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A multiresidue analytical method using solid-phase extraction and high-pressure liquid chromatography tandem mass spectrometry to measure pharmaceuticals of different therapeutic classes in urban wastewaters

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Abstract

An analytical method with two extraction steps has been developed and validated for the simultaneous determination of 30 pharmaceuticals belonging to various therapeutic categories in urban wastewater. The aim was to boost the little available information on drugs' fates in sewage treatment plants (STPs) and in the receiving surface water. Aqueous samples were divided into two aliquots, each extracted by a different solid-phase extraction (SPE) method and analysed by reversed-phase liquid chromatography tandem mass spectrometry (HPLC–MS–MS). Recoveries of the pharmaceuticals were mostly greater than 70% and the overall variability of the method was below 8%. The instrumental quantification limit (IQL) varied between 30 and 400 pg injected, and the limits of quantification (LOQ) were in the low ng/L range. Nineteen pharmaceuticals were detected in concentrations between 0.5 and 2000 ng/L in effluents collected from several STPs in Italy. Atenolol, ciprofloxacin, furosemide, hydrochlorothiazide, ofloxacin, ranitidine and sulphamethoxazole were the most abundant compounds. The present analytical method was useful to check for pharmaceuticals in various Italian STPs and to identify the most abundant compounds.

Keywords: High-pressure liquid chromatography tandem mass spectrometry (HPLC–MS–MS); Pharmaceuticals; Urban wastewaters; Sewage treatment plant (STP)

1. Introduction

Modern society makes available a wide range of pharmacologically active substances that are used in significant amounts to treat or prevent diseases. These substances are commonly excreted as the parent compound and/or metabolites in urine and faeces and discharged into domestic wastewaters continuously. The likelihood of a drug entering the environment depends mostly on the amount sold, and on its metabolic and physical–chemical properties. Several pharmaceuticals, widely used for human and veterinary medicine, are excreted unchanged or as active metabolites in high percentages. Antibacterial drugs such as fluoroquinolones (about 4 tonnes/year are sold in Switzerland and 14 tonnes/year in Italy) [1,2] are excreted mostly unchanged [1]. Diuretics like furosemide and hydrochlorothiazide (6.40 and 14.66 tonnes/year usage in Italy in 2001) are excreted 90–95% unchanged, and the β-blocker atenolol (22.07 tonnes/year in Italy in 2001) is excreted 90% unchanged [2]. Several other pharmaceuticals from different therapeutic classes such as bezafibrate (lipid regulating), ranitidine (ulcer healing) and lincomycin (antibacterial) are excreted as the parent compound for about 50% [2,3]. Therefore, hundreds of tonnes of pharmacologically active substances enter sewage treatment plants (STPs) each year, where they can escape degradation, and can eventually contribute to widespread environmental pollution.

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Recent analytical studies [3–7] show that some pharmaceuticals are poorly removed in STPs and are consequently detectable in surface waters (rivers, lakes and seas) in the ng/L up to the μg/L range [2,8,9,10]. STPs might therefore be important point sources of contamination, but for the majority of pharmaceuticals little information is available on their behaviour and ultimate fate in STPs and in the receiving surface water.

A multiresidue analytical method is a prerequisite to provide reliable figures on the fate of pharmaceuticals in STPs and surface water and to assess drug removal, partition and fate in the environment. Several analytical methods have been set up in Europe and USA for the detection of specific therapeutic categories [11–16]. Other methods are aimed at a wide range of compounds possibly present [17–22]. In Italy a multiresidue analytical method is already available [2] for the simultaneous determination of a limited number of pharmaceuticals.

This paper describes an improved analytical method to measure an extended list of 30 drugs, belonging to several therapeutic classes, at low concentrations in surface waters (about 1 ng/L). Pharmaceuticals were divided in two groups, extracted by different solid-phase extraction (SPE) methods, and analysed by reversed-phase liquid chromatography tandem mass spectrometry. The method was specifically designed to measure with accuracy a list of priority drugs, predicted to cause most of the pollution from pharmaceuticals in Italy. Pharmaceuticals for human use were selected according to their predicted environmental loads in Italy and the top pharmaceuticals by annual tonnage were included in the list. A group of historical drugs with long environmental persistence were also included, together with a group of molecules with high activity and potential toxicity, like estrogens and anti-cancer drugs. Some drugs widely used in animals [2] are also included, to assess the contribution of veterinary medicines. The analytical method was then applied to measure pharmaceuticals in effluents of STPs of several major Italian towns, in the framework of a national project funded by MIUR (Ministry of University and Research).

2. Experimental

2.1. Chemicals and materials

Table 1 shows the list of drugs selected for analysis, with their therapeutic category. It includes several antibacterial drugs, belonging to the penicillins, quinolones, macrolides, lincosamides and sulfamides class, some diuretics, cardiovascular, gastrointestinal and central nervous system drugs, an anti-inflammatory, a bronchodilator, a lipid regulator drug, some estrogens and two anticancer drugs. Two metabolites, clofibric acid, a metabolite of clofibrate, and demethyl diazepam, a metabolite of diazepam, and the natural estrogens 17β-estradiol and estrone were also included.

The reference standards of amoxycillin, atenolol, bezafibrate, carbamazepine, clofibric acid, cyclophosphamide, demethyl-diazepam, diazepam, enalapril, erythromycin, estrone, 17α-ethinylestradiol, 17β-estradiol, furosemide, hydrochlorothiazide, ibuprofen, lincomycin, methotrexate, ofloxacin, oxytetracycline, salbutamol, spiramycin and sulphamethoxazole were purchased from Sigma-Aldrich (Steinheim, Germany); ciprofloxacin was purchased from ICN Biochemicals (Meckenheim, Germany), ranitidine from

Table 1
Pharmaceuticals selected for analysis, with therapeutic class

Therapeutic class	Pharmaceutical	Therapeutic class	Pharmaceutical
Antibiotics for human use			
Macrolides–lincosamides	Clarithromycin Erythromycin Spiramycin Lincomycin	Cardiovascular	Atenolol Enalapril
Quinolones	Ciprofloxacin Ofloxacin	CNS drugs	Diazepam Carbamazepine
Penicillins	Amoxycillin	Diuretics	Furosemide Hydrochlorothiazide
Sulfamides	Sulfamethoxazole	Estrogens	17α-Ethinylestradiol
Antibiotics for veterinary use			
Macrolides	Oleandomycin Tilmicosin Tylosin	Gastrointestinal	Omeprazole Ranitidine
Tetracyclines	Oxytetracycline	Lipid regulators	Bezafibrate
Anticancer	Cyclophosphamide Methotrexate	Metabolites	Clofibric acid Demethyl diazepam
Anti-inflammatory Bronchodilator	Ibuprofen Salbutamol	Natural estrogens	17β-Estradiol Estrone

Glaxo SmithKline (Philadelphia, USA); clarythromycin and omeprazole were obtained from pharmaceutical preparations for intravenous injection, respectively, from Abbott, Latina, Italy (Klacid® i.v. 500 mg) and Malesci, Florence, Italy (Omeprazen®, 40 mg). Oleandomycin, tylosin and tilmicosin were kindly provided by the Istituto Zooprofilattico Sperimentale of Brescia. The reference compounds, used as I.S., salbutamol-D_3 (99.1% D) and ibuprofen-D_3 (99.7% D) were purchased from CDN Isotopes (Quebec, Canada). The I.S. 17β-estradiol-D_2 was obtained by labelling 17β-estradiol as previously described [23]. All the reference standards were stored at 4 °C.

Standards were dissolved in methanol (except those for i.v. injection that were dissolved in saline) up to a concentration of 1 mg/mL and subsequently diluted to 10 ng/μL (stock solutions). Three standard mixtures, containing all the pharmaceuticals to be analysed, were prepared before each analytical run by diluting stock solutions in methanol to concentrations of 1, 0.1 and 0.01 ng/μL. The stock solutions of amoxycillin, ciprofloxacin, ofloxacin, omeprazole, oxytetracycline and sulphamethoxazole were renewed monthly because of their limited stability. Purity of the stock solutions was checked before each analytical run by HPLC–MS–MS.

I.S. were dissolved in methanol (1 mg/mL) and subsequently diluted to 10, 1 and 0.1 ng/μL. All stock and I.S. solutions were stored at −20 °C in the dark.

The cartridges used for solid-phase extraction were: 3-mL disposable OASIS MCX (60 mg, Waters Corp., Milford, MA), and 3-mL disposable Lichrolut EN (200 mg, Merck, Darmstadt, Germany). Other cartridges tested were: OASIS HLB (60 mg, Waters Corp., Milford, MA) and 3-mL disposable Bakerbond C18 (500 mg, Baker, Phillipsurg, NJ). pH values of the water samples were monitored by a pH-meter Piccolo Plus, HANNA Instruments (Carlo Erba, Italy).

All the solvents used were of reagent grade or higher. Acetone, methanol, ethyl acetate were for pesticide analysis (Carlo Erba reagents, Italy), acetonitrile for HPLC analysis (Carlo Erba reagents, Italy) or for LC–MS (Riedel de Haen, Seelze, Germany). Ammonium hydroxide solution RPE (30%) and sodium chloride were from Carlo Erba reagents (Italy). Hydrochloric acid (37%), sodium hydroxide pellets and triethylamine (TEA) were from Merck, Darmstadt, Germany. Acetic acid was from Fluka (Buchs, Switzerland); formic acid (98–100%) and EDTA were from Riedel de Haen (Seelze, Germany). HPLC grade Milli-Q water was obtained with a MILLI-RO PLUS 90 apparatus (MILLIPORE, Molshelm, France).

2.2. Sample collection

Aqueous samples were effluents collected from various STPs in Italy: Cagliari (Cagliari Is Arena), Cosenza (Settimo di Montalto Uffugo), Palermo (Acqua dei Corsari), Latina, Naples, Cuneo (ACDA Cuneo), Varese Olona (Pravaccio) and Varese Lago (Gavirate). For each plant a 24-h composite sample was obtained by pooling effluent collected every 20 min by an automatic sampling device. Water samples (2 L each) were then stored at 4 °C until filtration and analysis. Before extraction, samples were filtered on a glass microfiber filter GF/D 2.7 μm (Whatman, Kent, UK).

Table 2
Grouping of pharmaceuticals according to the SPE method adopted for analysis, with recoveries and standard deviations

OASIS MCX (pH 2)	Recovery ± SD (%)	Lichrolut EN (pH 7)	Recovery ± SD (%)
Amoxycillin	36 ± 5.8	Carbamazepine	98 ± 7.2
Atenolol	106 ± 6	Clarithromycin	47 ± 6.1
Bezafibrate	76 ± 2.6	Cyclophosphamide	106 ± 7.5
Ciprofloxacin	32 ± 4.3	Erythromycin	50 ± 5.1
Clofibric acid	81 ± 1.8	Hydrochlorothiazide	56 ± 7.5
Demethyl diazepam	92 ± 5.1	Spiramycin	56 ± 2.1
Diazepam	96 ± 5.1	Tylosin	64 ± 7.6
Enalapril	96 ± 6.3		
17β-Estradiol	92 ± 4.6		
Estrone	97 ± 6.4		
17α-Ethinylestradiol	81 ± 5.9		
Furosemide	81 ± 4.2		
Ibuprofen	92 ± 3.7		
Lincomycin	76 ± 6.1		
Methotrexate	76 ± 6.6		
Oleandomycin	84 ± 2.5		
Ofloxacin	31 ± 5.7		
Omeprazole	49 ± 6.4		
Oxytetracycline	73 ± 3.4		
Ranitidine	95 ± 3.7		
Salbutamol	76 ± 4.4		
Sulphamethoxazole	65 ± 2.3		
Tilmicosin	131 ± 4.9		

2.3. Solid-phase extraction

To optimise the extraction method, in preliminary experiments we tested the extraction efficiency of some solid-phase extraction cartridges at various pH and elution conditions. The cartridges were Oasis MCX, tested at pH 1.5–2.0 and 3.0 for all the compounds, and at pH 7.0/7.5 for omeprazole; Lichrolut EN, tested at pH 3.0, 5.0, 7.0 and 9.0 for all the compounds; Bakerbond C18, tested at pH 8.0 and 9.5 for the extraction of amoxycillin, and Oasis HLB, tested at pH 7.0 for omeprazole and pH 8.5/9.0 for amoxycillin.

In the light of the results of these preliminary trials, for further experiments we selected Oasis MCX at pH 1.5–2.0 for the extraction of a first group of pharmaceuticals and Lichrolut EN at pH 7.0 for a second group (Table 2). For the first group, 500-mL water samples were spiked with 10 ng of the I.S. salbutamol-D_3, ibuprofen-D_3 and 17β-estradiol-D_2; 500 mg of Na_4-EDTA were added to prevent tetracyclines complexing with Ca^{2+} and Mg^{2+} ions and residual metals on the SPE cartridges. The pH was then adjusted to 2.0 with 37% HCl. The Oasis MCX cartridges were conditioned before use by washing with 6 mL methanol, 3 mL Milli-Q water and 3 mL water acidified to pH 2. Samples (500 mL) were then passed through the cartridges under vacuum, at a flow rate of 20 mL/min. Cartridges were vacuum-dried for 5 min and eluted with 2 mL methanol, 2 mL 2% ammonia solution in methanol and 2 mL 0.2% sodium hydroxide in methanol. The eluates were pooled and dried under an air stream.

To extract the second group of pharmaceuticals with the Lichrolut EN cartridges, 500-mL aqueous samples were adjusted to pH 7.0 with 30% ammonium hydroxide and spiked with 10 ng of the I.S. salbutamol-D_3 and ibuprofen-D_3. The cartridges were conditioned before use by washing with 6 mL methanol and 6 mL Milli-Q water. The aqueous samples (500 mL) were subsequently passed through the cartridges under vacuum at a flow rate of 16 mL/min. Cartridges were vacuum-dried for 10 min and eluted with 3 mL methanol and 3 mL ethyl acetate. Eluates were pooled and dried under an air stream.

The extraction efficiency of the two columns was checked in various conditions, extracting different volumes of a sample (100, 250 and 500 mL of mineral water) spiked with fixed concentrations of the pharmaceuticals, or extracting various samples (500 mL of mineral water) spiked with different concentrations of the pharmaceuticals (10, 100 and 1000 ng each).

2.4. Liquid chromatography tandem mass spectrometry (HPLC–MS–MS)

2.4.1. Liquid chromatographic separation

Before analysis, samples were dried under an air stream and redissolved in 100 μL of acetic acid 0.01% in Milli-Q water (pH 3.5), then centrifuged (Megafuge 1.0, HERAEUS Instruments), transferred into glass vials, and aliquots of 10 μL were injected with an auto sampler. The HPLC system consisted of two Perkin-Elmer Series 200 pumps and a Perkin-Elmer Series 200 auto sampler. A Luna C8 column 50 mm × 2 mm i.d., 3 μm particle size (Phenomenex, Torrance, CA, USA) was used for the chromatographic separation. For analysis in the positive ion mode eluent A was formic acid 0.1% in Milli-Q water (pH 2) and eluent B was acetonitrile. The elution started with 100% of eluent A, followed by a 10-min linear gradient to 100% of eluent B, 2-min isocratic elution and a 2-min linear gradient to 100% of eluent A, which was maintained for 6 min to equilibrate the column. Analysis in the negative ion mode was done with TEA (pH 8) 0.05% in water as eluent A and acetonitrile as eluent B and the elution gradient was the same as above.

Estrogens were analysed in the negative ion mode with the same eluents but a different elution gradient. Analysis started with 100% of eluent A, followed by a 6-min gradient to 70% of eluent A and 30% of eluent B and a 7-min gradient to 100% of eluent B, maintained for 2 min and then back to the initial conditions within 1 min. The column is then equilibrated for 6 min before another injection.

During analysis the flow rate was 200 μL/min and the column was kept at room temperature.

2.4.2. Mass spectrometry (MS–MS)

An API 3000 triple quadrupole ($Q_1q_2Q_3$) mass spectrometer equipped with a turbo ion spray source (Applied Biosystems – Sciex, Thornhill, Ont., Canada) was used. The analyses were done in the negative ion mode for clofibric acid, bezafibrate, ibuprofen, furosemide, hydrochlorothiazide, sulphamethoxazole and estrogens and in the positive ion mode for the other compounds. Analysis in positive ion mode was done with a spray voltage (IS) of 5.4 kV, while the orifice skimmer voltage varied from 30 to 56 V and the ring electrode voltages from 120 to 380 V. Analysis in negative ionisation mode was done with a spray voltage (IS) of −4.4 kV, the orifice skimmer voltage varied from −31 to −76 V and the ring electrode voltages from −130 to −280 V (Table 3). The mobile phase at a flow rate of 200 μL/min was directly introduced into the ion source, without splitting. Mass spectrometry analyses were done in the multiple reaction monitoring (MRM) mode, measuring the fragmentation products of the protonated or deprotonated pseudo-molecular ions of each drug and internal standard. The choice of fragmentation products for each substance and the optimization of energy collisions and other instrument parameters were done in continuous-flow mode, using standard solutions at concentrations of 1–10 ng/μL.

2.5. Quantification and method validation

Each compound was quantified by MRM, using the two highest characteristic precursor ion/product ion transitions. Comparison of the retention times with the corresponding reference standards also helped identify the compounds. The I.S. were salbutamol-D_3, which was used to quantify the phar-

Table 3
Optimum orifice and ring voltage, precursor and product ions with the respective collision energy (eV) for MS–MS determination of pharmaceuticals analysed in this investigation

Pharmaceuticals	Orifice voltage (V)	Ring voltage (V)	Precursor ion (*m*/*z*)	Product ion I (*m*/*z*) and collision energy (eV)	Product ion II (*m*/*z*) and collision energy (eV)
Amoxycillin	30	130	366.2	208.1 (−18)	113.1 (−28)
Atenolol	30	180	267.1	190.1 (−26)	145.2 (−36)
Bezafibrate	−46	−222	360.2	274.1 (22)	154.1 (40)
Carbamazepine	30	120	237.1	194.2 (−28)	192.1 (−30)
Ciprofloxacin	51	140	332.1	288.1 (−26)	231.3 (−50)
Clarithromycin	31	380	748.2	590.4 (−26)	158.1 (−38)
Clofibric acid	−31	−150	213.1	127.1 (18)	85.0 (14)
Cyclophosphamide	50	210	261.1	233.1 (−22)	140.1 (−30)
Demethyl diazepam	46	200	271.1	165.2 (−38)	140.2 (−38)
Diazepam	56	230	285.2	193.1 (−44)	154.1 (−38)
Enalapril	26	220	377.2	303.1 (−26)	234.1 (−28)
17β-Estradiol	−56	−280	271.1	183.0 (54)	154.0 (52)
Estrone	−56	−280	269.1	159.0 (48)	145.1 (52)
17α-Ethinylestradiol	−56	−280	295.1	159.0 (46)	145.1 (50)
Erythromycin	56	280	734.5	576.4 (−26)	158.2 (−40)
Furosemide	−41	−170	329.1	285.1 (22)	205.1 (32)
Hydrochlorothiazide	−76	−160	296.1	269.1 (30)	205.1 (32)
Ibuprofen	−36	−160	205.1	161.1 (12)	–
Lincomycin	56	280	407.2	359.1 (−26)	126.1 (−38)
Methotrexate	40	200	455.2	308.1 (−28)	175.2 (−52)
Ofloxacin	36	190	361.2	318.1(−28)	261.1 (−38)
Omeprazole	31	170	346.4	198.2 (−16)	151.2 (−26)
Oleandomycin	50	240	688.5	544.4 (−22)	158.1 (−40)
Oxytetracycline	40	200	461.2	426.2 (−26)	350.1 (−56)
Ranitidine	46	200	315.2	176.1 (−24)	130.1 (−36)
Salbutamol	30	180	240.1	166.1 (−20)	148.1 (−26)
Spiramycin	38	180	842.8	699.5 (−18)	540.5 (−21)
Sulphamethoxazole	−36	−200	252.1	156.1 (22)	92.1 (38)
Tilmicosin	38	180	869.1	695.5 (−24)	340.3 (−24)
Tylosin	50	240	916.4	772.5 (−40)	174.1 (−54)
Internal standards					
Salbutamol-D_3	31	180	243.1	169.1 (−20)	151.1 (−26)
Ibuprofen-D_3	−21	−130	208.2	164.1 (10)	–
17β-Estradiol-D_2	−56	−280	273.1	185.0 (54)	147.0 (52)

maceuticals analysed in the positive ion mode, ibuprofen-D_3 which was used for clofibric acid, bezafibrate, ibuprofen, furosemide, hydrochlorothiazide and sulphamethoxazole in the negative ion mode, and 17β-estradiol-D_2 used for estrogens in the negative ion mode.

Five-point calibration curves were generated for each pharmaceutical by injecting pooled solutions prepared from the standard mixtures. An instrumental blank containing only the I.S. was used as control for analytical interference.

Quantitative analysis was done by calculating the ratios between the peak area of each substance and the peak area of the relative I.S. for each sample. The same procedure was used to plot calibration curves from the standard solutions. To better reflect the sample conditions and to reproduce matrix effects, the standards for the calibration curves were diluted using a SPE eluate of a blank extracted in the same analytical batch. Procedural blanks were added to each set of samples to control for contamination, with a standard sample (500 mL of mineral water spiked with 10 ng of each pharmaceutical) to control for recovery.

Instrumental detection limits (IDL) and instrumental quantification limits (IQL) were determined by direct injection of decreasing amounts of each pharmaceutical, down to 10 pg. The detection limits (LOD) and quantification limits (LOQ) of the whole method were calculated from the chromatograms of the STP effluents, to take into account the matrix effect. The IDL and LOD were the concentrations for which the signal:noise ratio was 3, and the IQL and LOQ were the concentrations for which the signal:noise ratio was 10 (Table 4).

We tested the repeatability of the extraction methods by spiking 500 mL of mineral water with 10 ng of each pharmaceutical before extraction, and 10 ng of I.S. after extraction. Variability was investigated by running five replicate analyses of a single sample. The mineral water used to calculate recoveries had a conductibility of 569 μS, a pH of 7.5 at 20 °C and a residual of 381 mg/L at 180 °C.

We tested the linearity of the calibration curves for concentration ranges that are normally measured in waste and surface waters. Nine pooled solutions, with concentrations

Table 4
Linear ranges and inter-day correlation factors of the calibration curves, instrumental quantification limits (IQL) and quantification limits of the method (LOQ)

Pharmaceuticals	Linearity range 0.1–3000 ng/100 μL	Inter-day correlation factor (r^2) and SD	IQL (pg/injected)	LOQ (ng/L) in STP effluents
Amoxycillin	0.1–3000	0.999 ± 0.001	98	2.08
Atenolol	0.1–1800	0.9999 ± 0.000	61	1.07
Bezafibrate	0.1–1600	0.999 ± 0.001	6	0.1
Carbamazepine	0.1–500	0.997 ± 0.002	174	1.3
Ciprofloxacin	0.1–3000	0.996 ± 0.003	73	1.8
Clarithromycin	0.1–600	0.998 ± 0.001	20	0.15
Clofibric acid	0.1–3000	0.999 ± 0.001	16	0.36
Cyclophosphamide	0.1–2000	0.999 ± 0.000	348	1.9
Demethyl diazepam	0.1–1800	0.999 ± 0.001	70	0.62
Diazepam	0.1–1800	0.999 ± 0.001	63	1.08
Enalapril	0.1–600	0.999 ± 0.001	12	0.71
17β-Estradiol	0.1–600	0.9999 ± 0.000	394	5.2
Estrone	0.1–500	0.9999 ± 0.000	85	1.5
17α-Ethinylestradiol	0.1–600	0.9999 ± 0.001	194	4.6
Erythromycin	0.1–3000	0.999 ± 0.000	45	0.4
Furosemide	0.1–2000	0.999 ± 0.001	53	0.8
Hydrochlorothiazide	0.1–3000	0.998 ± 0.003	31	0.9
Ibuprofen	0.1–1800	0.9999 ± 0.000	196	1.38
Lincomycin	0.1–500	0.998 ± 0.002	12	0.31
Methotrexate	0.1–3000	0.999 ± 0.001	102	0.83
Ofloxacin	0.1–3000	0.998 ± 0.001	145	1.3
Omeprazole	0.1–3000	0.998 ± 0.001	154	1.57
Oleandomycin	0.1–600	0.999 ± 0.001	11	0.31
Oxytetracycline	0.1–2200	0.998 ± 0.001	58	1.19
Ranitidine	0.1–1800	0.999 ± 0.002	42	1.06
Salbutamol	0.1–600	0.999 ± 0.001	35	0.90
Spiramycin	0.1–3000	0.997 ± 0.001	114	1.4
Sulphamethoxazole	0.1–3000	0.999 ± 0.001	340	1.48
Tilmicosin	0.1–1800	0.996 ± 0.004	177	0.71
Tylosin	0.1–3000	0.998 ± 0.002	180	0.77

between 0.1 and 3000 ng of each pharmaceutical, were prepared from the standard mixtures in 100 μL of 0.01% acetic acid. The instrumental repeatability and precision were then assessed by repeated injections of standard mixtures (1, 10 and 100 ng/injected).

3. Results and discussion

3.1. Solid-phase extraction

Among the extraction materials tested were two polymeric sorbents (Lichrolut EN and Oasis HLB), a mixed polymeric and cation exchange sorbent (Oasis MCX) and an apolar sorbent (C18). Various pH conditions were tested. On the basis of these preliminary investigations, we extracted the pharmaceuticals using two different SPE methods, with an Oasis MCX at pH 1.5–2.0 or with a Lichrolut EN at pH 7.0. The Oasis MCX is a mixed reversed phase-cation exchange cartridge, in which the strong cation exchanger sulfonic acid groups are placed on the surface of a poly-divinylbenzene-co-*N*-vinylpyrrolidone copolymer. This column can therefore extract acidic, basic and neutral compounds at low pH values, since the cation-exchanger binds the basic compounds, which are in the ionized form, and the reversed phase can retain both acidic and neutral compounds. Drugs bearing amino groups, which are positively charged at pH 2, such as ciprofloxacin, ofloxacin, furosemide, hydrochlorothiazide, methotrexate, salbutamol and ranitidine are therefore bound by the cation exchanger, while neutral and acidic compounds, such as diazepam, bezafibrate, clofibric acid, estrogens and ibuprofen are retained by the polymeric phase.

The Lichrolut EN cartridge is an ethylvinylbenzene–divinylbenzene copolymer that can extract polar organic compounds. Through hydrophobic interactions, this polymeric sorbent can also retain neutral drugs at pH 7, such as carbamazepine, cyclophosphamide and the macrolides.

Table 2 reports recoveries and standard deviations calculated in mineral water with the two methods. Recoveries were mostly greater than 70%, with some exceptions. For instance, extraction of omeprazole by Oasis MCX at pH 2.0 gave 50% recovery. This is probably because the stability in the omeprazole solution is poor and highly affected by pH and salinity [24,25]. However, this recovery is an improvement on the method we previously published, where we only achieved <10%. Recoveries for amoxycillin were best with Oasis MCX at acidic pH, and were 36 ± 5.8%. Here again the low recovery is probably due to poor stability of the molecule in aqueous solution [26].

Erythromycin, spiramycin and tylosin recoveries were, respectively, 50%, 56% and 64%, with good improvements over the previous method, where the recoveries were, respectively, 26%, 28% and 45%. However, for ciprofloxacin and ofloxacin recoveries with the present method (about 30%) were lower than previously reported. A critical point in this result might be the extraction efficiency of these molecules, which is reported to be highly sensitive to the step of evaporation to dryness [1]. Recoveries of hydrochlorothiazide and sulphamethoxazole were 56% and 65%.

Among the other variables considered, the addition of EDTA to the samples enhanced the recovery of amoxycillin, methotrexate, omeprazole and oxytetracycline. Also crucial were the elution conditions for the Oasis MCX, which were optimized by using consecutively 2 mL methanol, 2 mL 2% ammonia and 2 mL 0.2% sodium hydroxide.

3.2. Liquid chromatography tandem mass spectrometry (HPLC–MS–MS)

The Luna C8 column gave a good chromatographic separation of the compounds, with R_t values in the range from 2 to 7 min, except for estrogens which had a R_t of about 10 min. Fig. 1 gives examples of chromatograms of water samples from STPs. The mass spectrometry parameters (orifice skimmer voltage and ring electrode voltage) are reported in Table 3, together with the precursor and products ions and the collision energies employed.

3.3. Quantification and method validation

We used three deuterated I.S. (salbutamol-D_3, ibuprofen-D_3 and 17β-estradiol-D_2) to analyse about 30 different molecules with various chemical structures and properties, and this is a limitation of the method. However, this choice was a good compromise between the need to obtain concentration data on a wide array of drugs, with acceptable data quality, and the difficulty or the impossibility of obtaining labeled I.S. for each substance. The isotope labeling of drugs in the laboratory can be difficult and time-consuming.

We tested the linearity of the chromatographic response by using nine pooled solutions of 0.1–3000 ng of each pharmaceutical in 100 μL of 0.01% acetic acid (0.01–300 ng of each pharmaceutical injected, injection volume 10 μL) (Table 4). There was linearity in the whole range except for carbamazepine, estrone and lincomycin where linearity was limited to the range 0.1–500 ng. The repeatability and precision of the calibration curves were determined by injecting the pooled solutions three times during the same day and on different days. The correlation factors r^2 of the calibration curves were then calculated for the intra and inter-day injections and the inter-day values are reported in Table 4. Values are greater than 0.998, except for ciprofloxacin and tilmicosin which have a correlation factor of 0.996. The inter-day standard deviations were lower than 0.002, except for tilmicosin (0.004), ciprofloxacin and hydrochlorothiazide (0.003).

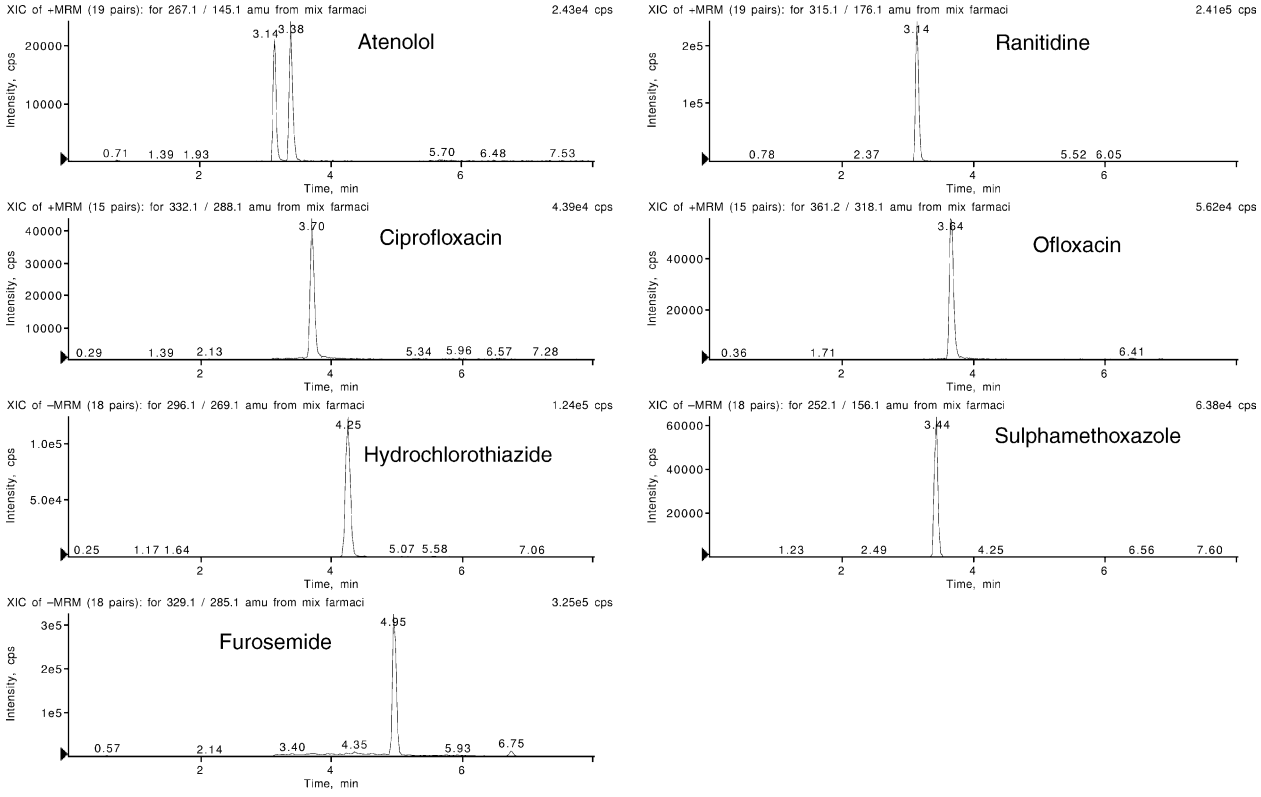

Fig. 1. Chromatograms of samples from STP effluents, referring to the most abundant compounds measured.

Table 5
Intra-day relative standard deviation (RSD%) measured by injecting 1, 10 and 100 ng and inter-day instrumental RSD% for 1 ng of each compound on different days

Pharmaceuticals	Intra-day RSD% 1 ng/injected	Intra-day RSD% 10 ng/injected	Intra-day RSD% 100 ng/injected	Inter-day RSD% 1 ng/injected
Amoxycillin	7.94	16.87	6.42	18.91
Atenolol	7.77	7.20	1.90	6.77
Bezafibrate	5.89	3.27	3.73	7.21
Carbamazepine	10.80	0.70	2.88	6.27
Ciprofloxacin	5.59	0.39	1.69	4.16
Clarithromycin	9.50	0.37	1.95	7.41
Clofibric acid	11.24	3.53	2.51	4.39
Cyclophosphamide	14.83	0.29	3.16	7.03
Demethyl diazepam	7.16	6.64	2.50	3.15
Diazepam	10.69	8.05	2.54	6.56
Enalapril	8.82	7.05	3.25	4.77
17β-Estradiol	11.01	1.55	1.96	4.31
Estrone	11.01	2.99	3.32	8.97
17α-Ethinylestradiol	5.77	1.17	3.36	10.65
Erythromycin	15.36	3.59	2.79	5.04
Furosemide	4.37	4.53	1.22	6.76
Hydrochlorothiazide	9.92	4.79	1.58	13.10
Ibuprofen	0.97	1.41	1.03	4.26
Lincomycin	5.84	1.99	2.59	5.92
Methotrexate	5.51	5.29	1.54	5.28
Ofloxacin	4.50	16.91	13.65	8.57
Omeprazole	–	–	–	38.48
Oleandomycin	7.36	5.72	2.33	6.43
Oxytetracycline	10.97	2.03	1.43	6.60
Ranitidine	1.62	5.29	2.05	15.79
Salbutamol	9.77	6.77	1.11	3.21
Spiramycin	11.81	0.74	1.65	5.42
Sulphamethoxazole	8.91	7.83	13.22	6.54
Tilmicosin	5.74	3.87	2.73	6.83
Tylosin	7.21	4.55	3.26	8.84

Table 4 reports the instrumental limits of quantification (IQL) and the limit of quantification (LOQ) of the method, calculated in STP effluents. The instrumental limits of quantification (IQL) are expressed as pg injected and varied between 30 and 400 pg injected. The limits of quantification (LOQ) of the whole method ranged from 0.1 to 5.2 ng/L. Most pharmaceuticals had LOQ of about 1 ng/L or lower, except for amoxycillin (2.08 ng/L), 17β-estradiol (5.2 ng/L) and 17α-ethinylestradiol (4.6 ng/L).

The effect of the sample volume on the recoveries was better when extracting samples of 500 mL, the volume which was adopted for the analysis. The extraction efficiency did not seem to be affected by the concentrations of the pharmaceuticals, with no significant changes in recoveries in the range 10–1000 ng (further recoveries were calculated by spiking samples with pools of 10 ng of each pharmaceutical). The repeatability of the method was therefore assessed by spiking 500 mL of mineral water with 10 ng of each pharmaceutical. The overall variability of the method is indicated by the standard deviations (Table 2) obtained from the analysis of five replicates, which was below 8%.

Instrumental repeatability and precision were measured by injecting different concentrations of standard mixtures (1, 10 and 100 ng of each pharmaceutical). Relative standard deviations (RSD%) are reported in Table 5. The intra-day RSD% were generally below 20%. The inter-day instrumental precision was assessed by injecting 1 ng three times on different days. Results are reported in Table 5. RSD% were generally below 20%, with exception of omeprazole, where it was 38%, probably because of the limited stability of this substance.

3.4. Analysis of STP effluents

The method was used to measure the pharmaceuticals in effluents of STPs in an analytical campaign in Italy. Com-

Table 6
Flow rates and population equivalents of the STPs analysed

STPs	Flow rates (m^3/d)	Population equivalents
Cagliari	86,700	270,000
Cosenza	80,000	180,000
Palermo	25,525	440,000
Latina	19,000	45,000
Cuneo	31,000	140,000
Varese Olona	30,000	120,000
Varese Lago	40,000	110,000
Naples	181,354	840,000

The STPs collected mostly urban wastes, processed through a primary settling and an activated sludge secondary treatment.

Table 7
Pharmaceuticals measured in STP effluents

Pharmaceuticals	Sampling stations							
	Cagliari	Cosenza	Palermo	Latina	Naples	Cuneo	Varese Lago	Varese Olona
Amoxycillin	nd	nd	120	15	nd	nd	nd	25
Atenolol	254	27	260	70	955	1168	554	466
Bezafibrate	9.2	0.3	8	15	117	55	55	87
Carbamazepine	1318	nd	nd	33	382	333	956	179
Ciprofloxacin	146	27	179	91	251	514	378	322
Clarithromycin	59	8	18	12	73	15	12	52
Clofibric acid	nd	nd	0.7	2.1	82	1.7	0.5	5.1
Cyclophosphamide	2.1	nd	nd	nd	nd	9.0	nd	nd
Diazepam	nd	nd	nd	nd	nd	nd	nd	nd
Demethyl diazepam	5	1	4	3.6	62	nd	22	25
Enalapril	nd	nd	nd	nd	nd	nd	nd	nd
17β-Estradiol	nd	nd	nd	nd	nd	nd	nd	nd
Estrone	48	nd	nd	47	nd	30	nd	42
17α-Ethinylestradiol	nd	nd	nd	nd	nd	nd	nd	nd
Erythromycin	161	27	64	20	353	27	47	9
Furosemide	585	26	560	289	602	2102	1178	650
Hydrochlorothiazide	431	60	654	261	256	1253	986	877
Ibuprofen	nd	nd	nd	nd	nd	nd	nd	nd
Lincomycin	28	43	639	25	846	30.5	11	22
Methotrexate	nd	nd	nd	12.6	nd	nd	nd	nd
Ofloxacin	600	150	474	347	1081	864	964	738
Oleandomycin	nd	nd	nd	nd	nd	nd	nd	nd
Omeprazole	nd	nd	nd	nd	nd	nd	nd	nd
Oxytetracycline	nd	nd	nd	nd	nd	nd	nd	nd
Ranitidine	263	36	260	77	610	517	335	288
Salbutamol	10	1.1	9.3	6.5	18	11	5.7	6
Spiramycin	75	1.4	129	59	161	11	19	91
Sulphamethoxazole	97	46	127	110	317	230	212	253
Tilmicosin	nd	nd	nd	nd	nd	nd	nd	nd
Tylosin	nd	nd	nd	nd	nd	nd	0.9	nd

Concentrations are expressed in ng/L. nd = not detected (pharmaceuticals concentrations below the LOQ).

posite samples of 24-h effluent were collected from STPs of some Italian cities (Table 6) during January and February 2004. The STPs mostly collected urban wastes processed through a primary settling and an activated sludge secondary treatment. Population equivalents of the STPs were from 45,000 to 270,000 and the flow rates were between 19,000 and 86,700 m^3/d, except for Naples, where the population equivalent was 840,000 and the flow rate 181,000 m^3/d. Nineteen pharmaceuticals were detected in concentrations from 0.5 to 2000 ng/L (Table 7). Atenolol, ciprofloxacin, furosemide, hydrochlorothiazide, ofloxacin, ranitidine and sulphamethoxazole were the most abundant compounds. This study therefore confirms that pharmaceuticals may be directly discharged in large amounts through STPs into surface water, in agreement with results from other countries. For instance, sulphamethoxazole was detected in the same order of magnitude in Germany [8], and comparable concentrations of ciprofloxacin were measured in STPs in Switzerland [1]. Pharmaceuticals we found in low concentrations, such as bezafibrate, clarithromycin, clofibric acid and erythromycin, have also been detected by others in Europe, USA and Canada. However, several papers focused only on antibiotics. Antibiotics were the most frequently drugs we measured too, but several others from various therapeutic categories, were detected in high concentrations, such as the β-blocker atenolol (27–1170 ng/L), the diuretics furosemide and hydrochlorothiazide (25–2000 and 60–1250 ng/L) and the ulcer healing drug ranitidine (36–610 ng/L), showing their widespread presence in STPs and surface waters.

4. Conclusions

Pharmaceuticals have a variety of different structures and physical–chemical properties, requiring complex methods for their simultaneous analysis. In this study, we divided pharmaceuticals into two groups, extracted from aqueous samples with two different SPE methods, and analysed by HPLC–MS–MS in positive and negative ionisation mode. This method was subsequently used to measure pharmaceuticals in effluent of STPs of some Italian towns. Nineteen pharmaceuticals were detected in concentrations from 0.5 to 2000 ng/L. In our hands, SPE and HPLC–MS–MS proved to be specific and precise for measuring pharmaceuticals in wastewaters. The method was useful to check for pharmaceuticals in effluents of various Italian STPs and to identify the most abundant compounds.

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