartan; LOS MTB: losartan metabolite; PER: perindopril; PET: perindoprilate; TEL: telmisartan; TRA: tramadol; VAL: valsartan; VEN: venlafaxine. Column: Ascentis RP-Amide (150 mm × 2.1 mm, 5 μm); mobile phase: (A) 5 mM aqueous ammonium acetate with 0.1% formic acid and (B) acetonitrile; flow: 0.2 mL/min; gradient conditions.

trace was acquired for TRA, which has a specific fragmentation with only one strong product ion at m/z 58.

3.3. Quantification and method validation

Table 2 gives an overview of the performance of the developed analytical method. The calibration curves, using the internal standard method, were linear in the investigated ranges with correlation coefficients >0.99 for all compounds. All calibrators met the limits of accuracy and precision as earlier described. For the accuracy, the requirements of 85–115% were fulfilled for all analytes and for all concentration levels, within-run as well as between-run. The within-run precision limit of <15% RSD was met for all analytes and concentration levels, whereas the between-run precision for the QC low concentration was for DIA and LIS just above these limits (16% RSD). For QC medium and high concentration levels, all analytes fulfilled widely the between-run precision requirements.

The lowest level of the calibration curve (calibrator 1) for each investigated compound was considered as the LLOQ and ranged between 0.5 ng/L for CLO and 25 ng/L for CAR, LIS and VAL. For some analytes, such as ATO, BIS, CAR, CLO, CLO MTB, FLU, LOS, TEL, TRA and VEN, it was possible to quantify even lower concentrations than the lowest calibration point used for the curves, when considering the signal to noise ratio of calibrator 1. Yet, since the observed concentrations in real influent wastewater samples were generally much higher, there was no need to reach better LLOQs. The extraction recovery in surface water was >70% for all analytes, except for ATO (40%). However, the extraction recovery was reproducible and comparable between surface water and influent wastewater.

The matrix effects in surface water, compared with the same concentrations without matrix, varied between −73% for TRA and +21% for ENA (Table 3). Since calibration curves were prepared in surface water and were further used to calculate concentrations of the analytes in influent wastewater, it was very important to estimate the matrix effects in influent wastewater. For accurate results, the internal standard assigned to each analyte should compensate for the occurring matrix effects. Therefore, wastewater samples (n = 4) were analyzed with the validated method and in parallel the same wastewater samples were spiked with each analyte at the concentration of calibrator 7 and then analyzed. The peak areas obtained in the wastewater samples without addition of calibrator 7 were subtracted from the peak areas in the samples with calibrator 7. These data were then compared with the peak areas of calibrator 7 extracted from surface water as present in the calibration curves. Matrix effects in wastewater were also calculated for the deuterated internal standards. The results varied between −77% for ENT and 53% for ATO (Table 3). Together with a suitable retention time, these results were used to assign a suitable internal standard to each analyte (Table 1).

For CLO and LIS, deuterated analogues were not available in the laboratory when the method was developed, and it was not possible to find an internal standard that compensated entirely for the occurring matrix effects. For these compounds, we assigned the deuterated internal standard with similarity in retention time and/or chemical structure. Then, a correction factor accounting for the difference in matrix effects between the internal standard and analyte was estimated and further used to calculate concentrations in real wastewater samples. The method should be updated in the future by using deuterated analogues for these two analytes. The concentrations for CLO and LIS calculated with the calibration curves prepared in surface water were as a result divided by 1.7 and 5, respectively. For ATO, CLO and LIS a signal enhancement was observed with influent wastewater samples, an effect that has

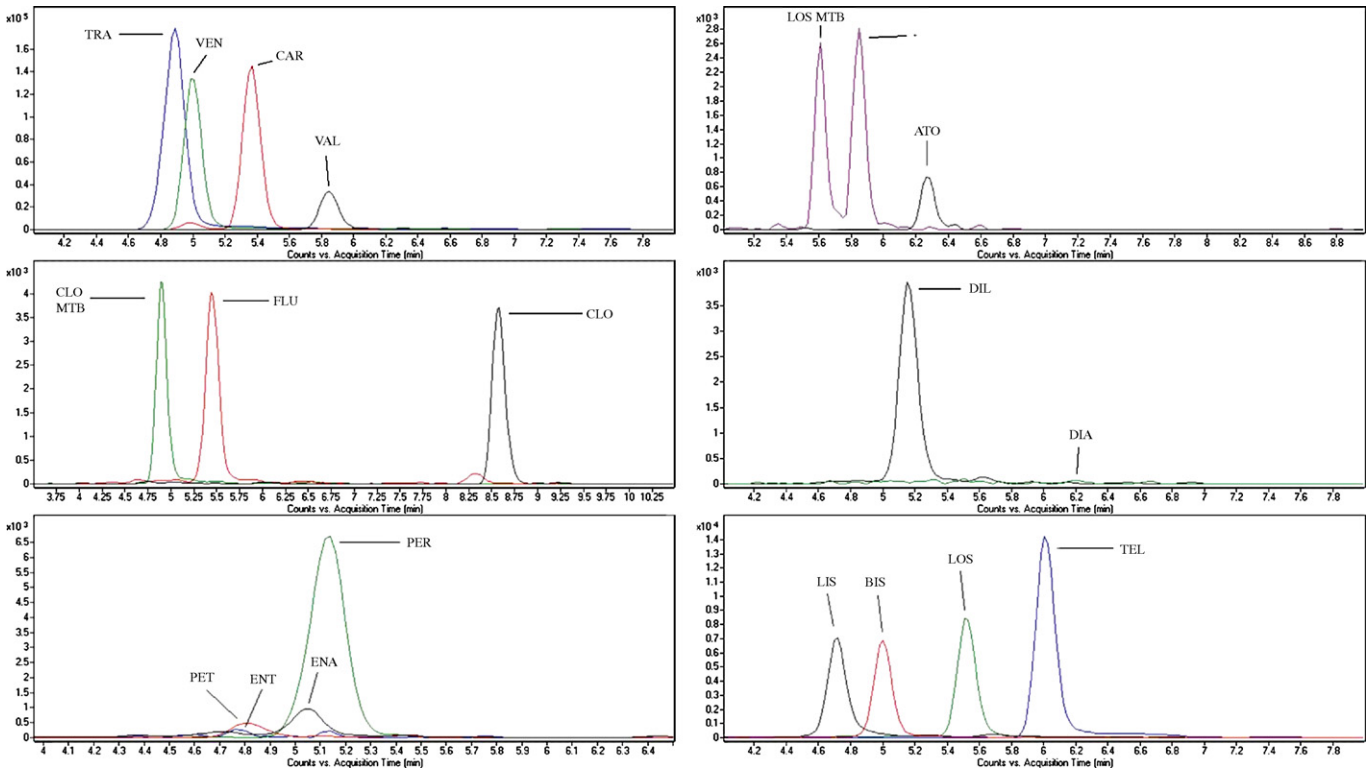

Fig. 3. Chromatogram of the quantifier MRM transition for all compounds for a wastewater sample from WWTP Ostend. ATO: atorvastatin; BIS: bisoprolol; CAR: carbamazepine; CLO: clopidogrel; CLO MTB; clopidogrel metabolite; DIA: diazepam; DIL: diltiazem; ENA: analapril; ENT: enalaprilate; FLU: fluoxetine; LIS: lisinopril; LOS: losartan; LOS MTB: losartan metabolite; PER: perindopril; PET: perindoprilate; TEL: telmisartan; TRA: tramadol; VAL: valsartan; VEN: venlafaxine. *: cross talk LOS MTB and VAL. Column: Kinetex C18 (100 mm × 2.1 mm, 2.6 μm); mobile phase: (A) 5 mM aqueous ammonium acetate with 0.1% formic acid and (B) acetonitrile; flow: 0.2 mL/min; gradient conditions.

been observed only sporadically when analyzing complex extracts with LC–MS/MS [18].

It can be concluded that an accurate estimation of the concentrations of pharmaceuticals in real influent wastewater samples can be obtained when a corresponding deuterated internal standard is used for each analyte. If for various reasons (availability or high costs) this is not possible and other internal standards are chosen, a thorough investigation of the matrix effects and appropriate correction is needed. Synthetic blank wastewater for obtaining exact matrix-matching spiked samples is at this moment not available. An alternative is using standard additions in a pooled lot of influent wastewater, but in this case, it will be difficult to estimate the LLOQ for the analytes with high concentrations in wastewater. On the other hand, physical and chemical properties of influent wastewater can differ between various treatment plants and therefore matrix effects need to be systematically investigated. In this study, we considered initially one deuterated internal standard for each class of compounds, considering the published literature, but several issues rose during method development. The results obtained here emphasize the complexity of wastewater as analytical matrix and suggest that the use of labelled internal standards is compulsory for the correct measurement of concentrations.

3.4. Application to real wastewater samples

The validated method was used for the analysis of 21 influent wastewater samples to demonstrate its applicability. The concentrations of pharmaceuticals in the investigated wastewater samples are shown in Table 4. Typical chromatograms are presented in Fig. 3 for the wastewater sample from WWTP Oostende.

All pharmaceuticals were detected in the wastewater samples. In the angiotensin II receptor antagonist group, the highest concentrations were observed for VAL (maximum of 1.8 μg/L) followed by LOS (maximum of 697 ng/L) and TEL (maximum of 424 ng/L), all in the wastewater sample from WWTP Brussels. LOS MTB was detected in all samples, with concentrations ranging from 17 to 360 ng/L. CLO was detected in 20 out of 21 samples with concentrations up to 6 ng/L. Until now, only one method to determine CLO in influent wastewater was reported [7], but CLO was not found in real samples. CLO MTB was here for the first time analyzed in influent wastewater and it was detected in concentrations ranging from 92 to 561 ng/L. ENA, ENT, PER and PET were measured in the range 2–71 ng/L, in almost all samples. LIS was present in higher concentrations up to 648 ng/L. The presence of LOS MTB, TEL ENT, PER and PET is here for the first time reported in influent wastewater.

The antidepressants FLU and VEN were found in all samples. CAR and TRA were found in all samples, in concentrations widely exceeding 200 ng/L, with maximum of 1 μg/L. DIA was only present in small amounts up to 9 ng/L. The levels of BIS were generally <200 ng/L. The cholesterol lowering drug ATO was detected in 19 of the 21 samples, the highest level being 58 ng/L. DIL was the only analyte found in <50% of the analyzed samples. The results were in agreement with the published literature, where applicable [4–7,19–21].

The wastewater samples used for this study were collected in the frame of another project that required pH 2 for a better stability of the analytes to be studied (illicit drugs). The newly validated method will be employed to investigate the stability of pharmaceuticals in influent wastewater at different temperatures, pHs, and in the presence of colloidal matter, therefore in the future, samples will also be collected at their natural pH. Further, the method will be applied for the analysis of large sets of wastewater samples to relate the concentrations in wastewater to the official sales figures.

4. Conclusion

A fast and sensitive RPLC–MS/MS method for the simultaneous determination of 15 top-sold prescription pharmaceuticals and some of their important metabolites was optimized and validated following guidelines. The chromatographic separation employed a newly developed Kinetex C18 (100 mm × 2.1 mm, 5 μm) column, which provided high separation efficiencies similar to those of sub-2 micron columns. Higher sensitivity was obtained due to peak narrowing. The LLOQs for the selected analytes were ranged between 0.5 and 25 ng/L. All compounds had a good retention and all critical aspects (cross-talk, peak splitting) were appropriately treated. Matrix effects were thoroughly studied. The complexity of influent wastewater as analytical matrix was evidenced once more, and the use of deuterated internal standards for quantification is the most suitable solution for obtaining correct results in real sample analysis. The method was applied to 21 influent wastewater samples in Belgium and all target compounds were detected in concentrations above LLOQ in agreement with published results.

Acknowledgements

Alexander van Nuijs and Dr. Adrian Covaci acknowledge the Flanders Scientific Funds for Research (FWO) for their grants. Dr. Isabela Tarcomnicu is grateful to the University of Antwerp for financial support.

References

[1] U.S. EPA, Pharmaceuticals and Personal Care Products (PPCPs): Relevant Literature, U.S. Environmental Protection Agency, Las Vegas, NV (a comprehensive database of literature references compiled and maintained by CG Daughton and MST Scuderi; first implemented 19 February 2008), 2009. Available from: http://www.epa.gov/ppcp/lit.html.
[2] S. Mompelat, B. Le Bot, O. Thomas, Environ. Int. 35 (2009) 803–814.
[3] J. Bones, K. Thomas, P.N. Nesterenko, B. Paull, Talanta 70 (2006) 1117–1128.
[4] M. Gros, M. Petrovic, D. Barcelo, Anal. Chem. 81 (2009) 898–912.
[5] M. Laven, T. Alsberg, Y. Yu, J. Adolfsson-Erici, J. Chromatogr. A 1216 (2009) 49–62.
[6] E. Gracia-Lor, J.V. Sanchez, F. Hernandez, J. Chromatogr. A 1217 (2010) 622–632.
[7] M. Huerta-Fontela, M.T. Galceran, F. Ventura, J. Chromatogr. A 1217 (2010) 4212–4222.
[8] N. Unceta, M.C. Sampedro, N.K. Abu Bakar, A. Gómez-Caballero, M.A. Goicolea, R.J. Barrio, J. Chromatogr. A 1217 (2010) 3392–3399.
[9] R.A. Trenholm, B.J. Vanderford, S.A. Snyder, Talanta 79 (2009) 1425–1432.
[10] Official Statistics of the Belgian Government. Available from: http://www.riziv.fgov.be/drug/nl/statistics-scientific-information/pharmanet/pharmaceu-tical-tables/pdf/2007/tables2007.pdf.
[11] WADA Technical Document TD2003IDCR: Identification Criteria for Qualitative Assays. Available from: http://www.wada-ama.org/rtecontent/document/criteria_1_2.pdf.
[12] European Union Decision 2002/657/EC, The Performance of Analytical Methods and the Interpretation of Results. Available from: http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2002:221:0008:0036:EN:PDF.
[13] U.S. Department of Health and Human Services, Food and Drug Administration. Guidance for industry: bioanalytical method validation, May 2001. Available from: http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM070107.pdf.
[14] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Anal. Chem. 75 (2003) 3019–3030.
[15] T. Kelly, T.R. Gray, M.A. Huestis, J. Chromatogr. B 867 (2008) 194–204.
[16] A. Kocijan, R. Grahek, D. Kocjan, L. Zupancic-Kralj, J. Chromatogr. B 755 (2001) 229–235.
[17] Phenomenex. http://www.phenomenex.com/cms400min/kinetex.aspx.
[18] R. Leverence, M.J. Avery, O. Kavetskaia, H. Bi, C.E.C.A. Hop, A.I. Gusev, Biomed. Chromatogr. 11 (2007) 1143–1150.
[19] B. Kasprzyk-Hordern, R.M. Dinsdale, A.J. Guwy, Water Res. 43 (2009) 363–380.
[20] A. Wick, G. Fink, A. Joss, H. Siegrist, T.A. Ternes, Water Res. 43 (2009) 1060–1074.
[21] R. Rosal, R. Rodriguez, J.A. Perdigon-Melon, A. Petre, E. Garcia-Calvo, M.J. Gomez, A. Aguera, A.R. Fernandez-Alba, Water Res. 44 (2010) 578–588.