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DETECTION OF CYTOSTATIC DRUGS IN MUNICIPAL WASTE WATER AND THEIR TRANSFER TO SURFACE WATER

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Abstract

A new sensitive and selective LC-MS/MS method with positive electrospray ionization was developed to detect seven cytostatic drugs and one metabolite from municipal waste water. The method separates the target analytes in only 16 minutes using a Hypersil Gold column (100 x 2.1 mm, 3.0 μm) kept at 20°C and a mobile phase made of aq. 0.2% HCOOH and ACN in the ratio 92/8 (v/v). A low mobile phase flow of 0.2 mL/min was used to increase MS ionization yield and hence method sensitivity. A complex two steps (10%/minute) and two plateau gradient elution program was applied for 12 minutes to achieve analyte separation from matrix and each other with highly efficient peaks. Collision energy, fragmentor and capillary voltages were optimized to increase MS sensitivity. Optimization of LC-MS parameters generated low instrumental LOQs situated in the range 0.1 ÷ 1.0 ng/mL. MS detector response was linear between 1 and 100 ng/mL with R^2 coefficients > 0.997 for all eight target analytes. SPE using Oasis HLB cartridges was employed to concentrate target analytes from water samples with MeOH as elution solvent. Intra-day and inter-day precision (RSD %) was situated between 5.4 ÷ 7.2% and 7.3 ÷ 10.8%, respectively. Cytostatics recovery after SPE was good due to internal standard correction with values between 70 ÷ 134%. Overall method LOQs were situated between 0.6 and 5.6 ng/L. The method was tested on four waste water samples from a WWTP plant in Bucharest, but none of the targeted cytostatic drugs were found above detection limit.

Keywords*: cytostatic drugs, LC-ESI-MS/MS, metabolites, municipal waste water*

Introduction

In the last decade, pharmaceuticals have attracted increasing attention from environmental scientists and are studied for their ecotoxicological effects in municipal wastewater treatment processes and occurrence in surface waters, sediments, along with ground and drinking water (Kovalova et al. 2009). From the various classes of pharmaceuticals, cytostatic drugs are amongst the most toxic ones, presenting serious adverse health effects for all living organisms (Johnson et al. 2008a). They are designed to interact with the genetic material and interrupt cell replication. Cytostatic drugs act unselectively on all growing cells and practically all eukaryotic organisms are vulnerable to damage, even at extremely low concentrations of ng/L as found in surface or waste water. Cytostatic compounds and their metabolites can have serious carcinogenic, mutagenic and teratogenic effects on living organisms, including humans. The most important effects, even at low concentrations, are found in foetuses, babies and children with a large number of rapidly growing cells (McKnight

2003). Many cytostatic compounds have very short half-lives and can be eliminated, as unchanged substances or as a mixture of metabolites, by urine and faeces and be directly discharged into the sewer system from hospitals and household discharge from outpatients (Lienert et al. 2007). It is well known that sewage treatment plants do not completely remove organic pollutants found in municipal waste water due to technological limitations and contamination of water with a growing number of new organic molecules, such as cytostatic (Buerge et al. 2006). Due to their low biodegradability, biodegradation of these compounds in municipal waste waters can be assumed to be low to moderate (Kosjek et al. 2011, Negreira et al. 2104). Significant increases in cancer cases over the past few years have led to an increased number of chemotherapy treatments with a related growing concern over the presence of cytostatic drugs in water systems putting humans and aquatic organisms at risk (Johnson et al. 2008b, Rowney et al. 2009). This is the main reason for which an ever-increasing number of studies focused on cytostatics occurrence in the waste water have been reported (Parrella et al. 2014, Busetti et al. 2009). The aim of the present study was to develop, optimize and validate a sensitive, selective, and accurate LC-ESI-MS/MS method able to determine seven cytostatic drugs (Methotrexate, Doxorubicin, Ifosfamide, Cyclofosfamide, Capecitabine, Docetaxel, Tamoxifen) and one metabolite (4-OH Tamoxifen) from municipal waste water at trace level concentration (ng/L).

Experimental

Reagents and chemicals

HPLC grade acetonitrile and methanol were acquired from Merck. HCOOH (p.a.) was obtained from Sigma-Aldrich. Ultra purified water was obtained in-house using a MilliQ instrument. High purity reference standards of cytostatic drugs: Methotrexate, Doxorubicin, Ifosfamide, Cyclophosphamide, Capecitabine, 4-hydroxytamoxifen, Docetaxel and Tamoxifen were acquired from Sigma-Aldrich. Isotopically labelled cytostatic compounds: Methotrexate-d_3, Cyclophosfamide-d_4, and EZ Tamoxifen-d_5 were obtained from Cambridge Isotope laboratories Inc. (Andover, MA, USA) and from Santa Cruz Biotechnologies. These compounds were used as surrogate/injection internal standards. Oasis HLB (500 mg/6 mL) cartridges used for SPE extraction were acquired from Waters (Milford, Massachusetts, USA).

LC-MS instrumentation and conditions

Experiments were performed using an Agilent 1260 series LC system (Agilent, Waldbronn, Germany) coupled with an Agilent 6410B triple-quadrupole mass spectrometer with electrospray ionization source (ESI). All chromatographic runs were carried out on a Hypersil Gold column (100 x 2.1 mm, 3.0 µm) from Thermo Scientific which was kept at 18°C. Initial mobile phase composition was a mixture of Aq. 0.2% HCOOH (A) and Acetonitrile (B) in the ratio 92/8. Separation was done in a complex gradient elution program at a flow-rate of 0.2 mL/min, as follows: time 0 ÷ 1 min (8%B - isocratic plateau); time 1 ÷ 5 min (8 ÷ 48%B); time 5 ÷ 7 min (48%B - isocratic plateau); time 7 ÷ 11 (48 ÷ 88%B); time 11 ÷ 12 min (88%B - isocratic plateau); time 12.1 ÷ 16 min (8%B isocratic plateau). Method injection volume was 20 µL using 0.2% HCOOH and Methanol 95/5 (v/v) as sample diluent. MS detection was achieved using Multiple Reaction Monitoring (MRM) acquisition mode.

Retention time, MRM transitions fragmentor voltages, collision energies and other MS parameters are given in Table 1. ESI ionization source was operated in positive mode with 300°C as the drying gas temperature, 10 L/min drying gas flow, 50 psi nebulizer pressure and 4000 V capillary voltage. Two MRM transitions were used, one for quantitation (quantifier) and another for analyte confirmation (qualifier).

Table 1. MS parameters for the detection of cytostatic drugs and labelled internal standards (MRM transitions, collision energy, fragmentor voltages)

Compound	Time segment (min)	MRM Transitions	Fragmentor voltage (V)	Collision energy (V)	Dwell time (msec)
Methotrexate-D_3	2.5 - 9.0	458.2→311.1 458.2→175.1	90 90	25 25	150 150
Methotrexate	2.5 - 9.0	455.2→308.2 455.2→175.1	90 90	25 25	150 150
Doxorubicin	9.0 - 11.6	544.3→361.0 544.3→397.1	90 90	15 15	80 80
Ifosfamide	9.0 - 11.6	261.1→92.0 261.1→154.0	85 85	25 25	80 80
Cyclophosphamide-D_4	9.0 - 11.6	265.1→140.0 265.1→106.0	90 90	30 30	80 80
Cyclophosphamide	9.0 - 11.6	261.1→140.0 261.1→106.0	90 90	30 30	80 80
Capecitabine	9.0 - 11.6	360.2→244.2 360.2→174.1	95 95	13 13	80 80
4-OH Tamoxifen	11.6 - 13.2	388.2→72.1 388.2→44.1	90 90	45 45	200 200
Docetaxel	13.2 - 15.9	830.3→549.4 830.3→304.1	95 95	25 25	120 120
EZ tamoxifen-D_5	13.2 - 15.9	377.3→72.1 377.3→44.1	90 90	45 45	120 120
Tamoxifen	13.2 - 15.9	372.3→72.1 372.3→44.1	90 90	45 45	120 120

Results and Discussion

LC separation optimization

Taking into account the different polarities of the studied cytostatics (log Kow = -1.85 ÷ 7.88), a gradient elution was chosen starting from a mobile phase with a high aqueous solvent concentration, allowing adequate retention and separation of the most polar compound (Methotrexate, log Kow = -1.85). Increasing the concentration of organic solvent will also allow elution in a reasonable time of higher hydrophobic compounds (Docetaxel, Tamoxifen, log Kow = 2.83, 7.88). Thus, the starting composition of the mobile phase was 92/8 aqueous solvent / organic solvent. The low

flow rate of 0.20 mL/min favored analytes ionization in the electrospray source, as it is known that this type of ionization gives better yields at low flow-rate. The reduced column temperature (20°C) was chosen for a better separation between the pair of E and Z geometric isomers of the 4-hydroxy tamoxifen metabolite. The chromatographic column, with 2.1 mm internal diameter, allowed working at a reduced flow rate of 0.2 mL/min without loss of chromatographic peak efficiency, and the small particle size of the stationary phase (3 μm) led to narrow peaks with high chromatographic efficiency. Different additives in the aqueous component of the mobile phase were tested in order to improve MS Electrospray ionization (MS response) and also the peak shape of the compounds. Formic acid (0.1 ÷ 0.3%), Ammonium formate (5 ÷ 10 mM), and Ammonium acetate (5 ÷ 20 mM) were used as aqueous component of the mobile phase. The best ESI ionization was observed for the mobile phase with 0.2% formic acid and thus it was finally chosen for the optimized method. A representative chromatogram showing separation of the target analytes is given in Fig. 1.

MS detection optimization

MS detection parameters were optimized to obtain maximum sensitivity for the cytostatic compounds quantitation. The detection was performed using MS/MS mode Multiple Reaction Monitoring (MRM) to increase selectivity and sensitivity. All analytes responded best in MS detector when using positive polarity for the electrospray source ionization and thus it was chosen for the final method. All ESI ionization source parameters were optimized by modification in a wide range to obtain highest ionization efficiency for all compounds. The final chosen values, giving maximum peak area response, were: 4000V capillary voltage, 300°C drying gas temperature, 50 psi nebulizer pressure and 10 L/min drying gas flow. Collision energy (CE) applied in the collision cell (Q2) to the precursor ions to dissociate them and obtain product ions was varied between 5 and 60V. CE between 13 - 45V generated highest dissociation yield of the precursor ions for all cytostatic compounds. CE showing the highest S/N ratio values for each analyte was chosen for the MRM transitions (Table 1). After the entire procedure of MS optimization, the obtained instrumental quantitation limits (IQL) for all compounds were lower than 0.4 μg/L, except for Docetaxel with a value of 1 μg/L (see Table 2).

Automated SPE optimization

All parameters of the automated SPE extractor were varied to determine the most efficient procedure to concentrate target analytes from waste and surface water. The optimized extraction procedure is given in the following: SPE cartridge conditioning was done with 2 x 5 mL MeOH and then 2 x5 mL H_2O. Water samples (250 mL) were filtered (0.45 μm cellulose). Samples were loaded on the SPE cartridge (Oasis HLB - 500 mg/5 mL) at a constant flow-rate of 5.0 mL/min. Next, the cartridges were washed with 10 mL H_2O to remove endogenous polar matrix compounds and purify the extract.

After washing, the cartridges were air-dried for 40 min and then the analytes were desorbed with 2 portions of 5 mL MeOH. Extracts are then evaporated to dryness under a gentle N_2 stream (45°C) and re-dissolved in 1 mL with aq. 0.2% HCOOH / methanol 95/5 (v/v). The final samples were filtered through 0.45 μm cellulose filters and then injected into the LC-MS system.

Automated SPE provided a 250 fold increase in analyte concentration allowing a detection of target compounds down to few ng/L (LOQs between 0.6 – 5.6 ng/L).

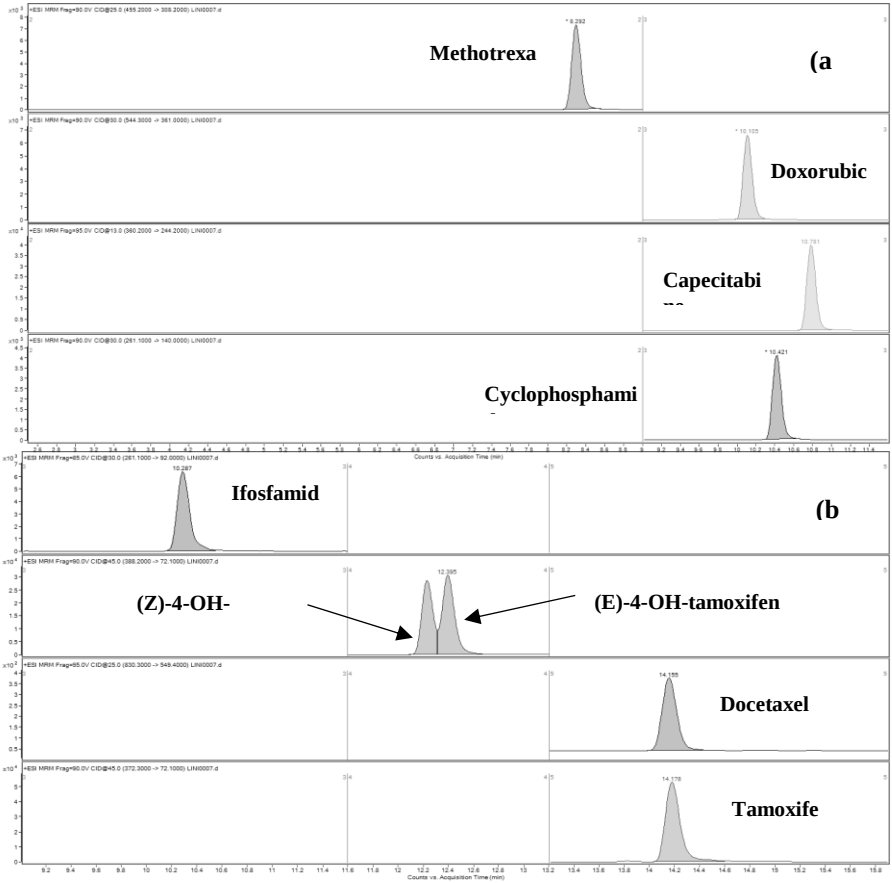

Figure 1. Representative MS/MS chromatograms (MRM) for the target analytes (7 cytostatics and 1 metabolite) obtained after injection of a 50 µg/L mixed solution: (a) Methotrexate, Doxorobicin, Capecitabine, Cyclophosphamide, (b) Ifosfamide, (Z) and (E) 4-OH-tamoxifen, Docetaxel, and Tamoxifen.
MRM transitions are given also in chromatograms legend.

SPE-LC-MS/MS method validation

The performance parameters of the developed method followed during the validation were: selectivity, linearity, precision, limit of quantitation, accuracy and recovery. MS detector response was linear in the range 1 ÷ 100 µg/L with high determination coefficients ($R^2 > 0.997$). Intra-day and inter-day method precision and accuracy were tested on 12 sample replicates. The procedure involved spiking a mixture of cytostatic drugs and internal standards (50 ng/L) in a waste water sample previously tested to

be free from the all target analytes. Method precision was expressed as RSD% and it was situated between 5.4 - 7.2% (repeatability procedure or intra-day precision) and between 7.3 - 10.8% (reproducibility procedure or inter-day precision). The obtained analyte recovery (method accuracy) was situated between 70 and 134% due to surrogate internal standard addition which compensated for sample preparation losses (SPE extraction, evaporation, re-dissolution, etc.). Validation data are presented in detail in Table 2.

Table 2. Determination coefficients (R^2), intra-day and inter-day precision, analyte recovery, instrumental quantitation limit (IQL) and overall method quantitation limit (LOQ) obtained for the proposed method

Analyte	R^2	Precision (RSD %)		Overall method recovery (% ± RSD %)	IQL (µg/L)	LOQ (ng/L)
		Intra-day	Inter-day			
Methotrexate	0.9998	6.1	9.6	98 ± 9.6	0.21	1.3
Doxorubicin	0.9996	6.9	8.4	111 ± 8.4	0.22	1.4
Ifosfamide	0.9999	5.4	7.3	94 ± 7.3	0.30	1.8
Cyclophosphamide	0.9998	5.7	7.9	106 ± 7.9	0.38	2.4
Capecitabine	0.9992	7.2	10.8	93 ± 10.8	0.10	0.6
4-OH-Tamoxifen	0.9975	7.1	9.9	134 ± 9.9	0.40	2.5
Docetaxel	0.9972	6.6	8.0	70 ± 8.0	0.98	5.6
Tamoxifen	0.9999	6.0	8.5	96 ± 8.5	0.18	1.1

In order to test the ability of the method to detect the targeted cytostatics at ng/L level, it was further applied on four different waste water samples from Bucharest, Glina WWTP plant. None of the targeted cytostatics were detected (< LOD) and thus these samples were spiked with a known mixture solution of cytostatics (25 ng/L) and analysed. The obtained results were included in a recovery range between 62 and 118% which is in agreement with that obtained when method accuracy was evaluated. It was observed that no significant matrix effects were generated with respect to the target analytes except for Docetaxel which did not have an isotopically labelled compound as the surrogate internal standard. For this compound a matrix effect of 35-40% expressed as absolute MS signal decrease was established due to interfering compounds from the waste water.

Conclusions

A new sensitive, selective and accurate LC-ESI(+)MS/MS method coupled with automated SPE extraction was developed to detect seven cytostatic drugs (Methotrexate, Doxorubicin, Ifosfamide, Cyclophosphamide, Capecitabine, Docetaxel and Tamoxifen) and one metabolite (4-hydroxytamoxifen) from waste water samples. All chromatographic conditions (mobile phase nature and composition, column temperature, gradient intensity) and MS parameters were

optimized to separate and detect the target analytes from a complex matrix like waste water. Samples were concentrated 250 times using SPE on Oasis HLB cartridges with methanol as desorption solvent. MS detector response was linear in the range 1 ÷ 100 ng/mL with determination coefficients higher than 0.997 for all eight cytostatics. Method accuracy was good with recovery values between 70 and 134% due to very good error compensation by using surrogate internal standard addition (previous to sample extraction). Method precision generated RSD values up to 10% (inter-day precision) indicating an adequate method from precision point of view taking into account that SPE, waste water matrix, MS analysis may generate all-together similar or even higher values. Optimization of LC-MS parameters generated very low instrumental LOQs situated in the range 0.1 ÷ 1 ng/mL. Coupled with the sample extraction and concentration procedure (SPE), the overall method quantitation limits (LOQ) for the targeted cytostatic drugs were determined to be below 6 ng/L - more precisely in the range of 0.6 ÷ 5.6 ng/L. The obtained values are comparable with results generated by other similar studies already reported for these compounds. The developed LC-ESI(+)MS/MS method coupled with automated SPE extraction allows for adequate detection and confirmation for the seven cytostatic drugs and one metabolite from waste water samples at trace level concentrations (ng/L). The optimized method was tested on four waste water samples from Glina WWTP plant in Bucharest, but none of the targeted cytostatics were found above detection limit.

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