Article

Comprehensive Characterization of 76 Pharmaceuticals and Metabolites in Wastewater by LC-MS/MS

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Abstract: Wastewaters are considered one of the main sources of pollution in the aquatic environment as release a large number of contaminants every day. Emerging contaminants such as pharmaceuticals have special interest due to the high levels of consumption by the global population, their bioactive properties and because actual directives do not include the monitoring of pharmaceuticals. Moreover, it is well-known that pharmaceuticals can be degraded to metabolites or transformation products (TPs), which could be more toxic than the parental compound. In this study, we have developed an analytical method based on solid-phase extraction (SPE) and liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) to determine 76 highly consumed pharmaceuticals, including metabolites and TPs in wastewater effluents. In the 10 wastewaters analysed, the mean concentrations were in the $\mu g\ L^{-1}$ levels, being mycophenolic acid, levodopa, ibuprofen, 4-aminoantypirine, losartan, amylmetacresol, amoxicillin, fluticasone, tramadol, budesonide, chlorpheniramine and diclofenac the pharmaceuticals with the highest concentrations. This study provides a comprehensive optimization on the MS conditions to determine pharmaceutical compounds and their metabolites and provides a spectral characterization to be used for the identification of these compounds in water.

Keywords: pharmaceuticals; metabolites; wastewater; LC-MS/MS

1. Introduction

In the last years, pharmaceuticals and personal care products (PPCPs), classified as Emerging Organic Contaminants (EOC), have affected the environment integrity because of their pseudo-persistent properties and because they are continuously discharged to the environment as they are non-regulated contaminants [1]. PPCPs of different families are detected in treated or incompletely treated wastewater and in receiving waters in a recurrent way [2]. Sewage waters collected across the urban sewage system contain PPCPs that have been excreted by the population and are directed to wastewater treatment plants (WWTPs), which are generally not equipped to remove these pollutants. For this reason, those contaminants remain biologically active for long periods and have been found in effluents of WWTPs and in surface waters [3–6]. Actual Directives do not include the monitoring of PPCPs, although the European Commission aims at implementing a Strategic Approach to Pharmaceuticals in the Environment to counteract the negative effects of pharmaceuticals on the environment, covering their whole lifecycle from design and production to disposal (http://www.waterjpi.eu/resources/newsletter/copy_of_2019/newsletter-december-2020/pharmaceuticals-in-the-environment-aquaticpollutants-expected-to-contribute-to-the-eu-strategic-approach-to-reduce-their-adverse-effects, accessed on 22 June 2021).

Pharmaceuticals have been investigated in the aquatic environment throughout the last 20 years [7–9]. However, less attention has been paid to the analysis of metabolites or

transformation products (TPs) [10–12]. The chemical structure of Active Pharmaceutical Ingredients (APIs) can be changed by biotransformation, biodegradation and non-biotic transformation, resulting in a change in their physicochemical and pharmaceutical properties. Metabolites are defined as compounds resulting from structural changes of pharmaceuticals within the human body, as biochemical processes. Moreover, metabolites are formed by enzymes and bacterial activity [13]. The biotransformation of APIs is the introduction of a functional group such as hydroxy, carboxyl, amine or sulfhydryl which increase hydrophilicity and then is followed by the conjugation with polar molecules like glucuronic acid, acetate esters, carboxamides or sulphate [14]. On the other side, TPs are molecules formed under environmental conditions after the excretion of parent compounds and their metabolites through processes of hydrolysis, light, oxidation or biotic process. In addition, compounds resulting from chemical reactions in technical facilities like sewage and drinking water treatment plants are considered TPs.

The advances in the sensibility of the analytical techniques have played an important role in the detection of EOCs at very low concentrations in water [2]. Different instrumental techniques are used for the identification and determination of pharmaceuticals in the environment. However, the most used analytical technique to identify PPCPs is LC-MS/MS with electrospray ionization (ESI), due to the high selectivity and sensitivity [15].

Thus, the aim of this study was to develop an analytical method for the simultaneous determination of 76 pharmaceuticals including related metabolites and a TP using solid-phase extraction (SPE) and liquid chromatography coupled to mass spectrometry (LC-MS/MS). Moreover, the presence of these pharmaceuticals in effluents of WWTP was determined as a means to prove the applicability of the developed methodology.

2. Experimental

2.1. Chemicals and Reagents

Table S1 displays the 76 pharmaceuticals, metabolites and a TP studied classified following their Anatomical Therapeutic Chemical Classification System (ATC) code. All pharmaceutical standards of 98–99% of purity were purchased from Sigma-Aldrich (St. Louis, MO, USA). Moreover, ibuprofen-d_3, diclofenac-acetophenyl ring-$^{13}C_6$, atenolol-d_7, lidocaine-diethyl-d_{10}, acetaminophen-methyl-d_3, acetylsalicylic acid-methyl-d_3, propranolol-d_7, carbamazepine-$^{13}C_6$, gabapentin-$^{13}C_3$ and L-dopa-phenyl-d_3 were also acquired from Sigma and were used as internal standard mixture (ISM). HPLC grade methanol (MeOH) and acetonitrile (ACN) were supplied by VWR Chemicals Prolabo (Leuven, Belgium). Ammonium hydroxide (NH_4OH) and ammonium formate (NH_4COOH) were supplied by Sigma-Aldrich (St. Louis, MO, USA). Formic acid (HCOOH) and hydrochloric acid 37% (HCl) were supplied by Fisher Scientific Chemical (Bridgewater, MA, USA) and Panreac AppliChem (Darmstadt, Germany), respectively. Finally, ultra-pure Milli-Q water was obtained through a Millipore purification system (Millipore, Bedford, MA, USA). Stock standard solutions were prepared at a concentration of 1000 ng μL^{-1} in methanol and working solutions were prepared at 10 and 100 ng μL^{-1} in 90% of Milli-Q® water and 10% of methanol. Table S2 shows the main physicochemical properties of all target compounds.

2.2. Wastewaters: Sampling, Pretreatment and Extraction

Ten effluent wastewaters (WW1–WW10) were sampled on different days in April 2021 in a WWTP located in a small coastal village close to Barcelona (41°14′20″ N 1°46′2″ E), Catalonia, Spain. The sampling consisted of twenty-four-hour composite samples collected using an automated position sample collector. Wastewater samples were kept in the fridge until analysis, to prevent the possible degradation of pharmaceuticals. Once in the laboratory, wastewaters were filtered using 0.22 μm nylon filters to remove the solid particulate phase.

The method was optimized using wastewater spiked at 4 μg L^{-1} of a mix of all target compounds and the ISM at 0.25 μg L^{-1}, which were used as extraction and analytical control. Then, solid-phase extraction (SPE) Oasis HLB cartridges (200 mg, 6 cc, Waters, Mildford, MA, USA) were used to preconcentrate target analytes. Two different pHs (2 and 7, adjusted with HCl 37% with a pH meter SensionTM + PH3, HACH®, Colorado, CO, USA) and three different elution conditions were tested to concentrate 50 mL of water. The cartridges were conditioned with 6 mL of MeOH and 6 mL of Milli-Q® water, and the sample was loaded at flow 1 mL min^{-1}. Once preconcentrated, cartridges were dried over 15–20 min at room temperature and then eluted with different conditions. Test A consisted of the elution of 2 × 6 mL MeOH; Test B consisted of the elution with 6 mL MeOH and 6 mL MeOH + 0.1% formic acid; and Test C consisted of the elution of 4 mL MeOH, 4 mL MeOH + 0.1% formic acid and 4 mL MeOH + 0.1% NH$_4$OH. At pH 7, only the elution Test A and Test C were tested. After the elution, the extracts were evaporated to almost dryness under a current of N$_2$, and then, transferred to a chromatographic vial with 1 mL MeOH as washing solvent. Finally, samples were evaporated with a ReactiVap® until dryness and reconstituted with 200 μL of a 10:90 (*v/v*) MeOH:Milli-Q® water solution.

2.3. LC-MS/MS Analysis

Pharmaceuticals were measured using a liquid chromatography connected to a triple quadrupole mass spectrometer (Xevo TQS, Acquity H-Class, Waters, Milford, CT, USA) (LC-MS/MS). For the chromatographic separation, a CORTECS T3 column (100 mm × 2.1 mm, particle size 1.6 μm, Waters, Milford, CT, USA) was used. Mobile phase composition consisted of binary mixtures with 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B), following a previously published study [16]. Gradient elution started at 95% A and 5% B, increasing to 60% B in 7 min, to 50% B in 12 min and to 100% of B in 3 min, held for 3 min and returned to initial conditions in 2 min, with a holding time of 5 min. Flow rates of 300, 350 and 400 μL min^{-1} were tested in order to improve the resolution and selectivity of target compounds and 300 μL min^{-1} was the best option. In all cases, 5 μL were injected. Most of the compounds were measured under positive electrospray ionization (ESI+), except acetylsalicylic acid, furosemide and hydrochlorothiazide that were detected in negative electrospray ionization (ESI−). Cone voltage (C.V.) was optimized from 1 to 90 V to obtain the precursor ion for each target compound using flow injection analysis (FIA). Moreover, in order to obtain the two intense fragment ions, the collision energy (C.E.) was optimized from 1 to 40 eV. Following the acquisition by selected reaction monitoring (SRM), two transitions from the precursor ion to the product ion were used in order to identify each compound. The optimal parameters are displayed in Table 1 for pharmaceuticals, metabolites, the TP and internal standards. The desolvation temperature was set at 350 °C whereas the desolvation gas flow and the cone gas flow were optimized at 900 L h^{-1} and 150 L h^{-1}, respectively. The system and data management were processed using MassLynx v4.1 software package.

Table 1. LC-MS/MS optimized parameters for selected pharmaceuticals (ordered by ATC code), metabolites (*), transformation product (**), and their internal standards (ˆ). C.V.: cone voltage (V); C.E.: collision energy (eV); q1: product ion for quantification; q2: product ion for confirmation.

Target Compound	Retention Time (Rt)	Precursor Ion [M + H]$^+$	C.V. (V)	q1 Transition	C.E. (eV)	q2 Transition	C.E. (eV)
Acetylsalicylic acid	6.63	137 [M − H]$^-$	6	137 > 93	14	137 > 65	26
Aspirin-d$_3$ ˆ	6.63	137 [M − H]$^-$	10	137 > 93	16	137 > 65	26
Salicylic acid *	6,10	137 [M − H]$^-$	6	137 > 93	16	137 > 65	28
Ranitidine	3.18	315	18	315 > 176	18	315 > 130	24
Omeprazole	6.07	346	35	346 > 198	11	346 > 136	35
Pantoprazole	6.93	384	26	384 > 200	12	384 > 138	37
Esomeprazole	6.07	346	14	346 > 198	10	346 > 136	32
Vildagliptin	3.48	304	30	304 > 154	26	304 > 97	28
Amiodarone	14.90	646	30	646 > 201	40	646 > 100	30
Hydrochlorothiazide	4.15	296 [M − H]$^-$	40	296 > 205	23	296 > 269	17
Furosemide	8.51	329 [M − H]$^-$	43	329 > 285	13	329 > 205	20
Pentoxifylline	5.87	279	54	279 > 181	15	279 > 138	23
Lidocaine	4.81	235	27	235 > 86	23	235 > 58	36
Lidocaine-d$_{10}$ ˆ	4.81	245	24	245 > 96	10	245 > 64	38
Propranolol	7.28	260	40	260 > 183	15	260 > 157	17
Propranolol-d$_7$ ˆ	7.26	267	22	267 > 161	26	267 > 116	18
Atenolol	3.03	267	41	267 > 145	25	267 > 74	23
Atenolol-d$_7$ ˆ	3.01	274	34	274 > 145	26	274 > 74	22
Verapamil	9.08	455	31	455 > 165	30	455 > 150	40
Enalapril	4.79	377	45	377 > 234	19	377 > 117	40
Enalaprilat *	4.87	349	20	349 > 206	16	349 > 91	40
Losartan	9.27	423	31	423 > 377	9	423 > 207	20
Valsartan	11.80	436	20	436 > 291	16	436 > 235	18
Simvastatin	16.01	419	34	419 > 285	8	419 > 199	10
Rosuvastatin	10.18	482	32	482 > 300	34	482 > 258	30
Ezetimibe	12.39	392	12	392 > 133	22	392 > 105	40
Estrone	11.44	271	40	271 > 253	12	271 > 133	20
Cyproterone	14.80	417	28	417 > 357	15	417 > 313	22
Dutasteride	15.54	529	72	529 > 461	32	529 > 69	44
Prednisone	7.81	359	7	359 > 341	11	359 > 147	36
Amoxicillin	4.51	349	56	349 > 255	16	349 > 107	40
Sulfapyridine	4.05	250	40	250 > 156	13	250 > 95	21
Sulfamethoxazole	6.34	254	20	254 > 108	20	254 > 92	28
N^4-acetyl sulfamethoxazole *	6.78	296	21	296 > 198	17	296 > 65	40

Table 1. *Cont.*

Target Compound	Retention Time (Rt)	Precursor Ion [M + H]$^+$	C.V. (V)	q1 Transition	C.E. (eV)	q2 Transition	C.E. (eV)
Sulfadiazine	3.56	251	30	251 > 156	13	251 > 96	20
Erythromycin	7.93	734	44	734 > 158	30	734 > 116	44
Clarithromycin	9.24	748	20	748 > 158	16	748 > 116	46
Ciprofloxacin	5.00	332	40	332 > 314	20	332 > 245	20
Norfloxacin	4.88	320	22	320 > 302	18	320 > 276	16
Levofloxacin	4.90	362	44	362 > 318	18	362 > 261	26
Cyclophosphamide	6.75	261	26	261 > 140	20	261 > 106	16
Ifosfamide	6.55	261	42	261 > 154	20	261 > 78	18
Megestrol	14.83	385	8	385 > 325	12	385 > 267	16
Bicalutamide	12.04	431	33	431 > 217	14	431 > 95	50
Mycophenolic acid	10.15	321	14	321 > 207	20	321 > 159	36
Diclofenac	13.71	296	26	296 > 250	12	296 > 215	20
Diclofenac-$^{13}C_6$ ^	13.71	338	38	338 > 303	14	338 > 111	20
N-(2,6-dichlorophenyl)-2-indolinone **	12.80	278	40	278 > 208	20	278 > 214	20
Ibuprofen	13.98	161	54	161 > 119	12	161 > 105	12
Ibuprofen-d$_3$^	13.98	164	40	164 > 122	12	164 > 108	10
1-hydroxy ibuprofen/2-hydroxy ibuprofen *	14.89	223	96	223 > 207	11	223 > 193	11
Tramadol	5.82	264	9	264 > 246	10	264 > 58	17
4-aminoantipyrine *	3.14	204	20	204 > 94	17	204 > 56	20
Paracetamol	2.98	152	32	152 > 110	20	152 > 93	20
Acetaminophen-(methyl-d$_3$) ^	2.98	155	30	155 > 110	38	155 > 93	20
Carbamazepine	8.47	237	30	237 > 194	20	237 > 179	36
Carbamazepine-$^{13}C_6$ ^	8.47	243	40	243 > 200	16	243 > 185	34
Carbamazepine 10,11-epoxide *	7.16	253	29	253 > 236	9	253 > 210	10
Topiramate	7.64	340	18	340 > 264	6	340 > 127	20
Gabapentin	3.50	172	27	172 > 154	10	172 > 137	14
Gabapentin-$^{13}C_3$ ^	3.50	175	30	175 > 157	12	175 > 140	12
Levetiracetam	3.27	171	25	171 > 154	5	171 > 126	13
Pregabalin	3.54	160	29	160 > 142	8	160 > 83	14
Levodopa	1.07	198	45	198 > 152	7	198 > 107	25
Levodopa-d$_3$ ^	1.07	201	24	201 > 184	6	201 > 155	10
Rasagiline	3.60	172	70	172 > 117	14	172 > 91	32
Diazepam	10.53	285	30	285 > 193	33	285 > 154	25
Clomethiazole	6.41	162	40	162 > 126	18	162 > 112	34
Scopolamine	4.07	304	27	304 > 156	15	304 > 138	21

Table 1. *Cont.*

Target Compound	Retention Time (Rt)	Precursor Ion [M + H]$^+$	C.V. (V)	q1 Transition	C.E. (eV)	q2 Transition	C.E. (eV)
Fluoxetine	9.38	310	27	310 > 148	7	310 > 44	13
Citalopram	7.98	325	10	325 > 262	16	325 > 109	22
Escitalopram	7.98	325	54	325 > 263	17	325 > 109	23
Trazodone	6.98	372	31	372 > 176	22	372 > 148	36
Venlafaxine	6.66	278	47	278 > 121	31	278 > 58	17
Caffeine	4.13	195	22	195 > 138	18	195 > 110	22
Donepezil	7.45	380	36	380 > 243	36	380 > 91	39
Memantine	6.69	180	30	180 > 163	11	180 > 107	21
Dichlorobenzyl alcohol	9.42	159	45	159 > 123	19	159 > 89	30
Amylmetacresol	15.40	179	32	179 > 109	8	179 > 71	8
Budesonide	11.28	431	29	431 > 147	30	431 > 73	30
Fluticasone	15.00	501	58	501 > 313	12	501 > 293	18
Tiotropium bromide	6.27	393	55	393 > 170	29	393 > 152	27
Acetylcysteine	1.70	164	24	164 > 122	6	164 > 76	16
Dextromethorphan	7.48	272	46	272 > 171	38	272 > 147	30
Cloperastine	9.50	332	42	332 > 203	18	332 > 166	45
Chlorpheniramine	6.02	275	6	275 > 230	18	275 > 167	43

2.4. Quality Assurance

Calibration was performed over a concentration range from 0.01 to 1 ng μL^{-1} using six calibration points in MeOH:Milli-Q® water 10:90 (*v/v*) and ISM at 0.25 μg L^{-1}. Recoveries of pharmaceuticals were estimated using wastewater samples spiked at 4 μg L^{-1} with the mixture of pharmaceuticals and the ISM. Ten pharmaceutical internal standards were used as extraction and analytical control and external calibration which offered a better response for some pharmaceuticals was used. The instrumental detection limit (IDL) is the minimum amount of analyte required to produce a signal that is statistically distinguishable from the background noise level within a specified confidence level. IDL was determined using the lowest concentration of a standard solution that generated an S/N ratio equal to 3. On the other hand, method detection limit (MDL) was calculated from the injection of spiked wastewater samples at 4 μg L^{-1} using the minimum concentration of analyte providing an S/N ratio of 3 for the MDL and a ratio of 10 for the LOQ. Moreover, the precision of the method was determined by the estimation of the intra-day assay, expressed as the percentage relative standard deviation (%RSD) of replicate measurements. The variation was assessed by five consecutive injections of 0.1 ng μL^{-1} standard solution. Finally, matrix effect (ME) was assessed in order to evaluate the degree of signal suppression or enhancement. The ME was calculated by dividing the areas of each pharmaceutical in a solution in wastewater following Equation (1). Values of 100% indicate that there is no matrix effect. However, values higher than 100% means ion enhancement whereas values lower than 100% indicate ion suppression.

$$ME\ (\%) = \frac{A - B}{C} \times 100 \qquad (1)$$

where *A* is the peak area of each analyte from spiked wastewater samples; *B* is the peak area of each analyte from non-spiked wastewater; and *C* is the peak area of each analyte in the standard solution. Table 2 displays the quality parameters studied.

Table 2. Quality parameters obtained for 76 compounds ordered following de ATC code for the pharmaceuticals, metabolites (*) and transformation product (**).

Compound	Linearity (ng µL^{-1})	R^2	IDL (pg)	Intra-Day Precision (%)	%R $\pm$ RSD (WW)	MDL (ng L^{-1})	LOQ (ng L^{-1})	Matrix Effect (%)
Acetylsalicylic acid/Salicylic acid *	0.01–1	0.9921	0.73	3	60 $\pm$ 11	21	70	53
Ranitidine	0.01–1	0.9953	2.10	18	100 $\pm$ 11	5	16	63
Omeprazole/Esomeprazole	0.01–0.1	0.9938	0.91	1	61 $\pm$ 2	2	5	89
Pantoprazole	0.01–0.5	0.9975	0.13	5	87 $\pm$ 2	1	2	68
Vildagliptin	0.01–1	0.9989	0.23	11	93 $\pm$ 7	0.4	1	66
Amiodarone	0.01–1	0.9928	1.74	11	53 $\pm$ 5	1	2	29
Hydrochlorothiazide	0.01–1	0.9900	0.01	16	160 $\pm$ 6	4	13	92
Furosemide	0.01–1	0.9926	0.48	11	45 $\pm$ 22	8	28	60
Pentoxifylline	0.01–1	0.9914	0.56	4	94 $\pm$ 8	1	3	63
Lidocaine	0.01–1	0.9945	0.03	4	140 $\pm$ 9	1	2	94
Propranolol	0.01–1	0.9933	0.02	3	90 $\pm$ 10	5	18	65
Atenolol	0.01–1	0.9920	1.12	5	109 $\pm$ 12	4	14	115
Verapamil	0.01–1	0.9949	1.04	4	90 $\pm$ 7	2	5	59
Enalapril	0.01–1	0.9929	0.47	7	85 $\pm$ 5	1	5	81
Enalaprilat *	0.01–1	0.9952	2.12	8	86 $\pm$ 12	1	4	77
Losartan	0.01–1	0.9934	1.50	9	160 $\pm$ 20	4	12	108
Valsartan	0.01–1	0.9923	0.49	3	70 $\pm$ 6	2	8	177
Simvastatin	0.01–1	0.9982	0.26	9	78 $\pm$ 6	3	9	102
Rosuvastatin	0.01–1	0.9924	1.10	7	126 $\pm$ 5	6	21	96
Ezetimibe	0.01–1	0.9903	1.22	1	59 $\pm$ 15	9	30	43
Estrone	0.01–1	0.9952	1.73	3	74 $\pm$ 9	13	42	69
Cyproterone	0.01–1	0.9940	1.04	1	84 $\pm$ 6	2	8	66
Dutasteride	0.01–1	0.9955	0.19	3	41 $\pm$ 7	2	5	34
Prednisone	0.01–1	0.9929	0.44	1	77 $\pm$ 9	14	47	101
Amoxicillin	0.01–1	0.9945	2.81	13	102 $\pm$ 5	8	15	98
Sulfapyridine	0.01–1	0.9900	0.21	6	98 $\pm$ 6	0.5	2	68
Sulfamethoxazole	0.01–1	0.9957	1.54	7	84 $\pm$ 6	16	53	66
N^4-acetyl sulfamethoxazole *	0.01–1	0.9985	1.44	4	82 $\pm$ 3	2	6	51
Sulfadiazine	0.01–1	0.9905	0.76	5	98 $\pm$ 5	2	8	62
Erythromycin	0.01–0.5	0.9996	0.44	1	67 $\pm$ 6	2	8	63
Clarithromycin	0.01–0.5	0.9998	2.12	1	74 $\pm$ 9	9	29	73
Ciprofloxacin	0.01–1	0.9966	1.59	12	79 $\pm$ 13	16	53	54
Norfloxacin	0.01–1	0.9927	1.60	13	65 $\pm$ 11	11	37	63
Levofloxacin	0.01–1	0.9910	0.40	10	80 $\pm$ 10	3	11	81
Cyclophosphamide	0.01–1	0.9916	0.96	4	69 $\pm$ 13	22	73	69

Table 2. *Cont.*

Compound	Linearity (ng μL^{-1})	R^2	IDL (pg)	Intra-Day Precision (%)	%R ± RSD (WW)	MDL (ng L^{-1})	LOQ (ng L^{-1})	Matrix Effect (%)
Ifosfamide	0.01–1	0.9935	1.16	1	106 ± 3	31	102	75
Megestrol	0.01–1	0.9926	0.38	4	66 ± 12	7	23	65
Bicalutamide	0.01–1	0.9939	1.42	5	78 ± 9	2	7	68
Mycophenolic acid	0.01–1	0.9905	2.19	1	81 ± 3	4	14	63
Diclofenac	0.01–1	0.9959	2.27	1	72 ± 12	1	3	154
N-(2,6-dichlorophenyl)-2-indolinone **	0.01–1	0.9923	1.20	2	89 ± 9	2	6	70
Ibuprofen	0.01–1	0.9971	2.07	1	100 ± 8	14	47	120
1-hydroxy ibuprofen/2-hydroxy ibuprofen *	0.01–1	0.9906	5.84	12	86 ± 10	19	65	57
Tramadol	0.01–1	0.9925	0.04	8	176 ± 7	1	2	117
4-aminoantipyrine *	0.01–1	0.9813	0.59	5	26 ± 11	23	78	115
Paracetamol	0.01–1	0.9958	1.09	1	52 ± 10	22	74	31
Carbamazepine	0.01–1	0.9914	0.67	4	117 ± 12	2	8	84
Carbamazepine 10,11-epoxide *	0.01–1	0.9900	0.24	1	109 ± 6	0.4	1	73
Topiramate	0.01–1	0.9949	0.47	3	93 ± 4	15	50	75
Gabapentin	0.01–1	0.9921	0.04	3	86 ± 14	2	5	30
Levetiracetam	0.01–1	0.9953	0.76	2	92 ± 7	0.4	1	68
Pregabalin	0.01–1	0.9913	0.24	7	51 ± 6	3	9	95
Levodopa	0.01–1	0.9975	1.61	7	101 ± 11	7	18	99
Rasagiline	0.01–1	0.9993	1.06	9	32 ± 5	26	87	84
Diazepam	0.01–1	0.9945	1.44	1	81 ± 6	12	41	86
Clomethiazole	0.01–1	0.9942	0.98	1	63 ± 14	26	87	38
Scopolamine	0.01–1	0.9920	1.55	1	70 ± 9	26	85	69
Fluoxetine	0.01–1	0.9959	1.49	5	68 ± 12	14	48	63
Citalopram/Escitalopram	0.01–1	0.9987	0.20	1	102 ± 15	4	12	61
Trazodone	0.01–1	0.9969	0.06	4	97 ± 6	4	15	60
Venlafaxine	0.01–1	0.9997	0.44	9	52 ± 10	10	15	79
Caffeine	0.01–1	0.9913	1.10	12	52 ± 5	8	18	77
Donepezil	0.01–0.5	0.9967	0.15	2	74 ± 8	21	69	67
Memantine	0.01–1	0.9951	0.96	1	104 ± 9	30	101	67
Dichlorobenzyl alcohol	0.01–1	0.9932	3.57	1	75 ± 5	40	135	89
Amylmetacresol	0.01–1	0.9936	5.87	4	62 ± 6	18	59	93.
Budesonide	0.01–1	0.9960	1.62	14	86 ± 3	7	25	76
Fluticasone	0.01–1	0.9904	0.95	7	58 ± 6	17	57	38
Tiotropium bromide	0.01–1	0.9906	0.75	2	70 ± 20	30	130	36
Dextromethorphan	0.01–1	0.9958	0.34	6	98 ± 8	3	8	71
Cloperastine	0.01–1	0.9950	0.14	6	85 ± 8	3	11	59
Chlorpheniramine	0.01–0.5	0.9958	0.88	15	100 ± 2	20	68	65

3. Results and Discussion

3.1. Optimization of the Ionization Parameters

Optimization of the ionization conditions was performed using LC-MS/MS in ESI+ and ESI−. For the large screening of PPCPs, the pH of the mobile phase affects the ionization, where acidic pH obtains molecular ions that are charged positively ($[M + H]^+$) and basic pH leads to negative ions ($[M − H]^-$). Both modes can work together, as some molecules may be ionized in positive and negative mode simultaneously, obtaining both mass spectrums, one with higher intensity than the other. Table 1 summarizes the precursor ions and the two most intense fragments, including the optimum C.V. and C.E. voltages for each target compound. C.V. was the major parameter influencing the intensity of signal ions and it was adjusted from 6 to 96 V to achieve the best response for the molecular ion. Then, the molecular ion was fragmented to produce intense product ions, using the C.E which was optimized from 5 to 46 eV. The two most abundant fragments were monitored for each compound, one for quantification purposes and the second for confirmation. Mass spectral characterization for target compounds is included in the Supplementary information, classified by ATC code for selected pharmaceuticals, including their metabolites, and the internal standards. Table S3 shows the MRM transition of the compounds studied. The section "Mass spectral characterization" has been added in the supplementary information describing the fragmentation pattern of each compound.

3.2. Optimization of the Chromatographic Conditions

In order to achieve the optimum resolution and identification of target compounds, the mobile phase composition and gradient conditions were tested using a CORTECS T3 column (Waters, Mildford, OH, USA) using a standard solution at a concentration of 1 ng μL^{-1}. In all the experiments, the mobile phase composition was formed by 0.1% formic acid in water (A) and 0.1% formic acid in ACN (B). Firstly, the composition of the standards was 100% of methanol but results showed that part of the compounds were not retained in the stationary phase, and presented wide peaks. In addition, for some compounds, two or three peaks were formed which means low interaction between the stationary phase and the mobile phase with the compounds. Moreover, for acetylsalicylic acid and hydrochlorothiazide analysed in ESI−, no-signal was observed. Secondly, different proportions of formic acid (0.1%, 0.5% and 1.0%) were added to the standard made in methanol in order to increase the affinity between the stationary phase and the analytes, and the ionization of target compounds. Although the shape of the chromatographic peaks was improved, most of the compounds were not well-retained presenting retention times <1 min. Finally, the last tests were performed changing the proportion of MeOH (10%) and Milli-Q® water (90%), and also, adding 0.1% formic acid at the aqueous phase of standards, providing better results in terms of resolution when high proportions of aqueous solvent were used. However, the addition of formic acid did not represent any improvements in the chromatographic resolution. For this reason, a composition of 10% MeOH and 90% Milli-Q® water was employed, solving the peak splitting, too.

Once the standard solution conditions were optimized, the gradient of the mobile phase composition was tested, following a previously published paper about the presence of pharmaceuticals in wastewaters from senior residences [16]. As indicated before, the mobile phase composition consisted of 0.1% formic acid in water (A) and 0.1% formic acid in ACN (B), testing different gradients. Firstly, Test 1 started at 100% A and increased to 70% B in 12 min, and to 100% of B in 3 min, holding for 5 min with a total run time of 27 min, using a flow of 0.3 mL min^{-1}. Moreover, in this gradient, small variations were performed with the proportion of the mobile phases at 12 min being 70%, 50%, 40%, and 30% of solvent B. On the other hand, Test 2 started at 100% A and increased to 30% B in 7 min, holding 1 min, and increasing to 70% of B in 7 min, holding 1 min, and finally at 100% of B in 4 min with a total run time of 27 min using a flow rate of 0.3 mL min^{-1}. The initial high proportion of the aqueous phase allowed the elution of most water-soluble analytes, whereas low and continuously increasing of the organic mobile phase offer lowest

retention time for compounds very soluble in water such as levodopa or paracetamol. Thus, Test 2, reported better results in terms of efficiency and resolution. On the other hand, Test 3 started at 100% A and decreased to 70% A in 7 min, followed by 50% of solvent B in 5 min and to 100% of B in 4 min with a total run time of 28 min using a flow of 0.3 mL min^{-1}. Based on it, some variations were carried out to obtain a good separation and peak resolution. Firstly, different proportions of the mobile phases (95%, 90%, 80%, 70% and 60% of solvent A) were used in minute 7 to adjust the best gradient increase of the organic phase. Secondly, the flow rate of the chromatographic analysis was optimised at 300, 350 and 400 μL min^{-1}, with the same gradient as Test 3. Finally, the best results were obtained with Test 4, starting at 95% A and 5% B, increasing to 40% B in 7 min, followed by 50% of solvent B in 5 min and to 100% of B in 3 min with a total run time of 25 min using a flow of 300 μL min^{-1}, producing better efficiency, resolution and sensitivity for most of pharmaceuticals and metabolites. However, the structural isomers acetylsalicylic acid/salicylic acid, omeprazole/esomeprazole, citalopram/escitalopram and 1-hydroxy ibuprofen/2-hydroxy ibuprofen could not be chromatographically separated due to the same MS transitions. The ion chromatogram of a mixed solution at 1 ng μL^{-1} containing all the target analytes and using the CORTECS T3 column is shown in Figure 1.
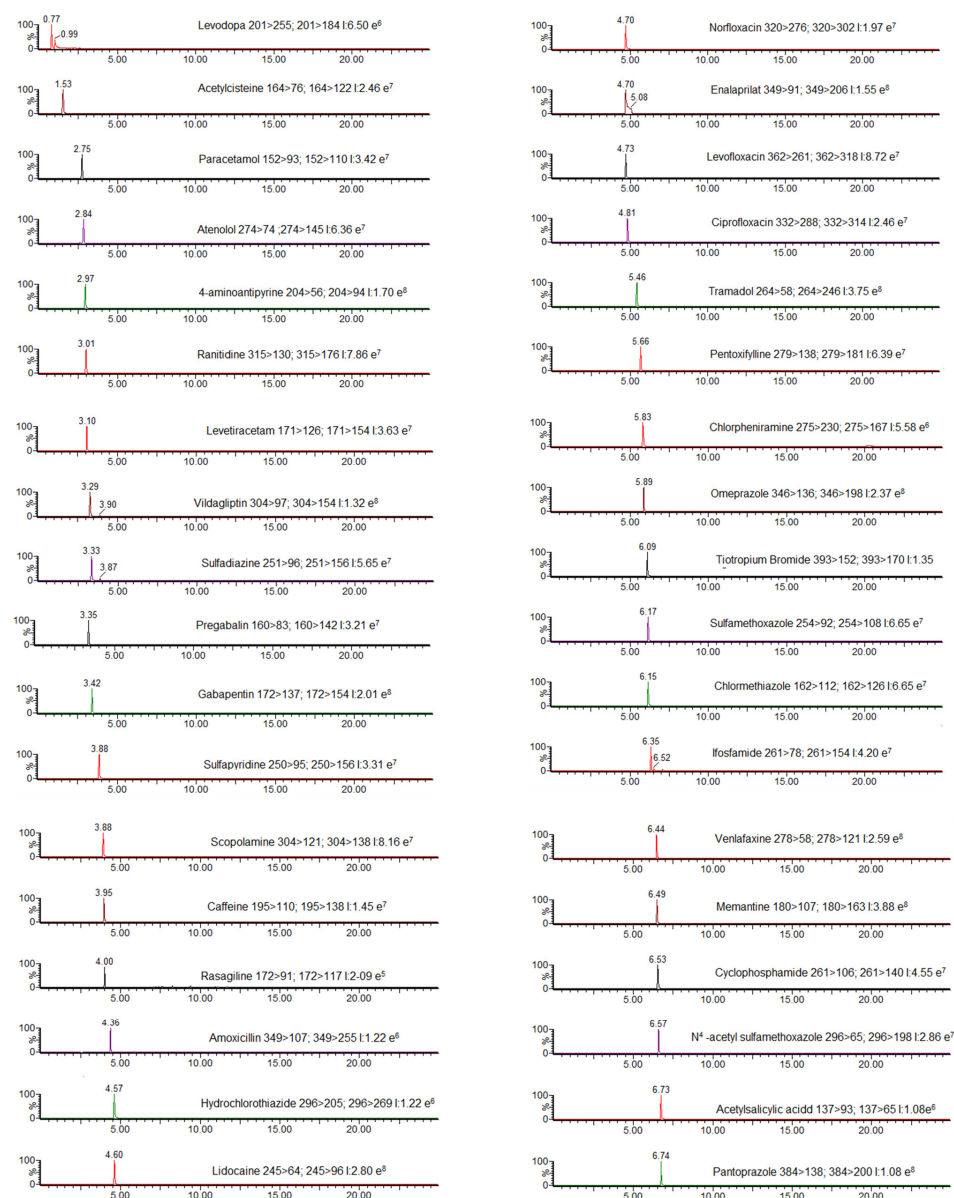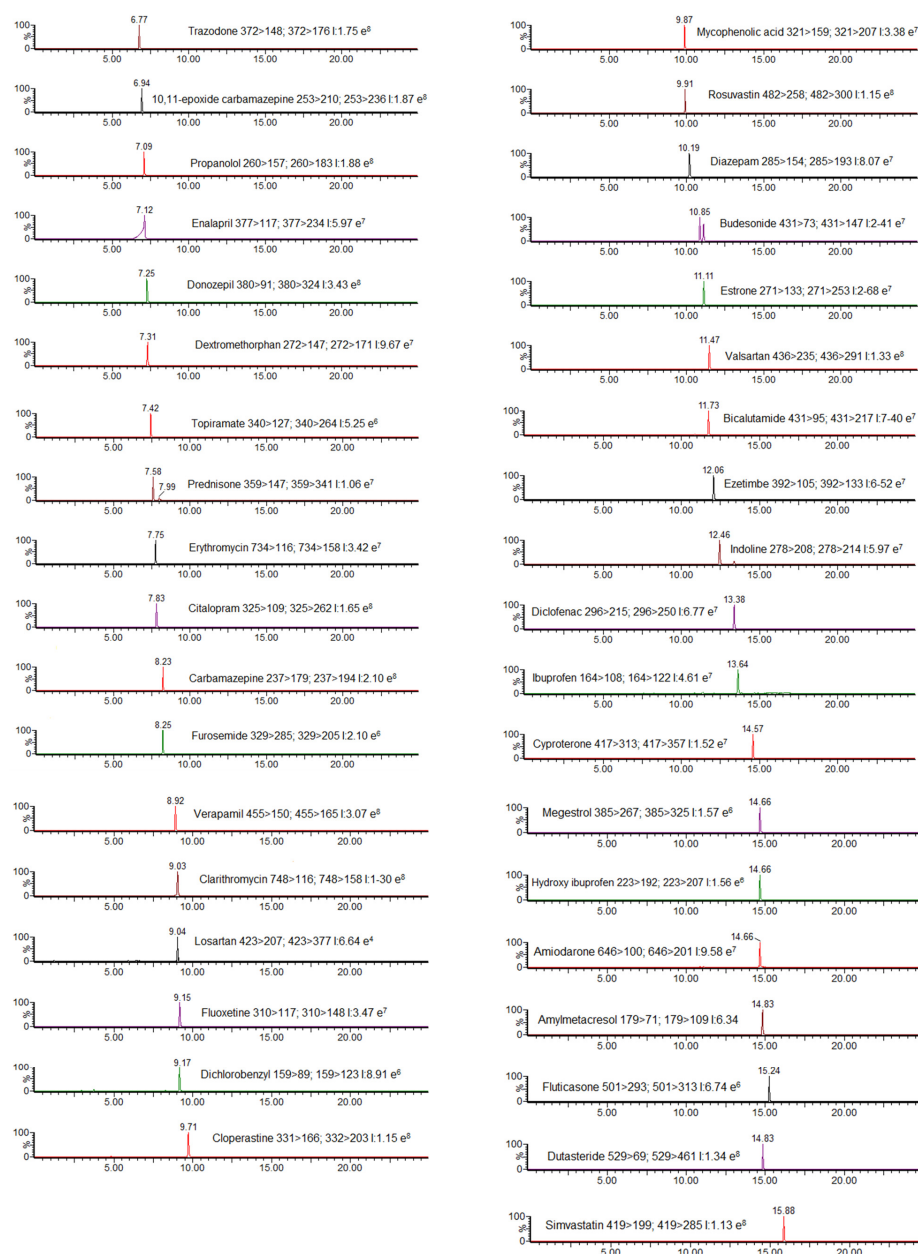

Figure 1. *Cont.*

Figure 1. LC-MS/MS separation of the 76 pharmaceuticals, metabolites and transformation product (TP) with CORTECS T3 column. The compounds acetylsalicylic acid/salicylic acid, omeprazole/esomeprazole, citalopram/escitalopram and 1-hydroxy ibuprofen/2-hydroxy ibuprofen coeluted at the same retention times due to the same MS transitions.

3.3. Optimization of Extraction Procedure and Quality Parameters

Table 2 shows the linearity studied that was in the range of 0.01–1 ng μL^{-1} for all selected compounds, but different linear responses were obtained, depending on the sensitivity for each analyte: 65 compounds were linear from 0.01 to 1 ng μL^{-1} with good correlation ($R^2 \geq 0.99$); pantoprazole, erythromycin, clarithromycin, donepezil and chlorpheniramine were linear in the range of 0.01 to 0.5 ng μL^{-1}; and finally, omeprazole/esomeprazole were linear in the range of 0.01 to 0.1 ng μL^{-1}. The IDLs ranged from 0.02 (propranolol) to 5.87 pg (amylmetacresol), the MDL ranged from 0.4 (levetiracetam) to 40 ng L^{-1} (dichlorobenzyl alcohol) and the LOQ ranged between 1 to 135 ng L^{-1}. Moreover, the intra-day precision (repeatability) of the method ranged between 1 to 18% ($N = 3$).

For SPE, different extraction procedures were tested at pH 2 and pH 7 using wastewater. Target compounds present different physicochemical properties linked with their solubility in aqueous samples, so developing two extraction procedures will increase the possibilities to retain most of them. As commented before, at pH 2, three different elution methods were tested to evaluate the efficiency of the elution solvents with Oasis HLB cartridges (200 mg, 6cc, Waters, Mildford, MA, USA); and at pH 7, two different elution methods were tested with the same cartridges. The high complexity of the type of water analysed (wastewater) affected considerably the results. Results showed that Test C allowed achieving the best efficiency as elution solvent at both pH 2 and pH 7, where 76 compounds were recovered in the range of 26% (4-aminoantipyrine) to 160% (hydrochlorothiazide) (Table 2). The matrix effect has significant relevance in LC-MS/MS analysis as signal intensity may be considerably changed affecting the recovery rates of pharmaceuticals in complex wastewater samples. For some target compounds such as acetylsalicylic acid/salicylic acid, amiodarone, verapamil, ezetimibe, dutasteride, N4-acetylsulfamethoxazole, ciprofloxacin, 1-hydroxyibuprofen/2-hydroxyibuprofen, paracetamol, gabapentin, clomethiazole, fluticasone and tiotropium, the presence of matrix compound in the wastewater results in a strong signal suppression presenting values lower than 60%. However, valsartan and diclofenac had signal enhancement, presenting matrix effect values over 120% (see Table 2).

3.4. Presence of Pharmaceutical Residues and Metabolites in Wastewater Samples

To validate the extraction method, 10 wastewater samples were analyzed by SPE and Table 3 displays the levels of the 76 pharmaceuticals and metabolites studied. All compounds were detected in wastewaters except acetylsalicylic acid/salicylic acid, hydrochlorothiazide, furosemide, cyproterone and rasagiline. Figure 2 shows a boxplot in logarithmic scale (in $\mu g\ L^{-1}$) of the pharmaceuticals detected at the highest concentrations in the wastewaters sampled. Mycophenolic acid, an immunosuppressant medication used to prevent rejection following organ transplantation and to treat autoimmune conditions such as Crohn's disease and lupus, was detected at levels between 1.3 and 23.2 $\mu g\ L^{-1}$ (Table 3 and Figure 2). Recently, Santos et al. developed and validated a single analytical methodology to identify and quantify seven cytostatics in waters and reported levels of mycophenolic acid in effluent samples from a WWTP equipped with a UV disinfection process located in Porto between 0.4 and 0.8 $\mu g\ L^{-1}$ [17]. The authors concluded that the UV disinfection step could contribute to increasing the removal/degradation of mycophenolic acid and other pharmaceuticals in wastewater. Another pharmaceutical with high concentrations was levodopa (L-DOPA), which was detected in wastewaters at levels between <LOQ and 8.8 $\mu g\ L^{-1}$ (see Table 3 and Figure 2). Levodopa is a drug used in the clinical treatment of Parkinson's disease and dopamine-responsive dystonia. Another pharmaceutical detected with high concentrations was ibuprofen, a nonsteroidal anti-inflammatory drug (NSAID) class widely used for treating pain, fever, and inflammation. Ibuprofen was detected in the 10 wastewater samples at levels between 1.1 and 7.3 $\mu g\ L^{-1}$ (Table 3). A recent review indicates the widespread presence of ibuprofen in wastewaters at $\mu g\ L^{-1}$ [18]. In 2015, Caballo et al. reported levels of ibuprofen between 0.63 and 0.68 $\mu g\ L^{-1}$ in 3 different WWTPs in Córdoba (South of Spain) [19]. Recently, Whang et al. published levels of the enantiomeric fraction of ibuprofen of 0.32 $\mu g\ L^{-1}$ in a wastewater treatment plant located in Yuhong District of Shenyang, China [20]. On the other hand, 4-aminoantipyrine, also known as ampyrone, is a metabolite of aminopyrine with analgesic, anti-inflammatory, and antipyretic properties. 4-aminoantipyrine ranged from 1.9 to 7.4 $\mu g\ L^{-1}$ (Table 3 and Figure 2). To the best of the author's knowledge, this is the first time that 4-aminoantypirine has been detected in wastewater. Losartan, a medication mainly used to treat high blood pressure and amylmetacresol, with mean levels of 1.82 and 1.81 $\mu g\ L^{-1}$, respectively (Table 3) were also detected. Other pharmaceuticals with mean values over 1 $\mu g\ L^{-1}$ were amoxicillin, fluticasone, tramadol, budesonide, chlorpheniramine and diclofenac.

Table 3. Levels of 76 pharmaceuticals, metabolites (*) and TP (**) in effluents of wastewater ($\mu g\ L^{-1}$). WW: wastewater.

	WW1	WW2	WW3	WW4	WW5	WW6	WW7	WW8	WW9	WW10	Mean
Acetylsalicylic acid/Salicylic acid *	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Ranitidine	0.045	0.048	0.048	0.097	0.081	0.036	0.098	0.026	<LOQ	0.026	0.056
Omeprazole/Esomeprazole	0.013	0.010	0.010	0.011	0.010	0.011	0.008	0.012	0.010	0.011	0.011
Pantoprazole	0.0038	0.0098	0.0065	0.0197	0.0024	0.0057	0.0047	0.0038	0.0024	0.0075	0.0066
Vildagliptin	0.173	0.170	0.243	0.314	0.253	0.261	0.260	0.240	0.212	0.206	0.233
Amiodarone	0.032	0.025	0.022	0.115	0.056	0.016	0.033	<LOQ	<LOQ	<LOQ	0.043
Hydrochlorothiazide	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Furosemide	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Pentoxifylline	<LOQ	<LOQ	0.038	0.083	0.099	0.179	0.168	0.058	0.082	0.073	0.098
Lidocaine	0.122	0.114	0.131	0.153	0.203	0.206	0.262	0.196	0.195	0.245	0.183
Propranolol	<LOQ	<LOQ	0.038	0.083	0.099	0.179	0.168	0.058	0.082	0.073	0.098
Atenolol	0.456	0.153	0.209	0.146	0.899	0.650	1.51	0.946	1.39	0.304	0.666
Verapamil	0.017	0.048	0.029	0.278	0.145	0.041	0.113	0.046	0.054	0.049	0.082
Enalapril	0.300	0.150	0.140	0.190	0.250	0.210	0.070	0.040	0.230	0.250	0.183
Enalaprilat *	0.076	0.036	0.026	0.361	0.230	0.079	0.385	0.053	0.205	0.047	0.150
Losartan	2.19	0.894	1.67	1.12	1.04	2.37	2.85	2.09	2.93	1.09	1.825
Valsartan	0.918	0.377	0.861	1.216	0.632	0.178	2.483	1.520	1.577	0.856	1.062
Simvastatin	0.011	0.023	0.011	0.005	0.033	0.005	0.004	0.006	0.007	0.053	0.016
Rosuvastatin	0.061	0.076	0.082	0.219	0.144	0.085	0.331	0.053	0.052	0.029	0.113
Ezetimibe	0.015	0.041	0.010	0.266	0.110	0.029	0.061	<LOQ	<LOQ	<LOQ	0.076
Estrone	0.027	0.028	0.033	0.168	0.093	0.029	0.231	0.012	0.020	0.014	0.066
Cyproterone	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Dutasteride	<LOQ	0.006	0.005	0.017	0.022	0.015	0.127	0.014	0.017	0.024	0.027
Prednisone	0.038	0.022	0.018	0.353	0.217	0.097	0.351	0.077	0.137	0.103	0.141
Amoxicillin	0.844	2.51	1.14	1.67	1.08	2.56	1.82	2.13	0.676	0.925	1.535
Sulfapyridine	0.170	0.055	0.071	0.094	0.122	0.164	0.220	0.080	0.077	0.124	0.118
Sulfamethoxazole	0.223	0.198	0.366	0.266	0.187	0.210	0.255	0.265	0.492	0.268	0.273
N^4-acetyl sulfamethoxazole *	<LOQ	0.045	0.019	0.324	0.167	0.050	0.241	0.017	0.045	0.041	0.105
Sulfadiazine	0.014	0.017	0.010	0.026	0.067	0.034	0.066	0.016	0.063	0.044	0.036
Erythromycin	0.070	0.060	0.045	0.026	0.074	0.024	0.020	0.042	0.036	0.045	0.044
Clarithromycin	0.149	0.115	0.157	0.039	0.200	0.066	0.190	0.091	0.065	0.031	0.110
Ciprofloxacin	0.134	0.096	0.030	0.107	0.111	0.124	0.074	0.144	0.114	0.082	0.102
Norfloxacin	<LOQ	0.324	0.213	0.102	0.171	0.196	0.054	0.304	<LOQ	0.098	0.183
Levofloxacin	0.236	0.144	0.222	0.273	0.139	0.064	0.084	0.107	0.139	0.132	0.154
Cyclophosphamide	<LOQ	0.267	<LOQ	0.648	0.135	0.128	<LOQ	0.235	0.082	0.275	0.253
Ifosfamide	0.029	0.045	0.021	0.251	0.219	0.080	0.364	0.047	0.030	0.025	0.111

Table 3. *Cont.*

|	WW1	WW2	WW3	WW4	WW5	WW6	WW7	WW8	WW9	WW10	Mean
Megestrol	0.028	0.042	<LOQ	0.319	0.232	0.132	0.262	0.039	0.056	0.051	0.129
Bicalutamide	0.051	0.066	0.037	0.432	0.156	0.095	0.193	0.031	0.035	0.038	0.113
Mycophenolic acid	4.95	1.32	4.23	9.99	10.40	6.32	23.24	4.37	15.26	3.39	8.35
Diclofenac	0.528	1.84	0.849	0.310	0.259	0.321	0.321	1.02	0.686	0.953	0.709
N-(2.6-dichlorophenyl)-2-indolinone **	0.444	0.302	0.236	0.502	0.322	0.412	0.438	0.090	0.086	0.117	0.295
Ibuprofen	5.23	6.14	6.65	7.28	6.27	4.34	5.06	1.06	2.20	1.35	4.56
1-hydroxy ibuprofen/2-hydroxy ibuprofen *	0.726	0.509	0.575	0.606	0.141	0.462	1.20	0.724	0.611	0.633	0.618
Tramadol	0.693	0.624	1.41	1.48	0.612	0.633	1.16	3.06	1.19	1.52	1.24
4-aminoantipyrine *	2.73	2.01	3.39	6.30	4.40	3.94	7.43	1.93	2.45	2.48	3.70
Paracetamol	0.140	0.060	0.130	0.200	0.330	0.150	0.230	0.160	0.200	0.090	0.169
Carbamazepine	0.135	0.190	0.322	0.348	0.241	0.421	0.743	0.308	0.195	0.223	0.313
Carbamazepine 10.11-epoxide *	0.695	0.569	0.658	0.810	0.655	0.523	0.506	0.623	0.583	0.695	0.632
Topiramate	0.135	0.190	0.322	0.348	0.241	0.421	0.743	0.308	0.195	0.223	0.313
Gabapentin	0.698	0.496	0.808	0.601	0.765	0.516	1.326	0.485	1.12	0.782	0.760
Levetiracetam	0.052	0.051	0.049	0.324	0.106	0.052	0.456	0.047	0.129	0.062	0.133
Pregabalin	0.178	0.102	0.238	0.262	0.275	0.227	0.331	0.202	0.323	0.275	0.241
Levodopa	7.95	6.10	8.76	7.48	5.30	6.77	<LOQ	8.50	6.96	6.29	7.12
Rasagiline	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Diazepam	0.309	0.112	0.058	0.190	0.014	0.024	0.059	0.018	0.023	0.049	0.086
Clomethiazole	0.006	0.006	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.009	0.012	0.010	0.009
Scopolamine	0.143	0.024	0.012	0.092	0.086	0.024	0.223	0.024	0.036	0.020	0.068
Fluoxetine	0.026	0.078	0.043	0.199	0.163	0.073	0.090	0.099	0.098	0.059	0.093
Citalopram/Escitalopram	<LOQ	0.218	0.309	0.034	0.217	0.298	0.039	<LOQ	0.155	0.286	0.195
Trazodone	0.096	0.035	0.102	0.146	0.088	0.067	0.163	0.115	0.099	0.104	0.102
Venlafaxine	0.559	0.581	0.597	0.820	0.648	0.277	0.646	0.104	0.550	0.797	0.558
Caffeine	0.450	0.089	0.201	0.643	0.882	0.388	1.158	0.476	0.994	0.445	0.573
Donepezil	0.009	0.017	0.008	0.205	0.069	0.041	0.154	0.016	0.019	0.017	0.056
Memantine	0.056	0.028	0.055	0.089	0.133	0.052	0.134	0.053	0.088	0.039	0.073
Dichlorobenzyl alcohol	0.709	0.622	0.812	0.669	0.745	0.790	0.861	0.540	0.534	0.540	0.682
Amylmetacresol	2.72	2.28	1.93	1.35	1.26	1.66	2.25	1.78	1.75	1.15	1.81
Budesonide	0.930	0.946	0.947	1.16	1.04	0.971	1.10	1.34	1.21	1.22	1.08
Fluticasone	1.24	1.30	<LOQ	1.73	1.55	1.30	1.32	1.26	0.77	1.24	1.30
Tiotropium bromide	0.551	0.533	0.533	0.718	0.531	0.527	0.572	0.535	0.551	0.530	0.558
Dextromethorphan	0.731	0.730	0.728	0.780	0.858	0.788	0.978	0.766	0.793	0.803	0.796
Cloperastine	0.726	0.728	0.725	1.18	0.831	0.776	0.899	0.742	0.775	0.742	0.812
Chlorpheniramine	0.550	1.00	0.960	1.00	1.27	1.31	1.06	1.27	1.22	1.01	1.07
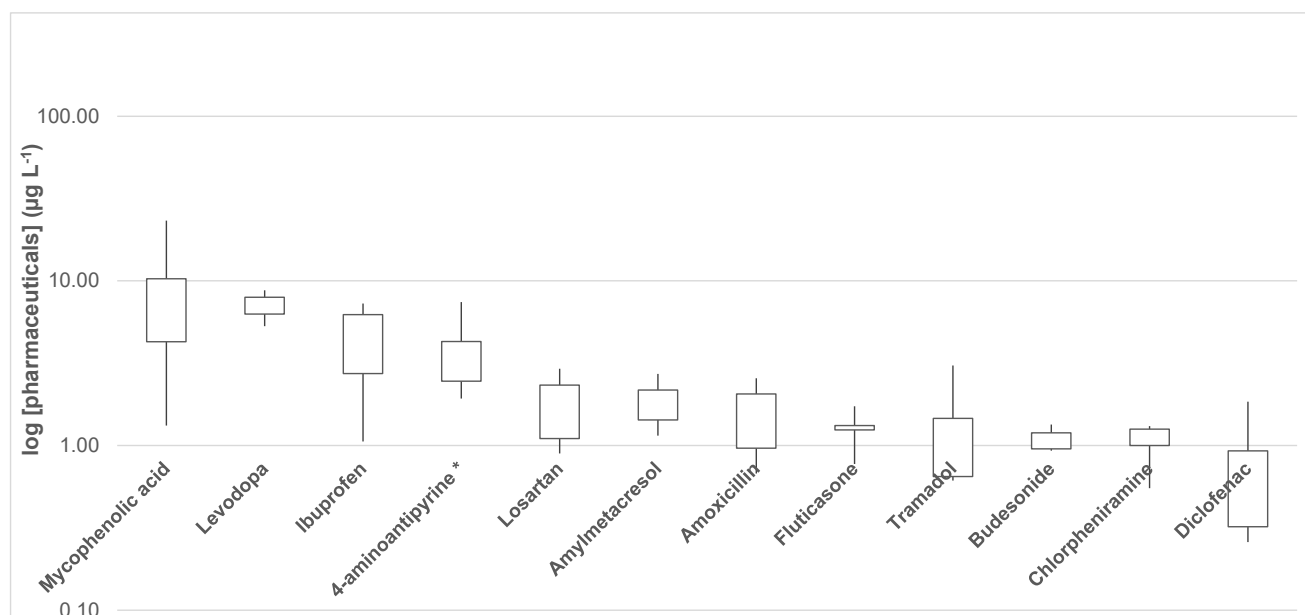
Figure 2. Boxplot in logarithmic scale (in $\mu g\,L^{-1}$) of eleven pharmaceuticals with highest concentrations in the 10 wastewater samples. * 4-aminoantipyrine is a metabolite of aminopyrine.

The novelty of the present study is the monitoring of pharmaceutical metabolites such as salicylic acid, enalaprilat, N_4-acetylsulfamethoxazole, N-(2,6-dichlorophenyl)-2-indoline, 1-hydroxy ibuprofen, 2-hydroxy ibuprofen, 4-aminoantipyrine and carbamazepine 10,11 epoxide. Figure 3 displays the levels of the target metabolites in the wastewater samples. In environmental monitoring, the importance of identifying pharmaceutical metabolites is often disregarded, where biotransformation is assumed to be compared with bioremoval, despite the fact that metabolites can be more harmful than the parent compound [21]. In the present study, the metabolites monitored have been detected at levels of $\mu g\,L^{-1}$, being 4-aminoantipyrine detected at the highest concentrations. On the other hand, carbamazepine 10,11 epoxide, the active metabolite of carbamazepine had levels between 0.50 and 0.81 $\mu g\,L^{-1}$ (Table 3), being the second metabolite with the highest concentrations. In this case, carbamazepine 10,11 epoxide had mean levels (0.63 $\mu g\,L^{-1}$), higher than its parental compound carbamazepine (0.31 $\mu g\,L^{-1}$). Leclercq et al. studied the presence of carbamazepine, oxcarbazepine, and seven of their metabolites (carbamazepine-10,11-epoxide, 10-hydroxy-10,11-dihydrocarbamazepine, 10,11-dihydro-10,11-trans-dihydroxycarbamazepine, 2-hydroxycarbamazepine, iminostilbene, acridine, and acridone) at three different treatment plants in France. Authors reported levels of carbamazepine-10,11-epoxide between 0.008 and 0.029 $\mu g\,L^{-1}$, concluding that metabolites were released in surface water associated with the parent compounds, indicating that aquatic ecosystems are exposed to these molecules [22].

1-hydroxy ibuprofen and 2-hydroxy ibuprofen are the main metabolites of ibuprofen and were in the range of 0.14 to 1.2 $\mu g\,L^{-1}$ (Table 3 and Figure 3). Ferrando-Climent et al. monitored ibuprofen and ibuprofen metabolites in sewage and natural water samples from Girona (Catalonia, Spain), where they were found at higher levels than expected: the maximum concentration in effluent wastewater samples were 1.9, 1.4, 10.7, 5.9 $\mu g\,L^{-1}$ for ibuprofen, 1-hydroxy ibuprofen, ibuprofen carboxylic acid and 2-hydroxyibuprofen, respectively [12].

N-(2,6-dichlorophenyl)-2-indolinone is the main transformation product (TP) produced by the degradation of diclofenac when exposed to UV light [23] and was detected between 0.086 and 0.50 $\mu g\,L^{-1}$ (Table 3 and Figure 3). To the best of the author's knowledge, this is the first time that this TP is monitored in effluents of wastewater. On the other hand, enalaprilat is the active metabolite of the orally available pro-drug, enalapril. It is an antihypertensive agent used for the management of hypertension when oral therapy is not

practical. Enalaprilat was detected at levels between 0.026 and 0.39 µg L^{-1}. In 2014, Kostich et al. measured concentrations of 56 active pharmaceutical ingredients (APIs) in effluent samples from 50 large wastewater treatment plants across the US. The authors reported limits of enalaprilat around 0.009 µg L^{-1} and predicted environmental concentrations (PECs) of 0.369 µg L^{-1} [24,25]. Finally, N$_4$-acetylsulfamethoxazole, which is a metabolite of the sulfonamide bacteriostatic antibiotic sulfamethoxazole excreted in urine and can be used as a probe for the molecular percentage enrichment of liver extramitochondrial acetyl-CoA. In the present study, N4-acetylsulfamethoxazole has been detected in the range of <LOQ to 0.32 µg L^{-1} (Table 3).
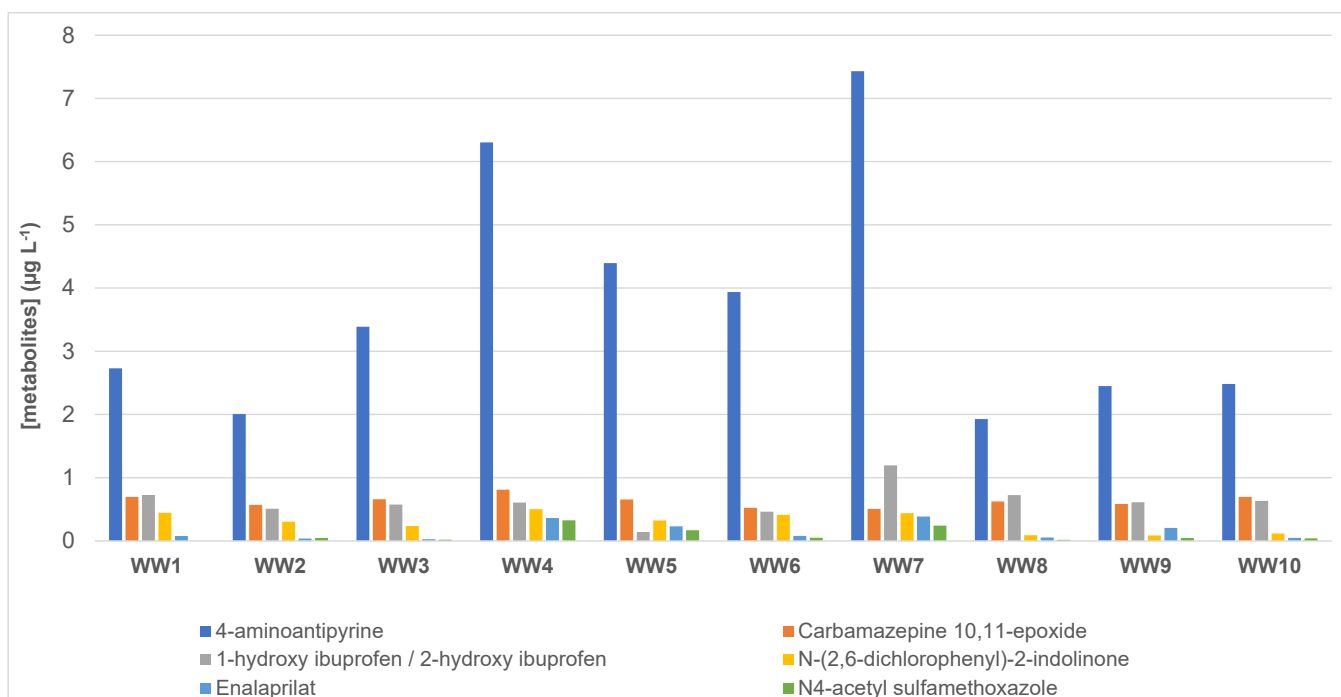
Figure 3. Concentrations (in µg L^{-1}) of the target metabolites and N-(2.6-dichlorophenyl)-2-indolinone (TP of diclofenac) in wastewater samples. WW: wastewater.

4. Conclusions

A comprehensive optimization of a solid phase extraction method followed by LC-MS/MS was performed for the unequivocal identification of 76 pharmaceuticals, metabolites and N-(2,6-dichlorophenyl)-2-indolinone as the main TP of diclofenac in wastewater effluents. The analytical performance of the LC-MS/MS method provides high selectivity and sensitivity and enhanced identification capabilities. In the 10 wastewater samples analyzed, only five drugs gave results below the limit of quantification. Mycophenolic acid, a cytostatic drug, was the pharmaceutical detected at the highest concentrations with levels between 1.3 and 23.2 µg L^{-1}, followed by levodopa and ibuprofen with levels between 1.1 and 8.8 µg L^{-1}. Moreover, pharmaceutical metabolites such as enalaprilat, N$_4$-acetylsulfamethoxazole, 1-hydroxiibuprofen, 2-hydroxiibuprofen, 4-aminoantipyrine and carbamazepine 10,11 epoxide and N-(2,6-dichlorophenyl)-2-indoline as TP have been detected in effluents of wastewater at levels of µg L^{-1}. Thus, the presence of pharmaceuticals and related metabolites in wastewater represents a high impact source of pollution of the aquatic environment.

from EPI Suite, Table S3: MS/MS fragmentation of the pharmaceuticals ordered following de ATC code for the pharmaceuticals; and including their metabolites, transformation product and internal standard (*).

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