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Multi-residue analytical method for human pharmaceuticals and synthetic hormones in river water and sewage effluents by solid-phase extraction and liquid chromatography–tandem mass spectrometry

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ABSTRACT

Pollutants such as human pharmaceuticals and synthetic hormones that are not covered by environmental legislation have increasingly become important emerging aquatic contaminants. This paper reports the development of a sensitive and selective multi-residue method for simultaneous determination and quantification of 23 pharmaceuticals and synthetic hormones from different therapeutic classes in water samples. Target pharmaceuticals include anti-diabetic, antihypertensive, hypolipidemic agents, β2-adrenergic receptor agonist, antihistamine, analgesic and sex hormones. The developed method is based on solid phase extraction (SPE) followed by instrumental analysis using liquid chromatography-electrospray ionization-tandem mass spectrometry (LC–ESI-MS/MS) with 30 min total run time. River water samples (150 mL) and (sewage treatment plant) STP effluents (100 mL) adjusted to pH 2, were loaded into MCX (3 cm^3, 60 mg) cartridge and eluted with four different reagents for maximum recovery. Quantification was achieved by using eight isotopically labeled internal standards (I.S.) that effectively correct for losses during sample preparation and matrix effects during LC–ESI-MS/MS analysis. Good recoveries higher than 70% were obtained for most of target analytes in all matrices. Method detection limit (MDL) ranged from 0.2 to 281 ng/L. The developed method was applied to determine the levels of target analytes in various samples, including river water and STP effluents. Among the tested emerging pollutants, chlorothiazide was found at the highest level, with concentrations reaching up to 865 ng/L in STP effluent, and 182 ng/L in river water.

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1. Introduction

The presence of residues of human pharmaceuticals and synthetic hormones has been acknowledged as one of the most urgent emerging environmental issues, particularly in the aquatic environment. Pharmaceuticals and synthetic hormones comprise a very diverse spectrum of chemicals that is continually expanding as new chemicals are discovered and brought to the market. In September 2009, Chemical Abstracts Service (CAS) recorded the 50 millionth substance, arylmethylideneheterocycle with analgesic properties [1]. Pharmaceuticals released to the environment have received very little attention because they are neither regulated as environmental pollutants nor listed as pollutants in WHO guidelines for drinking water quality [2].

The major source of pharmaceuticals and synthetic hormones into the environment is raw or inadequately treated sewage effluents. Other sources include production residues, improper disposal of expired medicines and unused drugs, landfill leachates and accidental spillage during manufacturing and distribution. Pharmaceuticals in the environment can potentially affect aquatic life forms and produce changes that threaten the sustainability of the ecosphere. Continuous releases and chronic exposure to these chemicals can result not only in subtle effects on aquatic species but also could pose a risk to human health associated with consuming contaminated drinking water over a lifetime [3].

Over the past decade, reports of widespread pharmaceutical contamination of lakes, streams, and ground waters have been increasingly documented [4–6]. Therefore, in recent years, the occurrence and fate of pharmaceutical residues in the environment has become a subject of much public interest and awakened great concern among scientists worldwide [7–13]. However, the occurrence of human pharmaceuticals and synthetic hormones in tropical aquatic environment is little documented [5]. To date, no data has been reported about the occurrence of these pollutants in Malaysian aquatic environment. Beyond the dampening effects of the lack of regulation, this gap can mainly be attributed to the lack of a comprehensive analytical method that can simultaneously detect in water and quantify the vast number (13065) of approved

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pharmaceuticals currently in use [14]. Presently, there is also no priority list for pharmaceuticals in the environment. The consumption pattern differs from country to country, and so far, no single analytical method has been universally agreed upon in the literature. Therefore, the knowledge about the environmental occurrence and fate of the majority of pharmaceuticals in environment still limited which continuously required development of new multi-residue analytical methods. The vast majority of available analytical methods in the literature for the determination of pharmaceutical compounds are focused on specific therapeutic classes particularly the antibiotics [15,16] and sex hormones [17,18]. There is no a single method available that can simultaneously measure all the pharmaceuticals of interest. The multi-residue analytical methods available generally involve multi extraction protocols with long preparation procedures designed to extract large volume of samples [19,20] and several instrumental analytical procedures are also required [21–23]. As a result, these methods are time consuming and resource intensive. Moreover, none of these methods includes some of the pharmaceuticals that widely consumed in Malaysia such as amlodipine, chlorpheniramine, chlorothiazide, perindopril and gliclazide. Consequently, there is a need to develop a comprehensive multi-residue analytical method, which enables rapid, sensitive and selective determination of the pharmaceuticals of interest in the aquatic environment for routine monitoring.

The analysis of pharmaceutical pollutants in water matrices generally involve the extraction of a suitable volume of water for enrichment of analytes and clean up of matrix component followed by instrumental analysis for separation and quantification of analytes. Developing analytical methods for pharmaceuticals analysis is very challenging due to the low, trace level concentration in environmental samples. In addition, unlike the majority of conventional pollutants such as heavy metals, PCBs and PAHs, pharmaceuticals usually occur as a complex mixture of pollutants from different chemical classes with different chemical and physical properties, and are present in very complex matrix [20].

Since the first detection of pharmaceutical pollutants in water [24], several analytical approaches have been developed including the use of GC–MS, GC–MS/MS, LC-UV, LC–MS and LC–MS/MS [23,25–28]. LC can separate almost any mixture and when coupled with mass spectrometry, LC–MS/MS, it is one of the most powerful analytical techniques available for quantification due to its sensitivity and selectivity. Its reproducibility, robustness and ability to determine ultra trace concentrations have made it useful for the routine analysis of many pharmaceuticals in aquatic environment [29–31]. Although the limits of detection achieved with LC–MS/(MS) methods were only slightly higher than those obtained with GC–MS methods, LC–MS methodology has shown advantages in terms of its versatility and its less complicated and labor intensive sample preparation (i.e., derivatizationis not needed) for less volatile and thermolabile compounds such as the majority of pharmaceuticals [19,32,33].

When using the LC–MS/(MS) systems, ESI is used as the ionization source because it allows rapid, accurate and sensitive analysis. ESI, a low-energy ionization source, generally does not cause fragmentation of molecular ions and is therefore recommended for polar and thermally labile compounds such as the pharmaceuticals [34] as well as for less polar compounds such as hormones. ESI was found to afford detection limits for progestogens such as levonorgestrel and norethindrone about ten times better than those achieved with APCI. ESI was also found to be the only ionization mode capable of detecting estrogens such as 17α-ethinylestradiol in the ng/mL range in the negative mode [3]. However, the matrix effect (ME) caused by co-eluting compounds during chromatographic separation is the common drawback of ESI source that could lead to relatively high detection limits and decreased reproducibility [35]. Therefore, accurate quantification of pharmaceuticals in complex matrices such as river water and sewage effluents requires intensive clean-up procedures and the use of appropriate internal standards.

Extraction of pharmaceutical pollutants from water samples is usually performed by SPE utilizing different types of sorbent materials such as silica-based C18 and polymer sorbents [23,36]. Polymer sorbents such as Oasis© HLB and MCX have been widely utilized for pharmaceuticals extraction in environmental samples [19,20,27,30]. Oasis© HLB, with its hydrophilic–lipophilic balance, is versatile and efficient for the extraction of analytes with a wide range of polarities and pH values. However, being universal, HLB is less selective resulting either in lower SPE recovery or more likely, higher ME in ESI source [37]. MCX, built upon HLB copolymer with additional presence of sulfonic groups, is a strong cation-exchanger. MCX therefore, provides both ion-exchange and reversed-phase retention and can adsorb polar, non-polar, neutral and cationic compounds simultaneously from aqueous media and has a wider spectrum of retention, more reproducible and more stable than all silica-based mixed-mode media. MCX has been successfully employed to extract a wide range of pharmaceuticals and synthetic hormones from water matrices [20,37,38].

In the light of these concerns, the aim of this paper is to present a new, fast and sensitive multi-residue analytical method for detection and quantification of a broad range of pharmaceuticals and synthetic hormones in both river water and STP effluents. The method is based on a single SPE extraction protocol for small sample volumes of 100–150 mL for fast sample preparation followed by LC–ESI-MS/MS instrumental analysis with 30 min total run time. In this work, fast chromatographic analysis was ensured using LC column with sub-2 μm particle size utilizing the conventional HPLC apparatus to improve separation, which is necessary for high sensitivity, low signal suppression and faster analysis time. The problem of ME has been minimized in this study by utilizing more selective SPE sorbent (MCX) and utilization of a group of eight I.S. The method presents a significant improvement for the analysis of polar compounds such as metformin in compared to previously published method [19] .The method has the advantageous of being simple, fast, and cost effective without compromising the selectivity and sensitivity. Several key points such as the utilization of ion-pairing agents to improve the chromatography of polar compounds and optimization of ESI parameters for optimum sensitivity were also discussed.

The target compounds were selected from the list of the top 40 highly used drugs in Malaysia [39] (Table 1) and top over the counter (OTC) and synthetic hormones sold in pharmacies in Malaysia (Table 2). Due to budget constraint, only the top 20 pharmaceuticals were selected. Of the synthetic hormones, desogestrel and drospirenone were excluded as their reference standards are not commercially available. The compounds studied included 18 pharmaceuticals, 4 synthetic hormones and one metabolite, their CAS number and physic-chemical properties are listed in Table 3.

2. Materials and method

2.1. Reference standards, reagents and materials

Reference standards were obtained from commercial sources and were at highest purity available. Reference standards of glibenclamide ≥99%, atenolol ≥98%, (±)-metoprolol (+)-tartrate salt at ≥98%, nifedipine ≥98%, simvastatin ≥98%, salbutamol 99%, gliclazide ≥98%, diclofenac sodium salt >99%, mefenamic acid >99%, loratadine >98%, furosemide 99%, chlorothiazide 99%, lovastatin ≥98%, D(−)-norgestrel (levonorgestrel) ≥99%, cyproterone acetate ≥98%, 17α-ethinylestradiol 99.4%, metformin HCl 97% and 19-norethindrone 99.5% were purchased from Sigma–Aldrich

Table 1
Top 40 pharmaceuticals by utilization in Malaysia, 2004.

#	Pharmaceutical	Therapeutic class	DDD/1000 *P*/day[a]
1	Glibenclamide	Anti-diabetic	14.4913
2	Atenolol	Antihypertensive	13.0782
3	Metformin	Anti-diabetic	11.7436
4	Metoprolol	Antihypertensive	10.9895
5	Nifedipine	Antihypertensive	9.8874
6	Simvastatin	Lipid lowering agent	7.9016
7	Amlodipine	Antihypertensive	6.5788
8	Salbutamol	Bronchodilator (inhalational)	6.3364
9	Chlorpheniramine	Antihistamine	5.7326
10	Gliclazide	Anti-diabetic	5.6477
11	Salbutamol	Bronchodilator (systemic)	5.4231
12	Diclofenac	Non-steroidal anti-inflammatory	5.3498
13	Mefenamic acid	Non-steroidal anti-inflammatory	4.7901
14	Loratadine	Antihistamine	4.6098
15	Furosemide	Antihypertensive	4.4716
16	Insulins and analogus	Anti-diabetic	4.4376
17	Chlorothiazide	Antihypertensive	4.0854
18	Lovastatin	Lipid-lowering drug	4.0799
19	Amoxicillin	Antibiotic	4.0243
20	Perindopril	Antihypertensive	4.0141
21	Atorvastatin	Blood lipid lowering agent	3.9146
22	Captopril	Antihypertensive	3.8928
23	Enalapril	Antihypertensive	3.8315
24	Prednisolone	Anti-inflammatory	3.5837
25	Ranitidine	Inhabit stomach acid	3.1843
26	Hydrochlorothiazide	Antihypertensive	3.0603
27	Amoxicillin+ Enzyme inhibitor	Antibiotic	2.9569
28	Cetirizine	Antihistamine	2.6469
29	Budesonide	Corticosteroid	2.5996
30	Prazosin	Antihypertensive	2.4520
31	Promethazine	Antihistamine	2.2757
32	Indapamide	Diuretic Antihypertensive	2.1897
33	Trimetazidine	Anti-ischemic agent	2.0636
34	Losartan	Antihypertensive	1.9803
35	Theophylline	Bronchodilator	1.8599
36	Insulins and analogus (Fast acting)	Anti-diabetic	1.7708
37	Doxycycline	Antibiotic	1.7350
38	Tiotropium Bromide	Anticholinergic bronchodilator	1.7158
39	Lisinopril	Antihypertensive	1.6354
40	Allopurinol	Angiotensin-converting enzyme inhibitors	1.5786

Malaysian Statistics on Medicine 2004 [39].
[a] DDD/1000 *P*/day: defined daily dose/1000 population/day.

(Germany). Salicylic acid >99.5% and acetaminophen 99.2% were purchased from Sigma (Missouri, USA). Amlodepine 98% and chlorpheniramine maleate salt 98% were purchased from Toronto Research Chemicals Inc. (Canada). Perindopril arginine salt 99.8% was provided courtesy of Technology Services (Orléans, France).

The isotope internal standards glibenclamide-d_{11} 98%, atenolol-d_7 98%, diclofenac-d_4 98%, amlodipine-d_4 maleic acid 98%, simvastatin-d_6 98%, 17α-ethinylestradiol-2,4,16,16-d_4 98% and chlorpheniramine-d_6 maleate salt 98% were purchased from Toronto Research Chemicals Inc. (Canada) and 2-hydroxybenzoic acid-d_6 97% from Cambridge Isotope Laboratories, Inc. (MA, USA).

HPLC grade methanol (MeOH) and acetonitrile (ACN) were purchased from Fisher Scientific (UK). Heptafluorobutyric acid (HFBA) 98%, dimethylchlorosilane (DMCS) >99% and HPLC grade dichloromethane (DCM) and tributylamine (TrBA) 99% were purchased from Fluka (Germany) while ammonium acetate was obtained from Systerm© (Spain). Extra pure formic acid and hydrochloric acid (HCl) 37% were purchased from Merk (Germany), HPLC grade methyltertbutylether (MTBE) and ascorbic acid >99.0% from Sigma–Aldrich (Germany). Sodium azide was purchased from Sigma–Aldrich (USA) and ammonia solution 25% and sodium hydroxide from Scharlau (Spain). The ultrapure water was produced by a MILLI-Q ADVANTAGE A10 (France).

Stock solution (1000 mg/L) of each individual standard was prepared by dissolving an appropriate amount of each substance in HPLC grade MeOH and further diluted as necessary to obtain the required concentration. To minimize degradation of standards, stock and working standards solutions were stored at −18 °C immediately after preparation.

Glassware used throughout the experiment were all deactivated by silanization to minimize sample loss through absorption of polar compounds onto active –OH sites present on glass surfaces. Silanization was conducted by rinsing the inner surface of the glassware with 5% (v/v) DMCS in DCM followed by rinsing twice with analytical grade DCM and MeOH sequentially, then drying at 160 °C for three hours. Deactivated injection amber glass vials (2 mL) were purchased from Agilent Technologies (USA).

2.2. Samples

For method development purposes and subsequent validation steps, pristine water samples from the upper reaches of the Langat River at the Langat river dam (Selangor, Malaysia), were used as control samples. Grab river water samples were collected from the Langat River in Bangi town (2°55′05.92 N″ and 101°45′33.37″E) downstream from the STPs. Effluent samples were collected from one of the STPs in Kajang town operating in extended aeration with 2785 population equivalent. Samples were collected in April 2009 and there was no rain episode for at least three days prior to samples collection. All samples were collected in 1 L amber glass bottles using a stainless steel bucket previously rinsed with distilled water and methanol. Sampling bottles were then labeled and stored in an icebox at 4 °C to minimize degradation of pollutants during transport to the laboratory.

2.3. Sample preparation and solid phase extraction

In the laboratory, 1 L aliquot of samples were preserved by adjusting the pH to 2 with HCl 37% solution and adding 50 mg of ascorbic acid to quench any residual oxidant and 1 g of sodium azide to prevent microbial degradation. Aliquots of 200 mL of control water, 150 mL of river water and 100 mL of STP effluents were filtered through 0.45 μm GF/F filter papers from Whatman (UK) to remove particulate matters.

SPE of samples was carried out with a 10-sample VacMaster SPE vacuum manifold from ISOLUTE (Mid Glamorgan, UK). The SPE protocol was optimized through several preliminary experiments involving the following variables: size of SPE sorbent, sample size and elution solvents. Two types of Oasis© MCX cartridges (3 cm^3,

Table 2
Top OTC pharmaceuticals and hormonal contraceptives sold in pharmacies in Malaysia.

Pharmaceutical	Therapeutic Class
Acetaminophen	Analgesic
Aspirin	Analgesic
Desogestrel + ethinylestradiol	Hormonal contraceptive
17α-Ethinylestradiol + levonorgestrel	Hormonal contraceptive
Drospirenone + 17α-ethinylestradiol	Hormonal contraceptive
Cyproterone + 17α-ethinylestradiol	Hormonal contraceptive
Norethisterone	Hormonal contraceptive
17α-Ethinylestradiol	Hormonal contraceptive

Survey conducted in pharmacies in Kajang, Bangi and Cheras in April 2007.

Table 3
Pharmaceuticals under study (name, CAS-No, therapeutic class, chemical structure, Log *P* and excretion rate% (unchanged)).

Name (CAS-No) Therapeutic class	Chemical structure	Log *P*[a,b]	Excretion[c] % (unchanged)
Glibenclamide (Glubride) 10238-21-8 Anti-diabetic		4.8	0%
Atenolol 29122-68-7 Antihypertensive (beta-blocker)		0.5	50%
Metformin 1115-70-4 Anti-diabetic		−0.5	90%
Metoprolol 37350-58-6 Antihypertensive (beta-blocker)		1.6	3–10%
Nifedipine 21829-25-4 Antihypertensive (calcium canal blocker)		2.0	1%
Simvastatin 79902-63-9 Hypolipidemic agent		4.7	10–15%

Table 3 (*Continued*)

Name (CAS-No) Therapeutic class	Chemical structure	Log P[a,b]	Excretion[c] % (unchanged)
Amlodipine 88150-42-9 Antihypertensive (calcium canal blocker)		1.9	10%
Salbutamol (albuterol) 18559-94-9 β2-Adrenergic receptor agonist		1.4	60–70%
Chlorpheniramine 132-22-9 Antihistamine (H_1 receptor antagonist)		3.2	
Gliclazide 21187-98-4 Anti-diabetic		2.6	60–70%
Diclofenac sodium 15307-79-5 Non-steroidal anti-inflammatory		3.9	15%
Mefenamic acid 61-68-7 Non-steroidal anti-inflammatory		4.2	50%

Table 3 (*Continued*)

Name (CAS-No) Therapeutic class	Chemical structure	Log P[a,b]	Excretion[c] % (unchanged)
Loratadine 79794-75-5 Antihistamine		3.8	40%
Furosemide 54-31-9 Antihypertensive (loop diuretic)		1.4	90%
Chlorothiazide 58-94-6 Antihypertensive (thiazide diuretic)		−0.5	10–15%
Lovastatin 75330-75-5 Hypolipidemic agent		4.5	10–15%
Perindopril 107133-36-8 Antihypertension (ACE inhibitor)		2.6	10%
Acetaminophen 103-90-2 Non-steroidal anti-inflammatory		0.46	2–5%
Salicylic acid 69-72-7 Metabolite of aspirin		2.26	

Table 3 (*Continued*)

Name (CAS-No) Therapeutic class	Chemical structure	Log P[a,b]	Excretion[c] % (unchanged)
Norethindrone (norethisterone) 68-22-4 (Sex hormone) oral contraceptive		3.2	
Cyproterone acetate 427-51-0 (Sex hormone) oral contraceptive		3.37	
Levonorgestrel 797-63-7 (Sex hormone) second generation oral contraceptive		3.8	
17α-Ethinylestradiol 57-63-6 (Sex hormone) oral contraceptive		4.3	

[a] Log P: octanol–water partition coefficient (hydrophobicity).
[b] Experimental value from DrugBank Database version 2.5. http://www.drugbank.ca.
[c] From RxList, the internet drug index 2009. http://www.rxlist.com.

60 mg) and (6 cm^3, 150 mg) purchased from Waters (MA, USA) were tested for best recovery with 1 L, 500 mL, 200 mL, 150 mL and 100 mL sample size of each matrix. 200 μL of I.S. mixture (50 ng/mL) was added to the sample prior to extraction and mixed thoroughly. SPE cartridges were sequentially conditioned with 3 mL MTBE, 3 mL MeOH, 3 mL ultrapure water and 3 mL ultrapure water acidified with formic acid to pH 2 to activate the sorbent by protonation of the sulfonic group in the sorbent surface. Samples were then loaded at 1 mL/min flow rate under vacuum with the aid of samplers (Teflon tubes) to automatically deliver large volume samples. Cartridges were then washed with 3 mL ultrapure water acidified with formic acid to pH 2 and dried under vacuum for 15 min. Finally, analytes were eluted to 12 mL glass tubes by sequentially passing (3× 2 mL MeOH), 2 mL (90/10 MTBE/MeOH), 2 mL (2% ammonium hydroxide in MeOH) and finally 2 mL (0.2% NaOH in MeOH). Finally, the combined eluents were evaporated to dryness under a gentle stream of N_2 gas with the aid of a hot plate heated at 45 °C. Dry extracts were reconstituted with 200 μL of 25% MeOH in ultrapure water, then transferred to 100 μL deactivated glass insert with polymer feet inserted in amber glass vials from Agilent Technologies (USA). 10 μL of the extract was automatically injected into LC–ESI-MS/MS system for analysis. If not analyzed immediately, dry extracts were stored at −18 °C until analysis within two weeks of extraction. For each batch of samples, an extra one sample of control water, which was spiked only with isotope standards, was used to check for any possible background concentration of pollutants. In addition, with each batch of samples, one sample of ultrapure water was included as a procedural blank which was accorded all extraction procedures along side with the water samples to check for any possible cross contamination.

2.4. Liquid chromatography–tandem mass spectrometry

Separation of analytes was carried out using Waters Alliance HPLC System (2695 Separations Module) from Waters (MA, USA). Identification and quantification of analytes were carried out using a Micromass Quattro Ultima Pt tandem QQQ mass spectrometer (MS/MS) system from Waters (MA, USA). The MS/MS system is equipped with ESI and atmospheric pressure chemical ionization (APCI) interfaces. For method simplicity and efficiency, only ESI source was utilized as ionization source. The ESI interface consists of the standard Z-spray^{TM} ion source fitted with an electrospray probe. Heated nitrogen gas (N_2) was used as both nebuliser and desolvation gas in the ESI source while argon (99.99% pure) was used as a collision gas in the second quadruple for collision induced dissociation (CID). N_2 was generated by a nitrogen generator from Peak Scientific (MA, USA). MassLynx V 4.0 software from Waters (MA, USA) was used for data acquisition and instrument control.

ESI-MS/MS parameters were optimized by direct infusion of 100 ng/mL of each individual standard in continuous flow injection analysis mode using external pump from Harvard apparatus 11 (MA, USA) at a flow rate ranging from 5 to 15 μL/min. Optimization of ESI-MS/MS parameters involves the determination of the best ESI mode (+) or (−), cone voltage and of the best fragmentation pattern for each analyte. CID energy that gave the best fragmentation pattern was also optimized. For higher sensitivity, product ions were monitored at a series of 5–40 eV and optimization was performed in product ion scan mode. The two most intense product ions were then selected for multiple reaction monitoring mode (MRM) analysis (Table 4). During MS/MS optimization, source and desolvation temperature were set at 80 and 160 °C, respectively. Flow rate of N_2 gas as both nebulising gas and desolvation gas were set at 50 and 550 L/h, respectively, and capillary voltage was set at 3.5 kV for both (+) and (−) modes.

Analytes detected in ESI (+) mode were separated on a ZORBAX SB-C18, RRHT Threaded Column 600 Bar 2.1 mm × 100 mm, 1.8 μm with low dispersion in-line filter (0.5 μm) from Agilent Technologies (USA) at 60 °C at a flow rate of 0.25 mL/min. In the final method, the mobile phase was a binary mobile phase solvent system consisting of a mixture of ACN/MeOH (66/34, v/v) as mobile phase B and 5% of mobile phase B in ultrapure water with 0.1% HFBA as mobile phase A. Both phases contain 10.0 mM ammonium acetate as a mobile phase additive in order to improve ESI performance. The elution gradient program was as follows: the column was equilibrated using 100% mobile phase A and held isocratically for 0.5 min. The gradient then was increased to 2% mobile phase B over 2 min, 30% by 3.5 min, 40% by 5 min, 70% by 8 min, and to 100% by 11 min and then held at 100% B for 4 min before returning to the starting conditions by 15.1 min and equilibrated for 5 min prior to the next run. The representative chromatogram of total MRMs of analytes is presented in Fig. 1.

Analytes detected in ESI (−) mode were separated on a ZORBAX Extend-C18, 2.1 mm × 100 mm 3.5 μm with low dispersion in-line filter (0.5 μm) from Agilent Technologies (USA) held at 30 °C with a flow rate of 0.25 mL/min. In the final method, the mobile phase was a binary mobile phase solvent system consisting of a mixture of ACN/MeOH (66/34, v/v) as mobile phase B and 5% of B in ultrapure water with 0.05% TrBA (pH 10.5) as mobile phase A. The column was equilibrated using 90% mobile phase A and held isocratically for 0.2 min. The gradient then increased to 50% mobile phase B over 1 min, 65% by 2.5 min, 85% for 4 min, and 100% for 5 min, then held at 100% B for 2 min before returning to the starting conditions by

Table 4
ESI-MS/MS experimental parameters.

Compound	C.V.[a]	CID[b]	MRM 1	MRM 2
ESI (+)				
Acetaminophen	35	18	152.00 > 110.00	–
Amlodipine	55	8/11	409.60 > 238.30	409.60 > 294.40
Atenolol	35	21/23	267.30 > 145.20	267.30 > 190.20
Chlorpheniramine	55	11/32	275.10 > 167.10	275.10 > 230.10
Cyproterone	35	25/25	417.60 > 279.40	417.60 > 147.10
Loratadine	75	30/20	383.50 > 337.40	383.50 > 281.30
Lovastatin	45	18/8	405.00 > 199.30	405.00 > 285.50
Metformin	55	20/14	130.20 > 71.40	130.2 > 60.0
Metoprolol	65	15/21	268.20 > 116.30	268.2 > 159.10
Nifedipine	45	25/8	347.40 > 254.40	347.40 > 315.50
Norethindrone	35	30/20	299.50 > 109.30	299.50 > 145.10
Levonorgestrel	35	20/27	313.50 > 245.50	313.50 > 109.00
Perindopril	35	30/25	369.60 > 172.30	369.60 > 170.30
Salbutamol	35	14/9	240.16 > 148.1	240.1 > 222.2
Simvastatin	45	20/13	419.50 > 199.30	419.50 > 225.30
Atenolol-d7[c]	35	23	274.40 > 145.20	–
Amlodipine-d4[c]	55	8	413.00 > 238.00	–
Chlorpheniramine-d6[c]	55	15	281.30 > 230.10	–
Simvastatin-d6[c]	45	20	425.50 > 199.30	–
ESI (−)				
Chlorothiazide	60	33/35	294.20 > 214.20	294.20 > 179.10
Diclofenac	45	14/32	293.98 > 250.10	293.98 > 178.20
17α-Ethinylestradiol	120	35/35	295.10 > 145.10	295.10 > 159.00
Furosemide	35	14/30	329.40 > 205.00	329.40 > 285.30
Glibencalmide	45	26/18	492.30 > 169.90	492.30 > 367.40
Gliclazide	45	24/35	322.40 > 170.10	322.40 > 106.40
Mefenamic acid	35	16/25	240.20 > 196.30	240.20 > 180.20
Salicylic acid	40	10	137.00 > 93.20	
Diclofenac-d_4[c]	45	14	298.2 > 254.00	–
Glibencalmide-d_{11}[c]	45	26	503.50 > 170.10	–
Salicylic acid-d_6[c]	40	10	142.10 > 98.00	–
Ethinylestradiol-d_4[c]	80	40	299.20 > 147.00	–

[a] C.V.: cone voltage (V).
[b] CID: collision induced dissociation (eV).
[c] Isotope internal standard.

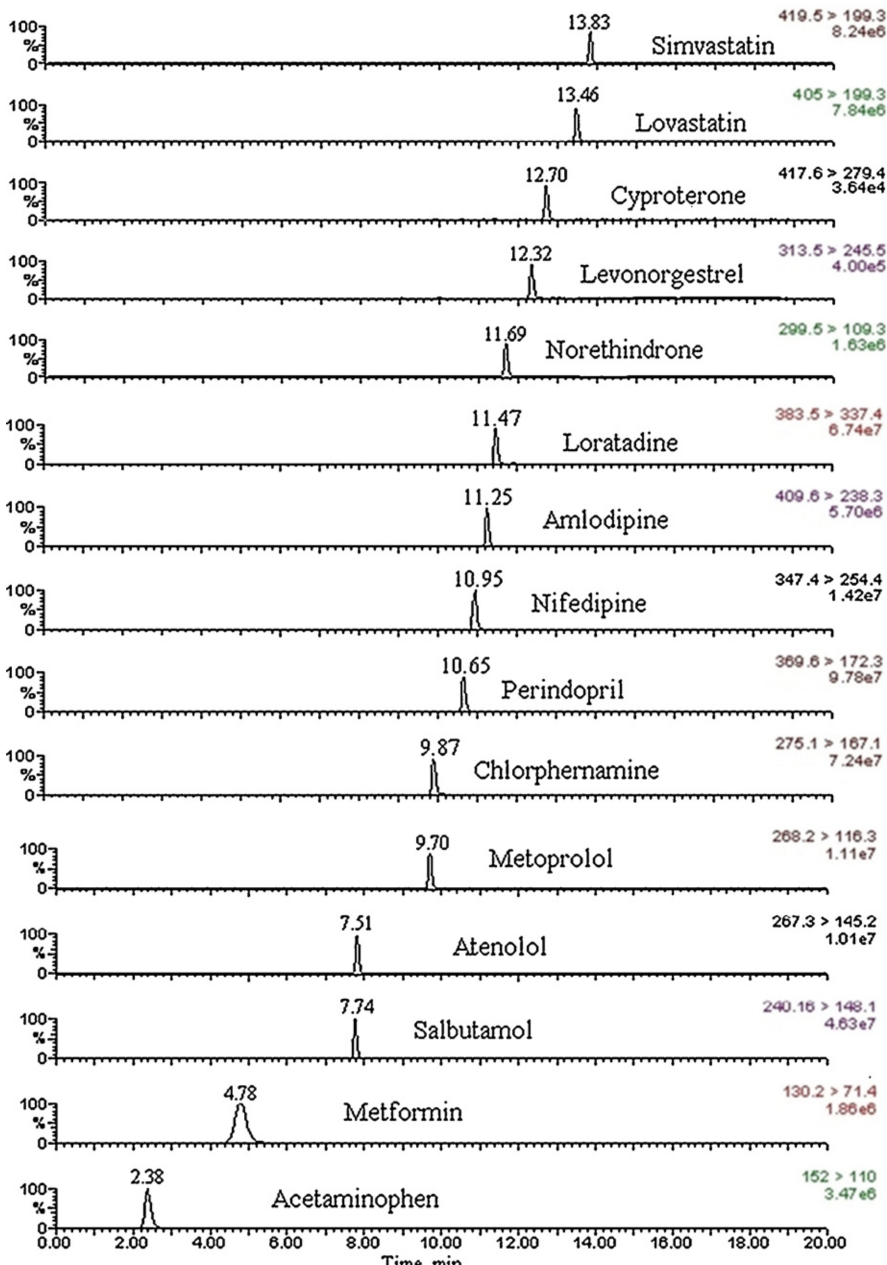

Fig. 1. MRM chromatogram for analytes detected in ESI (+).

7.1 min and equilibrated for 3 min prior to the next run. The representative chromatogram of total MRMs of analytes is presented in Fig. 2.

2.5. Quantification and method validation

Comparison of the t_R of analytes with the corresponding reference standards and detection of both first and second MRM transitions were used to identify the analytes. Each analyte was quantified using the highest characteristic precursor ion/product ion MRM transition (MRM 1) by means of internal standardization. Five-point calibration curves were generated for each analyte by injecting pooled solutions prepared from the standard mixtures. An instrumental blank containing only the I.S. mixture was used as control to check for analytical interference and for carryover. Calibration curves were built by calculating the ratios between the peak area of each analyte and the peak area of the relative I.S. using weighted $1/x^2$ least square model. Instrumental detection limits (IDL) and instrumental quantification limits (IQL) were determined by direct injection of decreasing amounts of each analyte down to 0.001 ng/mL. The IDL and IQL for each analytes were the concentrations for which the S/N was 3 and 10, respectively, of the chromatographic response (Table 5). R^2 was used to assist the linearity for each analyte at concentration ranged from its IQL to 500 ng/mL.

Extraction recoveries of each analyte were determined for control water, surface water and STP effluents. Recovery was evaluated by spiking four replicates of each matrix with 200 μL of analyte standard mixtures (100 μg/L) containing I.S. mixture (50 μg/L). An additional sample of each matrix was processed as a reference sample by adding the same concentration of spiked standard mixture to the sample extract after the extraction procedure. For each matrix,

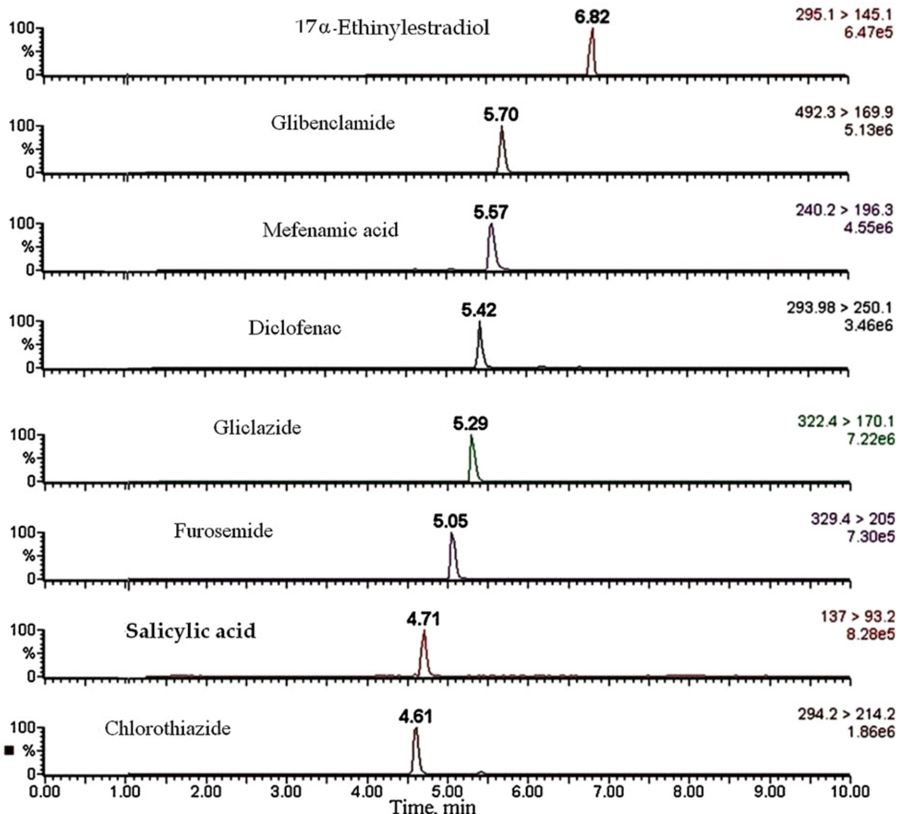

Fig. 2. MRM chromatogram for analytes detected in ESI (−).

percent recovery was calculated by comparing the concentration of each analyte found in spiked matrix (*Cs*) to its concentration found in the reference sample of the same matrix (*Cr*) using Eq. (1). The recovery calculated represents the loss resulting from SPE extraction, excluding any losses by matrix interferences in ESI interface or other instrumental fluctuation (Table 6). Precision of recovery test was evaluated as the RSD% of the replicate measurements.

$$\text{Recovery\%} = \frac{Cs\ \ (\mu g/L)}{Cr\ \ (\mu g/L)} \times 100 \qquad (1)$$

Method detection limit (MDL) of each analyte was determined using a statistical approach established by the USEPA [40]. The procedure involves spiking seven replicates of each matrix with each analyte at a concentration resulting in an instrumental S/N between 2.5 and 5. The MDL was then calculated with 99% confidence that the result was greater than zero by multiplying the SD of replicate measurements by 3.14, the Student's *t*-value for 6 degrees of freedom (Eq. (2)). This value of MDL for each analyte (Table 6) was used to judge the significance of measurement of future samples. For all matrices, the samples were processed along with QC samples as a single set through the entire extraction and analytical procedures.

$$\text{MDL} = t_{(n-1,1-\alpha=0.99)} \times \text{SD} \qquad (2)$$

In order to investigate the influence of matrix components on analyte signal during the ionization in the ESI source, the matrix effect (ME) and the efficiency of each I.S. to correct for it were evaluated using the procedure suggested by Vieno et al. [30]. The ME was calculated using Eq. (3) as a percentage of analyte signal suppression or enhancement.

$$\text{ME\%} = \frac{As - (Asp - Ausp)}{As} \times 100 \qquad (3)$$

where *Asp* is the peak area of matrix extracts spiked with the analyte standard mixtures (100 ng/mL); *Ausp* is the background concentration of the analytes in the same extract matrix; *As* is the peak area of the ultrapure water sample extract spiked with standard mixtures (100 ng/mL). In this procedure the losses of analytes caused during ionization can be evaluated, yet excluding any losses

Table 5
Instrumental performance and validation data.

Analyte	IDL (ng/mL)	IQL (ng/mL)	Linearity (linear fit)	
			R^2	Range (ng/mL)
Acetaminophen[a]	0.5	1	0.9995	1–500
Amlodipine[a]	0.005	0.01	0.9975	0.01–500
Atenolol[b]	0.05	0.5	0.9986	0.5–500
Chlorpheniramine[c]	0.1	0.5	0.9977	0.5–500
Cyproterone[a]	0.5	1	0.9938	1–500
Loratadine[a]	0.05	0.1	0.9992	0.1–500
Lovastatin[d]	0.1	1	0.9971	1–500
Metformin[a]	1	2	0.9958	2–500
Metoprolol[b]	0.05	0.5	0.9978	0.5–500
Nifedipine[a]	0.1	0.5	0.9986	0.5–500
Norethindrone[a]	0.1	10	0.9953	10–500
Levonorgestrel[a]	0.1	1	0.9982	1–500
Perindopril[a]	0.005	0.01	0.9948	0.01–500
Salbutamol[b]	0.001	0.05	0.9997	0.05–500
Simvastatin[d]	1	10	0.9967	10–500
Chlorothiazide[e]	0.1	5	0.9977	5–500
Diclofenac[g]	0.1	1	0.9991	1–500
17α-Ethinylestradiol[h]	0.1	5	0.9997	5–500
Furosemide[e]	0.05	0.1	0.9941	0.1–500
Glibencalmide[e]	0.001	0.1	0.9927	0.1–500
Gliclazide[e]	0.05	1	0.9957	1–500
Mefenamic acid[e]	0.001	0.1	0.9927	0.1–500
Salicylic acid[f]	0.1	5	0.9929	5–500

Internal standards: [a]Amlodipine-d_4; [b]Atenolol-d_7; [c]Chlorpheniramine-d_6; [d]Simvastatin-d_6; [e]Glibencalmide-d_{11}; [f]Salicylic acid-d_6; [g]Diclofenac-d_4; [h]Ethinylestradiol-d_4.

Table 6
Analytical method performance and validation data.

Analyte	Recovery % (RSD%) ($n = 4$)			MDL (ng/L)			ME %					
	Control water	River water	STP effluent	Control water	River water	STPs effluent	Control water		River water		STPs Effluents	
							Absolute	Relative	Absolute	Relative	Absolute	Relative
Acetaminophen[a]	32 (7)	18 (15)	39 (10)	7	9	31	17.1	−4.6	11.1	−6.4	56.5	23.4
Amlodipine[a]	102 (15)	63 (11)	51 (3)	1	2	3	7.8	23.1	58.1	24.7	44.2	9.1
Atenolol[b]	99 (8)	74 (8)	82 (3)	0.6	14	9	19.6	7.6	−14.4	−2.8	7.3	6.5
Chlorpheniramine[c]	85 (16)	75 (6)	52 (3)	2	3	16	9.2	0.0	14.3	9.4	1.5	−14.2
Cyproterone[a]	77 (6)	87 (5)	60 (3)	47	68	20	−30.3	−32.3	−23.5	−23.3	1.7	−18.4
Loratadine[a]	74 (19)	79 (3)	41 (19)	9	3	5	−19.8	−21.7	−24.5	−29.4	56.9	34.4
Lovastatin[d]	44 (18)	62 (8)	57 (12)	47	48	6	−171.1	17.7	29.7	33.2	−142.1	−29.6
Metformin[a]	26 (18)	43 (48)	25 (30)	2	281	9	−15.2	−16.5	89.5	21.3	25.7	1.1
Metoprolol[b]	76 (16)	71 (4)	87 (5)	0.8	67	106	12.3	−2.4	−31.3	−16.2	38.5	−19.4
Nifedipine[a]	41 (1)	61 (20)	42 (12)	27	9	8	2.7	−21.5	17.6	0.8	3.7	−10.3
Norethindrone[a]	67 (9)	87 (6)	76 (15)	9	46	162	0.2	−8.3	−20.7	−25.3	36.1	−8.3
Levonorgestrel[a]	79 (16)	81 (13)	65 (3)	22	31	66	7.7	2.7	44.1	19.4	50.5	33.4
Perindopril[a]	75 (15)	25 (12)	55 (7)	2	4	8	8.4	−0.2	13.7	9.8	29.8	11.5
Salbutamol[b]	64 (3)	49 (9)	74 (1)	0.4	1	1	23.1	12.1	1.5	7.1	47.2	16.3
Simvastatin[d]	82 (17)	48 (13)	56 (12)	77	140	131	44.5	7.7	−67.6	35.2	46.5	−26.6
Chlorothiazide[e]	75 (18)	108 (9)	100 (6)	2	1	14	−19.6	−24.3	−37.5	−25.7	67.3	29.5
Diclofenac[g]	87 (14)	68 (5)	119 (12)	0.9	10	33	−144.6	6.3	−235.6	−26.7	−158.2	34.5
17α-Ethinylestradiol[h]	98 (3)	59 (9)	61 (4)	4	32	22	0.8	−0.8	−37.4	−28.1	2.6	−19.2
Furosemide[e]	110 (4)	96 (10)	77 (19)	6	5	26	22.7	13.7	4.8	6.7	−3.5	−15.5
Glibencalmide[e]	98 (8)	81 (8)	88 (14)	0.3	0.25	0.4	13.5	3.7	2.3	−4.5	12.3	0.8
Gliclazide[e]	103 (17)	70 (20)	63 (14)	0.2	1.6	4	49.7	17.6	33.2	−6.4	18.2	−33.2
Mefenamic acid[e]	98 (14)	93 (15)	86 (13)	0.5	2	1	11.9	4.4	45.8	17.3	40.7	3.7
Salicylic acid[f]	90 (17)	89 (15)	82 (6)	2	15	6	18.3	−14.3	−11.2	19.2	0.7	22.8

Internal standards: [a]Amlodipine-d_4; [b]Atenolol-d_7; [c]Chlorpheniramine-d_6; [d]Simvastatin-d_6; [e]Glibencalmide-d_{11}; [f]Salicylic acid-d_6; [g]Diclofenac-d_4; [h]17α-Ethinylestradiol-d_4.

caused by SPE and further sample preparations. ME% > 0% suggests ionization suppression and ME% < 0% suggests ionization enhancement. Absolute and relative ME% for each analyte are summarized in (Table 6). Absolute ME% was based on the peak area of analyte without the correction of I.S. area, and relative ME% was calculated relative to I.S. area.

3. Results and discussion

3.1. Solid-phase extraction

The most challenging part of the multi-residue analysis of pharmaceuticals from different therapeutic classes concerns the choice of the best SPE sorbent, resulting in acceptable recovery for all compounds. To make the extraction step more cost effective, productive and less labor intensive, our main criterion for SPE sorbent was that it be able to extract all analytes present using only one SPE cartridge and protocol.

Oasis© HLB is a sorbent widely utilized for pharmaceuticals extraction in environmental samples. With its hydrophilic–lipophilic balance, it has proven versatility and efficiency in the extraction of analytes of a wide range of polarities and pH values. However, it has been suggested that HLB, being universal, is less selective and can adsorb many more matrix components, resulting in lower SPE recovery or, more likely, higher ME in ESI source [37].

Oasis© MCX is another widely used polymer sorbent which has been employed to extract the wide range of pharmaceutical pollutants from water matrices [20,37,38]. MCX is a strong cation-exchanger with mixed mode copolymer sorbent as it is built upon HLB copolymer with additional presence of sulfonic groups that allows for cation-exchange interaction (electrostatic interaction). MCX therefore, provides both ion-exchange and reversed-phase retention and can adsorb polar, non-polar, neutral and cationic compounds simultaneously from aqueous media and has a wider spectrum of retention, more reproducible and more stable than all silica-based mixed-mode media.

Extraction of analytes with MCX requires acidic pH of the sample solution in order to ionize basic analytes and to minimize the dissociation of acidic analytes. Therefore, MCX can extract acidic, basic and neutral compounds at low pH values as the cation-exchanger binds the basic compounds, which are in the ionized form, and the reversed phase can retain both acidic and neutral compounds. These characteristics can eliminate the need for different sample pH values to achieve the selectivity required for sample preparation. Many of the pharmaceuticals under investigation have either an acidic or a basic functional group that can interact with either the hydrophilic or the lipophilic portion of the MCX sorbent. Analytes bearing amino groups, which are positively charged at pH 2, such as atenolol, furosemide, hydrochlorothiazide, salbutamol and loratadine, interact with the sulfonic group of the MCX. On the other hand, anionic or acidic analytes, such as salicylic acid, mefenamic acid and diclofenac, are protonated at low pH and are no longer ionic. Neutral compounds such as these and the hormones are retained by the polymeric phase. Due to the weak acidity of the steroid hormones, it is also recommended to acidify water samples for better retention of the analytes for reversed phase SPE [41,42]. Hence, acidifying the samples for preservation will not pose any restriction for changing the pH of the samples for further treatments.

Preliminary results showed that MCX ($3\,cm^3$, 60 mg) cartridge gave acceptable recovery for all analytes and was cost effective, the cost of ($6\,cm^3$, 150 mg) sorbent being around 3 times the cost of ($3\,cm^3$, 60 mg) sorbent. Optimum recovery was ensured by passing through various eluting solvents. Elution of neutral and weakly acidic analytes was accomplished by three elutions of 2 mL MeOH. Hydrophobic compounds, such as hormones, were eluted with strongly nonpolar solvent (90/10 of MTBE/MeOH). The weakly basic compounds were eluted with 2 mL 2% ammonium hydroxide in MeOH, and finally, the strongly basic compounds were eluted with 2 mL 0.2% NaOH in MeOH. The use of eight isotope I.S. during the extraction to monitor the overall method performance was useful for quality control as well as serving as a data quality indicator for analytes under study.

3.2. LC–MS/MS

For fifteen analytes detected in ESI (+) mode, chromatographic separation was optimized with a series of preliminary experiments, testing different columns and utilizing various mobile phase consisting of MeOH, ACN and water with different additives, such as formic acid and HFBA at various concentrations. The separation and peaks shape of analytes using ZORBAX SB column with 1.8 μm particle size were found to be good except for metformin, which elutes very early with tailing peak shape. Metformin is a very polar small molecule that interacts weakly with C18 chain, leading to poor retention and usually to less sensitivity as a result of the small S/N ratio. Furthermore, the ME is more pronounced at regions where polar matrix components elute and can cause suppression of ions eluting early [43]. The tailing peak observed with metformin is another problem that can lead to less accurate quantification. Tailing of metformin peak is a result of secondary interaction between the charge on the ionized metformin and the stationary phase of the column. Employing HFBA as ion-pairing agent helped to improve the peak shape and symmetry and increase the t_R of metformin without need for a special column. Adding HFBA to mobile phase A only, the t_R for metformin increased from 1.22 to 4.78 min with symmetrical peak shape. Overall, the method was able to separate fifteen analytes efficiently with good peak shape (Fig. 1) in 20 min total run time, a short analysis time for conventional HPLC system.

Concentration of ammonium acetate was also optimized for maximum sensitivity at concentration levels 5.0 mM and 10.0 mM. Initial concentration of 5.0 mM was selected as concentration of ammonium acetate higher than 2 mM was hardly found to affect the t_R. Below 2.0 mM a strong influence could be observed, even resulting in a change of the retention order [44]. 10.0 mM concentration was tested to investigate any enhancement in analyte signals because higher concentrations have also been reported in the literature [45]. At 10.0 mM, peak area increased dramatically for salbutmol, perindopril and atenolol (Fig. 3) without any change in the separation pattern. Some analytes such as chlorphernamine and nifidipine, however, suffered a slight decrease in peak area; therefore no higher concentration was tested.

Finally, for optimum sensitivity, the dependent parameters for ESI source, desolvation temperature and capillary voltage, were optimized during chromatographic separation. Although several parameter adjustments were found to be detrimental to some compounds while being beneficial to others, parameters were optimized to benefit the majority of analytes. In ESI source, N_2 gas is used as desolvation gas and is heated to assist converting the mobile phase to become charged aerosols during the ionization process. Heating N_2 gas at the proper temperature is crucial for optimum sensitivity. Higher desolvation temperature increases the sensitivity but can reduce the stability of the ion beam. Moreover, some analytes are thermo labile and can dissociate at very high temperature. The optimum desolvation temperature is dependent on the mobile phase composition, so optimization of the desolvation temperature during the chromatographic separation is recommended. The impact of desolvation temperature on the peak area of analytes was tested at 300, 350, 375, 400 and 450 °C. Fig. 4 shows only the analytes with less intense response (metformin, acetaminophen, nifidipine, cyproterone, northindrone, lovengestrel, lovastatin, and simvastatin) at tested disolvation temperature. It is obvious that the peak area increased with increasing desolvation temperature except for lovastatin and simvastatin, whose area decreased dramatically at temperatures greater than 375 °C. Some analytes such as metformin, acetaminophen and hormones however show better response at high temperatures. The optimum desolvation temperature was then selected to be 400 °C as it is a good compromise between the necessary increase of peak area of early peaks and hormones and the decrease of peak area of statins. Although the peak area of the statin compounds is not the optimum, it is still acceptable and comparable with that of the rest of the analytes. On

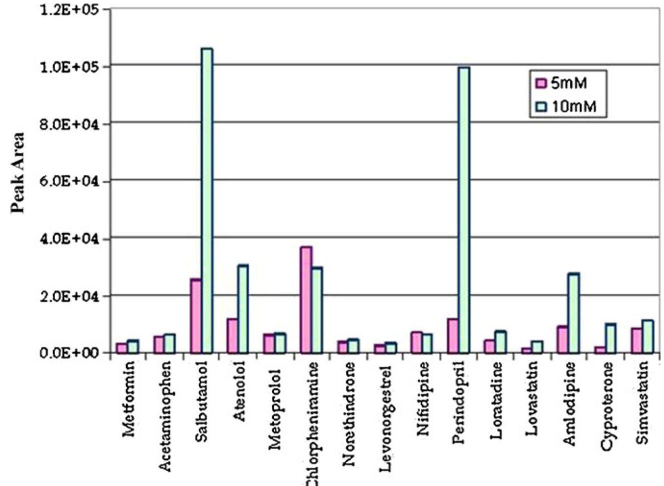

Fig. 3. Impact of increasing the concentration of ammonium acetate on the peak area of analytes detected in ESI (+) at 100 ng/mL.

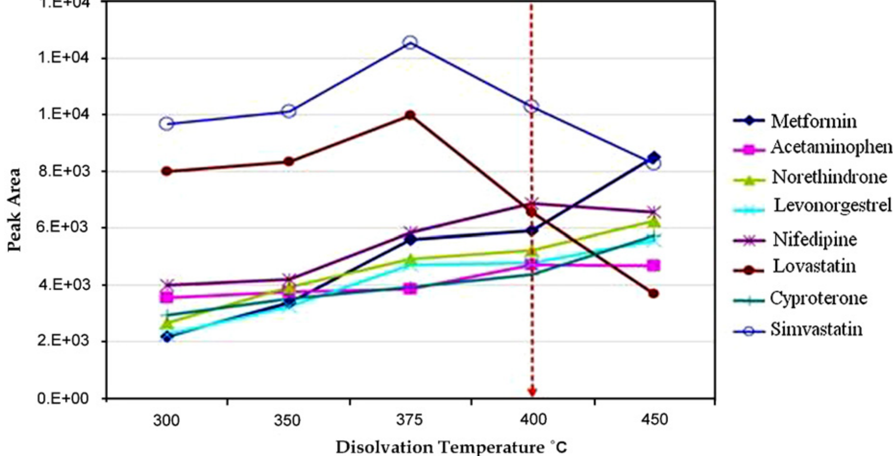

Fig. 4. Optimization of disolvation temperature for analytes detected in ESI (+) at 100 ng/mL.

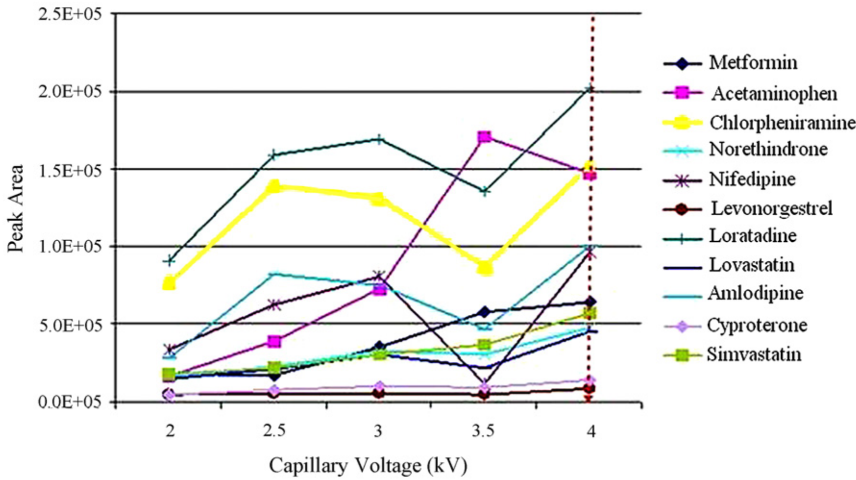

Fig. 5. Optimization of capillary voltage for analytes detected in ESI (+) at 100 ng/mL.

the other hand, the analytes with good response (e.g. salbutamol, metoprolol, and atenolol) did not suffer any serious suppression at this temperature.

Capillary voltage was optimized at different values starting from 2 to 4 kV to find the optimum value for all analytes. Fig. 5 indicates that at 3.5 kV the peak areas for all analytes decrease with increasing capillary voltage, then increase at 4 kV, the only exception being acetaminophen, which shows the best response at 3.5 kV. However, 4 kV was selected as the optimum value as it is a good compromise between getting the best response for all analytes and acceptable response for acetaminophen.

For the eight analytes detected in ESI (−) mode, ZORBAX SB was found to give sufficient separation of the analytes. However, very polar analytes such as chlorothiazide and salicylic acid eluted very early with distorted broad peak shape, which can partially be attributed to secondary interaction of the analytes with the column stationary phase. These problems necessitated the addition of a basic ion-pairing agent that can form bigger molecules with salicylic acid and chlorothiazide, which removed any free charges on the analyte and suppressed the secondary interaction, thus increasing the retention and improving the peak shape. However, the basic ion-pairing agent increased the pH of the mobile phase to a level which could not be tolerated by the column (maximum pH 6), especially under the high temperature (60 °C) applied to reduce the backpressure. The 17α-ethinylestradiol peak was also not detected under this analysis condition, indicating that this analyte was not ionized. 17α-Ethinylestradiol has been reported to ionize poorly because it is very weakly acidic, requiring addition of a basic agent to promote deprotonation [4,36,46]. High pH of the mobile phase posed an extra challenge and required use of another C18 column with different chemistry that can stand basic pH. The ZORBAX Extend column was selected for further method development as it can withstand pH up to 11.5, at maximum temperature of 40 °C.

Two basic agents were tested as ion-pairing agent namely ammonia (weakest ion pairing agent) and TrBA (strong ion pairing agent). Ammonia was found to increase the pH of the mobile phase to pH (10.5), but it did not solve the problems of poor retention and distorted peak shape of salicylic acid and chlorothiazide as it enhanced the dissociation of these compounds leading to less retention and more secondary interaction with the column stationary phase. On the other hand, TrBA, a big molecule known as a strong ion-pairing agent, very likely increased the hydrophobicity of the polar, early eluting compounds (e.g. chlorothiazide and salicylic acid), resulting in suppression of their dissociation, leading to longer retention on the column surface. This yields more symmetrical and sharper peaks of these compounds, which in turn enhances S/N ratio and improved their overall sensitivity and result better separation of all analytes in a short analysis time (10 min) (Fig. 2).

TrBA was found also to provide the basic pH (10.5) required for the ionization of 17α-ethinylestradiol. However, 17α-ethinylestradiol signal intensity was very weak. Careful investigation of the mobile phase composition revealed that 17α-ethinylestradiol signal could be totally suppressed by the presence of ammonium acetate, which is usually believed to enhance the signal intensity in ESI and reverse phase chromatography. Ionization suppression of 17α-ethinylestradiol by ammonium acetate has been also reported elsewhere [41]. Although the selective scan procedure by MRM mode in MS/MS detection does not require a complete baseline separation of all analytes, separation method was designed to separate the peaks as much as possible as separated peaks will have longer scan time, better S/N ratio and an overall improved sensitivity. It was found that adding TrBA not only eliminates the problem of distorted peaks but also increased the peak intensities for most of the analytes. On an average, more than twice-increased peak intensity of analytes were observed with 0.05% TrBA than without additives (Fig. 6). Since chlorothiazide suffers a slight decrease in the intensity in the presence of TrBA, no higher concentrations of TrBA were tested.

The overall LC–MS/MS method was found to be quick and efficient for determination of the 23 targeted wide spectrum analytes. To the best of the authors' knowledge, this study includes some pharmaceuticals (amlodipine, chlorpheniramine, chlorothiazide,

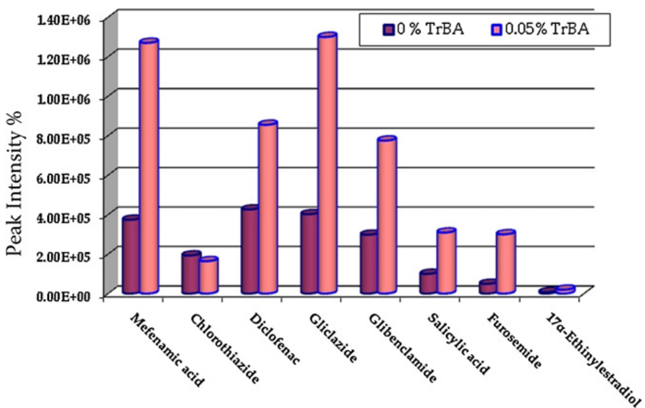

Fig. 6. Impact of adding 0.05% of TrBA on the peak intensity of analytes in ESI (−).

perindopril and gliclazide) that have not yet been investigated in environmental matrices.

3.3. Performance of the analytical method (quantification and method validation)

3.3.1. Instrumental performance

Values of IDL for analytes under study ranged from 0.001 to 1 ng/mL while the IQL ranged from 0.01 to 10 ng/mL (Table 5). Wide ranges of IDL and IQL were obtained because the detection limit depends on two factors, the sensitivity of the instrument and the ionization efficiency of the analyte in ESI source. The linearity of the calibration curve for each analyte was tested in the range shown in Table 5. Linearity was evaluated by statistical methods measuring the coefficient of determination (R^2) which quantify the goodness of fit of the linear regression. The developed LC–MS/MS procedure exhibits excellent linearity ($R^2 > 0.9927$) for most of the analytes.

3.3.2. Recovery

Good recoveries (>70%) for most of the analytes were obtained using MCX cartridge. Recovery varied among analytes and matrices as the percent recovery is analyte- and matrix-specific (Table 6). Recovery ranged from 26% to 110% in reference water matrix, 18% to 108% in surface water matrix and from 25% to 119% in STP effluents. It was found that recoveries of the analyte were, in some cases such as perindopril and simvastatin, severely reduced in the presence of natural waters. This reduction can be explained in part by the presence in the matrices of organic matter and other chemicals competing for binding sites and reducing the sorption efficiency of SPE cartridges, resulting in breakthrough of analytes and lower recoveries [37,46].

Acetaminophen and metformin had the lowest recoveries at 18% and 43% in river water and 39% and 25% in STP effluent, respectively. A possible explanation for these low recoveries could be the high background concentration of these analytes in the samples, hindering the evaluation of recovery from spiked samples [47]. However, the low recoveries for acetaminophen and metformin were also observed in control water (31% and 26%, respectively), which mitigates against the probability that high background concentrations cause this low recovery. This result indicates that the MCX sorbent was not able to retain acetaminophen and metformin on its surface. Metformin is less frequently investigated in the environmental matrices and low recovery using HLB cartridge was also reported [19,48]. In this study, higher recovery for environmental matrices compared to control samples was observed. For instance, metformin recovery improved from 26% in reference water to 43% in river water and lovastatin recovery was 44% in reference water and 62% in river water. This suggests recoveries improvements related to environmental sample matrix even though reference water recoveries typically were very low. Acetaminophen low recovery (12.2% for the control water) using MCX ($3\,cm^3$, 60 mg) was also reported [20]. However, using bigger MCX sorbent ($6\,cm^3$, 150 mg) enhanced recovery of acetaminophen up to 83% was obtained, indicating that MCX ($3\,cm^3$, 60 mg) does not have enough capacity for acetaminophen. Nevertheless, since other performance data, such as repeatability and sensitivity, were good, the low recovery was not considered an obstacle for the reliable determination of metformin and acetaminophen. In trace environmental quantitative analysis, low recovery is not obstacle for quantification as high recovery is required only when the sensitivity of method is poor [38]. Therefore, taken together, these results suggest that MCX ($3\,cm^3$, 60 mg) gave acceptable recoveries and hence was selected for SPE. It can be concluded that, in general, the obtained recoveries are higher than those reported in the literature for the same compounds such as acetaminophen using MCX ($3\,cm^3$, 60 mg) [20]. This can be attributed to lower volume loaded to the SPE cartridge in this study, 150 mL for river water and 100 mL for STP effluents, compared with a 1 L sample loaded to the same type of cartridge. In addition, utilizing more I.S. to correct for analyte loss could also account for the improved recoveries. It should be recalled that simultaneous analysis of a group of compounds with different physico-chemical characteristics generally necessitates a compromise in the selection of experimental conditions, which in some cases means not obtaining the best performance for each one of the compounds. However, developing a multi-residue method is rewarding as it can be applied in routine analysis, providing a large amount of data.

Precision of the recovery test was evaluated by calculating the RSD %. For most of the analytes the RSD % was less than 20% in all matrices which indicate good precision of the test. The only exception was metformin (48%) in river water matrix. This can be explained by the high background concentration of metformin in river water matrix that masks the small amount of metformin standard spiked to the matrix.

3.3.3. MDL

The MDL for an analytical procedure may vary as a function of sample matrix; therefore, the MDL was determined for all matrices. The resulting MDL for each analyte is listed in (Table 6) for control water, river water and STP effluent. The ranges for the MDL values were found to be 0.2–77 ng/L for control water, 0.25–281 ng/L for river water and 0.4–162 ng/L for STP effluents. MDL values for river water and STP effluents were higher than for control water because the calculated MDL value is affected by both ME and the variability in the total sample work-up [49]. High MDL for metformin in river water (281 ng/L), northindrone (162 ng/L), diclofenac (33 ng/L) and furosemide (26 ng/L) in STP effluents can be attributed to the high background concentration of these analytes in environmental sample used to evaluate MDL. High background concentrations mask the low concentration spiked to replicate samples, resulting in high SD of replicate measurements and hence higher MDL values. Additionally, the high MDL for metformin in river water can also be attributed to the variability in recovery (RSD% = 48%), resulting in a high SD of concentration and a resulting high MDL. Although a few analytes such as acetaminophen and metformin were poorly recovered, they have relatively low MDLs in control water (7 and 2 ng/L, respectively), perhaps because the MDL is determined using the absolute SD of concentration. These results are in good agreement with a previous investigation [19] where metformin was poorly recovered by HLB but had low MDL, confirming that the recovery and MDL need to be evaluated together to determine the performance of an analyte in this method. Low MDL for these analytes indicates that the method can be used to determine these compounds in the environmental matrices with good sensitivity.

3.3.4. ME

ME in LC–MS/MS analysis utilizing ESI occurs when molecules coeluting with the compounds of interest alter their ionization efficiency in the ionization interface, leading to ionization suppression or enhancement. ME is the main drawback of ESI and results from the fact that ESI is susceptible to organic and inorganic components that are present both in the sample together with analytes and in the mobile phase additives. Consequently, signal suppression or, less frequently, the enhancement of analyte signal might take place. Acetaminophen, for instance, suffered in this study 11% decrease in single intensity in river water extract and an up to 56% decrease in STP effluent extract when compared with the same amount of analyte in ultrapure water extract (Fig. 7). Therefore, the ME can be one of the mean factors that cause the decrease of method sensitivity and accuracy and if not well characterized may lead to erroneous quantification of analytes under study. In this study utilizing eight I.S.s for the analysis of 23 compounds found to be efficient for the

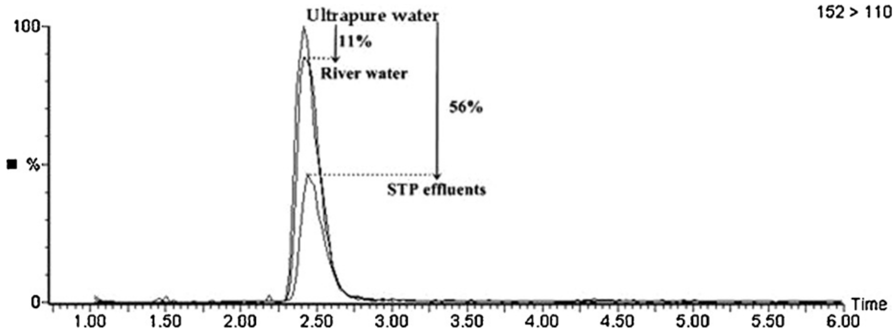

Fig. 7. Single suppression for acetaminophen in extracts of ultrapure water, river water and STP effluents spiked at 100 ng/mL.

correction for ME with no need for further treatment or calibration (Table 6). The presence of either an absolute or relative ME does not necessarily suggest that a method is not valid. As long as the analyte and I.S. suffer the same effect, the change in single intensity will be corrected by the I.S. Acetaminophen, for instant, suffer 56% absolute single suppression caused by STP effluents, however, using I.S. the single suppression reduced to 23% relative suppression. Although there is a variability of chemical structure or properties between some pharmaceuticals studied and the chosen I.S., the usage of eight I.S. for analysis of twenty three pharmaceuticals proved to be a good compromise between having acceptable quantitative result on a wide array of analytes and cost of analysis. This approach has been utilized by using only two I.S. to compensate the ME for 28 analytes and found also to be efficient and highly recommended [20]. Other strategies to reduce or correct the ME have been reported in the literature [36,46]. One way is to reduce the injected matrix quantity by a smaller concentration factor or a smaller injection volume to compromise between sensitivity and the occurrence of ME [22]. Minimizing ME was also achieved by using selective extraction cleanup and elution techniques and including size-exclusion chromatography [36]. However, sometimes these approaches are not the appropriate solutions because they could lead to analyte loss as well as long preparation times. One can also say most of these approaches become problematic when applied to the simultaneous analysis of a broad range of compounds that encompass many different classes and structures in matrices having varying degrees of suppression and enhancement. The most effective strategies reported in the literature is using isotope dilution technique in which the ME were negligible and reporting limits for all compounds were at or below 1 ng/L [46,50]. However, it has been characterized as being cost intensive and having a lack of availability as the appropriate I.S. are not always commercially available for each analyte under study or they are expensive.

3.4. Environmental application

The new multi-residue developed method was applied to real samples for determination of 23 pharmaceutical pollutants in surface water and STP effluents. This is the first report of human pharmaceutical pollutants in samples collected from Malaysian aquatic environment. Result of samples analysis is presented in Table 7. Twelve targeted analytes were detected in river water samples at measurable concentration namely acetaminophen, atenolol, metformin, levonorgestrel, perindopril, chlorothiazide, diclofenac, furosemide, glibenclamide, gliclazide, mefenamic acid and salicylic acid. Metformin and chlorothiazide found in surface water, at relatively high concentrations 293 and 182 ng/L, respectively. The lowest concentrations detected were for glibenclamide, perindopril and furosemide (2, 8 and 9 ng/L, respectively). For STP effluents amlodipine, cyproterone, loratadine, simvastatin, nifedipine, levonorgestrel, chlorpheniramine and 17α-ethinylestradiol

Table 7
Concentrations of pharmaceuticals detected in Langat River and STPs effluents (ng/L).

Analyte	Concentration (ng/L)	
	River water	STPs effluent
Acetaminophen	10	70
Amlodipine	<MDL	<MDL
Atenolol	46	107
Chlorpheniramine	<MDL	<MDL
Cyproterone	<MDL	<MDL
Loratadine	<MDL	<MDL
Lovastatin	<MDL	10
Metformin	293	16
Metoprolol	<MDL	364
Nifedipine	<MDL	<MDL
Norethindrone	<MDL	188
Levonorgestrel	38	<MDL
Perindopril	8	16
Salbutamol	<MDL	3
Simvastatin	<MDL	<MDL
Chlorothiazide	182	865
Diclofenac	17	217
17α-Ethinylestradiol	<MDL	<MDL
Furosemide	9	175
Glibenclamide	2	5
Gliclazide	4	65
Mefenamic acid	13	142
Salicylic acid	34	36

were not detected. The highest concentration detected was 865 ng/L for chlorothiazide followed by metoprolol (364 ng/L), diclofenac (217 ng/L), norethindrone (188 ng/L), mefenamic acid (142 ng/L) and atenolol (107 ng/L). Detecting these pollutants suggest incomplete elimination of most of the targeted analytes by STP. Therefore, these pollutants are continuously discharged to the Langat River through STP effluents. In this study chlorothiazide, perindopril and gliclazide are reported for the first time in the aquatic environment. Chlorothiazide was detected at the highest concentrations up to 865 ng/L in STP which can be explained as being very polar analyte ($\log P = -0.5$). Therefore, chlorothiazide tends to partionate more in the aqueous phase and escapes the microbial degradation during sledge treatment.

4. Conclusions

A single sample preparation method based on SPE and followed by two separate LC–MS/MS runs has been developed for simultaneous determination of the prescription and OTC pharmaceuticals most likely to be found in Malaysian waters. This method permits the accurate multi-residue determination of 23 pharmaceuticals and synthetic hormones in river water and effluents of STPs at ng/L levels. The method involved the usage of selective Oasis© MCX (3 cm^3, 60 mg) cartridges and 30 min LC–MS/MS analytical method. As a result, a fast and cost effective method was devel-

oped. The method performance data indicate that the techniques applied to routine analysis of surface and STP effluents samples for pharmaceuticals is selective and sensitive for the majority of compounds tested with MDL down to 0.2 ng/L. Good recovery and reproducibility for MCX sorbent was obtained for many of the analytes. ME was also evaluated and found to be the main factor affecting the sensitivity and accuracy of the method. However, utilizing eight deteriorated I.S. was found to compensate for losses of compounds during both the sample preparation procedure and ME. This method includes five pharmaceuticals to be analyzed in environmental matrices for the first time, amlodipine, chlorpheniramine, chlorothiazide, perindopril and gliclazide. The method was applied for the analysis of targeted pharmaceuticals in samples collected from surface water and STP effluents in Malaysia. The results confirmed its applicability in environmental monitoring.

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