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Contributions to risk assessment of food and water contamination with pharmaceutical residues

PhD Thesis Resume

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Experimental results

Chapter 2

Simultaneous analysis of four sulfonamides in chicken muscle tissue by HPLC

Materials and methods

Apparatus

The HPLC system consisted of a UV/VIS detector (Waters USA). The HPLC column used was Zorbax SB-C18, 5 μm 4.6 x 250 mm.

Chemicals and reagents

The marker residue for SAs is represented by parent drugs. Standards and reagents used reagents were HPLC grade.

Tissue samples

Blank matrix came from previously unmedicated chickens, which were provided by the Biobase S.N. Pasteur Institute (Bucharest, Romania). Incurred samples came from medicated chicken, under normal farm conditions, which were provided by the Biobase S.N. Pasteur Institute.

Muscle tissue samples were collected and stored frozen (-17°C) until they were analysed.

Sample preparation

A volume of 30 mL acetonitrile was added to 10 g of minced and homogenised muscle tissue was weighed in a glass centrifuge tube. The sample was homogenised for 1 min in a vortex and centrifuged at 3500 rpm for 10 min. The supernatant was transferred into a pearshaped

flask. A total of 20 mL of acetone was added to the sediment before the mixture was sonicated for 10 min. The solution was centrifuged once again and the supernatant added into the same pear-shaped flask. The mixed solution was evaporated at 40°C until near to dryness. Afterwards, 5mL of dichloromethane was added, homogenised by vortexing and transferred into a test tube. The step after the addition of dichloromethane was repeated three times and the combined dichloromethane was dried at 40°C. The residue was reconstituted with 1ml of 50% methanol in di-sodium hydrogen phosphate solution (6 g/1000 mL) and mixed properly by vortexing. n-Hexane (2 mL) was added into test tube for defatting and vortexed before being removed. The remaining solution was filtered through the filter of 0.2 mm or no more than 0.45 mm, and was ready for injection into the HPLC system.

HPLC analysis

HPLC analyses were performed by using as mobile phase disodium hydrogen phosphate solution: methanol (75:25 v/v). Detection was performed at 245 nm.

LC-MS/MS

Identification and quantification of analytes were carried out on Waters 2695 liquid chromatography, equipped with a MS-MS Quatro Micro (Micromass, USA) tandem mass spectrophotometer. The X Bridge Shield RP18 column, 150mm x 2.1, 3.5 mm (Waters) was used for separation. Data acquisition was made in multiple reaction-monitoring mode. The transitions of two common product ions were monitored.

Results and discussion

Calibration curves were linear with very good correlation coefficients for concentration ranging from 30 to 150 mg kg⁻¹. The limits of detection (LOD) for sulfonamides ranged from 6.5 to 0.14 mg kg⁻¹. The recovery for spiked chicken muscle with 50–150 mg kg⁻¹ was more than 70%. The relative standard deviations (RSDs) of the sulfonamides for six measurements at 50, 100 and 150 mg kg⁻¹ were less than 15%. These parameters met the European Union criteria for method validation.

Analysis of real samples

Thirty samples of chicken muscles collected from a local market in Romania were investigated for SA residues using the HPLC method. Twelve muscle samples were found to contain sulfadiazine with a concentration level ranging from 300 to 180 mg kg⁻¹, a level which exceeded the regulated tolerance.

Chapter 3

Ultrasonic or Accelerated Solvent Extraction followed by U-HPLC- high mass accuracy MS for screening of pharmaceuticals and fungicides in soil and plant samples

Experimental

Chemicals, reagents and solutions

A mixture of different compounds belonging to different drugs classes: benzimidazole, tranquilizers, macrolides, sulfonamides, quinolones, penicilines, tetracyclines, non-steroidal anti-inflammatory drugs (NSAIDs), antiepileptic, lipid regulator, azole antifungals, polyene antifungals, mitotic inhibitor; azole biocides and fungicides, was chosen to develop the methods.

Sample collection

Twenty real samples, ten different soil samples and ten plant material samples (grass and corn crop) were collected from agricultural areas within the Netherlands. The sampling was carried out in May 2011).

Methods

Accelerated solvent extraction (ASE) procedure

ASE was performed on an ASE 200 system equipped with a solvent selector (Dionex, ASE 350, USA). Approximately 5g of dried soil sample or 3 g of chopped plant material were weighed into 33 mL extraction cells (lined with glass microfiber filters from Whatman, Maidstone, UK) and mixed with 5 g Diatomaceous earth to increase the contact-surface between soil particles and extraction solvent and prevent clogging of the extraction cell. The optimized operating conditions were: extraction temperature 50°C; extraction pressure 1500 psi; two cycles of 5 min each, static extraction; 50% flush volume; and a 60-s purge with nitrogen. Extraction solvent used was acetone/citric acid 0.2M (50:50), (pH adjustment at 4.5

with sodium hydroxide) for soil sample and methanol/citric acid 0.2M (50:50), (pH adjustment at 4.5 with sodium hydroxide) for plant material sample.

After the extraction, 100 μL Na_2EDTA 1M was added to each sample extract and Milli-Q water was added until a final volume of 50 mL volume. The sample was centrifuged for 15 min at 2800 x g (Falcon 6/300 MSE Refrigerated Centrifuge, London, UK). An aliquot of the final extract, corresponding with 1 g of sample (10 mL soil extract and 16.6 mL plant material extract) was taken and diluted with Milli-Q water to a final concentration of 10% organic solvent (50 mL for soil and 85 mL for plant samples).

SPE procedure

An SPE procedure was applied in order to clean and concentrate the extract, using Strata X, 200 mg/6 mL, reverse phase, SPE cartridge.

Instrumentation

One LC–full scan MS configuration was used: Exactive LC-MS Mass Spectrometer powered by Orbitrap Technology from Thermo Fisher. For separation an ultra-performance Acquity U-HPLC Column C18 (100 x 2.1 mm, 1.8 μm). Detection was based on calculated exact mass and on retention time of target compounds.

Method parameters

In order to compare the two extraction method the following parameters were considered: the absolute recovery, level of detection, selectivity and mass error (Δppm) of the measurements for the selected analytes.

Validation

The level of interest, based on literature (EMEA/CVMP/ERA/418282/2005) was considered 100 $\mu\text{g kg}^{-1}$. A 50 $\mu\text{g kg}^{-1}$ value was considered the screening target concentration (1/2 of level of interest).

LC- MS/MS confirmation

Triple quadrupole based precursor scans were performed on a Micromass Quattro Ultima MS/MS (Waters, Milford, MA, USA) equipped with an electrospray interface, coupled to an LC-20AD (Shimadzu, USA). For the final confirmation of the identity the criteria described in EU 2002/657/EC were applied, including the detection of two fragment ions with the appropriate ion-ratio.

Validation and sample analysis

The recoveries from soil samples were >70% for more than 68% of the compounds. The levels of detection were $\leq 10 \mu\text{g kg}^{-1}$ for 93% of the compounds tested. The recoveries from

plant material were >70% for 64% of the compounds tested. The levels of detection were $\leq 10 \mu\text{g kg}^{-1}$ for 66% of the compounds.

The concentration of $100 \mu\text{g kg}^{-1}$, (the trigger value for phase two of the risk assessment in EU regulation - EMEA/CVMP/ERA/418282/2005), was set as a concentration to be relevant for the environment the screening target concentration was established at $50 \mu\text{g kg}^{-1}$ ($1/2$ of the interest value). For soil sample the evaluation of the result proves that the detection capability $\text{CC}\beta$ (concentration at with β error 5%) is lower than $50 \mu\text{g kg}^{-1}$ for all analyzed compounds. For plant material sample the evaluation of the result proves that the detection capability $\text{CC}\beta$ is lower than $50 \mu\text{g kg}^{-1}$ for all analyzed compounds, with the exception of itraconazole, fluquiconazole and prothioconazole.

Validation was based on real sample which may be contaminated. Not unexpectedly, because of the intensive usage in veterinary medicine, one soil sample was founded contaminated with oxytetracycline.

Chapter 4

Accurate mass screening of pharmaceuticals and fungicides in water by

U-HPLC – Exactive Orbitrap MS

Experimental

Chemicals, reagents and materials

A mixture of different compounds belonging to different drugs classes like: benzimidazole, tranquilizers, macrolides, sulfonamides, quinolones, penicilines, tetracyclines, non-steroidal anti-inflammatory drugs (NSAIDs), antiepileptic, lipid regulator, azole antifungals, polyene antifungals, mitotic inhibitors; azole biocides and fungicides, were chosen to develop the methods.

Samples collection and pre-treatment

Samples were selected in order to provide real environmental matrix for method development and validation. Random samples include samples of river, lakes, sewage, and ground water, from areas with important agricultural activities in The Netherlands and Romania A drinking water sample from Romania was included. The water samples were filtered through a paper filter (Whatman, Maidstone, England) and stored at 4°C in refrigerators until they were extracted.

Methods

Sample clean-up and concentration

An SPE procedure was applied in order to clean and concentrate the extract, using Strata X, 200 mg/6 mL, reverse phase, SPE cartridge. The cartridge was previously preconditioned with 6 mL methanol followed by 6 mL water. Before loading the cartridge, at the sample of 100 mL of water 10 µL of Na₂ EDTA solution at 0.5M was added and pH was adjusted to 3 with acetic acid. After sample application, the column was washed with 6 ml water, followed by 6 mL methanol/water 30% (v) and vacuum dried for 3 minutes. The analytes were eluted

with 6 mL methanol. The eluate was concentrated by evaporation under flow of high purity nitrogen, in a water bath at 42°C (Turbo Vap LV Evaporator, Zymark, USA), and resolved in 25 µL methanol and 225 µL water.

Instrumentation

One LC–full scan MS configuration was used: Exactive Orbitrap LC-MS Mass Spectrometer at 50,000 FWHM coupled to an U-HPLC system.

Validation study

Applying the final method, 74% of the compounds show recoveries higher than 80%, 15% of the compounds show recoveries between 60% and 80% and 7% of the compounds show recoveries between 40% and 50%. One of the compounds (itraconazole) had a recovery lower than 10% and nystatin wasn't detected. The level of detection was 10 ng L⁻¹ for 61% of the compounds, 50 ng L⁻¹ for 32% and 100 ng L⁻¹ for 5%.

In-house validation, based on EU guidelines, proves that the detection capability $CC\beta$ is lower than 10 ng L⁻¹ (for β error 5%) for 37% of the compounds, lower than 50 ng L⁻¹ for 35% of the compounds, and lower than 100 ng L⁻¹ for 14% of compounds.

For a total of 6 (14%) of compound could not be validated at maximum validation level of 100 ng L⁻¹: nystatin, dicloxacilin, itraconazole, fluquiconazole, terconazole and prothioconazole.

LC- MS/MS confirmation

The sample extracts suspected to contain residues of pharmaceuticals or fungicides by the screening method were (re)analysed by UPLC-MS/MS for the confirmation of the proposed identity.

Beyond the usual contaminants reported for water (Stolker et al., 2004): sulfamethoxazole, carabamazepine, diclofenac, erythromycin, this study was confirmed the presence in water of antifungal drugs like ketoconazole, fluconazole or thiabendazole. All groundwater samples were found to be contaminated with at least one pharmaceutical compound. Surface water was found to be most highly contaminated, but none of the tested analytes was present at levels of over 100 ng L⁻¹.

Screening results showed that Romanian rivers are contaminated with residues of pharmaceuticals, both for human use and veterinary worth mentioning is the presence of diclofenac in drinking water at a concentration approximately 50 ng L⁻¹, which exceeds the maximum tolerance for human use.

Chapter 5

Uptake of oxytetracycline, sulfamethoxazole and ketoconazole, from fertilized soils by plants

Experimental

Tested Soil

Two sandy soils were used in the study. The soil was collected in the summer of 2011 from two farms located close to Harskamp and Wageningen, The Netherlands.

Test plants

A commercial available mixture of grass species seeds (75% English Ray Grass, *Lolium perenne*, 25% Field Meadow Grass, *Poa pratensis*, *Poa trivialis*) and watercress (*Nasturtium officinale*) were selected for the study. The plants were planted from the seeds.

Test Chemicals and Soil Spiking

Three test substances were selected to cover different veterinary classes and different environmental properties.

Uptake studies

For this study plastic containers were used filled with soil. To each 2.5 L plastic container (Ø18.5cm) 2 kg of soil was added. The experiment was designed in duplicate. For each soil sample/plant combination, three pots were prepared (two spiked and one control soil), which resulted in a total number of 24 pots.

Compounds analysis

Extraction was performed on an accelerated solvent extractor, ASE 200 system equipped with a solvent selector. The optimized operating conditions described in Cap. 3.

An SPE procedure was applied in order to clean and concentrate the extract. The validated procedure is described Cap. 4.

Recovery of the three compounds investigated by spiking plant material was: 53% for oxytetracycline, 59% for ketoconazole, and 60% for sulfamethoxazole.

The sample extracts were analysed by LC-MS/MS for the confirmation of the proposed identity. The detection limit for sulfamethoxazole, oxytetracycline and ketoconazole, based on calibration curves, were $4.4 \mu\text{g kg}^{-1}$, $2.6 \mu\text{g kg}^{-1}$, $1.1 \mu\text{g kg}^{-1}$ respectively.

Results

Experimental uptake by plants

Both grass and watercress took up sulfamethoxazole and ketoconazole.

Sulfamethoxazole was detected in all samples, in concentration levels ranging from 7 to $21 \mu\text{g kg}^{-1}$ for grass and 4 to $7.5 \mu\text{g kg}^{-1}$ for watercress. No major differences were observed between the uptake from the two spike level. Watercress presented a lower absorption.

For ketoconazole, the results were less homogenous. In two samples of grass and five sample of watercress the compound wasn't detected. The rest of the samples showed low absorption. Oxytetracycline wasn't detected in any sample.

The study demonstrates the ability of plants to uptake pharmaceutical compounds from fertilised soils.

The effective concentration of test pharmaceuticals available for plant uptake is considered to be the concentration in soil interstitial (pore) water, which can be estimated and calculated. Soil composition influences by soil organic matter (SOM) the concentration of the pharmaceutical in pore water. The concentration of the pharmaceutical in different part of the plants may or may not be in equilibrium with external water.

The partition limited model gives satisfactory results for the passive transport of sulfamethoxazole. Taking into account the oxytetracycline capacity to form strong complexes with the soil mineral low absorption of oxytetracycline can be explained. In case of highly lipophilic compounds like ketoconazole, there is a reverse correlation between the quasi-equilibrium factor (α_{pt}) and K_{ow} value, and therefore low absorption from the soil.

Chapter 6

A chain modeling approach to estimate the impact of soil pharmaceuticals pollution on human exposure

Scenarios parameters.

Considering the agricultural practice of using manure as fertilizer in this chapter, through mathematical modeling, pharmaceuticals transfer to food chain was evaluated.

In a critical analysis of the models proposed by the EMEA, Montfort (2005), adjusted these models with regard to the pharmacokinetic data, degradation of the substance, life cycle duration of different species selected, and specific agricultural practices, proposing different formulas for each situation.

An algorithm for the model proposed by Montfort was adapted in this study for calculating the predicted concentration of contaminant in the soil

The scenario parameters and variables refer to contaminants emission slurry production, storage time, immission into soil, plant uptake and transfer to meat and milk and bioaccumulation process.

Scenarios chosen for this modelling study includes three active substances from different therapeutic classes with different physicochemical properties, administered in therapeutic and sub therapeutic doses , three species of poultry: chicken broilers, turkeys, laying hens. It was assumed that there is a contamination of a grazing land due the fertilization with contaminated slurry from poultry farm.

The Dutch model assumes that within the period March-September the phosphate/nitrogen immission standard is filled in four events: one at the start of the season. The slurry spread at

the start of the season has been stored for 152 days, the other batches for 71 days (Montfort, 2005).

The model consider one treatment/cycle life for the poultry, and take in account the excreted fraction of active substance and it's degradation rate in soil and slurry. No metabolites were considered.

The model considered that a herd of cattle graze freely on contaminated land, and estimates the amount of pharmaceutical residues in meat and their milk.

Limited partition model was considered for plant uptake.

A logarithmic regression was used to estimate the steady-state concentrations for biotransfer of the chemicals from plants to beef meat and milk.

The results confirm the possibility of contamination of meat and dairy milk due the ingestion of contaminated feed by the animals. The estimated values obtained for meat ranging from 0 to 2 ng kg⁻¹ for oxytetroccline, from 0,824 to 0,11 µg kg⁻¹ for sulfamethoxazole and from 32,4 to 0,05 µg kg⁻¹ for ketoconazole. The estimated values obtained for milk ranging from 0 to 0,3 ng L⁻¹ for oxytetroccline, from 0,122 to 0,017 µg L⁻¹ for sulfamethoxazole and from 4,8 to 0,009 µg L⁻¹for ketoconazole.

The model is particularly sensitive to variations is log Kow and soil organic matter fraction.

Chapter 7

Risk characterization of dietary intake of food and water contaminated with residues of pharmaceutical compounds.

Pharmaceutical substances in drinking water

Toxicological limits of pharmaceutical contaminants in drinking water are considered as 10% of the ADI or MRL in milk (Montfort, 2005). An average consumption of 2 L water daily for a person of 60 kg weight is considered.

If ADI and MRL are not available, a temporary ADI is calculated from the lowest dose with pharmaceutical effect, using a safety factor of 100.

For the selected compound the following maximum limits in drinking water were considered:

Sulfamethoxazol - maximum residue level in drinking water: $75 \mu\text{g L}^{-1}$.

Oxytetracycline- maximum residue level in drinking water: $75 \mu\text{g L}^{-1}$.

Ketoconazol- maximum residue level in drinking water: $2 \mu\text{g L}^{-1}$.

Daily estimated dose

Estimate the EDI resulting from food basket amounts of relevant tissues/products containing residue water and vegetal contamination the result will be the following:

$$EDI = \sum(MRL_{carne} * 0,3 + MRL_{ficat} * 0,1 + MRL_{rinichi} * 0,05 + MRL_{grasime} * 0,05 + MRL_{lapte} * 1,5 + MRL_{apa} * 2 + C_{veg} * 0,5)$$

$$EDI = 357,5 \mu\text{g zi}^{-1}.$$

Not exceeding ADI, the daily amount ingested can have long term effects, cause allergic reactions or hypersensitivity, haematological complications in patients with glucose-6 phosphate dehydrogenase deficiency, or bacterial resistance development.

CONCLUSIONS

The major goal of this thesis was to contribute to the current research by developing new method for pharmaceuticals detection in food and in the environment and to evaluate their impact on food chain.

A multi-residue method was developed for the simultaneous determination of sulfadiazine, sulfamethoxazole, sulfadimethoxine and sulfaquinoxaline in chicken muscle tissue by HPLC-UV, after extraction with organic solvents. Analyses of real sample of chicken muscles collected from a local market demonstrated the potential exposure of the consumers to sulphonamides residue, in concentration level which exceeded the regulated tolerance.

Developing multimetode capable to determine different classes of pharmaceuticals and biocide in environmental matrix is a requirement of the current environment risk assessment.

A multi-method was developed and validated for screening purposes for forty-three pharmaceutical and fungicides compounds, in water, soil and plant material, in one single full scan MS method, using bench top U-HPLC-Exactive Orbitrap MS.

The study was confirmed the presence of pharmaceutical compounds and antifungal drugs in water collected from The Netherland and Romania.

Oxytetracycline was detected in one soil sample collected from The Netherlands, indicates that fertilisation probably affect the contamination of the area.

The potential for a range of veterinary medicines to be taken up from soil by plants was investigate. Plant analyses data indicated absorption of sulfamethoxazole and ketoconazole by both plants species used in study, in the concentration range of 5-10 $\mu\text{g kg}^{-1}$. The uptake is compound specific.

Through the ubiquitous of pharmaceutical compounds in the environment, indirect contamination evaluation of consumer exposure is needed.

A modelling approach was applied to evaluate the impact of soil contamination on introduction of this compound in food chain. The chain of models was applied to nine different scenarios.

From the predictive data it can be concluded the level of the human exposure do not exceed ADI.

Original contributions and future research directions

During the doctoral studies the following analytical methods were developed:

- method for the determination of four sulfonamides in chicken muscle tissue using high-performance liquid chromatography (HPLC) with UV detection
- method for the simultaneous scanning of forty two compounds (pharmaceuticals,azole biocides and fungicides) in surface and groundwater sample using U-HPLC-Exactive Orbitrap MS technologie.
- method for the simultaneous scanning of forty two compounds (pharmaceuticals,azole biocides and fungicides) in soil and fresh vegetal material samples, using U-HPLC-Exactive Orbitrap MS technologie.

Different extraction techniques used (liquid-liquid extraction by ultrasonification or accelerated solvent extraction), purification and concentration by solid phase extraction were optimized to obtain the highest recovery and to minimize the matrix effect.

All analytical methods presented in this thesis are original and have been validated by following the legislation requirements:

- Prospective validation of the sample preparation and detection methods.
- Data processing of the control samples to determine accuracy (recovery).
- Retrospective validation to assess repeatability and variability during the study.

Antibiotics plant uptake study has been contribute to research in the field by using a new compound (ketoconazole), and by the comparative approach. The experimental values were compared with the estimated values of a limited partition model. The relation between physicochemical properties of the compounds and plant uptake was highlighted.

A chain modeling approach was elaborated to estimate the impact of soil pharmaceuticals contamination on human exposure. The model adapted equations from the literature to concrete situations, running different scenarios.

The research has revealed that pharmaceutical residues can cause a hazard in food, due the non-compliances to good veterinary practice requirements and / or environmental contamination.

Considering the detection of residues as: diclofenac, sulfamethoxazole, carbendazim, carbamazepine, tebuconazole thiabendazol in Danube water samples, including drinking water, a future direction for study may be regular monitoring of the pharmaceutical

compounds, leading to contamination assessment of the river and the sediments. Also the contamination possible effects on adjacent ecosystems, including impacts on human health should be evaluated.