ITEMS OF Ph.D. THESIS

Monitoring the most often prescribed drugs' residues in surface waters: determination of anti-ulcers and cardiovascular drugs with SPE-LC-MS/MS method

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2012.

1. Introduction

Due to the aging society, increasing industrial environmental pollution and consumption of processed foodstuff - doped with preservatives and coloring agents - increasing number of people suffer from different illnesses. As a result of these phenomena and the development of pharmaceutical industry nowadays the consumption of prescribed and non-prescribed drugs – however usage of the latter can hardly be followed – increases both in Europe and Hungary.

Due to improper waste treatment and low efficiency of waste water treatment procedures purchased drugs and their possibly active metabolites can enter into surface waters. However this type of pollution is not directly harmful for the people, it affects the development and subsistence of the aqueous organisms. At the same time several compounds can accumulate in fish tissue that can also be dangerous for people.

Therefore monitoring of pharmaceutical residues in surface waters is important to protect the environment. The target compound lists of available methods are usually based on drugs' consumption frequency, route of elimination and their effect on different kind of aqueous organisms.

Recently several groups of pharmaceuticals are increasingly investigated. These are hormones, antibiotics and non-steroidal anti-inflammatory drugs. Formerly the developed methods were mainly based on gas chromatography – mass spectrometry (GC-MS) but nowadays liquid chromatography – mass spectrometry (LC-MS) methods are more current.

The main objective of my PhD study was the extension of the above described methods. The list of target compounds was based on a pharmaceutical sale-statistic from 2006. In the first table the first ten most sold products are listed along with their active substances and ATC-codes. The ATC-code serves for grouping pharmaceutical compounds according to their usage.

Product	Active substance	ATC-code	Manufacturer
Plavix	clopidrogel	B	Sanofi-Aventis
Coverex	perindopril	C	Egis
Glivec	imatinib	L	Novartis
Neorecormon	B-epoetin	B	Roche
Sortis	atorvastatin	C	Pfizer
Controloc	pantoprazole	A	Altana Pharma
Normodipine	amlodipine	C	Richter Gedeon
Nexium	esomeprazole	A	AstraZeneca
Tritace	ramipril	C	Sanofi-Aventis
Quamatel	famotidine	A	Richter Gedeon

Table 1. – The most often purchased drugs in Hungary (2006) [1]

It can be seen that most of the ten best selling products belong to group C which are cardiovascular drugs. They are followed by compounds from group A which serve for the treatment of lead and metabolism diseases. Further on compounds from group B serve for blood-type diseases, while group L is for anti-cancer and immune-modulator drugs.

Based on Table 1 my investigations targeted active substances from those groups to which the most often purchased drugs in Hungary belong. These can have remarkable effect on aqueous organisms in surface waters.

[1] A legnagyobb forgalmú gyógyszerek Magyarországon (2006), IMS Health, Marketing Pirula, 2007, http://www.euuzlet.hu/tablazatok/gyogyszerek.html

2. Scope

I aimed at the development of a multi-component LC-MS/MS method for the determination of pharmaceutical residues of the most often prescribed drugs in Hungary. Method requirements were the following: fast sample preparation without handling extremely large sample volumes and fast analytical method with low quantitation limits.

After validation I intended to apply the method for Danube river water samples' measurements. I also planned to carry out an environmental loading investigation. The two main streams surrounding Budapest from Buda side (Aranyhegyi- and Hosszúréti-streams) were sampled seasonally at different sampling sites. Sampling was done for a year at eleven points. From estuary samples' concentration and from runoff calculations annual environmental loading of my target compounds were estimated.

Finally identification of the target compounds' metabolites with predictive methods and further confirmation by accurate mass measurements was the forth objective of my work.

3. Methods

3.1 Target compounds

The 26 target compounds can be grouped as follows:

- H_2-receptor antagonists (cimetidine, famotidine, nizatidine, ranitidine)
- Protonpump inhibitors (lansoprazole, omeprazole, pantoprazole)
- Beta-blockers (acebutolol, atenolol, betaxolol, carvedilol, esmolol, metoprolol, oxprenolol, propranolol, sotalol)
- Selective Ca-channel blockers (amlodipine, nifedipine, nimodipine)
- Angiotensin-converting enzyme inhibitors (enalapril, lisinopril, ramipril)
- HMG CoA reductase inhibitors (atorvastatin, fluvastatin, lovastatin, simvastatin)

Four deuterium-labeled internal standards were also applied (atenolol-d_7, cimetidine-d_3, enalapril-d_5, lansoprazole-d_4).

3.2 Conditions of the validated SPE-LC-MS/MS method

Samples of 500 ml were prepared by solid phase extraction on Waters Oasis HLB (500 mg, 12 cc) tubes, after their pH had been adjusted to 10.0 with 25% aqueous ammonium-solution. The extraction procedure was as follows:

Conditioning	5 ml hexane 5 ml aceton 10 ml methanol
Equilibrating	5 ml Millipore water, pH = 10.0
Sample loading	Through PTFE tubes, with ~3-4 ml/min flow rate
Washing	5 ml (5%methanol/2% aqueous NH_4OH)
Drying	10 min
Elution	2x2.5 ml methanol
Evaporation	To dryness, under N_2 flow
Reconstitution	500 µl 10% methanol / Millipore water

Table 2. – The SPE procedure

Liquid chromatographic separation was carried out on an Agilent 1200 LC system, on a Zorbax Eclipse Plus C_{18} (2.1 x 100 mm, 1.8 µm) column, at 50 °C. Eluents were 10 mM ammonium-acetate, pH = 5.0 („eluent A") and acetonitrile + 0.15% glacial acetic acid („eluent B") in combination with a flow rate of 0.25 ml/min. Sample aliquots of 5 µl were injected each time. Gradient elution started with 10% eluent B, which increased to 80% in 4 min, and up to 100% in another 4 min. This final composition was held for 4 min, then immediately changed back to the starting composition which was held for 8 min as equilibration.

Detection of the compounds was carried out with an Agilent 6460 triple quadrupole (QqQ) mass spectrometer, applying electrospray ionization (ESI) in positive mode. The component specific parameters were determined individually. These were the fragmentor voltage (which rules the entering into the analyzer), parent ions and the two most intensive fragment ions (the so-called MRM-transitions) along with their collision energy values. The separation was divided into two segments: in the first segment there were 7 target compounds and 2 internal standards while in the second one there were 19 target compounds and 2 internal standards.

Quantitation was based on matrix-matched calibration in a 5 – 300 ng/l concentration range.

3.3 Conditions of the predictive LC-QqQ-MS method

For the supposed metabolites predictive methods were developed based on the parent molecules fragmentation pattern and on the biotransformation reactions. I supposed that the mass defect caused by the biotransformation reaction can be seen on either one of the fragments while the other fragment remains unchanged. Thus for each metabolite four MRM-transitions could be described. Investigations were carried out as described in section 3.2 with the following changes: altogether 5 liters of sample were extracted which meant a 10,000-fold enrichment; the final elution composition was held for 7 min instead of 4 min.

3.4 Conditions of the LC-TOF-MS method

Confirmation of the identified compounds was carried out with accurate mass measurements by an Agilent 6210 TOF instrument. Measurements were carried out as described in section 3.3 sample preparation, with the extended gradient and with the injection of 10 µl of sample aliquots due to the lower sensitivity of this instrument. In this case measurements were carried out at three pre-defined fragmentor voltages. Spectra were taken

in the range of 50-1650 amu among which every five spectra were averaged. Extracted ion chromatograms for the supposed metabolites' calculated accurate masses were used for confirmation. Acceptance criteria were the following: difference between the calculated and measured accurate mass should be less than 10 ppm, and high similarity of the isotopic distribution.

3.5 Details of the environmental loading investigation

The two main streams encompassing Budapest from Buda side (Aranyhegyi- and Hosszúréti-stream) were sampled at eleven points in every three months, during a year. By seasonal sampling effects of different weather conditions and by sampling at different sites the contribution of nearby waste-water treatment plants and tributaries to the streams' pollution were investigated. In each case 3x500 ml of samples were taken and measured by the validated analytical method. Samples were spiked on-site with internal standard solution. Based on the initial measurements the calibration range was extended by one-fold.

When sampling estuaries the size of the concreted estuary and flow rate of the streams were also measured. From these data the actual run-off could be calculated. Based on the measured concentrations in the estuary samples and the calculated run-off data annual loading were estimated.

4. Summary of results

- Out of eleven different SPE tubes three were chosen experimentally.
- Applying post-extraction spike method a solid phase extraction method was developed for fast and effective sample preparation for 26 compounds from surface water.
- A selective and sensitive LC-MS/MS method was developed for the determination of 26 compounds simultaneously.
- The developed method was validated by means of determining selectivity, linearity, precision, accuracy, and limit of detection and of quantitation for each compound.
- The validated method has been applied for Danube river water samples.
- 21 metabolites were identified from surface water samples by predictive methods.
- Six of the identified metabolites were confirmed by accurate mass measurements.
- By seasonal sampling the target compounds were followed in stream water samples for a year. No seasonal variation could be observed.
- By sampling at different sites contribution of waste-water treatment plants to the streams' pollution was confirmed.
- Estimation of annual loading was based on the measured estuary concentrations and on the calculated run-off data. From these data several compounds' loading were estimated to be higher than a kilogram per year.

5. Scientific results of the PhD thesis

1. An SPE-LC-MS/MS method has been developed for the simultaneous determination of 26 compounds which are the active substances of the most often prescribed drugs' in Hungary.
2. Post-extraction spike method – formerly only applied in bio-analytical studies - was applied for the matrix effect investigation.
3. The developed method was successfully applied for the investigation of Danube river water samples.
4. By predictive mass spectrometry methods – without standards – twenty-one of the metabolites of my target compounds were identified and six of them were confirmed by accurate mass measurements.
5. By sampling stream waters contribution of waste-water treatment plants to the streams' pollution was confirmed.
6. Annual loading of the target compounds was estimated.

6. Publication list

6.1 Articles

Determination of antihypertensive and anti-ulcer agents from surface water with solid-phase extraction – liquid chromatography – electrospray ionization tandem mass spectrometry, R. Varga, I. Somogyvári, Zs. Eke, K. Torkos, Talanta 83 (2011) 1447-1454.

Identification of phase I metabolites of cardiovascular and anti-ulcer drugs in surface water samples with liquid-chromatography – mass spectrometry methods, R. Varga, Zs. Eke, K. Torkos, Talanta 85 (2011) 1920-1926.

Seasonal monitoring of cardiovascular and anti-ulcer agents' concentration in stream waters encompassing a capital city, R. Varga, I. Somogyvári, Zs. Eke, K. Torkos, Environ. Sci. Poll. Res., *submitted*

6.2 Posters

SPE-LC-MS/MS Method for the Determination of Cardiovascular- and Anti-ulcer Agents from Surface Water
Varga, R., Tölgyesi, L., Eke, Z., Torkos, K.
Balaton Szimpózium, Siófok, 2009

Solid Phase Extraction Methods for Pharmaceutical Residues from Surface Water Samples: Difficulties and Solutions
Renáta Varga, I. Somogyvári, Zs. Eke, K. Torkos
HTC-11 and HTSP Conference, Bruges, Belgium, 2010

Environmental Loading Studies: How to Estimate the Real Impact of Growing Pharmaceutical Consumption on Aquatic Fauna and Flora?
Renáta Varga, Iván Somogyvári, Zsuzsanna Eke, Kornél Torkos
HPLC-2011, Budapest, 2011