Analysis and Fate Assessment of Sulphonamides in the Environment

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Abstract. Since the last ten years there has been a growing interest in the research focused on the residues of all pharmaceuticals in the environment. It has been proven, that the residues of these substances may pose a real threat not only to ecosystems, but also to human health, as for example antimicrobials lead to the formation of the dangerous phenomenon of bacteria resistance and thereby decrease in efficacy of the treatment of many bacterial diseases. For these reasons there is an ongoing research aimed at better understanding of the potential adverse environmental effects, including the degree of contamination, mobility, bioavailability and effects on the environment. Therefore, the aim of our study is to present the overview on our previous studies concerning the development of analytical methods used in the exposure assessment as well as on the evaluation of the environmental fate (including soil sorption, hydrolysis and ecotoxicological studies) of the residues of sulphonamides (SAs) – pharmaceuticals widely used in veterinary.

Keywords: Environment, sulphonamides, analysis, soil sorption, hydrolysis, ecotoxicity.

1. Introduction

Thanks to their low cost and their broad spectrum of activity in preventing or treating bacterial infections, sulphonamides (SAs) are one of the oldest groups of veterinary chemotherapeutics, having been used for more than fifty years. To a lesser extent they are also applied in human medicine. After tetracyclines, they are the most commonly consumed veterinary antibiotics in the European Union. As these compounds are not completely metabolized, a high proportion of them are excreted unchanged in feces and urine. Therefore, both the unmetabolized antibiotics as well as their metabolites are released either directly to the environment in aquacultures and by grazing animals or indirectly during the application of manure or slurry [1]-[3].

SAs are fairly water-soluble polar compounds, the ionization of which depends on the matrix pH. All the sulphonamides, apart from sulfaguanidine, are compounds with two basic and one acidic functional group. The basic functional groups are the amine group of aniline (all the SAs) and the respective heterocyclic base, specific to each SA. The acidic functional group in the SAs is the sulfonamide group. With such an SA structure, these compounds may be described by the pK_{a1}, pK_{a2} and pK_{a3} values corresponding to the double protonated, once protonated and neutral forms of SA [3], [4].

Due to their properties, after disposal in soils, these compounds may enter surface run-off or be leached into the groundwater, which explains why in the last ten years they have been regularly detected not only in terrestrial environments but mainly in aquatic compartments [1]-[4]. Although SA concentrations in environmental samples are quite low (at the ppt or ppb level), they are continuously being released [1]-[3]. Therefore, the kind of exposure organisms may be subjected to will resemble that of traditional pollutants (e.g. pesticides, detergents), even those of limited persistence. Consequently, SAs as well as other pharmaceuticals may be considered pseudo-persistent. Although their presence in the environment has been widely discussed in the scientific community in recent years, we still know very little not only about the

behaviour and fate of these compounds in the environment but also of their effects on whole ecosystems and human health.

These compounds are designed to exhibit a specific pharmacological action (SAs competitively inhibit the conversion of *p*-aminobenzoic acid, PABA) by inhibiting the biosynthetic pathway of folate (an essential molecule required by all living organisms), so they not only affect bacteria (target organisms) but can also have unknown effects on environmentally relevant non-target organisms, such as unicellular algae, invertebrates, fish and plants. Belonging to different trophic levels, these taxonomic groups may be exposed to by SAs to various extents [5]-[8].

For all the above mentioned reasons the detection, determination and analysis of the fate of these pharmaceutically active compounds in different compartments of the environment are some of the main tasks of modern analytical and environmental chemistry. Hence, they were the main objectives of our study.

2. Analytics of Sulphonamides in the Environmental Samples

According to the literature data, the most frequently used analytical technique for determining pharmaceuticals in the environment is liquid chromatography coupled with mass spectrometry (LC-MS) or tandem mass spectrometry (LC-MS/MS) [9]. Undoubtedly, its great advantages are its universality and the much less complicated sample preparation procedure; moreover, it can fairly easily be coupled on-line with sample preparation techniques (e.g. SPE). Much effort has been expended on increasing the number of analytes determined in a single measurement run, improving the sensitivity and selectivity of the methods, and shortening the time of analysis. Moreover, special emphasis should be placed on the development of reliable qualification methods that take into account the identification criteria recommended by Commission Decision 2002/652/EC [10]. On the other hand, liquid chromatography with spectrophotometric detection, despite not fulfilling the above requirements, is applicable to the study of the environmental fate of pharmaceuticals, and – in view of the wide availability of environmental laboratories – can be used for the routine determination of drug residues in less complex environmental samples. For these reasons, our aim was to develop a method for determining twelve sulphonamides in environmental samples using LC–MS/MS in multiple reaction monitoring (MRM) mode, which fulfils the requirements of Directive 2002/657/EC [11], and a method based on HPLC-UV for determining the above mentioned compounds for routine analysis and fate/modeling studies [12].

Depending on the class of pharmaceuticals, EU Commission Decision 2002/652/EC requires a minimum of 4 identification points (IP) for confirming the presence of Group A drugs, and a minimum of 3 IPs for Group B drugs, when GC-MS or LC-MS are used for their determination (IPs correspond to precursor and product ions). For antibiotics and chemotherapeutic agents, which include sulphonamides, 3 IPs are required. By using a low-resolution mass spectrometer (QqQ, IT) and identifying a pseudo-molecular ion one can obtain 1 IP and 1.5 IP for every fragmentation ion. This means that when working in MRM mode and selecting for every compound a pseudo-molecular ion as a precursor ion and two pseudo-molecular ion → product ion transitions, four IPs can be obtained, which fulfils the above requirements [10].

The development of a method for determining twelve sulphonamides in environmental samples using LC-MS/MS in MRM mode [11] was based on the selection of the optimal conditions for the mass spectrometric detection as well as chromatographic separation conditions. The parameters were optimized manually, separately for each compound. Working in full scan MS, all the MS/MS parameters were tested in order to obtain the most intensive precursor ions $[M+H]^+$ for every compound. Finally, Electrospray Ionization (ESI) and the positive ion mode were chosen for the LC-MS/MS measurements, because in such conditions the high-intensity pseudo-molecular ion → product ion transitions took placed for every compound and could be used in MRM mode. It should be pointed out that for each sulphonamide, three transitions were suggested instead of two, which yielded 5.5 IP [11], thereby significantly enhancing the reliability of the qualitative analyses. Next, experiments involving the selection of the different mobile phases, elution programme, eluent flow rate, injection volume, column temperature and mobile phase pH were carried out to establish the optimal chromatographic separation conditions. Although complete chromatographic separation is not necessary for selective MS–MS detection in MRM mode, it considerably improves detectability and reduces ion suppression; this is one of the main reasons for performing such

experiments. Moreover, the different retention time for each analyte is very useful for identifying target compounds in the samples investigated. Each MRM transition was tested for its usefulness not only for the qualitative but also for the quantitative analysis of sulphonamides in the environmental samples. Finally, the method based on the highest-intensity transition for each compound was proposed and validated in order to confirm its satisfactory validation parameters [11]. Moreover, in order to determine sulphonamides in the environmental samples at this stage of our study different extraction procedures (based on solid phase extraction (SPE) technique) were tested. Furthermore, this method was fully characterized with the respect to matrix effects and was successfully applied to the analysis of different environmental (drinking waters, surface waters, sea waters, soil and marine sediments) samples collected from the north part of Poland or from the Gulf of Gdańsk (Baltic Sea). These results confirmed that the final determination by LC-MS/MS in MRM mode is suitable for the trace analysis of sulphonamides in environmental samples.

As already mentioned, LC-MS/MS is an extremely valuable tool for the trace analysis of pharmaceuticals in the environment, but unfortunately, despite its many advantages, this technique has one serious drawback: it is expensive, so the relevant instruments in laboratories are much less widely available. Hence, the method for the determination of ten sulphonamides using HPLC-UV (which is the main analytical tool in most commercial and research laboratories) was also developed. This method was also used to test the influence different variables affecting the extraction process (determined by the absolute recoveries) such as sea water salinity and the humic acid content. It was shown that the presence of humic acids influences the average absolute recoveries of analytes: the average recovery for 10 sulphonamides extracted from artificial seawater was 92.9 % in comparison to 85.8 % for water containing 2 mg/L humic acids and 82.4 % for water containing 10 mg/L of these acids [12]. Furthermore, it was found that the absolute recovery with one washing run was sufficient to recover the whole salinity spectrum (7, 15 and 35 PSU). Very good absolute recoveries were obtained for all salinities with a single such run (80-113%), which indicates that this SPE procedure can be very effective, even in highly saline waters and in the presence of humic acid.

This HPLC-UV method developed here, with minor modifications, was subsequently used for analysing the fate of sulphonamides in the environment [4], [12], [13].

3. Fate Assessment of Sulphonamides in the Environment

In recent years, there has been growing interest in the analysis of the occurrence and fate of pharmaceuticals, the continual entry of which into the environment may have significant, long-term effects on the stability of ecosystems. It has been proven, for example, that the residues of antibiotics may pose a real threat not only to ecosystems, but also to human health, as they lead to the formation of the dangerous phenomenon of bacterial resistance, thereby diminishing the efficacy of the treatment of many bacterial diseases. According to the European Medicines Agency (EMEA) reliable data for environmental risk assessments (ERA) of pharmaceuticals should fulfil the regulatory demands given in the EMEA guideline on environmental impact assessment for veterinary medicinal products [14]. According to these regulations and the data quality criteria given by Klimisch *et al.* [15] such information is classified into three categories of reliability: Category I (RI 1) - reliable data, Category II (RI 2) - less reliable information, and Category III (R1 3) - unreliable data. It was also stipulated that the environmental effects/fate of pharmaceuticals must be investigated using validated methods, preferably from Category I, which includes data which were obtained or generated according to internationally accepted test guidelines (e.g. OECD, ISO), or which were based on a specific testing guideline (e.g. DIN) or in which all the parameters described are closely related/comparable to a guideline method. Taking into account the above information, the main objective of our further studies was to: (*i*) gain an understanding of the mechanism of sorption to soil of the three selected sulphonamides, (*ii*) determine the hydrolytic stability of twelve of them, (*iii*) evaluate the risks that they might pose to different organisms as well as to human health. In order to obtain the most reliable ERA data (Category I), it was decided to perform these experiments according to the recommended procedures (e.g. OECD, ISO, DIN).

3.1. Sorption Study

This study was performed in accordance with procedure OECD 106, in which a static model was applied. Initially, two sulphonamides – sulphadimethoxine (SDM) and sulphaguanidine (SGD) [13] – were selected for this investigation, the former because of its widespread use in veterinary medicine, the sparse data regarding its sorption potential and the highest toxicity to duckweed (*Lemna minor*) among the twelve sulphonamides tested [16], and the latter because, according to the report of the National Veterinary Institute in Puławy on the use of antimicrobials in the treatment of pigs in Poland in 2010, it is the most frequently used sulphonamide in medicated feeds [17]. Moreover, there are no studies describing SGD behaviour in soils. Furthermore, even though these two compounds belong to the same group of pharmaceuticals, they differ in their chemical structures and physico-chemical properties (especially their pK_{a2} values: 5.9 for SDM and 12.1 for SGD. To achieve a better understanding of the influence of soil properties on SDM and SGD sorption, three natural soil types – sandy-clayey silt, alluvial soil and beach sand– differing in their pH, organic content, particle size distribution and cation exchange capacity were used in this study [13]. The shapes of the sorption isotherms of SDM and SGD in the three soils indicated a decreasing tendency to be sorbed with increasing initial concentration. This was probably due to the fact that the high energy sites in the soil are occupied first, followed by adsorption at lower energy sites, which can result in total saturation of the active centres or in block access to other centres. The equilibrium sorption coefficients (Kd) for SDM and SGD differed not only from each other but also depended on the type of soil [13]. Thus, the estimated Kd values ranged from 0.31 to 107.53 mL/g for SDM and from 1.03 to 30.99 mL/g for SGD, depending on the soil characteristics. A relationship between the organic content of the soils and the sorption/desorption potential of SDM and SGD was observed. Desorption of these compounds was inversely proportional to their sorption, which means that the compounds strongly sorbed to the soils (high Kd values) had a lower desorption potential. Determination of sulphonamide concentrations in the aqueous and soil fractions enabled the shape of the Freundlich and Langmuir isotherms to be established for SGD and SDM in the three soils. It was established that both models were suitable for describing the sorption behaviour of these compounds in the soils, as indicated by the high regression coefficient for linear. The 1/n values for all soils were below 1 (in the 0.67-0.89 range), which confirmed the earlier assumption that the sorption of these compounds to soil has a saturated character – the sorbates are bound first at the high energy sites of the soil, and then at lower energy sites.

The influence of pH and ionic strength on the sorption coefficients of SDM and SGD on the three soils was also investigated. It was found that a decrease in pH enhanced sorption of sulphonamides onto soils, whereas the use of natural fertilizers (usually alkaline) or introducing these compounds in manure increased their mobility in the environment. On the other hand, the results showed that the equilibrium sorption coefficients of these sulphonamides decreased with increasing ionic strength. The decrease in ionic strength strongly influenced SDM and SGD sorption to soils with a high organic content, but had only a minor effect on their sorption onto soils with the lowest organic content. In summary, the results demonstrated that sulphaguanidine and sulphadimethoxine belong to a class of drugs with a relatively low potential sorption to soils. These results indicated the significant bioavailability of these drugs in soils and their ability to rapidly reach surface waters and/or infiltrate ground water once they have entered soils [13].

3.2. Hydrolytic Studies

The majority of the earth's surface is covered by water in the form of oceans, seas, lakes or rivers. Hence, chemical pollutants (e.g. pharmaceuticals) entering the environment can be degraded via hydrolysis [18]. Because water is present in great excess compared to the concentrations of the chemicals, this type of reaction is usually described as a pseudo-first order reaction at fixed pH and temperature, and may be influenced by acidic or basic species H_3O^+ and OH^-. Even though hydrolysis is one of the most common reactions controlling abiotic degradation, including that of pharmaceuticals, the available information on this process is very limited, especially that obtained in accordance with OECD procedure 111, which is obligatory in any environmental risk assessment (ERA). Therefore, hydrolytic stability of twelve sulphonamides was evaluated since, as already stated, can easily enter the aquatic ecosystem [4]. During hydrolysis, persistent and toxic conversion products may form, which should be accounted for in the ERA of the parent compounds. For this reason, knowledge of the structures formed in this process is an important part of such a study. The investigations were based on OECD procedure 111. This consists of two steps:

a) a 5-day preliminary test performed at 50 °C and pH 4.0, 7.0 and 9.0,

b) a higher tier (advanced) test performed for the substances found to be unstable by the preliminary test.

These experiments are carried out at three different temperatures but at the same pH values at which the target substances in the preliminary test were degraded $\geq$ 10%. In the next step of this study, to test the kinetics of sulphonamide hydrolysis, the logarithms of the sulphonamide concentrations ln C_t/C_0 were plotted against time (t) and the slopes of the resulting straight lines were analysed. Sulphanilic acid, sulphanilamide or aniline were identified as the degradation products of these compounds [4]. Nevertheless, in summary, it was found that under typical environmental conditions (pH 6.0 to 8.5; temperature from 0 to 35 °C) sulphonamides are hydrolytically stable and may therefore accumulate in aquatic systems.

3.3. Ecotoxicity Evaluation

Unfortunately, relevant ecotoxicity data for SAs are not available. Knowledge of the potential effects of SAs on the environment is very limited. Moreover, most current studies investigate acute effects and have dealt mainly with sulfamethoxazole, one of the most common SAs, which is used in both veterinary and human medicine [5], [6], [8]. However, since many drugs are designed to affect specific biological pathways in the target organism at relatively low doses and exposure concentrations, the consequences of the subacute effects of pharmaceuticals could be of much greater concern than the acute effects in non-target animals. Therefore, in an attempt to enrich our hitherto limited knowledge of the potentially deleterious effects of the twelve SAs most commonly used in veterinary medicine on the environment a flexible (eco) toxicological test battery was used [16]. This included enzymes (acetylcholinesterase and glutathione reductase), luminescent marine bacteria (*Vibrio fischeri*), soil bacteria (*Arthrobacter globiformis)*, limnic unicellular green algae (*Scenedesmus vacuolatus*) and duckweed (*Lemna minor*), in order to take into account both the aquatic and terrestrial compartments of the environment, as well as different trophic levels. It was found that SAs are not only toxic towards green algae (EC_{50} = 1.54 − 32.25 mg/L) but have even stronger adverse effect on duckweed (EC_{50} = 0.02 − 4.89 mg/L) than atrazine − herbicide (EC_{50} = 2.59 mg/L). As a result first comparative (eco) toxicological analysis investigating different SAs, enabling the potential effects and origins of these compounds in the environment was presented [16].

4. References

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