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Analysis of Anticancer Drugs in Sewage Water By Selective SPE and UPLC–ESI-MS–MS

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Abstract

A trace analytical procedure was developed to assay the anticancer drugs methotrexate, azathioprine, doxorubicin, doxorubicinol, vincristine, ifosfamide, cyclophosphamide, etoposide, and procarbazine in water samples from sewage treatment plants. After concentration and purification using Oasis HLB solid-phase extraction cartridges and Oasis WAX cartridges, the analytes were separated using ultra-high performance liquid chromatography coupled with the electrospray ionization tandem mass spectrometry operating in the positive ion mode. The method showed good precision and accuracy. Recoveries of all analytes were in the range of 45.3–108.9% with relative standard deviations between 2.4–24.5%. The limits of detection for influent and effluent sewage water were in the range of 0.6–7.0 ng/L and 0.5–3.5 ng/L, respectively. It is expected that this method will be applied to investigate the environmental occurrence of anticancer drugs in sewage water.

Introduction

Cancer has become the leading cause of death in China and the second most prominent in the western world (1,2). Among the remedies, chemotherapeutic drug administration is the most common form. Both the number of anticancer agents and the amount of their consumption has increased considerably during the last decade. In China, their production was estimated to be 9.8 tons in 2002 with increases of 16% per year (3). Anticancer drugs act by either inhibiting cell growth or killing cells. Due to the fact that they cannot distinguish between healthy cells and cancerous cells, these drugs often give rise to secondary side effects and health risks. Carcinogenic, mutagenic, and teratogenic properties are of particular interest. These dangerous side effects have been confirmed in animal experiments and/or epidemiological studies (4–8). According to the International Agency for Research on Cancer (IARC) (9), nine anticancer drugs are classified as carcinogenic to humans (Group 1). Several anticancer drugs are classified in Groups 2A and 2B by IARC, probably and possibly carcinogenic to humans, respectively. Others are not classifiable as to their carcinogenicity to humans (Group 3); however, most of these are mutagenic and teratogenic.

In recent years, the occurrence of pharmaceuticals in the aquatic environment is an emerging issue in environmental chemistry and will be further exacerbated by the constantly increasing consumption of drugs in the developing healthcare system (10). The possible pollution of the aquatic environment can be attributed to many different sources. However, the main sources of anticancer drugs in the environment are emissions from hospitals and industrial productions (11). Many of the anticancer drugs applied to patients for the medical treatment are excreted via urine and faeces, partially transformed or even unchanged (12). Therefore, anticancer drugs can enter the hospital wastewater, reach sewage treatment plants (STPs), and even the effluent due to inefficient elimination and consequently be released into aquatic environment. Given these putative risks, researchers have been monitoring anticancer drugs in the different aquatic environment samples. Steger-Hartmann et al. found cyclophosphamid and ifosfamide in the hospital effluent samples at concentrations of 146 ng/L and 24 ng/L, respectively (13). Mahnik et al. determined the concentrations of 5-fluorouracil and doxorubicin in the wastewater from an oncologic inpatient treatment ward, which ranged from < 8.6–124 µg/L and < 0.26–1.35 µg/L, respectively (14). In the influents of municipal STPs, ifosfamide was measured at concentrations of 6.2–8.5 ng/L without significant reduction during sewage treatment (15). Cyclophosphamide was detected in the influents and effluents of a communal STP with concentration levels ranging from 7–143 ng/L and 6–15 ng/L, respectively (16). Methotrexate, a widely used anticancer drug for treatment of acute lymphoblastic leukemia, was observed in STP effluent by Castiglioni et al. at a concentration of 12.6 ng/L (17). Lake water was found to contain 0.07 ng/L cyclophosphamide (18). No anticancer agents have yet been found in the groundwater.

Despite China's enormous consumption of anticancer drugs, no scientific reports on the determination of anticancer drugs in the aquatic environment of China were available. Therefore, it is necessary to develop an effective method for future investigation into the occurrence of multi-antineoplastics in STPs. Currently, a variety of solid-phase extraction (SPE) cartridges have been successfully used in determination of anti-

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cancer drugs in aqueous samples, including the C_{18} (13,19), C_8 (14,19,20), Oasis MCX (17,21), and LiChrolut EN, an ethylvinylbenzene–divinylbenzene copolymer (17,19,21). The selection of the SPE cartridges was mainly based on their capacity, selectivity, regenerability, cost of the adsorbents material, and the properties of the studied drugs. As to the instrumental analysis, several methods including gas chromatography (13), capillary electrophoresis (14), and high-performance liquid chromatography (HPLC) (17–22) were used. The following confirmation and qualification were commonly performed by mass spectrometry (13) or tandem mass spectrometry (17,18,21,22) owing to their better sensitivity and selectivity for typical ng/L levels of pollutants in the complex matrices. However, the UV (19) and fluorescence detectors (20) were also used in the analysis as they were much more accessible. To the best of our knowledge, most of the published studies were limited to simultaneous determination of no more than three anticancer drugs in aqueous samples. This was probably due to the distinct differences of chemical structures between the different anticancer drug groups (alkylating agents, antimetabolites, antibiotics, plant alkaloids, hormones, and other compounds).

In this work, we have developed a liquid chromatography coupled with tandem mass spectrometry (LC–MS–MS) method for the analysis of nine anticancer drugs (Figure 1) in raw and treated wastewater samples. The drugs studied are frequently used in Chinese hospitals and belong to four different groups. The method is applied to determination of these drugs in sewage samples from local STPs.

Experimental

Chemicals and reagents

The anticancer drugs, methotrexate, azathioprine, doxorubicin, doxorubicinol, vincristine, ifosfamide, cyclophosphamide, etoposide, and procarbazine (purity > 99%) were purchased from Sigma (St. Louis, MO). HPLC-grade solvents such as acetonitrile and methanol were purchased from Fisher Scientific (Fair Lawn, NJ). Ultra-pure water was obtained using an in-house Milli-Q Ultra-pure water system (Millipore, Bedford, MA). HPLC-grade formic acid (HCOOC, 99%) was purchased from Acros Organics (Morris Plains, New Jersey). Sodium hydroxide, hydrochloric acid (36% HCl), and ammonia (25–28% NH_3) were analytical-grade and obtained from Beijing Chemical (Beijing, China).

A methotrexate stock standard solution of 1000 mg/L was prepared in methanol–water (50:50, v/v) containing 0.01 M hydrochloric acid. Standard stock solutions of the other drugs were prepared in methanol at a concentration of 1000 mg/L. These solutions were stored at –18ºC. Spiking and calibration mixtures at various concentrations were obtained by combining aliquots of stock solutions with methanol–water (50:50, v/v).

Liquid chromatography

LC analysis was performed on a Waters ACQUITY Ultra Performance LC system consisting of a binary pump, a seal wash pump, a solvent degasser, an automatic sample manager, and a thermostatted column compartment. Samples were automatically injected with a 10-µL syringe (injection volume: 10 µL), and the analytes were separated on an ACQUITY UPLC BEH C_{18} column (2.1 mm × 150 mm, 1.7 µm) with a guard column of the same material at 40ºC. Eluents were 0.01% aqueous formic acid at pH 4 (A) and acetonitrile (B). The flow rate was maintained at 0.4 mL/min. Gradient conditions were as follows: 0–2.50 min, linear from 5% to 35% B; 2.50–4.00 min, linear from 35% to 70% B; 4.00–4.50 min, linear from 70% to 100% B; 4.50–6.50 min, isocratic 100% B; 6.50–7.00 min, linear from 100% to 5% B; 7.00–10.00 min, isocratic 5% B.

Mass spectrometry

Under the described LC conditions, the column eluate was monitored by a Waters Quattro Premier XE triple quadrupole mass spectrometer equipped with an ESI interface operated in the positive mode. Nitrogen gas (purity 99.9%) was used as both cone and desolvation gas at flow rates of 50 and 650 L/h, respectively. Source and desolvation temperatures were set at 100ºC and 450ºC, respectively. A capillary voltage of 3.0 kV was employed, and the extractor voltage was held at 4.0 V. Ion energy 1 and ion energy 2 were set to 0 and 1.0 V, respectively. The entrance and exit slits were held at 0 and 2, respectively. The multiplier voltage was 650.0 V. During tandem mass spectrometric analysis, argon was used as the collision gas and the pressure of the collision chamber was kept at 3.3×10^{-3} mbar. Acquisitions of the samples were made in multiple reaction

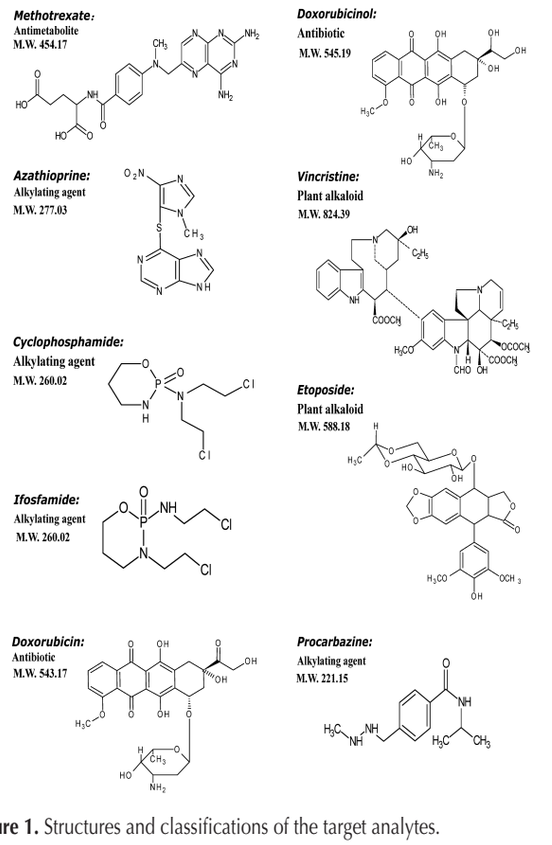

Figure 1. Structures and classifications of the target analytes.

monitoring (MRM) mode with dwell time varying between 50 and 500 ms. The cone voltages and collision energies used for MRM acquisitions are presented in Table I. Two transitions were selected for identification but only one was used for quantification. The chromatograms of analytes at the given elution gradient are shown in Figure 2.

Sample collection

In November 2007 and May 2008, influent and effluent water samples were collected from several STPs located in Beijing, China. A 24-h composite sample was obtained by pooling wastewater collected every 60 min using an automatic sampling device. No rain events were registered either during the previous seven days or on the sampling days. Water samples were stored at 4ºC in the dark and extracted within 24 h. Before extraction, samples were filtered with a Whatman GF/A glass fiber membrane (1.6 µm, Kent, UK) under a vacuum of less than 0.1 Mpa.

Solid-phase extraction

In preliminary experiments, the extraction efficiencies of three SPE cartridges were tested under various pH and elution conditions. The cartridges tested were Oasis HLB (200 mg, 6 mL), Oasis MCX (150 mg, 6 mL) (Waters, Milford, MA), and Supelclean Envi-Carb (500 mg, 6 mL) (Supelco, Bellefonte, PA). The HLB and MCX cartridges were sequentially conditioned with 6 mL methanol and 6 mL water. The Envi-Carb cartridge was sequentially conditioned with 6 mL elution solvent (described below), 6 mL methanol, and 6 mL water. Aliquots of 500 mL ultra-pure water were spiked with 25 ng of each analytes for the recovery test, obtaining a concentration of 50 ng/L. The pH values of the water samples were adjusted to 1.0–10.0 and 1.0–6.0 for Oasis HLB and MCX, respectively, using 1 mol/L of sodium hydroxide or hydrochloric acid solution. All the water samples were passed through the cartridges at a flow rate of 5 mL/min under vacuum. After sample loading, the HLB and MCX cartridges were vacuum-dried for 5 min and eluted separately with 6 mL methanol and 6 mL methanol containing 5% ammonia for MCX cartridge. The Envi-Carb cartridge was vacuum-dried for 30 min and eluted using 6 mL dichloromethane–methanol solution with different ratios (1/9–10/0, v/v). The eluates were evaporated to near dryness under a gentle stream of nitrogen at room temperature. The residues were reconstituted in 1 mL methanol–water (50:50, v/v) for LC–MS–MS analysis.

In the light of the results of these preliminary experiments, Oasis HLB cartridges at pH 2.0 were selected for the extraction of target analytes in further experiments. The final method was optimized as follows: Oasis HLB cartridges were preconditioned with 6 mL methanol and 6 mL water at pH 2. Water samples (500 mL) were acidified to pH 2 and siphoned through the cartridges at a flow rate of 5 mL/min under vacuum. The cartridges were vacuum-dried for 5 min and then washed with 3 mL methanol–water (30:70, v/v). This was followed by an elution with 6 mL methanol–water (80:20, v/v).

Table I. LC–MS–MS Acquisition Parameters for the Nine Anticancer Drugs

Compound	Retention time (min)	MRM transitions*	Cone voltage (V)	Collision energy (eV)
Methotrexate	1.74	**455.0 → 308.0**	30	20
		455.0 → 174.8	30	38
Azathioprine	1.89	277.9 → 232.0	25	15
		277.9 → 141.9	25	11
Doxorubicinol	2.72	**546.0 → 399.1**	17	13
		546.0 → 363.1	17	25
Doxorubicin	2.98	**544.3 → 397.0**	18	12
		544.3 → 129.7	18	18
Cyclophosphamide	3.08	**260.9 → 140.0**	30	20
		260.9 → 106.1	30	20
Ifosfamide	3.00	260.9 → 153.9	27	24
		260.9 → 92.1	27	24
Vincristine	3.23	825.4 → 807.5	70	37
		825.4 → 765.4	70	37
Etoposide	3.36	589.0 → 435.1	20	8
		589.0 → 229.1	20	14
Procarbazine	3.54	**221.9 → 179.9**	25	14
		221.9 → 162.8	25	21

*The bold MRM transitions were used for quantification.

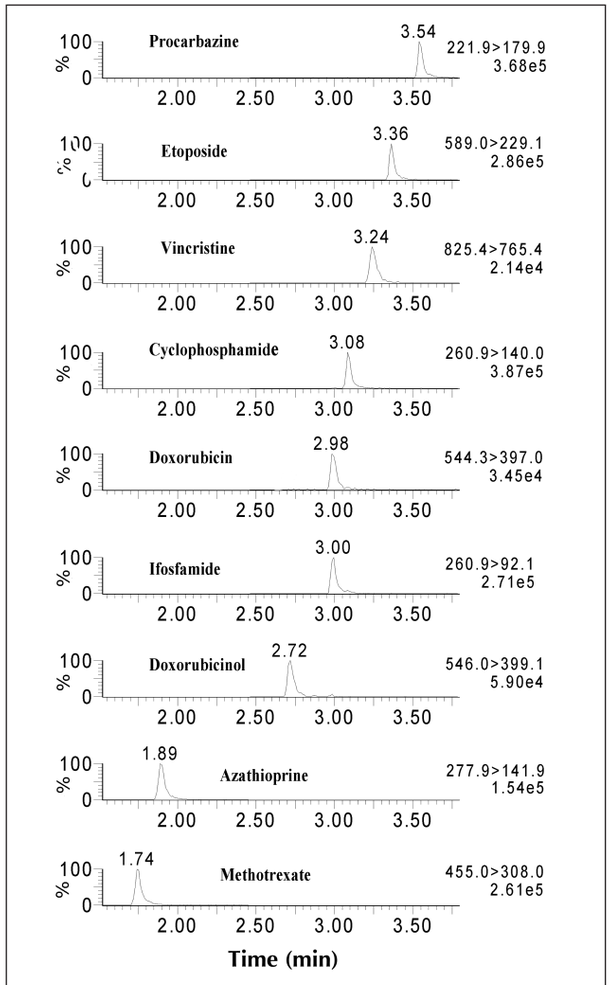

Figure 2. MRM chromatograms of nine anticancer drugs in standard solution at 25 µg/L.

The eluates were dried under a gentle stream of nitrogen at room temperature. The residuals were reconstituted to 0.5 mL with methanol.

For further clean-up, the extracts were diluted to 2 mL with water and then applied to the Oasis WAX cartridges (150 mg, 6 mL) (Waters), which had been conditioned with 6 mL methanol and 6 mL water. The target compounds were eluted consecutively with 7 mL methanol–water (60:40, v/v) and 5 mL methanol–water (40:60, v/v) containing 0.1% formic acid. This eluate was evaporated to near dryness in a gentle stream of nitrogen and the residual was reconstituted to 1 mL with methanol–water (50:50, v/v) for LC–MS–MS analysis.

Method validation

As the matrix components of the influent and effluent water samples were quite different and could lead to different sensitivities of the analytical method, all the validation studies were performed using influent and effluent water samples, respectively.

The linearity in the response was studied by using matrix-matched calibration standards prepared by dissolving nitrogen-dried sample extracts with solvent-based standard mixtures at six different concentration levels. Integrated peak areas of the selected quantification MRM transitions were used to construct six-point matrix-matched calibration curves, which were used for quantitative determinations. Each point on the calibration curves was obtained as the average of three injections.

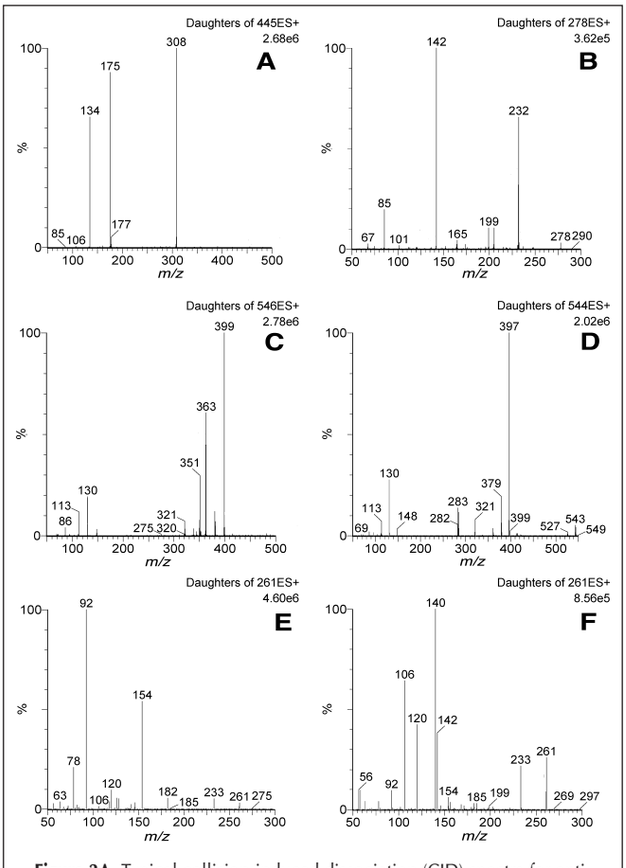

Figure 3A. Typical collision-induced dissociation (CID) spectra for anticancer drugs: (A) methotrexate, (B) azathioprine, (C) doxorubicinol, (D) doxorubicin, (E) ifosfamide, and (F) cyclophosfamide.

Recoveries were determined for each analyte at low (20 ng/L) and high (200 ng/L) concentrations in both influent and effluent. Five samples (500 mL) spiked with 10 ng of each pharmaceutical, and five samples (500 mL) spiked with 100 ng of each pharmaceutical were extracted and analyzed. Individual recoveries for the entire methods were calculated by comparing the integrated peak areas of five replicates per extracted sample to the calibration counterparts representing 100% recovery. Extracts of unspiked wastewater (500 mL) were examined first, and the peak areas were blank subtracted if target analytes were detected. The precision expressed as percent relative standard deviation (RSD %) was determined for each compound from five replicates of spiked water samples.

The limit of detection (LOD) and limit of quantification (LOQ) for each compound were calculated by determining signal-to-noise ratio (S/N) of the lowest measured concentrations and extrapolating to S/N values of 3 and 10, respectively.

Matrix effects

Evaluation of matrix effects is very important during LC–MS–MS method development. Typical signal suppression or enhancement effects are often observed when determining analytes in wastewater by LC–ESI-MS–MS system. Thus, experiments to evaluate the extent of the matrix effects have been performed in this study. With this aim, matrix-matched and solvent-based standard calibration curves were drawn, and the corresponding slope in matrix/slope in solvent ratios was calculated. The calibration solutions were prepared at six different concentrations before assays. The ratio [(slope in matrix/slope in solvent) × 100] is defined as the absolute matrix effect. A value of 100% indicates the absence of absolute matrix effect. There is signal enhancement if the value is > 100% and signal suppression if the value is < 100%.

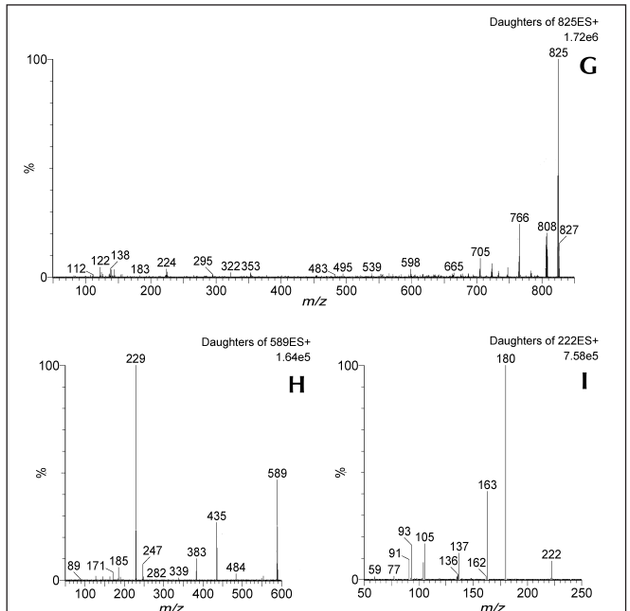

Figure 3B. Typical collision-induced dissociation (CID) spectra for anticancer drugs: (G) vincristine, (H) etoposide, and (I) procarbazine.

Statistical analysis

One-way analysis of variance (ANOVA) was used for statistical evaluation of matrix effects in sewage water samples collected from different STPs. In the tests, the hypotheses of normality and variance homogeneity were successful. The differences among means were considered to be significant if the *P* value, the statistical significance of comparing values, was less than 0.05. This method was also used to evaluate the differences between the matrix effects of the HLB SPE method and the extended HLB+WAX SPE method.

Results and Discussion

HPLC–ESI-MS–MS

Acquisition parameters of the mass spectrometer were optimized in ESI positive and negative mode by directly infusing each individual standard solution (1 mg/L) using a Harvard syringe pump at a flow rate of 10 µL/min. Full-scan spectra data were collected preliminarily to choose an abundant precursor ion for each compound. The cone voltages of the drugs were optimized to maximize the response of their precursor ions. In our experiments, $[M+H]^+$ ions were the most abundant and stable peaks in the mass spectra for all compounds and were, therefore, selected as precursor ions. The collision gas was then turned on and collision-induced dissociation (CID) spectra for each analyte were acquired under different collision energies. Figure 3 shows the CID spectra of the target analytes. For methotrexate, the $[M+H\text{-}C_5H_9NO_4]^+$ and $[M+H\text{-}C_5H_9NO_4\text{-}C_8H_7NO]^+$ ions were abundant in the spectra and selected for MRM experiments. For azathioprine, the molecular ion at *m/z* 232 and the ion at 141.9 were found with relatively high abundance in the mass spectra, corresponding to $[M+H\text{-}NO_2]^+$ and $[M+H\text{-}H_2O\text{-}C_5H_2N_4]^+$. The main fragmentation pathways were similar for doxorubicinol and doxorubicin with loss of water and $C_6H_{13}NO_3$, corresponding to the $[M+H\text{-}C_6H_{13}NO_3]^+$ and $[M+H\text{-}C_6H_{13}NO_3\text{-}2H_2O]^+$ ions for doxorubicinol and the $[M+H\text{-}C_6H_{13}NO_3]^+$ and $[M+H\text{-}C_{21}H_{16}O_8\text{-}H_2O]^+$ ions for doxorubicin. For cyclophosfamide, the ion at *m/z* 140.0 and the ion at 106.1 could be attributed to the fragments $[M+H\text{-}C_3H_8NO_2P]^+$ and $[M+H\text{-}C_3H_6NO_2P\text{-}HCl]^+$, indicating loss of the six-member heterocycle and hydrochloride. For ifosfamide, the ions at *m/z* 153.9 and 92.1 were found in the spectra, corresponding to $[M+H\text{-}C_2H_6NCl\text{-}C_2H_4]^+$ and $[M+H\text{-}C_2H_6NCl\text{-}C_2H_4\text{-}C_2H_3Cl]^+$. The molecular ions at *m/z* 807.5 and 765.4 due to the loss of water and CH_3CO_2H from the precursor ion were present in mass spectra of vincristine. The $[M+H\text{-}C_8H_{10}O_3]^+$ and $[M+H\text{-}C_8H_{10}O_3\text{-}C_8H_{14}O_6]^+$ ions were formed for etoposide, which was evidenced by the presence of molecular ions at *m/z* 425.1 and 229.1. Procarbazine produced the $[M+H\text{-}C_3H_6]^+$ ion (*m/z* 179.9) as the major ion (100% abundance) and the ion fragment $[M+H\text{-}C_3H_9N]^+$of *m/z* 162.8 with 40% abundance.

In an effort to increase ion production in MS and improve the peak shapes in LC, the pH of the mobile phase was decreased by the addition of formic acid to the water. Several concentrations of formic acid from 0.005–0.2% (v/v) were evaluated. The results indicated that the addition of formic acid improved the signal intensity compared to pure water and that 0.01% (v/v) formic acid was the most appropriate for all compounds except for vincristine. A significantly higher response

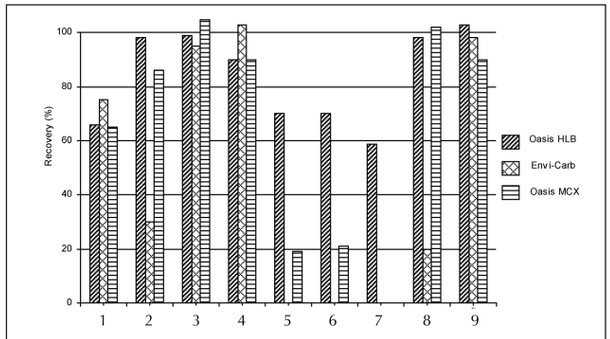

Figure 4. Recoveries of nine compounds in spiked ultra-pure water using three different sorbents (*n* = 3): 1, Methotrexate; 2, Azathioprine; 3, Cyclophosphamide, 4, Ifosfamide; 5, Doxorubicin; 6, Doxorubicinol; 7, Vincristine; and 8, Procarbazine.

Table II. Matrix Effects of Analytes Using Solid-Phase Extraction*

	Influent				Effluent			
	Matrix effect (RSD, %) *n* = 5		ANOVA results		Matrix effect (RSD, %) *n* = 5		ANOVA results	
Compound	HLB	HLB + WAX	F†	P	HLB	HLB + WAX	F†	P
Methotrexate	187 (6)	217 (6)	16.5	< 0.01	169 (4)	93 (3)	639.1	< 0.01
Azathioprine	17 (11)	42 (9)	168.5	< 0.01	23 (7)	63 (6)	548.7	< 0.01
Doxorubicinol	n.d.‡	22 (10)	–	–	6 (13)	42 (6)	962.1	< 0.01
Doxorubicin	n.d.‡	25 (7)	–	–	6 (10)	43 (8)	466.5	< 0.01
Cyclo-phosphamide	20 (9)	36 (6)	164.1	< 0.01	47 (7)	64 (7)	49.4	< 0.01
Ifosfamide	30 (11)	48 (6)	114.8	< 0.01	54 (5)	74 (8)	47.2	< 0.01
Vincristine	n.d.‡	82 (3)	–	–	215 (7)	146 (4)	162.2	< 0.01
Etoposide	5 (7)	22 (6)	643.6	< 0.01	12 (8)	42 (9)	296.6	< 0.01
Procarbazine	17 (8)	25 (8)	43.0	< 0.01	39 (6)	65 (4)	220.1	< 0.01

* With and without a clean-up step on WAX, in both influent and effluent and the results of one-way analysis of variance.
† F-value, which is used to calculate the *p*-value in ANOVA statistics, is the ratio of the variance between groups to the variance within the groups.
‡ Not detected at the maximal concentration of the calibration curve.

was obtained with an acetonitrile–water mobile phase than with a methanol–water mobile phase. Therefore, a mixture of water containing 0.01% (v/v) formic acid and acetonitrile was selected as mobile phase. Under these LC conditions, good separation was obtained for all target compounds except for doxorubicin and ifosfamide. Although these two compounds can be separated by change the gradient program, ifosfamide and cyclophosphamide were then coeluted. These two drugs can also be efficiently separated when acetonitrile was replaced by methanol, but the sensitivities were decreased. Therefore, the mobile-phase gradient program and composites were used as described previously by a compromise between resolution and MS response. This would not impact the determinations of these two drugs in our study by using the good selectivity of MS–MS.

Optimization of solid-phase extraction

Comparison of the SPE cartridges

Three types of SPE cartridges were tested in order to obtain good recoveries for the widest range of compounds in a single extraction step. The extraction materials tested included a macroporous poly (divinylbenzene-co-*N*-vinylpyrrolidone) copolymer (Oasis HLB), a graphitized carbon black, (Envi-Carb), and a copolymer of poly (divinylbenzene-co-*N*-vinylpyrrolidone) with strong cation exchanger sulfonic acid groups on the surface (Oasis MCX).

Oasis HLB was studied first because of its proven versatility and efficiency in the extraction of analytes of a wide range of polarities. Furthermore, this sorbent is more flexible because it can dry out during the extraction procedures without diminishing its ability to retain analytes. Experiments were performed by adjusting the pH of the spiked ultra-pure water samples to several different values (1.0–10.0). Better recoveries were obtained at pH 2.0 for the majority of analytes.

Envi-Carb, a non-specific and non-porous sorbent, was investigated for analyte retention because it has been extensively used in the past few years for the SPE of organic polar and non-polar compounds from water samples (23–25). In our case, the cartridges were eluted with different mixtures of dichloromethane and methanol after sample loading. Better recoveries were obtained for the majority of analytes when 6 mL dichloromethane–methanol (70:30, v/v) was used as elution solvent. However, recovery was low for etoposide and doxorubicin. Doxorubicinol and vincristine were not detected probably due to their stronger retention on the graphitized carbon black. Other elution solvents such as isopropanol, acetone, ethyl acetate and their mixtures were further tested, but no improvement was observed.

The Oasis MCX cartridge is a mixed reversed-phase cation-exchange cartridge. The cation exchanger of this sorbent can bind drugs bearing amino groups, which are positively charged at low pH values. Neutral and acidic compounds can be retained by the polymeric phase. As most of the target analytes in this paper contain amino groups in their structure, this sorbent was also considered and evaluated under acidic conditions. The recovery values for most analytes were greater than 60% at pH 1 with exceptions of doxorubicin, doxorubicinol and vincristine, where the recoveries were less than 20%.

Figure 4 shows that Oasis HLB retained the compounds better than the other two sorbents tested. Based on these preliminary investigations, target analytes were extracted using Oasis HLB at pH 2 in the further experiments.

Optimization of the Oasis HLB SPE procedure

First, 1 mL of methanol–water solution with different ratios was tested for removal impurities. Results indicated that no target analyte was eluted when the percentage of methanol in the solution was ≤ 30%. Therefore, methanol–water (30:70, v/v) was used as the washing solvent, and the washing volume was further optimized as 3 mL. All target analytes were found to be partially eluted when 1 mL of methanol–water (80:20, v/v) was used as eluting solvent. Optimal recoveries were achieved when the eluting volume of methanol–water (80:20, v/v) was increased to 6 mL.

The use of a second SPE cartridge

When the Oasis HLB SPE procedure was applied to real sewage water samples, co-eluting compounds originating from the matrix caused severe signal suppression on the target analytes and greatly reduced the LC–MS–MS sensitivity. Thus, the eluate from the HLB cartridge cannot be directly analyzed, and a further cleanup procedure with a second SPE cartridge was conducted to reduce the matrix suppression.

Based on the structure of the target analytes and the increased selectivity of anion exchange phases for compounds with acidic groups, the Oasis WAX cartridge (mixed-mode reversed-phase/weak anion-exchange polymeric sorbent) was selected for further purification. All the pharmaceuticals were retained on this cartridge when 2 mL methanol–water (25:75, v/v) was used as the loading solvent. Under this condition, all analytes were adsorbed by the reverse-phase mechanism with an additional interaction for methotrexate via electrovalent bonding between its carboxylic acid moieties and the quaternary amine ions of WAX. All analytes, except methotrexate, were completely eluted from WAX with 7 mL methanol–water (60:40, v/v). The elution of methotrexate was investigated using methanol–water solutions containing different percentages of formic acid (0.05%–0.3%). Results indicated that nearly 100% methotrexate was recovered using 5 mL methanol–water (40:60, v/v) containing at least 0.1% formic acid.

The matrix effects in both influent and effluent water samples using HLB and HLB+WAX cartridges were compared using one-way ANOVA. A significant decrease was observed in the matrix effects for most of the compounds in both influent and effluent water samples (Table II) with the exception of methotrexate in influent water. Therefore, the use of WAX cartridges for clean-up was effective.

Validation of the overall procedure

The linearity range of each analyte is summarized in Table III and IV. The correlation coefficients (r^2) of the matrix-matched calibration curves (Table III and IV) were all greater than 0.99 for both influent and effluent samples. The results indicate that the matrix-matched calibration curves could be effectively applied to quantify the nine drugs in the wastewater from STPs.

Recoveries for the entire method are reported in Table III and Table IV. Average recoveries of each compound in both influent and effluent samples were greater than 50% with the exception of vincristine in effluent at 20 ng/L (recovery 45.3%). The recovery of less than 50% is not ideal but is acceptable (26). The precision of this method at each fortification level, represented by the RSD % obtained from the analysis of five replicates, were generally below 20% (Table III and IV). Again, vincristine at 20 ng/L was an exception with precisions of 20.5% for influent and 24.5% for effluent, possibly because the spiking level was at the LOQ.

For each analyte, the LOD and LOQ calculated in STP influents and effluents are listed in Table III and IV, respectively. The LODs and LOQs for the analytes varied from influent to effluent because of the different matrices in these samples. The LODs for the whole method ranged from 0.6–7.0 ng/L for influent and 0.5–3.5 ng/L for effluent. The LOQs for the whole method ranged from 1.7–20 ng/L for influent and 1.5–10 ng/L for effluent. The LOQs of methotrexate and cyclophosphamide were approximately the same as those published previously, where the LOQs were 0.8 ng/L for methotrexate and 1.9 ng/L for cyclophosphamide in STP effluents (17). Moreover, for doxorubicin, ifosfamide, and etoposide, the LOQs in our method are much lower than in previously reported methods, where the LOQs were determined at 260 ng/L for doxorubicin in hospital wastewater (14), 14 ng/L for ifosfamide in ground water (22), and 0.09 mg/L for etoposide in surface water (19). Extraction of the other compounds from surface or ground water has not been described in previously published literature.

Matrix effect

Different sewage water samples collected from three different STPs (A, B, and C) were analyzed to evaluate matrix impact on the final LC–ESI-MS–MS method. The matrix effects of different wastewater samples for each analyte were calculated and compared using one-way ANOVA. For the majority of drugs, significant differences were observed in the matrix effects between different water samples (Table V). Thus, problems could arise when quantifying the target analytes in different wastewater samples using calibration curves as the slopes of the curves may be significantly different from one sample to another. The best strategy to solve this problem is to use isotopically labeled internal standards. However, it is difficult to obtain so many isotopically labeled internal standards commercially. Therefore, in our experiments, the standard addition method recommended by the U.S. Food and Drug Administration was applied to determine the concentrations of analytes in real sewage samples with a minor amendment (27). For that, the extract was divided into three aliquots of 300 µL, and each aliquot was dried under a gentle nitrogen stream. The residues were then reconstituted with 300 µL of standard solutions at three different concentrations (0, 50, 100 µg/L). These solutions were analyzed, and the peak areas of analytes (y) were plotted against the concentrations of each standard solution (x) to construct matrix-matched calibration curves. These curves were extrapolated to intersect the x-axis, and the absolute values of x-intercepts represented the concentrations of analytes in the blank sample extracts.

Real water samples

This method was used to investigate the influent and effluent samples from five local STPs. Three analytes, cyclophosfamide, ifosfamide, and methotrexate, were detected in samples from three of the STPs. Cyclophosfamide was present in both

Table III. Analytical Performance of the LC–ESI-MS–MS Method Applied to Influent

Compound	Linearity range (ng/mL)	Correlation factors (r^2)	Recovery 20 ng/L (RSD, %) n = 5	Recovery at 200 ng/L (RSD, %) n = 5	LOD (ng/L)	LOQ (ng/L)
Methotrexate	1.00–200	0.9984	61 (2)	67 (5)	0.6	1.7
Azathioprine	2.50–200	0.9992	102 (3)	95 (3)	1.6	5.0
Doxorubicinol	10.0–400	0.9995	66 (11)	72 (4)	6.5	20.0
Doxorubicin	10.0–400	0.9979	70 (11)	68 (5)	6.5	20.0
Cyclophosphamide	1.25–200	0.9990	93 (6)	96 (10)	0.8	2.5
Ifosfamide	5.00–200	0.9998	80 (12)	80 (17)	2.5	7.0
Vincristine	10.0–400	0.9998	51 (21)	56 (13)	7.0	20.0
Etoposide	8.00–320	0.9987	97 (8)	99 (7)	5.0	15.0
Procarbazine	8.00–320	0.9981	104 (7)	98 (6)	5.5	16.0

Table IV. Analytical Performance of the LC–ESI-MS–MS Method Applied to Effluent

Compound	Linearity range (ng/mL)	Correlation factors (r^2)	Recovery 20 ng/L (RSD, %) n = 5	Recovery at 200 ng/L (RSD, %) n = 5	LOD (ng/L)	LOQ (ng/L)
Methotrexate	1.00–200	0.9942	61 (5)	57 (5)	0.6	1.7
Azathioprine	1.25–200	0.9992	100 (10)	91 (6)	1.2	3.5
Doxorubicinol	2.50–400	0.9992	70 (7)	71 (3)	1.6	5.0
Doxorubicin	2.50–400	0.9986	68 (8)	67 (3)	2.5	6.5
Cyclophosphamide	0.63–200	0.9967	109 (6)	96 (4)	0.5	1.5
Ifosfamide	2.50–200	0.9977	92 (4)	83 (4)	1.6	5.0
Vincristine	5.00–400	0.9986	45 (25)	50 (6)	3.5	10.0
Etoposide	4.00–320	0.9980	95 (10)	91 (4)	2.5	7.0
Procarbazine	4.00–320	0.9991	106 (4)	100 (4)	2.6	8.0

influents and effluents with concentrations of 8.5–14.5 ng/L. Ifosfamide was also detected in both influents and effluents, and the concentrations ranged from 9.0–16.4 ng/L. Methotrexate was observed only in influents with concentrations of 1.6–18.1 ng/L. These three compounds have been applied to clinic use for decades and are the most frequently used anticancer drugs. The other compounds were not detected in the studied wastewater samples, which may reflect their lower usage and resulting lower amounts received at these STPs.

Figure 5 shows the mass chromatograms of one influent sample. Three peaks were found with the same retention time as methotrexate (1.74 min), cyclophosphamide (3.08 min), and ifosfamide (3.00 min) in these chromatograms. The area ratios of the chromatograms obtained for 260.9 > 140.0/260.9 > 106.1, 260.9 > 92.1/260.9 > 153.9 and 455.0 > 308.0/455.0 > 174.8 in this influent sample were close to those in the standard sample. This study confirmed that the anticancer drugs such as cyclophosfamide and ifosfamide could be directly discharged in ng/L levels through STPs into surface water in China. This is in agreement with results from other countries (16,18).

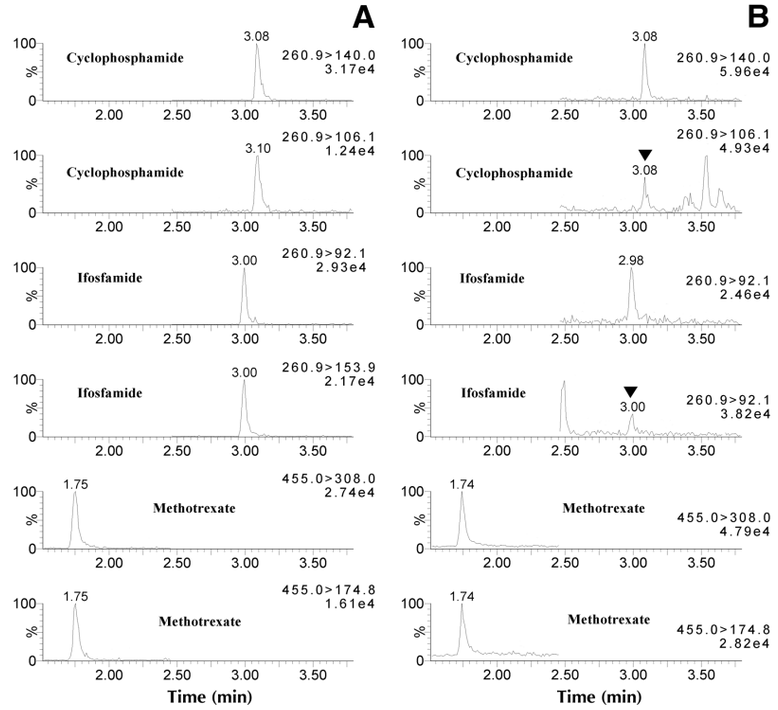

Figure 5. MRM chromatograms of methotrexate, ifosfamide, and cyclophosphamide in (A) 2.5 µg/L standard and (B) an influent sample extract (two small peaks of ifosfamide and cyclophosphamide are indicated by ▼.

Conclusion

A comprehensive analytical method was developed for simultaneous analysis of nine anticancer drugs in sewage water using two-step SPE and a UPLC–MS–MS system. The method demonstrates good linearity, accuracy, and precision. The LOD of this method was approximately the same or less than previously reported methods. This new method is expected to be applicable for investigations on environment occurrence and fate of the anticancer drugs in sewage water.

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Table V. Matrix Effects of Different Wastewater Samples Taken from Three STPs and the Results of One-Way Analysis of Variance

	Influent					Effluent				
	Matrix effect (RSD, %) *n* = 5			ANOVA results		Matrix effect (RSD, %) *n* = 5			ANOVA results	
Compound	A	B	C	F*	P	A	B	C	F*	P
Methotrexate	217 (6)	126 (5)	177 (1)	109.7	< 0.01	93 (3)	109 (5)	146(3)	154.0	< 0.01
Azathioprine	42 (10)	57 (4)	60 (2)	61.1	< 0.01	63 (6)	76 (12)	80 (10)	9.0	< 0.01
Doxorubicinol	22 (10)	44 (3)	24 (5)	159.6	< 0.01	42 (6)	78 (5)	63 (5)	130.8	< 0.01
Doxorubicin	25 (7)	41 (14)	19 (13)	46.7	< 0.01	43 (8)	78 (9)	48 (7)	63.4	< 0.01
Cyclophosphamide	36 (6)	56 (1)	62 (10)	46.7	< 0.01	64 (7)	81 (10)	77 (6)	11.8	< 0.01
Ifosfamide	48 (6)	65 (1)	94 (6)	171.1	< 0.01	74 (8)	89 (14)	85 (8)	3.7	0.06
Vincristine	82 (3)	132 (4)	206 (8)	160.2	< 0.01	146 (4)	154 (8)	141 (6)	2.2	0.16
Etoposide	22 (6)	33 (5)	29 (6)	61.9	< 0.01	42 (9)	60 (6)	49 (7)	23.6	< 0.01
Procarbazine	25 (8)	28 (3)	31 (6)	16.8	< 0.01	65 (5)	69 (8)	64 (9)	0.87	0.45

*F-value, which is used to calculate the *p*-value in ANOVA statistics, is the ratio of the variance between groups to the variance within the groups.

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