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Analysis of pharmaceuticals in indirect potable reuse systems using solid-phase extraction and liquid chromatography–tandem mass spectrometry

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ABSTRACT

A solid-phase extraction (SPE) LC–MS/MS method for 18 commercial drugs in secondary wastewater and product water from water recycling plants using microfiltration (MF) and reverse osmosis (RO) has been developed, optimised and validated. The method incorporates a range of multi-class pharmaceuticals including lipid lowering agents, analgesics, antipyretics, non-steroidal anti-inflammatory drugs, antidepressants, anticoagulants, tranquilizers, cytostatic agents, and antiepileptics. Method limits of quantitation (MLQs) in secondary wastewater ranged from 15 to 250 ng/L, while MLQs in post-RO water ranged from 1 to 25 ng/L. Results from analysis of secondary wastewater from Western Australia are presented, and represent the largest survey of non-antibiotic pharmaceuticals within Australia to date. Analysis of post-RO water from two MF/RO water recycling facilities also demonstrate that MF/RO treatment removes most pharmaceuticals to below the analytical limits of detection, and more importantly, up to seven orders of magnitude below health-based guideline values.

1. Introduction

The increase in the detection of pharmaceuticals in secondary wastewaters from municipal and industrial wastewater treatment plants (WWTPs) is an emerging issue because of a lack of knowledge of their sources, occurrence, fate and environmental effects [1,2]. Traditionally pharmaceuticals have not been viewed as environmental pollutants and there has been little consideration of their fate post-excretion. Indeed monitoring pharmaceuticals in the environment has only really been possible since the 1990s, when instruments were developed with sufficient chemical separation efficiency to distinguish these compounds, often present at ng/L concentrations, from other substances.

Pharmaceuticals are large and chemically complex molecules. The wide range of chemical classes represented in the group means that generalisations on their behaviour is impossible. Numerous environmental impacts may occur, including acute or chronic toxicity [3,4], endocrine disruption [5], interference with detoxification systems [6], stimulation of reproductive processes [7], and inhibition of primary productivity [8]. Their mobility in soils and sediments can also vary [9].

While wastewater from pharmaceutical manufacturing facilities has been regulated in the US since the 1990s [10], pharmaceuticals are most commonly derived from municipal wastewater sources and have the potential for direct release into the environment wherever humans live or visit. Raw household and hospital wastewaters both represent a significant source of pharmaceuticals. For example, Sacher et al. [11] has estimated that up to 10% of the total prescription volume of carbamazepine and diclofenac (ca. 100×10^3 kg/year) eventually ends up in the lower river Rhine, while Snyder et al. [12] have demonstrated that the discharge of highly treated wastewater into Lake Mead has resulted in elevated concentrations of numerous pharmaceuticals and other chemicals of concern. Hospital wastewaters entering sewerage networks without any pre-treatment may result in pharmaceuticals concentrations in WWTP primary influent in the order of μg/L [13–17]. Generally WWTPs are designed and are regulated to remove nutrients, and any chemical of concern (COC) removal is a side benefit only of the existing treatment. Consequently, many pharmaceuticals have been detected in secondary wastewater at measurable concentrations (10 μg/L down to 10 ng/L) [13,14,18,19], demonstrating that classical activated sludge treatment is not capable of removing all pharmaceuticals from the influent sewage. Indeed large variations in removal rates have been reported between different WWTP [20], and even within a single WWTP, particularly with variable WWTP efficiency or seasonality [14,21]. Drinking water treatments such as sorption, flocculation and chloramination have also been demonstrated having variable efficiency for pharmaceutical removal, although both ozonation and sorption onto granular activated carbon (GAC) were generally more efficient

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[19,22]. Alternately, however, tertiary treatments such as physical removal through microfiltration (MF), nanofiltration (NF), or reverse osmosis (RO), or chemical removal using advanced oxidation processes have been demonstrated to be effective in reducing pharmaceutical concentrations [19,23,24]. Reported concentration ranges of pharmaceuticals after tertiary treatment (10 ng/L down to pg/L) are orders of magnitude lower than health-based guideline values [25] and acute toxicity data for aquatic organisms [4]. However, there is still significant uncertainty regarding the long-term risks associated with pharmaceutical mixtures present at low concentration levels for non-target wildlife organisms as well as for human health [1,3,4].

Pharmaceuticals are not yet considered in drinking water quality guidelines in Australia [26] or elsewhere [27]. More recently, the presence of pharmaceuticals and other micropollutants in secondary wastewater has taken on further significance as wastewaters have become a resource for potable reuse, and consequently they are being considered in water reuse guidelines [25]. The potential for residual wastewater-derived chemicals in drinking water sourced from either deliberate or accidental potable reuse has triggered numerous studies [14,19,23,28,29]. There is therefore a need to assess the presence of pharmaceuticals in water produced for potable reuse, as well as to investigate their removal during tertiary treatment with a comprehensive monitoring plan. When considering the range of chemical classes represented in the group pharmaceuticals, achieving comprehensive monitoring is challenging [30]. While many analytical procedures for single pharmaceutical compounds or specific class groupings have been published in literature [13,18,30,31], greater flexibility and efficiency is achieved when pharmaceuticals from different classes can be measured in one procedure.

In this paper we present the development, optimisation and validation of an analytical method for the determination of 18 multi-class commercial drugs in secondary wastewater and RO treated water using solid-phase extraction (SPE) pre-concentration followed by liquid chromatography tandem mass spectrometry (LC–MS/MS). The pharmaceuticals targeted in this study span many classes, including lipid lowering agents, analgesics, non-steroidal anti-inflammatory drugs (NSAIDs), anticoagulants, antipyretics, cytostatics, antiepileptics, antidepressants and tranquilizers (Table 1). While many methods for multi-class pharmaceutical analysis of wastewater exist [21,32,33], incorporation of warfarin and morphine is relatively unusual. The work presented in this paper is part of a larger project investigating the effectiveness of MF/RO to treat secondary wastewater for indirect potable reuse, a key water conservation strategy for Western Australia. Lack of knowledge of health and environmental risks associated with micropollutants and their removal by advanced treatment processes have been major barriers preventing establishment of large reuse schemes to date [34]. As well as providing information on the efficacy of RO to remove specific pharmaceuticals, these results also provide the most extensive analysis of non-antibiotic pharmaceuticals in treated wastewater in Australia published to date.

2. Experimental

2.1. Sampling and sample pre-treatment

Samples were collected from the Kwinana Water Reclamation Plant (KWRP) and the Beenyup WWTP in Perth, Australia. Details of each have been previously published [28,35], but briefly, KWRP treats secondary treated wastewater from Woodman Point WWTP by MF/RO to produce approximately 16 ML/day of general process water for neighbouring industrial facilities, reducing Perth's total demand for scheme water by about 2%. The Beenyup WWTP is Perth's major northern metropolitan WWTP with a capacity of 120 ML/day. While most secondary wastewater from Beenyup WWTP is discharged to the Indian Ocean, a small volume of secondary wastewater (approximately 100 kL/day) is treated by MF/RO in the Beenyup Pilot RO Plant. This pilot plant comprises the first stage of a larger project investigating indirect potable reuse of Beenyup wastewater.

At Beenyup WWTP, duplicate composite samples were taken of Beenyup secondary wastewater and post-RO water from Beenyup RO Pilot plant on a single day (21 January 2008). These duplicate samples enabled the reproducibility of both the sampling procedure and the analytical method to be assessed. At KWRP, composite and grab samples were collected pre- and post-RO treatment on 3 days over a week-long period (30 May–7 June 2007) to determine whether there were significant differences between grab and composite samples and to investigate trends over a week. At both sites, composite samples were taken over 24 h using an automated and refrigerated ISCO 4700 sampler, while grab samples were collected from the relevant stream at the time of sampling. Field and trip blanks were also collected on each day of sampling. Samples were preserved with 100 mg/L of NaN_3, which was added as a solid to the amber glass sample bottles (4 L) before sampling. Samples were stored and at 4 °C until sample extraction which usually took place within 2 weeks of sampling.

2.2. Analytical standards and chemicals

Morphine hydrochloride, paracetamol, carbamazepine, cyclophosphamide monohydrate, fluoxetine hydrochloride, phenythoin, diazepam, ketoprofen, warfarin, bezafibrate, diclofenac, indomethacin, ibuprofen, naproxen, clofibric acid, and gemfibrozil were supplied by Sigma–Aldrich (Sydney, Australia); atorvastatin, calcium salt was supplied from Toronto Research Chemicals (North York, Canada) and ifosfamide were supplied by United States Pharmacopoeia-USP (Rockville, MD, USA). All analytical standards were ≥97% pure.

Surrogate standards, [^{2}H3] morphine (morphine-d_3) (100 μg/μL, 1 mL) and [^{2}H5] diazepam (diazepam-d_5) (100 μg/μL, 1 mL), were supplied by Cerilliant (Wellington, New Zealand); [^{2}H10] carbamazepine (carbamazepine-d_{10}), [^{2}H4] indomethacin (indomethacin-d_4), [^{2}H4] clofibric acid (clofibric acid-d_4), [^{2}H4] ketoprofen (ketoprofen-d_4), [^{2}H3] ibuprofen (ibuprofen-d_3), [^{2}H10] phenythoin (phenythoin-d_{10}) were supplied by CDN Isotopes (Quebec, Canada, distributed by SciVac, Hornsby, Australia); [^{2}H4] paracetamol (paracetamol-d_4), [^{2}H3] naproxen (naproxen-d_3), [^{2}H5] atorvastatin (atorvastatin-d_5), [^{2}H4] diclofenac (diclofenac-d_4) and [^{2}H6] gemfibrozil (gemfibrozil-d_6) were supplied by Toronto Research Chemicals; [^{2}H5] warfarin (warfarin-d_5) was supplied by Cambridge Isotope Laboratories (Andover, USA) and distributed by Novachem (Collingwood, Australia); [^{2}H5] fluoxetine (fluoxetine-d_5) (1 mg/mL, 1 mL) was supplied by Isotec (Sigma–Aldrich). Isotope enrichment was ≥98%.

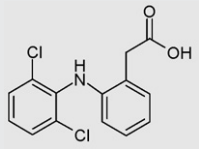
Methanol (MeOH) and acetonitrile (ACN) (ChromAR HPLC grade) were purchased from Mallinckrodt Baker (Phillipsburg, NJ, USA); ethyl acetate (EtAC), purity >99.8%, was purchased from Sigma–Aldrich; formic acid (purity 99%) was purchased from Ajax Finechem (Sydney, Australia). The ultra pure water (H_2O) used for laboratory purposes as well as LC mobile phase was purified using an IBIS Technology (Perth, Australia) Ion Exchange System followed by Elga (High Wycombe, UK) Purelab Ultra System.

Single compound stock solutions (nominal concentration of 1 μg/μL) were prepared by dissolving a known amount of an analytical standard or a surrogate standard in MeOH/H_2O 50:50 (v/v), except for diazepam which was prepared in ethanol due to its limited solubility in MeOH/H_2O mixtures. From serial dilution of the single compound stock solutions, two working solutions (nomi-

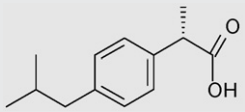
Table 1
Class, formula, molecular weight, and CAS number of the pharmaceuticals investigated.

Analgesics, antipyretics, and non-steroidal anti-inflammatory drugs (NSAIDs)

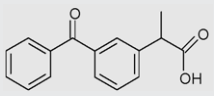
Diclofenac
$C_{14}H_{11}Cl_2NO_2$
MW: 296.1
CAS: 15307-79-6
NSAID and analgesic



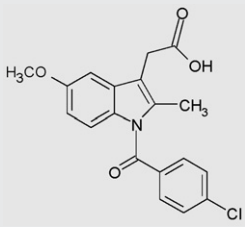
Ibuprofen
$C_{13}H_{18}O_2$
MW: 206.3
CAS: 15687-27-1
NSAID



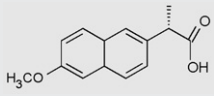
Ketoprofen
$C_{16}H_{14}O_3$
MW: 254.3
CAS: 22071-15-4
NSAID, analgesic and antipyretic



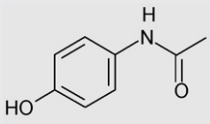
Indomethacin
$C_{19}H_{16}ClNO_4$
MW: 357.8
CAS: 53-86-1
NSAID



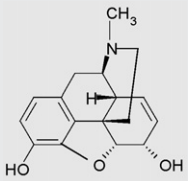
Naproxen
$C_{14}H_{14}O_3$
MW: 230.6
CAS: 22204-53-1
NSAID



Paracetamol
$C_8H_9NO_2$
MW: 151.2
CAS: 103-90-2
Analgesic and antipyretic

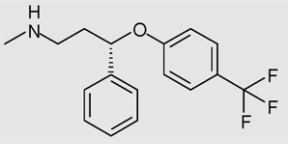


Morphine
$C_{17}H_{19}NO_3$
MW: 285.4
CAS: 57-27-2
Opiate analgesic

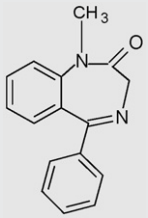


Antidepressants and tranquilizers

Fluoxetine
$C_{17}H_{18}F_3NO$
MW: 309.3
CAS: 59333-67-4
Antidepressant

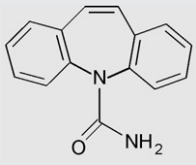


Diazepam
$C_{16}H_{13}ClN_2O$
MW: 284.7
CAS: 439-14-5
Benzodiazepine derivative

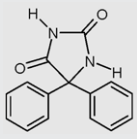


Antiepileptics

Carbamazepine
$C_{15}H_{12}N_2O$
MW: 236.3
CAS: 298-46-4

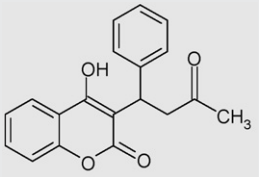


Phenytoin
$C_{15}H_{12}N_2O_2$
MW: 252.3
CAS: 57-41-0



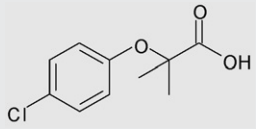
Anticoagulants

Warfarin
$C_{19}H_{16}O_4$
MW: 308.3
CAS: 81-81-2

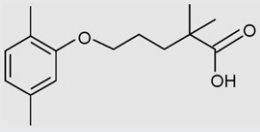


Lipid lowering agents

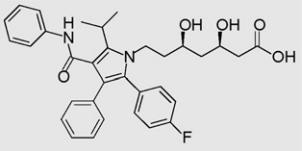
Clofibric acid
$C_{10}H_{11}ClO_3$
MW: 214.6
CAS: 882-09-7



Gemfibrozil
$C_{15}H_{22}O_3$
MW: 250.3
CAS: 25812-30-0



Atorvastatin
$C_{33}H_{35}FN_2O_5$
MW: 558.64
CAS: 134523-03-8



Bezafibrate
$C_{19}H_{20}ClNO_4$
MW: 361.8
CAS: 41859-67-0

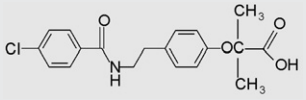
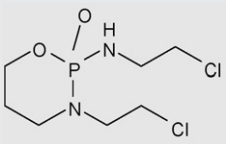
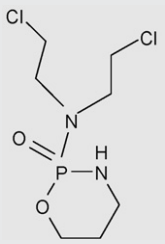


Table 1 (*Continued*)

Cytostatics Ifosfamide $C_7H_{15}Cl_2N_2O_2P$ MW: 261.1 CAS: 3778-73-2		Cyclophosphamide $C_7H_{15}Cl_2N_2O_2P$ MW: 261.1 CAS: 6055-19-2	

nal concentrations 10 and 1 ng/μL) containing all the analytical standards were prepared freshly for each analytical run, whilst a working solution (10 ng/μL) containing all the surrogate standards were prepared bimonthly. All solutions, as well as analytical and surrogate standards were kept refrigerated at 4 °C to avoid degradation.

2.3. Solid-phase extraction pre-concentration

Solid-phase extraction was chosen for pre-concentration and sample cleanup because it is known to provide sufficient sample concentration for sub-ng/L analysis in environmental and wastewater samples [36]. Prior to SPE enrichment/cleanup, secondary wastewater samples (250 mL) were filtered through 0.45 μm polyethersulfone membrane filters (PALL Life Sciences, East Hills, USA) and then diluted to 500 mL with ultra pure water to reduce matrix interactions on the SPE cartridges. Post-RO water samples (500 mL) had already been subject to microfiltration and therefore did not require further filtration before SPE cleanup. The SPE procedure used Strata-X (6 mL, 500 mg) cartridges (Phenomenex, Sydney, Australia), and an automated Aspec XLi extractor (Gilson, Middleton, USA) for the conditioning, washing, and elution of the cartridges. The Strata-X stationary phase is a surface-modified styrene divinylbenzene polymeric surface that has hydrophilic and hydrophobic properties that can efficiently extract acidic, neutral and basic analytes at a wide range of pH. Optimisation of the SPE method was undertaken after the analytes were grouped by their optimal LC–MS/MS electrospray ionisation (ESI) mode. Group one (G1) analytes, measured in positive ESI mode (ESI(+)), included both acidic, and neutral or slightly basic pharmaceuticals, and SPE cartridge conditioning and sample loading was conducted at neutral pH. The G1 analytes included atorvastatin, bezafibrate, carbamazepine, cyclophosphamide, diazepam, diclofenac, fluoxetine, ifosfamide, indomethacin, ketoprofen, morphine, paracetamol, phenytoin, and warfarin. Group two (G2) analytes, measured in negative ESI mode (ESI(−)), included acidic compounds only (clofibric acid, gemfibrozil, ibuprofen, and naproxen) and conditioning and sample loading was conducted at pH = 3.5. Conditioning, washing and elution procedures for both groups are listed in Table 2. Before sample loading, an appropriate surrogate standard spike (typically ranging between 50 and 100 ng/L for post-RO water and 200–500 ng/L for secondary wastewater) was added to all samples to determine recoveries and to correct for matrix effects. Samples were homogenized by shaking and then loaded onto the SPE cartridges using three 8-channel off-line peristaltic pumps (Gilson) at a flow rate of between 3 and 5 mL/min. After loading and washing, cartridges were gently dried under vacuum in a manifold system (Supelco, Bellefonte, USA) for 20–30 min. Analytes were subsequently eluted into 12 mL glass test tubes with a 3 min delay between each aliquot of eluting solvent. The delay between the dispensed aliquots ensures that the stationary phase was efficiently soaked with the eluting solvents. The extract (approximately 12 mL) was concentrated to near dryness in a dry block heater fitted with nitrogen blowdown (Ratek 30D, Boronia, Australia) set at 38 °C and under a gentle stream of nitrogen. The final extracts were redissolved in 500 μL of MeOH:H_2O 30:70 (v/v) and then stored in 2 mL PTFE-lined screw cap amber glass vials at 4 °C, until analysis.

Table 2
Solid-phase extraction (SPE) conditioning, washing and elution steps used.

SPE steps	Acidic/neutral/basic pharmaceuticals, ESI(+) Group 1	Acidic pharmaceuticals, ESI(−) Group 2
Conditioning	6 mL EtAC	4.5 mL ACN
Flow rate = 5 mL/min	6 mL ACN	4.5 mL MeOH
	6 mL MeOH	12 mL H_2O, pH = 3.5 (formic acid)
	12 mL H_2O, pH = 7	
Washing	4.5 mL 5% MeOH (v/v) in H_2O	4.5 mL 5% MeOH (v/v) in H_2O
Flow rate = 5 mL/min	12 mL H_2O	12 mL H_2O
Elution	4 mL ACN, 3 min delay	6 mL ACN, 3 min delay
Flow rate = 1 mL/min	4 mL MeOH, 3 min delay	6 mL MeOH, 3 min delay
	3 mL EtAC, 3 min delay	1.0 mL ACN, 3 min delay
	1.5 mL ACN, 3 min delay	

2.4. LC–MS/MS method

Liquid chromatography separations were performed using an Agilent 1100 HPLC system (Palo Alto, USA) equipped with a solvent degasser unit, a quaternary pump and a 100 well-plate autosampler. The details of parameters used in the LC separation for G1 and G2 compounds are given in Table 3. The LC was coupled to a Micromass Quattro Ultima Triple Quadrupole (Manchester, UK) system fitted

Table 3
LC conditions for Group 1 (acidic/neutral/basic) pharmaceuticals and Group 2 (acidic) pharmaceuticals.

	Group 1, ESI(+)		Group 2, ESI(−)	
Injection volume	25 μL		12.5 μL	
Flow rate	200 μL/min		100 μL/min	
Column	Phenomenex GeminiC18 (150 mm × 2 mm, 3 μm)		Phenomenex Synergi MAX-RP (100 mm × 2 mm, 2.5 μm)	
Guard column	Phenomenex GeminiC18 (4 mm × 2 mm, 3 μm)		Phenomenex Synergi MAX-RP (4 mm × 2 mm, 2.5 μm)	
Eluent A	MeOH with 1% (v/v) formic acid		MeOH with 0.01% (v/v) formic acid and 5% (v/v) ACN	
Eluent B	H_2O with 0.4% (v/v) formic acid		H_2O	
LC gradient	Time (min)	% Eluent B	Time (min)	% Eluent B
	0	70	0	50
	3	70	3	50
	25	10	10	5
	35	5	35	0
	36	0	36	50
	46	0	50	50
	47	70		
	67	70		

Table 4
General ESI and MS tuning parameters.

	Group 1, ESI(+)	Group 2, ESI(−)
Capillary voltage (V)	2800	2600
Cone voltage (V)	30	35
Hex 1, aperture, hex 2 (V)	0, 0.1, 0.1	0, 0.1, 0.1
Source temperature (°C)	130	130
Desolvation temperature (°C)	325	325
N_2 cone gas flow (L/h)	30	30
N_2 desolvation gas flow (L/h)	325	300
Ar collision gas pressure (kPa)	2×10^{-4}	2×10^{-4}
Q1 and Q3 mass resolution	1	1
Ion energy Q1, ion energy Q3	1.0, 1.5	1.0, 1.0

with an ESI operated in both in positive and negative ion mode for G1 and G2, respectively. For optimum signal in ESI(+), capillary and cone voltages were 2800 and 30 V, while optimum signal for ESI(−) required capillary and cone voltages of 2600 and 35 V. Otherwise MS parameters were generally the same for both ESI modes (see Table 4). The molecular weight distribution of the analytes targeted in this work range between 100 and 550 Da, thus Hexapole1, Aperture and Hexapole2 generally required low voltages (0, 0.1 and 0.1 V, respectively) when the ion block of the mass spectrometer was perfectly clean. When the ion block required cleaning, however, sensitivity enhancement could be achieved by increasing these voltages in the following ranges: Hexapole1 (0–1 V), Aperture (0.4–0.6 V) and Hexapole2 (0.4–0.6 V). Cryogenic liquid nitrogen gas (BOC Gases, Perth, Australia) was used as both desolvation and nebulizer gas, while high purity Argon (99.997% purity, BOC Gases) was used as collision gas ($P = 2 \times 10^{-4}$ kPa) in both single ion reaction monitoring (SIR) and multiple reaction monitoring (MRM) experiments. Prior to injection, the needle of the injector was rinsed thoroughly in the injection port with a mixture of MeOH:H_2O 50:50 (v/v) before and after each injection to minimise potential carryover. Instrumental and/or laboratory contaminations were also monitored by regular and methodical analysis of injector and procedural blanks, as well as field and trip blanks collected during field sampling. In general, about 33% of the samples analysed were blanks (i.e. trip blanks, procedural blanks and field blanks).

A minimum of three identification points are required to meet the identification performance criteria defined by the EU Commission for quantitative mass spectrometric detection [37]. Using LC–MS/MS to monitor one precursor ion and two daughter ions 'earns' four identification points and therefore fulfils these criteria. In this work, the most intense characteristic MRM transitions chosen for each analyte and surrogate standard and Table 5 lists the precursor and daughter ions monitored. In ESI(+) mode, two transitions were used for each G1 compound, except for phenytoin which showed poor fragmentation. The MRM ratio and retention time (t_R) was also monitored. In contrast G2 compounds analysed in ESI(−) mode were characterised by poor MS fragmentation spectra and only one transition and t_R could be monitored While this does not fulfil the EU identification criteria, comparisons of analyte t_R in the sample and in synthetic standards demonstrated that shifts in t_R were generally less than 2.1%.

The MRM transitions were grouped in five windows for ESI(+) mode analysis and two windows for ESI(−) mode, based on analyte t_R to ensure that at least 15–20 points were obtained to define each chromatographic peak and ensure reproducible integration results (see Table 5). The dwell time of each m/z monitored depended on the number of transitions in that window, with a maximum of 12 transitions at 100 ms dwell time in a single window.

Analytes were quantified using the ratio of the analyte peak area to surrogate standard peak area and using an external calibration curve obtained by diluting working standards with MeOH:H_2O 30:70 (v/v). A deuterated homologue was used as a surrogate standard for most analytes except for ifosfamide and cyclophosphamide, which both used phenythoin-d_{10}, and bezafibrate, which used warfarin-d_5. It was demonstrated that these surrogate standard accounted for matrix effects satisfactorily, as described in Section 3.6. Calibration curves with up to seven concentration points, and spanning 5 ng/mL up to 1000 ng/mL, were acquired at the beginning and at the end of analytical run. Data processing was carried out using MassLynx NT 4.0 software, while data quantitation was performed using QuanLynx 4.0.

3. Results and discussion

3.1. Optimisation of MS parameters and MRM transitions

Direct infusion experiments were used to determine whether pharmaceuticals produced a better signal to noise ratio (S/N) in ESI(+) or ESI(−) mode, as well as optimise MS tuning parameters for both modes. The ionisation mode selected for each compound is shown in Table 5. Single compound standard solutions (1–10 ng/μL) prepared in MeOH:H_2O 50:50 (v/v), in the presence or absence of formic acid, were introduced into the MS at a flow rate of 5 μL/min using a syringe pump (Harvard Apparatus, Australia). Pharmaceuticals characterised by neutral or slightly basic properties (i.e. carbamazepine, fluoxetine, cyclophosphamide, ifosfamide, paracetamol, diazepam, morphine, phenytoin) were only tested in ESI(+) mode because these compounds were unlikely to form $[M-H]^-$ parent ions. All other compounds were tested in both modes because of the presence of carboxylic functionalities and/or basic-to-neutral nitrogen-containing functional groups. Warfarin and atorvastatin had better S/N in ESI(+) mode, while naproxen, ibuprofen gemfibrozil and diclofenac clearly had better S/N in ESI(−) mode. Ketoprofen, bezafibrate, diclofenac and indomethacin showed very similar S/N ratio in both modes, however they were include in ESI(+) mode (G1) so that as many pharmaceuticals as possible were separated and detected in a single LC–MS run.

For ESI(+) mode, the presence of formic acid ensured that the most intense precursor ion observed was the proton adduct $[M+H]^+$. In the absence of formic acid, other characteristic precursor ions such as the sodium or potassium adducts ($[M+Na]^+$ or $[M+K]^+$) or the sodium–solvent adduct ($[M+MeOH+Na]^+$ formed but these were not suitable for MS/MS fragmentation because they yield unstable MS/MS spectra. In ESI(−) mode, the deprotonated $[M-H]^-$ ion was the only precursor ion present in the MS spectra. The intensity of selected MRM transitions were optimised by varying the cone voltage, which controls the introduction of the ions into the ion block, and the collision energy, which influences the formation of fragments in the collision cell. Cone voltage did not particularly influence sensitivity of the analytical determination, but collision energy required specific tuning for each analyte to ensure maximum sensitivity (see Table 5). Fragmentation patterns for most G1 ESI(+) and all G2 ESI(−) compounds were generally in agreement with those previously reported in literature in wastewaters and sludges [11,32,33,38–43]. Several compounds analysed in ESI(+) mode, including indomethacin, diclofenac, bezafibrate, warfarin and ketoprofen, are usually measured in ESI(−) mode [32,39,40], although typically the transitions varied from those reported here only by the addition or subtraction of H^+.

3.2. Development of the chromatographic separation

Due to the variety of pharmaceuticals selected for this project, a range of columns from Phenomenex containing different stationary phases (C8, C12, C18 and C6-phenyl) and a variety of mobile phases (H_2O, MeOH, ACN) containing different additives (formic acid, ammonium formate) in different percentages, were initially

Table 5
Retention time (t_R), specific MS/MS parameters, and precursor and daughter ions monitored for each analyte. MRM transitions were grouped in five windows for ESI(+) mode analysis and two windows for ESI(−) mode to ensure sensitivity and reproducible integration results.

Compound	t_R (min)	Precursor ion [m/z]	Product ions [m/z]	Cone voltage	Dwell time (ms)	Collision energy
			Group 1, ESI(+) mode			
			Window 1: 1.0–13.0 min			
Morphine	2.64	286.5	153.1	30	100	45
			165.1	30	100	45
Morphine-d_3	2.58	289.5	153.2	30	100	45
			165.1	30	100	45
Paracetamol	5.32	152.0	93.0	30	100	20
			110.0	30	100	15
Paracetamol-d_4	5.21	156.0	97.0	30	100	20
			114.0	30	100	15
			Window 2: 13.1–20.5 min			
Ifosfamide[a]	17.93	263.1	154.2	30	150	22
			156.2	30	150	22
Cyclophosphamide[a]	19.31	263.1	234.8	30	150	15
			141.9	30	150	22
			Window 3: 20.0–24.0 min			
Fluoxetine	21.62	310.3	44.0	20	100	10
			148.0	20	100	8
Fluoxetine-d_5	21.57	315.3	44.0	20	100	10
			153.0	20	100	8
Phenytoin	22.69	253.3	182.0	45	100	17
Phenytoin-d_{10}	22.60	263.3	192.0	45	100	20
Carbamazepine	23.13	237.3	192.0	35	100	20
			194.0	35	100	20
Carbamazepine-d_{10}	23.03	247.3	202.0	35	100	20
			204.0	35	100	20
			Window 4: 24.1–26.5 min			
Diazepam	25.81	285.4	154.1	60	100	25
			193.4	60	100	25
Diazepam-d_5	25.75	290.4	154.1	60	100	25
			198.4	60	100	25
Ketoprofen	25.75	255.3	105.4	35	100	20
			209.3	35	100	15
Ketoprofen-d_4	25.65	259.3	105.4	35	100	20
			213.3	35	100	15
			Window 5: 25.5–28.0 min			
Warfarin	26.71	309.2	163.1	30	100	15
			251.0	30	100	15
Warfarin-d_5	26.68	314.3	163.1	30	100	15
			256.0	30	100	15
Bezafibrate[b]	26.69	362.0	276.2	30	100	15
			316.3	30	100	15
Atorvastatin	27.48	559.1	440.2	45	100	20
			466.2	45	100	15
Atorvastatin-d_5	27.38	564.1	445.1	45	100	20
			471.1	45	100	15
			Window 6: 27.5–33.0 min			
Diclofenac	28.39	296.0	215.1	30	100	15
			250.2	30	100	15
Diclofenac-d_4	28.29	300.2	219.0	30	100	15
			254.0	30	100	15
Indomethacin	28.37	358.3	139.1	30	100	15
			174.1	30	100	15
Indomethacin-d_4	28.27	362.3	143.1	30	100	15
			174.1	30	100	15
			Group 2, ESI(−) mode			
			Window 1: 12.0–17.5 min			
Naproxen	14.96	229.1	170.0	50	150	15
Naproxen-d_3	14.83	232.1	173.1	50	150	15
Clofibric acid	16.38	213.1	127.0	50	150	12
Clofibric acid-d_4	16.27	217.1	131.0	50	150	12
			Window 2: 17.6–22.0 min			
Ibuprofen	18.81	205.1	161.1	50	150	7
Ibuprofen-d_3	18.82	208.1	164.1	50	150	7
Gemfibrozil	20.58	249.3	121.1	50	150	12
Gemfibrozil-d_6	20.47	255.3	121.1	50	150	12

[a] Phenytoin-d_{10} was used as surrogate standard.
[b] Warfarin-d_5 was used as surrogate standard.

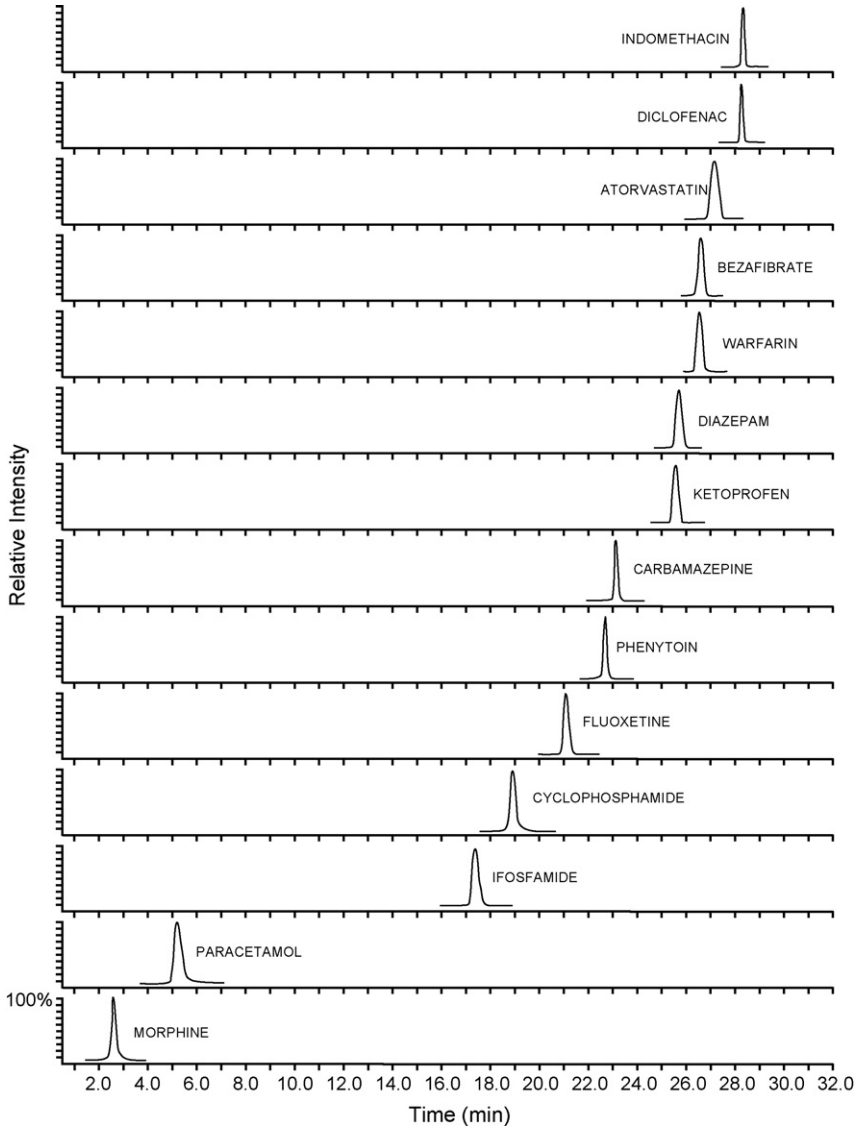


Fig. 1. A typical chromatogram for separation of a synthetic solution of 14 pharmaceuticals in ESI(+) mode obtained using the Phenomenex GeminiC18 column (150 mm × 2 mm, 3 μm particle size) at a flow rate of 200 μL/min.

tested. Neither the C6-phenyl or C8 columns provided satisfactory results for the 14 compounds analysed in ESI(+) mode, due to peak tailing and poor separation, respectively. However, good separation was achieved using the GeminiC18 column with a gradient mobile phase consisting of MeOH and ultra pure water, each containing a relative high percentage of formic acid (1% and 0.4%, respectively). Formic acid substantially promoted the formation of $[M+H]^+$ parent ions, leading to better sensitivity, as well as better resolution and peak shape. As demonstrated by separation of a 1 ng/μL standard solution in Fig. 1, morphine and paracetamol were the only compounds showing limited retention ($t_{R\,morphine}$ = 2.6 min and $t_{R\,paracetamol}$ = 5.3 min) but chromatography was satisfactory even in secondary wastewater samples. All the other compounds showed t_R > 15 min and were characterised by very satisfactory and reproducible chromatography.

Phenomenex Synergi MAX-RP (C12) was used to separate the four compounds analysed in ESI(−) mode. The column gave comparable separation to the GeminiC18 column but elution was quicker because of the shorter length and lower carbon load. Separation also used a gradient mobile phase of MeOH and ultra pure water. While the presence of formic acid substantially improved the separation of the four analytes, it was only added to the MeOH mobile phase at 0.01% (v/v). Higher concentrations (i.e. 0.05% and 0.025%) reduced MS signal by one to one and a half orders of magnitude because excess protons suppressed formation of negative ions in the ESI source. Addition of 5% (v/v) ACN to the MeOH mobile phase also minimised tailing of longer retained compounds. An example of a separation of a 1 ng/μL standard solution is shown in Fig. 2.

3.3. Instrumental performance: linearity, detection limits, and peak identification

Instrumental performance was assessed through the analysis of standard solutions (Table 6). Calibration curves for all analytes showed good linearity (R^2 > 0.998) up to either 12.5 or 25 ng on column, depending on the ionisation efficiency of each compound. Repeat injections (n = 10) of a 0.05 ng/μL standard solution (equivalent to 1.25 ng on column) were used to assess instrumental detection limits (IDLs) as well as to determine the variability of analyte t_R and MRM ratio. Instrumental detection limits (IDLs), estimated at signal-to-noise (S/N) ratio equal to 3, ranged from 0.004 to 0.142 ng, comparable to Gros et al. [32] who have also calculated IDL using this method. Instrumental quantitation limits (IQL) estimated at S/N = 10, correspondingly ranged from 0.013 to 0.475 ng on

Table 6
Typical linear ranges (pg on column) and regression values observed for calibration curves; instrumental detection limits (IDL) estimated at $S/N = 3$ and instrumental quantitation limits (IQL) estimated at $S/N = 10$, both also reported as pg on column; instrumental precision in terms of MRM ratio and t_R were obtained from repeated injections ($n = 10$) of 1.25 ng on column.

Compound	Linear range	R^2	IDL	IQL	MRM ratio (± RSD)	t_R (min ± SD)
Group 1 ESI(+)						
Morphine	250–25,000	0.9995	34	113	0.99 ± 3.8	2.6 ± 0.15
Paracetamol	250–25,000	0.9998	27	90	5.9 ± 3.7	5.3 ± 0.32
Ifosfamide	500–25,000	0.9982	136	454	0.90 ± 2.3	17.9 ± 0.09
Cyclophosphamide	500–25,000	0.9986	93	310	25.5 ± 5.5	19.3 ± 0.10
Fluoxetine	250–25,000	0.9999	13	45	0.91 ± 3.4	21.6 ± 0.25
Phenytoin	500–25,000	0.9990	142	475	n.a.	22.7 ± 0.53
Carbamazepine	250–25,000	0.9990	10	35	4.0 ± 5.0	23.1 ± 0.49
Ketoprofen	250–25,000	0.9987	50	166	10.5 ± 5.4	25.7 ± 0.05
Diazepam	250–12,500	0.9999	19	62	2.0 ± 4.5	25.8 ± .13
Warfarin	250–25,000	0.9994	4	13	0.81 ± 5.5	26.7 ± 0.16
Bezafibrate	250–12,500	0.9995	64	212	1.63 ± 5.2	26.7 ± 0.16
Atorvastatin	250–12,500	0.9993	15	51	5.6 ± 2.5	27.5 ± 0.11
Diclofenac	250–12,500	0.9997	24	80	1.65 ± 5.6	28.4 ± 0.19
Indomethacin	250–25,000	0.9995	14	47	4.58 ± 2.1	28.4 ± 0.10
Group 2 ESI(−)						
Naproxen	125–12,500	0.9998	4	13	n.a.	15.0 ± 0.22
Clofibric acid	125–12,500	0.9999	31	103	n.a.	16.4 ± 0.34
Ibuprofen	125–12,500	0.9999	22	73	n.a.	18.8 ± 0.28
Gemfibrozil	125–25,000	0.9999	5	17	n.a.	20.6 ± 0.31

n.a.: not available.

column. Generally the standard deviation (SD) of t_R was less than 30 s for both ESI(+) and ESI(−) modes, while the relative standard deviation (RSD) of the MRM ratios measured was generally less than 6%.

3.4. Accuracy and precision

The SPE-LC–MS/MS method was validated for both secondary wastewater and post-RO water. Accuracy, expressed as percentage recovery relative to the surrogate standards, and precision, expressed as RSD were determined by processing spiked ($n = 3$) and unspiked ($n = 3$) samples through the entire procedure (Table 7). Unspiked post-RO did not contain appreciable pharmaceutical concentrations and therefore blank correction was not required. In contrast unspiked secondary wastewater could contain considerable pharmaceutical concentrations. Therefore recoveries were corrected by subtracting the average unspiked analyte concentration ($n = 3$) from the corresponding spiked samples. Concentrations tested were 10, 50, and 100 ng/L in post-RO water and 100, 250, and 500 ng/L in secondary treated water. There were no substantial differences in the recovery of the analytes were observed spiking the water samples with different concentrations of pharmaceuticals, thus recoveries are averaged of all samples ($n = 9$).

The average percent recoveries of these spikes were generally greater than 71% in post-RO water and 74% in secondary wastewater. Precision expressed as RSD ($n = 9$) varied between 2–16% in post-RO water and 2–19% in secondary wastewater. We note that diazepam and morphine yielded significantly lower recoveries than other analytes in this validation experiment. However, recoveries for all other QC samples (see, for example Table 8) have been much closer to 100%, and so this low recovery value in this experiment has been attributed to a possible error in preparing the calibration solutions or the solution spiked in the samples. Other methods utilising SPE pre-concentration have reported similar recoveries and precision [32].

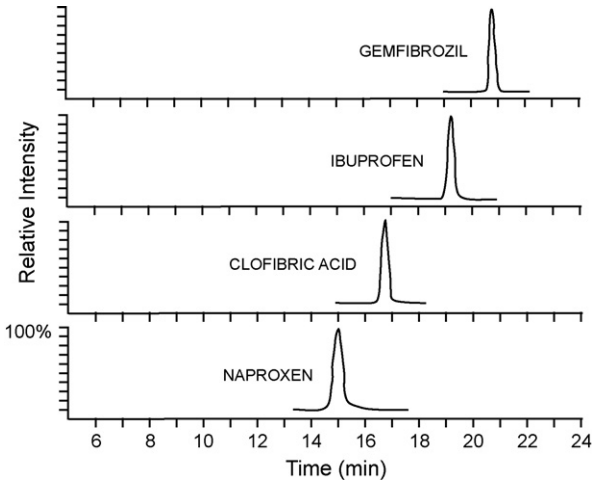


Fig. 2. A typical chromatogram for separation of a synthetic solution of four pharmaceuticals in ESI(−) mode obtained using the Phenomenex Synergi MAX-RP column (100 mm × 2 mm, 2.5 μm particle size) at a flow rate of 100 μL/min.

3.5. Method limits of detection and quantitation

Method limits of quantitation (MLQ) were calculated as the concentration equivalent to $S/N = 10$ using spiked post-RO and secondary wastewater samples [28,44], by manual S/N calculation on unsmoothed chromatograms using peak of a known concentration (Table 7). Samples were spiked at three different concentrations in triplicate (i.e. 10, 50, and 100 ng/L in post-RO water and 100, 250, and 500 ng/L in secondary wastewater) and the MLQ was then calculated from two lowest concentrations producing a peak with $S/N > 10$. Post-RO water MLQ ranged between 1 and 25 ng/L (average ~10 ng/L) and were typically calculated from samples spiked with 10 and 50 ng/L except when MLQ = 25 ng/L. In these cases MLQ were determined using the samples spiked with 50 and 100 ng/L. Average MLQs in secondary wastewater were calculated using the 100–500 ng/L spiked samples. Since secondary wastewater could contain pharmaceutical concentrations well above detection, the concentration corresponding to MLQ was calculated by downscaling the S/N ratio of the peak at the total measured concentration and assuming a linear correlation through zero [45]. Reported MLQ values are generally an average of six analyses, and ongoing QC samples (data not presented) remain consistent with these values.

On average, MLQ for secondary wastewater were 10 times higher than those reported in post-RO water, ranging between 15 and

Table 7
Accuracy, precision and MLQ in post-RO and secondary wastewater achieved with the proposed SPE-LC–MS/MS method. Accuracy and precision were determined from spiked and unspiked samples through the entire procedure and recoveries are presented as average value of all samples ($n = 9$). The health-based guidelines tabled have been proposed by the Western Australian Department of Health [35].

Compound	Post-RO water			Secondary wastewater			Target health-based guidelines (ng/L)
	% Accuracy ($n = 9$)	Precision (RSD, $n = 9$)	MLQ (ng/L)	% Accuracy ($n = 9$)	Precision (RSD, $n = 9$)	MLQ (ng/L)	
Morphine	78	12	25	89	14	100	50,000
Paracetamol	99	6	10	98	7	125	2,500
Ifosfamide	97	10	25	86	10	100	4,500,000
Cyclophosphamide	114	14	5	115	16	100	35,000
Fluoxetine	97	3	5	97	3	25	100,000
Phenytoin	107	6	5	109	2	55	1,400,000
Carbamazepine	103	3	1.5	102	6	40	1,000,000
Diazepam	71	7	5	74	12	30	25,000
Ketoprofen	91	5	25	113	3	275	35,000
Warfarin	89	8	5	102	2	15	5,000
Bezafibrate	101	4	15	92	2	65	3,000,000
Atorvastatin	100	2	20	83	9	150	50,000
Diclofenac	101	8	2.5	112	2	20	18,000
Indomethacin	95	16	5	92		40	250,000
Naproxen	105	8	13	110	19	250	2,200,000
Clofibric acid	105	6	1	107	2	15	7,500,000
Ibuprofen	110	14	12	89	11	500	4,500,000
Gemfibrozil	92	6	2.5	105	5	50	6,000,000

500 ng/L (average ~100 ng/L). However, the MLQs achieved were still an average of four orders of magnitude lower than health-based guidelines (Table 7) proposed by the Western Australian Department of Health [35] and developed using similar methods to the Australian guidelines for water recycling [25]. This demonstrates that the proposed SPE-LC–MS/MS is suitable of measuring pharmaceuticals at limits far below the limits required in secondary wastewater. For post-RO water the MLQs achieved were an average of five orders of magnitude lower than the guidelines.

3.6. Matrix effect and choice of surrogate standards

Because of the influence of ion suppression on ESI-based quantitative analysis, matrix effects are often a concern in SPE-LC–MS/MS, particularly when homologue deuterated standards are not available or are prohibitively expensive. Matrix components present in the water sample (and in the SPE extract) can enhance or suppress the absolute analyte response, resulting in variable detection limits and, more importantly, erroneous quantitative results. Ion suppression was observed for all compounds in secondary wastewater extracts in this study. Different approaches have been proposed in the literature to address these effects, including specific sample preparation strategies (i.e. SPE), use of surrogate standards, standard addition, dilution of the SPE extracts, as well as using an "echo peak", which involves injections of the sample and standard solution within a short time period so that standard and sample analytes elute in a similar chromatographic region and are subject to a similar degree of signal suppression/enhancement [28,36,46].

In this study, we chose to use deuterated standards to account and correct for matrix effects as the analyte and a co-eluting deuterated homologue are subject to almost identical matrix effects [36,46]. However, homologue deuterated standards were not available for ifosfamide, cyclophosphamide, or bezafibrate, and therefore matrix effects were specifically studied for these analytes. The surrogate standard chosen for ifosfamide and cyclophosphamide was phenytoin-d_{10} while warfarin-d_5 was used for bezafibrate. For a surrogate standard to correct for matrix effects, it must show a similar degree of ion-enhancement/ion suppres-

Table 8
Duplicate composite secondary wastewater and post-RO water samples from Beenyup WWTP testing in-house reproducibility of sampling and the SPE-LC–MS/MS method. Spike recovery from QC samples, consisting of three secondary effluent samples spiked at 100 ng/L and three ultra pure water spiked at 50 ng/L, is also shown.

Compound	Secondary wastewater				Post-RO water		
	Sample 1	Sample 2	Average (± %RSD)	Spike recovery (± %RSD)	Sample 1	Sample 2	Spike recovery (± %RSD)
Morphine	<150	<150	n.a.	112 ± 4	<25	<25	96 ± 3
Paracetamol	<135	<135	n.a.	104 ± 6	<25	<25	96 ± 5
Ifosfamide	<125	<125	n.a.	167 ± 16	<35	<35	96 ± 9
Cyclophosphamide	<125	<125	n.a.	106 ± 8	<10	<10	98 ± 5
Fluoxetine	23	22	22.5 ± 3	101 ± 3	<10	<10	102 ± 6
Phenytoin	71	71	71	102 ± 5	<20	<20	123 ± 10
Carbamazepine	938	957	947 ± 1	104 ± 5	<3	<3	103 ± 4
Diazepam	<25	<25	n.a.	100 ± 7	<8	<8	101 ± 2
Ketoprofen	<100	<100	n.a.	103 ± 5	<15	<15	97 ± 1
Warfarin	<15	<15	n.a.	111 ± 3	<5	<5	109 ± 7
Bezafibrate	<45	<45	n.a.	106 ± 3	<8	<8	97 ± 12
Atorvastatin	108	116	112 ± 5	99 ± 6	<8	<8	98 ± 9
Diclofenac	423	407	415 ± 3	94 ± 3	<2.5	<2.5	109 ± 7
Indomethacin	132	149	140 ± 9	106 ± 3	<5	<5	102 ± 6
Naproxen	<83	<83	n.a.	110 ± 4	<4.3	<4.3	106 ± 1
Clofibric acid	<8.6	<8.6	n.a.	102 ± 2	<1.3	<1.3	108 ± 2
Ibuprofen	160	162	161 ± 1	101 ± 4	<8.0	<8.0	100 ± 3
Gemfibrozil	59	56	57.5 ± 4	98 ± 1	<3.0	<3.0	106 ± 1

Table 9
Pharmaceuticals concentration in pre- and post-RO samples collected from the Kwinana Water Reclamation Plant (KWRP), Perth, Australia.

Compound	Sampling date: 30 May 2007				Sampling date: 04 June 2007				Sampling date: 07 June 2007			
	Pre-RO		Post-RO		Pre-RO		Post-RO		Pre-RO		Post-RO	
	Comp	Grab	Comp	Grab	Comp	Grab	Comp	Grab	Comp	Grab	Comp	Grab
Morphine	140	120	<25	<25	<100	130	<25	<25	150	120	<25	<25
Paracetamol	1500	2700	<100	<100	1400	1200	<100	<100	900	4500	<100	<100
Ifosfamide	<100	<100	<25	<25	<100	<100	<25	<25	<100	<100	<25	<25
Cyclophosphamide	<100	<100	<5	<5	<100	<100	<5	<5	<100	<100	<5	<5
Fluoxetine	29	32	<5	<5	30	18	<5	<5	27	13	<5	<5
Phenytoin	140	140	<5	<5	170	130	<5	<5	120	160	<5	<5
Carbamazepine	910	970	<1.5	<1.5	1000	900	<1.5	<1.5	960	900	<1.5	<1.5
Diazepam	<30	<30	<5	<5	<30	<30	<5	<5	<30	<30	<5	<5
Ketoprofen	160	180	<25	<25	180	180	<25	<25	150	160	<25	<25
Warfarin	<15	<15	<5	<5	<15	<15	<5	<5	<15	<15	<5	<5
Bezafibrate	<65	<65	<15	<15	<65	<65	<15	<15	<65	<65	<15	<15
Atorvastatin	<150	<150	<20	<20	<150	<150	<20	<20	<150	<150	<20	<20
Diclofenac	320	320	<2.5	<2.5	310	280	<2.5	<2.5	280	300	<2.5	<2.5
Indomethacin	130	130	<5	<5	120	130	<5	<5	140	130	<5	<5
Naproxen	1100	1300	<13	<13	890	1000	<13	<13	1200	1600	15	<13
Clofibric acid	<15	<15	1.6	<1	<15	<15	<1	<1	<15	<15	<1	<1
Ibuprofen	650	880	<12	<12	790	820	<12	<12	840	980	<12	<12
Gemfibrozil	420	560	<2.5	<2.5	380	390	<2.5	<2.5	360	430	<2.5	<2.5

Comp: 24 h composite sample; Grab: grab sample.

sion as the target analyte. To verify the efficiency of the chosen surrogate standards to correct for signal changes due to ion suppression, the peak area ratios to concentration ratio of standard calibration curves in 70:30 (v/v) MeOH:H_2O were compared to those in three different wastewater samples each spiked to a different concentration. The spiked wastewaters produced a linear response almost identical to the calibration curves, with less than 5% difference in slope. This is represented visually in Fig. 3, available as supplementary information. This similarity in response for spiked wastewater and calibration curves demonstrates that the ratio of analyte to internal standard was consistent for all three wastewaters, and that all wastewaters were consistent with the calibration curve. Therefore, the surrogate standards chosen do correct ion suppression and matrix effects despite the fact they are not deuterated homologues. Ongoing QC controls using secondary wastewater from several Perth WWTPs (data not shown) confirms that the surrogate standards chosen are appropriate.

3.7. In-house reproducibility

The in-house reproducibility of sampling and SPE-LC–MS/MS method was tested by measuring duplicate 24 h composite secondary wastewater and post-RO water samples from Beenyup WWTP and data from this sampling event is reported in Table 8. Only a small amount of reproducibility data was obtained from unspiked samples morphine, paracetamol, ifosfamide, cyclophosphamide, diazepam, ketoprofen, warfarin, bezafibrate, naproxen, and clofibric acid were all below detection in the secondary wastewater samples and all analytes were below detection in post-RO water. To ensure reproducibility data for all analytes, six QC samples were also analysed with the samples, consisting of three ultra pure water samples spiked to 50 ng/L and three secondary wastewater samples spiked to 100 ng/L and the results obtained for the QC samples are also reported in Table 8. There is good agreement between replicate samples for those analytes present in unspiked wastewater at concentrations above MLQ and precision is similar to both the QC samples, and the precision data in Table 7. This suggests that the composite sampling procedure is reproducible and does not contribute significantly to the uncertainty budget. As expected, most variation is therefore introduced during the SPE sample preparation. Overall variability of the method is better than 10%.

3.8. Pharmaceuticals in secondary wastewater and RO membrane rejection

Results from the 3 days sampling of secondary wastewater and post-RO water at KWRP are presented in Table 9. Data from field and trip blanks are not included because results for these samples were consistently below detection. Eleven pharmaceuticals (morphine, paracetamol, fluoxetine, phenytoin, carbamazepine, ketoprofen, diclofenac, indomethacin, naproxen, ibuprofen, and gemfibrozil) were above MLQ in secondary treated wastewater from KWRP, though concentrations were two to three orders of magnitude lower than the suggested guidelines for drinking water (Table 7). Results from secondary wastewater at Beenyup WWTP and KWRP were generally similar, although significantly higher concentrations of paracetamol, naproxen, ibuprofen and gemfibrozil were detected at KWRP. It is not expected that the catchments of the two WWTP should differ particularly and therefore these differences are attributed to WWTP performance, possibly in part due to higher removal efficiencies in the warmer summer months, when Beenyup was sampled. Further sampling to investigate seasonal variation and trends in distribution around the Perth metropolitan area is now underway.

When averaging over the three sampling days, there is little statistical difference in concentrations from KWRP secondary wastewater grab and composite samples except for paracetamol, gemfibrozil and naproxen. For all three, however, there was significantly less variation in concentrations from composite samples than grab samples. Variations between days may indicate that pharmaceutical concentrations can vary within the plant, related to changes in usage in the community. Joss et al. [15] have demonstrated diurnal variation for several pharmaceuticals in a small Swiss WWTP that was also correlated to nitrogen load, which is in line with human excretion being the major source of pharmaceuticals in wastewater. It is likely that composite sampling better represents overall plant performance compared to grab samples, where time of sampling may be a confounding factor.

In contrast to secondary wastewater, pharmaceutical concentrations in post-RO samples were almost always below detection. Only two compounds, clofibric acid and naproxen were detected post-RO at KWRP at concentrations very close to their MLQ. Naproxen has been detected in product water from the Luggage Point Water Reclamation Plant (LWRP), a Queensland water reclamation plant

that also produces high quality water for industry [17]. These preliminary results demonstrate that RO is an effective treatment for removal of most pharmaceuticals originally present in secondary treated wastewater. Further analysis is currently underway to investigate trends in concentration and study the effective rejection properties of the RO membranes.

3.9. Comparison to other studies

There are few studies of pharmaceuticals at other Australian WWTP or water recycling facilities [17,23,47] and work presented in this paper represents the largest survey of non-antibiotic pharmaceuticals within Australia published to-date. Our results are generally consistent with studies which include pharmaceuticals we have monitored, although direct comparisons are difficult because each study monitors different pharmaceuticals and because of the variations in WWTP processes, geography and climate.

Non-prescription drugs are commonly found in secondary wastewater and the frequent detection of the NSAIDs ibuprofen, naproxen, diclofenac and indomethacin in this study fits with past estimates of pharmaceuticals dispensed in Australia [47]. Ibuprofen was measured and detected in almost every study we cite [13,14,16–18,20,21,32,38,47,48] with diclofenac, naproxen, and ketoprofen also commonly monitored and detected [13,14,16–18,21,38,47,48]. Indomethacin is less commonly studied, but has been measured at comparable concentrations to those measured in this study [14,21]. Paracetamol has been determined as Australia's greatest dispensed drug by mass [47], but measured concentrations in our study and others ranged from below detection to greater than 5 μg/L [18,21,32,38]. This suggests that paracetamol degradation/removal in WWTP is highly variable.

When considering prescription drugs, carbamazepine is known to be ubiquitous and poorly removed in WWTP [1], and this is reflected in the large number of studies that measure it [13,16,17,21,32,48,49] and the concentrations reported in this study. Atorvastatin is the most commonly prescribed drug in Australia [50], but there are few reported detections in the international literature [51,52] and concentrations measured in this study were relatively low. In contrast, gemfibrozil was detected more consistently than atorvastatin and at significantly higher concentrations in the KWRP/'winter' sampling event, even though less gemfibrozil is prescribed than atorvastatin in Australia [50]. Gemfibrozil is also much more commonly measured in other studies [17,18,21,32,48]. Neither bezafibrate nor clofibric acid were detected in secondary wastewater from either KWRP or Beenyup, despite the low MLQ achieved, and despite being poorly removed in WWTP [53,54]. This would suggest that neither is commonly used in Australia and this is confirmed by prescription data from the most recently available Australian Statistics on Medicines [50]. Phenytoin, fluoxetine, and morphine were also detected in our study, but insufficient data exists to elucidate any particular trends. These compounds are only included in a few methods measuring a wide range of pharmaceuticals [36,55]. Morphine, in particular, is most often measured with other illicit drugs rather than prescription drugs [42,56].

Findings from this paper show that pharmaceuticals are efficiently removed by MF/RO which is in agreement with other studies investigating water recycling and RO particularly [17,19,23]. Removal efficiencies for RO can only be estimated from our data because most compounds were not detected in post-RO water. However, using the post-RO water MLQ as upper bound produces removal efficiencies >95% except for some pharmaceuticals present in wastewater at concentrations only slightly larger than the post-RO water MLQ (e.g. fluoxetine and phenytoin in Beenyup WW and morphine, fluoxetine and ketoprofen in KWRP WW). Even considering these lower removal efficiencies, the average removal efficiency for all pharmaceuticals is >90%, which compares well to those calculated for other water recycling schemes [17,19]. Sedlak et al. [57] have suggested that indicators to monitor pharmaceuticals attenuation during advanced wastewater treatment such as RO should be present at one to two orders of magnitude above MLQ in wastewater, and identified carbamazepine, diclofenac, gemfibrozil, naproxen, and ibuprofen as potential indicator compounds in their study. All these compounds, as well as phenytoin, are present in Perth WWTP at least one order of magnitude greater than MLQ for RO water and thus could also be used to monitor water treatment for indirect potable reuse in Western Australia.

4. Conclusions

The method presented incorporate pharmaceuticals from a wide variety of classes, including morphine and warfarin. The acidic and/or basic neutral nature of the compounds necessitates analysis in both ESI(+) and ESI(−) mode. However, optimisation of a single SPE extraction procedure for both neutral/basic and acidic compounds is planned to further streamline the method, as exemplified by Vanderford et al. [33]. The SPE extraction constitutes about 90% of sample preparation and therefore a single SPE extraction would be a significantly improve in sample throughput.

Generally results from Beenyup and KWRP WWTPs were similar, with variations probably due to season. Further fieldwork over the course of a year will hopefully enable a better understanding of seasonal and geographical trends. Analysis of post-RO water has confirmed previous findings that RO is an efficient method of pharmaceutical removal with average removal efficiency estimated here to be >90%. Potential indicators for monitoring water treatment for indirect potable reuse in Western Australia are phenytoin, carbamazepine, diclofenac, gemfibrozil, naproxen, and ibuprofen.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2009.06.001.

References

[1] C.G. Daughton, T.A. Ternes, Environ. Health Perspect. 107 (1999) 907.
[2] S.E. Jorgensen, B. Halling-Sorensen, Chemosphere 40 (2000) 691.
[3] O.V. Enick, M.M. Moore, Environ. Impact Assess. Rev. 27 (2007) 707.
[4] M. Cleuvers, Toxicol. Lett. 142 (2003) 185.
[5] J.P. Sumpter, Acta Hydrochim. Hydrobiol. 33 (2005) 9.
[6] D. Epel, T. Smital, in: C.G. Daughton, T.L. Jones-Lepp (Eds.), Pharmaceuticals and Personal Care Products in the Environment: Scientific and Regulatory Issues, American Chemical Society, Washington, DC, 2001, p. 244.
[7] P.P. Fong, in: C.G. Daughton, T.L. Jones-Lepp (Eds.), Pharmaceuticals and Personal Care Products in the Environment: Scientific and Regulatory Issues, American Chemical Society, Washington, DC, 2001, p. 264.
[8] B. Halling-Sorensen, S.N. Nielsen, P.F. Lanzky, F. Ingerslev, H.C.H. Lutzhoft, S.E. Jorgensen, Chemosphere 36 (1998) 357.
[9] J. Oppel, G. Broll, D. Loffler, M. Meller, J. Rombke, T. Ternes, Sci. Total Environ. 328 (2004) 265.

[10] Permit Guidance Document: Pharmaceutical Manufacturing Point Source Category (40 CFR Part 439), U.S. Environmental Protection Agency, Washington, DC, 2006.
[11] F. Sacher, M. Ehmann, S. Gabriel, C. Graf, H.J. Brauch, J. Environ. Monit. 10 (2008) 664.
[12] S.A. Snyder, K.E. Kelly, A.H. Grange, G.W. Sovocool, E.M. Snyder, J.P. Giesy, in: C.G. Daughton, T.L. Jones-Lepp (Eds.), Pharmaceuticals and Personal Care Products in the Environment: Scientific and Regulatory Issues, American Chemical Society, Washington, DC, 2001, p. 116.
[13] M. Carballa, F. Omil, J.M. Lema, M. Llompart, C. Garcia-Jares, I. Rodriguez, M. Gomez, T. Ternes, Water Res. 38 (2004) 2918.
[14] N.M. Vieno, T. Tuhkanen, L. Kronberg, Environ. Sci. Technol. 39 (2005) 8220.
[15] A. Joss, E. Keller, A.C. Alder, A. Gobel, C.S. McArdell, T. Ternes, H. Siegrist, Water Res. 39 (2005) 3139.
[16] J.L. Santos, I. Aparicio, E. Alonso, Environ. Int 33 (2007) 596.
[17] J.H. Al-Rifai, C.L. Gabefish, A.I. Schafer, Chemosphere 69 (2007) 803.
[18] J.T. Yu, E.J. Bouwer, M. Coelhan, Agric. Water Manage. 86 (2006) 72.
[19] J.E. Drewes, D. Sedlak, S. Snyder, E. Dickenson, WateReuse Foundation, Alexandria, VA, 2008, p. 146.
[20] R. Kanda, P. Griffin, H.A. James, J. Fothergill, J. Environ. Monit. 5 (2003) 823.
[21] T.A. Ternes, Water Res. 32 (1998) 3245.
[22] T.A. Ternes, M. Meisenheimer, D. McDowell, F. Sacher, H.J. Brauch, B.H. Gulde, G. Preuss, U. Wilme, N.Z. Seibert, Environ. Sci. Technol. 36 (2002) 3855.
[23] S.J. Khan, T. Wintgens, P. Sherman, J. Zaricky, A.I. Schafer, Water Sci. Technol. 50 (2004) 15.
[24] J. Radjenovic, M. Petrovic, F. Ventura, D. Barcelo, Water Res. 42 (2008) 3601.
[25] Australian Guidelines for Water Recycling: Managing Health and Environmental Risks Augmentation of Drinking Water Supplies, Environment Protection and Heritage Council, National Health and Medical Research Council, Natural Resource Management Ministerial Council, Canberra, 2007.
[26] Australian Drinking Water Guidelines 6, National Health and Medical Research Council, Natural Resource Management Ministerial Council, Canberra, 2004.
[27] Guidelines for Drinking-Water Quality: Incorporating First Addendum, World Health Organisation, Geneva, 2006.
[28] F. Busetti, K.L. Linge, J.W. Blythe, A. Heitz, J. Chromatogr. A 1213 (2008) 200.
[29] K.K. Barnes, D.W. Kolpin, E.T. Furlong, S.D. Zaugg, M.T. Meyer, L.B. Barber, Sci. Total Environ. 402 (2008) 192.
[30] T.A. Ternes, Trends Anal. Chem. 20 (2001) 419.
[31] F. Sacher, F.T. Lang, H.J. Brauch, I. Blankenhorn, J. Chromatogr. A 938 (2001) 199.
[32] M. Gros, M. Petrovic, D. Barcelo, Talanta 70 (2006) 678.
[33] B.J. Vanderford, R.A. Pearson, D.J. Rexing, S.A. Snyder, Anal. Chem. 75 (2003) 6265.
[34] S. Khan, A. Schafer, P. Sherman, Water 31 (2004) 114.
[35] Premier's Collaborative Research Program (2005-2008), Characterising Treated Wastewater For Drinking Purposes Following Reverse Osmosis Treatment, Technical Report, Water Corporation of Western Australia, Western Australian Department of Health, Perth, 2009.
[36] M. Gros, M. Petrovic, D. Barcelo, Anal. Bioanal. Chem. 386 (2006) 941.
[37] Commission Decision 2002/657/EC of 12 August 2002 Implementing Council Directive 96/23/EC Concerning the Performance of Analytical Methods and the Interpretation of Results, European Union, Brussels, 2002.
[38] M.J. Hilton, K.V. Thomas, J. Chromatogr. A 1015 (2003) 129.
[39] T.A. Ternes, M. Bonerz, N. Herrmann, D. Loffler, E. Keller, B.B. Lacida, A.C. Alder, J. Chromatogr. A 1067 (2005) 213.
[40] L. Barron, J. Tobin, B. Paull, J. Environ. Monit. 10 (2008) 353.
[41] M. Petrovic, M.D. Hernando, M.S. Diaz-Cruz, D. Barcelo, J. Chromatogr. A 1067 (2005) 1.
[42] S. Castiglioni, E. Zuccato, E. Crisci, C. Chiabrando, R. Fanelli, R. Bagnati, Anal. Chem. 78 (2006) 8421.
[43] B.J. Vanderford, S.A. Snyder, Environ. Sci. Technol. 40 (2006) 7312.
[44] J.P. Foley, J.G. Dorsey, Chromatographia 18 (1984) 503.
[45] A. Gobel, C.S. McArdell, M.J.F. Suter, W. Giger, Anal. Chem. 76 (2004) 4756.
[46] L.L. Jessome, D. Volmer, LC–GC N. Am. 24 (2006) 498.
[47] S.J. Khan, J.E. Ongerth, Chemosphere 54 (2004) 355.
[48] S.D. Kim, J. Cho, I.S. Kim, B.J. Vanderford, S.A. Snyder, Water Res. 41 (2007) 1013.
[49] S. Khan, Occurrence, Behaviour and Fate of Pharmaceutical Residues in Sewage Treatment, PhD Thesis, The University of New South Wales, Sydney, 2002.
[50] Australian Statistics on Medicines, Australian Government Department of Health and Aging, Canberra, 2006.
[51] X.S. Miao, C.D. Metcalfe, J. Mass Spectrom. 38 (2003) 27.
[52] X.S. Miao, C.D. Metcalfe, J. Chromatogr. A 998 (2003) 133.
[53] C. Zwiener, F.H. Frimmel, in: K. Kümmerer (Ed.), Pharmaceuticals in the Environment: Sources, Fate, Effects and Risks, Springer, Berlin, 2004, p. 121.
[54] T. Ternes, in: C.G. Daughton, T.L. Jones-Lepp (Eds.), Pharmaceuticals and Personal Care Products in the Environment: Scientific and Regulatory Issues, American Chemical Society, Washington, DC, 2001, p. 39.
[55] S.A. Snyder, E.C. Wert, D.J. Rexing, R.E. Zegers, D.D. Drury, Ozone-Sci. Eng. 28 (2006) 445.
[56] M.R. Boleda, M.T. Galceran, F. Ventura, J. Chromatogr. A 1175 (2007) 38.
[57] D.L. Sedlak, C.-H. Huang, K. Pinkston, in: K. Kümmerer (Ed.), Pharmaceuticals in the Environment: Sources, Fate, Effects and Risks, Springer, Berlin, 2004, p. 108.