Talanta 158 (2016) 330–341

Contents lists available at ScienceDirect

Talanta

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

Multiresidue trace analysis of pharmaceuticals, their human metabolites and transformation products by fully automated on-line solid-phase extraction-liquid chromatography-tandem mass spectrometry

María Jesús García-Galán [a,b,*], Mira Petrovic [b], Sara Rodríguez-Mozaz [b], Damià Barceló [b]

[a] LEQUIA, Institute of the Environment, University of Girona, Campus Montilivi, E-17071 Girona, Spain
[b] ICRA, Catalan Institute for Water Research, Emili Grahit 101, E-17003 Girona, Spain

ARTICLE INFO

Article history:
Received 21 March 2016
Received in revised form
23 May 2016
Accepted 24 May 2016
Available online 26 May 2016

Keywords:
LC-LC analysis
Metabolites
Transformation products
Environmental waters

ABSTRACT

A novel, fully automated analytical methodology based on dual column liquid chromatography coupled to tandem mass spectrometry (LC-LC-MS^2) has been developed and validated for the analysis of 12 pharmaceuticals and 20 metabolites and transformation products in different types of water (influent and effluent wastewaters and surface water). Two LC columns were used – one for pre-concentration of the sample and the second for separation and analysis – so that water samples were injected directly in the chromatographic system. Besides the many advantages of the methodology, such as minimization of the sample volume required and its manipulation, both compounds ionized in positive and negative mode could be analyzed simultaneously without compromising the sensitivity. A comparative study of different mobile phases, gradients and LC pre-concentration columns was carried out to obtain the best analytical performance. Limits of detection (MLODs) achieved were in the low ng L^{-1} range for all the compounds. The method was successfully applied to study the presence of the target analytes in different wastewater and surface water samples collected near the city of Girona (Catalonia, Spain). Data on the environmental presence and fate of pharmaceutical metabolites and TPs is still scarce, highlighting the relevance of the developed methodology.

1. Introduction

Thousands of tons of different classes of pharmaceutical active compounds (PhACs) are used on a regular basis in human and veterinary medicine worldwide. After their usage and excretion, it is highly probable that both the metabolites and the unchanged parent drug enter the environment [1]. Due to their physical-chemical properties, PhACs are generally hardly biodegradable and only partially removed by physical and standard biological treatment processes (conventional active sludge treatment, (CAS)) in wastewater treatment plants (WWTPs), and could also remain biologically active for long periods [2]. Consequently, several studies concluded that effluents from urban WWTPs should be considered one of the main entrance pathways of PhACs into the environment and therefore partly responsible for surface and marine water contamination [2–4]. The presence of PhACs in all kind of environmental waters has been widely documented during the last decades, at concentrations ranging from ng L^{-1} to μg L^{-1} [5–9]. Although information is still scarce on the ecotoxicity derived of PhACs under real environmental conditions, it is not expected that these concentrations levels for individual compounds could pose an acute risk. However, the combined effect of a mixture of compounds, sharing or not a common mechanism of action could be substantial [10]. Furthermore, the coexistence of the parent drugs with their human metabolites and transformation products (TPs) could also lead to additive, antagonistic and/or synergetic effects which are hard to predict and should be investigated. For instance, a photodegradation TP of DCF has proved to be phytotoxic against certain species of green algae [11], and the assessment of the ecotoxicity of other photoproducts of DCF and naproxen has provided the evidence that acute and chronic toxicity can be greater for these photoproducts than for the parent compounds [12,13]. Donner et al. [14] demonstrated that UV photoproducts of CBZ, acridine and acridone (ACRI, ACRO), were more toxic to certain aquatic organisms than the parent compound. Effective concentration values (EC_{50}) obtained after 15 min exposure for the antibiotic sulfapyridine (SPY) and its acetylated metabolite, N^4-acetylsulfapyridine (acSPY), demonstrated that the marine bacteria *Vibrio fischerii* was more sensitive to the presence of the metabolite than to the original drug, and according to the European Directive 93/67/EEC [15], acSPY could be categorized as toxic [16]. On the other hand, it has been demonstrated that antimicrobial activity of several antibiotics is fully eliminated after advanced treatments such as ozonation [17], but other environmental

* Corresponding author at: LEQUIA, Institute of the Environment, University of Girona, Campus Montilivi, E-17071 Girona, Spain.

http://dx.doi.org/10.1016/j.talanta.2016.05.061
0039-9140/

degradative processes may not be so efficient against the bioactivity of these micropollutants. Majewsky et al. demonstrated that TPs of sulfamethoxazole (SMX) modified in the para (amino) group, such as 4-hydroxy-SMX or 4-nitro-SMX, exhibited higher growth inhibiting properties than SMX against the marine bacteria *V. fischerii*, and that these effects were additive [18].

Only recently, the environmental presence of human metabolites and TPs of PhACs has started to be considered within the scope of monitoring studies, and eventually regarded as potential elements of risk [5,8,19]. During treatment in the WWTP or once released onto the environment, PhACs (and their metabolites) can undergo biotic and abiotic transformation processes (microbial degradation, hydrolysis, photodegradation, oxidation, etc.) yielding a potentially high number of new compounds of unknown elemental composition, stability and potency [5]. Human metabolites and TPs can also be identical; this is the case of 4′-OH-diclofenac (4-OH-DCF) and 5-OH-diclofenac, which account for approximately 22% of the excreted dose of DCF in the urine, but have also been detected as biodegradation products in DCF removal experiments by white rot fungi and identified also as photodegradation TPs [20–22]. The same has been observed for the human metabolites of CBZ, 2-OH-carbamazepine and 10,11-epoxy-carbamazepine (2-OH-CBZ, epo-CBZ), detected after CBZ treatment with fungi [23] and also after its natural biodegradation in soils [24]. However, in many cases degradation pathways are not identical for PhACs, yielding different TPs.

Nowadays, the challenge for PhACs analysis at environmental levels in water matrices has shifted from reaching enough sensitivity and selectivity for their detection, which is generally accomplished using liquid chromatography followed by tandem mass spectrometry (LC-MS^2) as analytical technique, to the reduction of the time of analysis, manipulation of the samples in a minimum number of steps and a reduced use of solvents. Analytical methodologies capable of detection at environmental levels (pg L^{-1}), usually require a clean-up of the sample and pre-concentration of the target analytes based on solid phase extraction (SPE) off-line; SPE involves a certain number of steps that imply several hours of preparation, requiring also sample volumes of up to 100 – 1000 mL to obtain the desired sensitivity and the use of significant amounts of solvents [25,26]. Taking this into account, on-line pre-concentration has become one of the most suitable sample preparation approaches available. Previous works account for the many advantages of on-line SPE procedure, such as minimum sample manipulation by the analyst (lower probability of error), reduced sample volume required, reduced time and solvents used and improved throughput [7,19,27]. By means of dual column liquid chromatography switching system (LC-LC), ordinary on-line SPE has also been improved, as only one pre-concentration column is used for all the set of samples, instead of one SPE cartridge per sample [28].

Although several analytical methods for the determination of pharmaceuticals and TPs are currently available in the literature, the majority is based on off-line SPE [29–31] and the few works dealing with on-line SPE perform analyses in PI and NI mode separately [8,32]. The aim of this work is the development and optimization of a new, fast, robust and high-throughput multi-residue analytical method, based on on-line pre-concentration of the target analytes by means of Equan™ Direct Injection Technology, which permits simultaneous monitoring in either PI and NI mode in the same chromatographic run of 12 pharmaceuticals and 20 of their metabolites and TPs, in surface and wastewaters. The target PhACs were selected considering both their high consumption rates and environmental relevance (high occurrence in the environment). Metabolites and TPs were selected depending on their commercial availability and also considering the little information available regarding their environmental presence.

2. Materials and methods

2.1. Chemicals and reagents

HPLC-grade solvents (water, methanol (MeOH), acetone and acetonitrile (ACN)) and formic acid (HCOOH) (98–100%) were supplied by Merck (Darmstadt, Germany) and Thermo Fisher Scientific (Franklin, MA, US). High purity standards (> 99%) of the pharmaceuticals acetaminophen (ACM), sulfamethoxazole (SMX), sulfapyridine (SPY), sulfamethazine (SMZ), venlafaxine (VFX), diazepam (DZP), carbamazepine (CBZ), diclofenac (sodium salt) (DCF), fluoxetine (FXT), metoprolol (MTP) and the metabolites norverapamil (norVPM), norfluoxetine (norFXT) and acridine (ACRI) were purchased from Sigma-Aldrich (St. Louis, MO, USA). High purity standards for the metabolites 4-nitro-sulfamethoxazole (4-nitro-SMX), 4′-hydroxy-diclofenac (4-OH-DCF), diclofenac amide (adDCF), diclofenac acyl-B-D-glucuronide (gluDCF), acridone (ACRO), D,L-N-desmethylvenlafaxine (N-desVFX), D,L-O-desmethylvenlafaxine (O-desVFX), N^4-acetylsulfapyridine (acSPY), N^4-acetylsulfamethazine (acSMZ), N^4-acetylsulfamethoxazole (acSMX), desmethyldiazepam (norDZP), 3-OH-acetaminophen (3-OH-ACM), α-hydroxymetoprolol (α-HMTP), metoprolol acid (MTPA), O-desmethylmetoprolol (O-DMTP), 2-OH-carbamazepine (2-OH-CBZ) and 10,11-epoxy carbamazepine (epoCBZ) were purchased from TRC (Toronto Research Chemicals Inc., Ontario, Canada). Verapamil (VPM) was obtained from the European Pharmacopoeia (EP). Desmethyl-sulfamethoxazole (des-SMX) was kindly provided by Dr. Tobias Licha, from the Geoscience Center of the University of Göttingen. Isotopically labeled compounds, used as internal standards were purchased from Sigma-Aldrich (atenolol-d_7, fluoxetine-d_5), TRC (verapamil-d_6, diclofenac-d_4, 4′-OH-diclofenac-d_4, sulfamethoxazole-d_4, N^4-acetylsulfapyridine-d_4, N,L-O-desmethylvenlafaxine-d_4 and acetaminophen-d_4), Cerilliant (Texas, USA) (diazepam-d_5) and from CDN isotopes (Quebec, Canada) (carbamazepine-d_{10} and venlafaxine-d_6). Stock standard solutions for each of the analytes were prepared in MeOH at 1 mg mL^{-1} and stored in the dark at −2 °C. Standard solutions of the mixtures of all compounds were made at appropriate concentrations and used to prepare the aqueous calibration curve and also to perform the recovery studies. Similarly, stock standard solutions of the internal standards were prepared. Aqueous standard solutions always contained < 0.1% of MeOH.

2.2. Sampling

For the application and final validation of the methodology, a total of 8 samples of surface water, 6 samples of influent and 6 samples of effluent wastewaters were taken.

Twenty-four hours-integrated samples of WWTP influent (6 samples) and effluent waters (6 samples) were taken in non-consecutive days during winter 2012 from the WWTP of the city of Girona (Spain) and during spring 2013 from the WWTP of Platja d'Aro (Spain), considering the hydraulic retention time in both cases. The WWTP of Girona carries out a secondary biological treatment based on conventional activated sludge (CAS) and serves 206,000 equivalent inhabitants. The, second WWTP counted with a membrane bioreactor (MBR) and serves 175,000 equivalent inhabitants (maximum capacity). Eight surface water samples were also taken: four of them corresponded to a section of the Segre river located upstream of the nearest urban center in a countryside area, and therefore with very low anthropogenic impact, and the other four were taken downstream the discharge of the WWTP of Girona, in the Ter river. All the different water matrices were collected in amber polyethylene terephthalate (PET) bottles and transported to the laboratory under cooled conditions (4 °C). Upon reception, samples were filtered through 0.45 μm Nylon filters (Whatman, Maidstone, UK) to eliminate suspended solid matter and then kept at −18 °C until analysis, which was always carried out within 48 h of collection to avoid degradation. All the analyses were carried out in triplicates.

2.3. Analytical methodology

2.3.1. LC-LC conditions

Fully automated on-line pre-concentration of samples, aqueous standards and operational blanks was performed using a Thermo Scientific EQuan™ system consisting of two quaternary pumps: a loading pump (Accela™ 600 pump) and an elution pump (Accela