Development of LC-MS/MS Methods for the Analysis of Chiral and Achiral Pharmaceuticals and Metabolites in Aqueous Environmental Matrices

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Abstract

This thesis describes the development of liquid chromatography tandem mass spectrometry (LC-MS/MS) methods for the trace analysis of active pharmaceutical ingredients (APIs) and their metabolites in aqueous environmental matrices. The research was focused on the development of chiral LC-MS/MS methods for the analysis of fluoxetine and metoprolol, as well as their chiral metabolites in environmental water samples. A method was also developed for the achiral compounds, diazepam and nordiazepam.

The LC-MS/MS methods were validated by the use of the isotope-labeled compounds. As these isotope-labeled compounds were not found in the wastewater samples, the validation could be assessed at trace level concentrations in the actual matrices in which the analytes were detected.

The analytes were extracted from the water samples using solid phase extraction methods. Different types of solid phase extraction sorbents were evaluated. Fluoxetine and norfluoxetine were extracted through the use of a mixed mode polymeric based extraction sorbent. A hydrophilic and lipophilic balanced sorbent was employed for the simultaneous extraction of metoprolol and its metabolites, the base α-hydroxymetoprolol and the acidic metabolite deaminated metoprolol. Moreover, silica based C18 extraction discs were applied for the sample preparation of diazepam and nordiazepam.

The chromatographic separations were conducted in reversed phase LC with MS compatible mobile phases. The enantiomers of fluoxetine and norfluoxetine were simultaneously separated using the chiral stationary phase (CSP), αα,α-acid glycoprotein (AGP). The Chiral AGP column was also applied for the separation of the enantiomers of deaminated metoprolol. For the simultaneous separation of the metoprolol enantiomers and the four stereoisomers of α-hydroxymetoprolol, the cerelaceinsehase (CBH) protein based CSP was used. An octadecyl silica based LC column was applied for the separation of diazepam and nordiazepam.

The analytes were detected by the use of tandem quadrupole mass spectrometry operating in selective reactive monitoring mode. High resolution MS, employing a quadrupole time-of-flight (QqTOF) mass analyzer, was utilized for the identification of an unknown compound in wastewater samples.

The APIs and their metabolites, as well as their respective enantiomers, were quantified in raw and treated wastewater from Uppsala, Sweden along with surface water from the River Fyris in Uppsala.

Keywords: Environmental water matrices, chiral separation, LC-MS/MS, trace analysis, method development, active pharmaceutical ingredients, metabolites

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Be fearless and
always put your best foot forward

Manolo Blahnik
List of Papers

This thesis is based on the following papers, which are referred to by the Roman numerals assigned below.


III  Barclay, Victoria, K.H., Tyrefors, Niklas, L., Johansson, I. Monika, and Pettersson, Curt, E. Chiral analysis of metoprolol and two of its metabolites, α-hydroxymetoprolol (H 119/66) and deaminated metoprolol (H 104/83), in wastewater samples using liquid chromatography-tandem mass spectrometry. In manuscript

IV  Barclay, Victoria, K.H., Tyrefors, Niklas, L., Johansson, I. Monika, Fransson, Dick, and Pettersson, Curt, E. A liquid chromatography-tandem mass spectrometry method for the trace analysis of diazepam and nordiazepam in environmental water samples. A comprehensive study of storage conditions and identification of an unknown compound. In manuscript

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## Contents

1. Introduction..................................................................................................................... 11
   1.1. The sources of pharmaceuticals and metabolites reaching the aquatic environment and the pathways taken ................................................................. 11
   1.2. The occurrence and ecotoxicological effects of APIs and metabolites in environmental matrices...................................................................................... 13
   1.3. Chiral pharmaceuticals and metabolites in the aquatic environment 14

2. Aims..................................................................................................................................... 17

3. The analytical method...................................................................................................... 18

4. Development of methods for the trace analysis of APIs and metabolites in aqueous environmental samples ........................................................................... 20
   4.1. Sample collection and storage conditions ................................................................. 21
       4.1.2. Storage conditions for diazepam and nordiazepam ................................. 22
   4.2. The solid phase extraction methods ......................................................................... 24
       4.2.1. Solid phase extraction of fluoxetine and norfluoxetine in raw and treated wastewater .......................................................... 25
       4.2.2. Solid phase extraction of metoprolol and its metabolites α-OH-met and COOH-met .......................................................... 28
       4.2.3. Solid phase extraction of diazepam and nordiazepam ............................ 28
   4.3. The liquid chromatographic separation systems ......................................................... 29
       4.3.1. Chiral separation methods ........................................................................... 30
       4.3.2. Achiral separation methods ......................................................................... 35
   4.4. Mass spectrometry detection methods ..................................................................... 35
       4.4.1. The tandem quadrupole MS detection methods ........................................ 36
       4.4.2. Identification of an unknown compound in treated wastewater ............. 37
   4.5. Method validation .................................................................................................... 39
       4.5.1. Method validation by the use of isotope-labeled standards ..................... 40
       4.5.2. Sample and instrumental contamination..................................................... 41
   4.6. Analysis of the target compounds in environmental water matrices .......................... 42
       4.6.1. Chiral analysis of fluoxetine and norfluoxetine ...................................... 42
       4.6.2. Chiral analysis of metoprolol and its metabolites in treated wastewater ....... 44
       4.6.3. Analysis of diazepam and nordiazepam in treated wastewater and surface water ................................................................. 45
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AGP</td>
<td>α₁-Acid glycoprotein</td>
</tr>
<tr>
<td>α-OH-met</td>
<td>α-Hydroxymetoprolol</td>
</tr>
<tr>
<td>API</td>
<td>Active pharmaceutical ingredient</td>
</tr>
<tr>
<td>β-blocker</td>
<td>β-adrenoceptor antagonist</td>
</tr>
<tr>
<td>C18</td>
<td>Saturated chain of 18 carbon atoms</td>
</tr>
<tr>
<td>CBH</td>
<td>Cellobiohydrolase</td>
</tr>
<tr>
<td>COOH-Met</td>
<td>Deaminated metoprolol</td>
</tr>
<tr>
<td>CSP</td>
<td>Chiral stationary phase</td>
</tr>
<tr>
<td>E1, E2</td>
<td>The 1\textsuperscript{st} &amp; 2\textsuperscript{nd} eluting enantiomers, resp.</td>
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<tr>
<td>EF</td>
<td>Enantiomeric fraction</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray ionization</td>
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<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
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<tr>
<td>LC</td>
<td>Liquid chromatography</td>
</tr>
<tr>
<td>MDL</td>
<td>Method detection limit</td>
</tr>
<tr>
<td>MF</td>
<td>Matrix factor</td>
</tr>
<tr>
<td>MQL</td>
<td>Method quantification limit</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>MS/MS</td>
<td>Tandem mass spectrometry</td>
</tr>
<tr>
<td>(m/z)</td>
<td>Mass-to-charge ratio</td>
</tr>
<tr>
<td>NSAID</td>
<td>Non-steroidal anti-inflammatory drug</td>
</tr>
<tr>
<td>q</td>
<td>Collision cell</td>
</tr>
<tr>
<td>QqQ</td>
<td>Tandem quadrupole mass analyzer</td>
</tr>
<tr>
<td>QTOF</td>
<td>Quadrupole time-of-flight mass analyzer</td>
</tr>
<tr>
<td>RP</td>
<td>Reversed phase</td>
</tr>
<tr>
<td>(R_s)</td>
<td>Peak resolution</td>
</tr>
<tr>
<td>RSD</td>
<td>Relative standard deviation</td>
</tr>
<tr>
<td>S1, S2, S3, S4</td>
<td>The 1\textsuperscript{st}, 2\textsuperscript{nd}, 3\textsuperscript{rd} &amp; 4\textsuperscript{th} eluting stereoisomers, resp.</td>
</tr>
<tr>
<td>SPE</td>
<td>Solid phase extraction</td>
</tr>
<tr>
<td>SRM</td>
<td>Selected reaction monitoring</td>
</tr>
<tr>
<td>(t_R)</td>
<td>Retention time</td>
</tr>
<tr>
<td>WWTP</td>
<td>Wastewater treatment plant</td>
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</table>
1. Introduction

1.1. The sources of pharmaceuticals and metabolites reaching the aquatic environment and the pathways taken

The essential aim of all production, distribution and usage of medicinal products is to uphold public health [1]. Medicinal products are defined as any substances that are administered for medical diagnosis or to restore, correct or modify physiological functions [1]. Thus, active pharmaceutical ingredients (APIs) are developed to treat and prevent diseases, and are intended to have a specific pharmacological effect in a biological system. During the last few decades the growing use of medicinal products has, however, become a potential environmental problem. Emerging contaminants include a wide range of different types of compounds, e.g. APIs, illicit drugs, personal care products, food additives, pesticides and nanomaterials, as well as metabolites and transformation products for the compounds listed. This group of contaminants is considered to pose a potential threat to environmental ecosystems, wildlife, and human health and safety [2-4]. In addition to recognized contaminants, the concept of “emerging contaminants” has been adopted to include recently developed compounds, as well as compounds that have only just been detected in environmental matrices, often as a result of developments in analytical chemistry [3].

APIs have been detected in the terrestrial environment in, e.g., soil, sediment and sludge samples [5], as well as in the aquatic environment [6]. The presumed sources and pathways for pharmaceuticals to reach the aquatic environment have been reviewed in the literature [3, 7-11]. Most APIs intended for veterinary and human use are excreted from the body in the urine together with their more polar metabolites (phase I and II metabolites) [12]. Thus, pharmaceutical residues from human use most often enter a wastewater treatment plant (WWTP) through the sewage systems, Fig. 1. Different types of APIs, and metabolites with more polar characteristics that are not eliminated during their passage through these systems, enter rivers and streams in the WWTP discharges. On the other hand, less polar compounds are more distributed to the WWTP sludge [7, 8]. Discharges
from hospital and industrial wastewater also contribute to the distribution of APIs in the aquatic environment [13].

Veterinary pharmaceutical residues can contaminate fields and groundwater without first passing a WWTP. For example, the APIs and their metabolites can be urinated or defecated directly on the fields by treated animals [7, 8]. Other exposure routes for veterinary APIs and metabolites are from the discharges from livestock farming and aquaculture (e.g., fish farms) [13]. Moreover, during rainfall, surface water can be contaminated with human or veterinary APIs and metabolites through run-off from fields treated with digested sludge or livestock slurries, respectively [7].

Secondary pathways, i.e., pathways that only contribute minor to the overall environmental loadings, are the release of pharmaceutical residues from the skin during personal hygiene related activities (bathing and washing) [10]. These residues would be in the form of remains from, e.g., dermal applications of pharmaceutical products and APIs excreted through transpiration. These residues are also released from fabrics during laundering. Another secondary route is the disposal of unused pharmaceutical products, or unused or partially used medical devices [10].

*Figure 1.* The suggested sources and pathways for APIs and metabolites to reach the aquatic environment.
1.2. The occurrence and ecotoxicological effects of APIs and metabolites in environmental matrices

In 1985, Richardson and Bowron [14] reviewed the concerns about pharmaceutical residues in the aquatic environment. Since then, studies have been carried out in environmental matrices for about approximately 150 different APIs [4]. This, however, is only a small fraction of the about 3200 APIs that are currently available on the market [15]. Numerous studies have been conducted on the occurrence of APIs in water samples and most parts of the world have now been covered [7, 16-20]. Compounds from different therapeutic classes have been detected in the aquatic environment, on some occasions together with their metabolites, e.g. antibiotics [21], NSAIDs [22], antidepressants [23], β-blockers [24], contraceptives[25], chemotherapeutics [26], lipid regulators [27, 28] and opiates [29].

There are several general concerns associated with the widespread occurrence of pharmaceuticals in the environment. The presences of antibiotic pharmaceuticals in the water systems are associated with the possible risk of the development of microorganisms that are resistant to antibiotics (the creation of “super bugs”) [30, 31]. A newer concern is that antibiotics could slow down the biodegradation of plant materials that serve as a food source in the aquatic environment [31]. Moreover, hormones could cause estrogenic or other effects in wildlife and humans. The detected APIs from the different therapeutic classes also present a general threat to our drinking water supplies [31]. However, the possible short and long term effects of APIs and metabolites in the aquatic environment in human are currently unknown. APIs have, however, been shown to give non-target effects in aquatic living organisms at different trophic levels and many APIs and metabolites are also persistent in the aquatic environment. Clofibric acid, a metabolite to the lipid-lowering compound clofibrate, has an estimated residence time in the aquatic environment of 1–2 years. Clofibric acid is mobile and has been detected in the open ocean of the North Sea at depths of up to 6 meters [27, 28]. One of the side-effects caused by clofibric acid in humans is impaired spermatogenesis, an adverse effect that has also been suggested to be the most pronounced effect in Pimephales promelas (fathead minnows) [32]. Egg production was also found to decrease in female Pimephales promelas when they were exposed to clofibric acid [33]. APIs do not necessarily need to be persistent in the aquatic environment to cause adverse effects, however, since high degradation rates might be counteracted by continuous introduction of the APIs to the water. The veterinary pharmaceutical ivermectin, for example, is classified as non persistent [34] but has been found to have negative effect on the crustean Gammarus pulex [35].
The “worst case of wildlife poisoning ever” [31] was that which struck the Oriental white-backed vulture in Pakistan. The population declined (> 95 %) as a result of renal failure associated with residues of the NSAID diclofenac [36]. The vultures were most probably exposed to diclofenac by feeding on diclofenac-treated livestock or by drinking diclofenac-contaminated water [36].

1.3. Chiral pharmaceuticals and metabolites in the aquatic environment

In 1848, Louis Pasteur discovered the principle of molecular asymmetry when he separated a racemic mixture of a tartaric salt. He made the groundbreaking assumption that chemical compounds could be related to each other as mirror images [37]. Pasteur also found that only one type of compound (i.e. one of the enantiomers) was degraded in mould, whereas the other compound was intact. These discoveries were the first important steps in the direction of modern biochemistry [37]. However, it was in 1883 that Lord Kelvin named the phenomenon discovered as chirality, after the Greek word for hand, with the following definition [38]:

“I call any geometrical figure or any groups of points chiral and say it has chirality if its image in a plane mirror, ideally realised cannot be brought to coincide with itself.”

Nowadays, the nature of chiral compounds is widely recognized and approximately 50 % of the marketed APIs are chiral molecules. Of these chiral APIs, about 50 % are marketed as racemic mixtures rather than single enantiomers [2]. The enantiomers (one of a pair of molecules which are mirror images of each other and non-superposable) of an API can differ considerably in their pharmacological and toxicological effects [39]. Consequently, in 1992, the US Food and Drug Administration (FDA) stated that enantiomers of APIs should be treated as separate compounds and, moreover, stated that they should be developed accordingly [40]. Thus, many different analytical methods have been developed for the enantiomeric separation of chiral pharmaceuticals in biological matrices. The chiral aspects of pharmaceutical residues in environmental matrices have, however, been ignored in the past [2].

“Neglect of stereoselectivity of biological objects and stereo-specificity of bioactive agents never the less is a still persisting aspect of to-day’s pharmacology and toxicology. It results in heavily biased “scientific” data such as half life times, biological availabilities, concentration response relationships etc. for racemates and mixtures of isomers in general. It is like
presenting the age or body weight of a married couple or even of a 4 person family (a mixture of 4 stereoisomers) in one figure.”

The criticism by Ariëns from 1988 [41] is left for the reader to evaluate. Nevertheless, to some extent it has been rendered historical, as, as a result of developments in analytical methodologies, the trace level environmental occurrence and fate of the separate enantiomers of chiral APIs in complex matrices can be studied nowadays [4]. At present, however, knowledge of the environmental occurrence and fate of the enantiomers of APIs and metabolites is still far from complete [42]. Indeed, it is only recently that the chiral environmental contaminants have been addressed, however most studies have been focused on e.g. chiral pesticides, insecticides and plasticizers, i.e., not on chiral APIs [2]. The abiotic environmental processes (e.g. abiotic transformation, sorption and air-water exchange) of chiral compounds are mostly non-enantioselective, since enantiomers of a compound have identical physical and chemical properties. This is in contrast to biological processes (e.g. biotransformation), which usually are enantioselective [42, 43]. However, early research on the chiral degradation of pesticides indicates that the environmental fate of the enantiomers cannot always be predicted. Some environmental degradation processes are not enantioselective to a particular compound, even if microorganisms are involved. The microbial degradation can be rapid for both enantiomers. In other cases microbial populations can change or even reverse the enantiomeric ratios of the chiral compound. In addition, racemization or enantiomerization might also occur where, in the latter case, one enantiomer is converted to the other [4, 43, 44]. The compounds might also be degraded by a process not involving microorganisms.

Any change in the enantiomeric composition of an API in an environmental matrix is a good indicator of biological degradation [43]. Enantioselective analyses of APIs are, therefore, powerful tools with which to obtain insights into the environmental fate of pharmaceuticals in the environment and, moreover, to determine their sources [42]. In 1999, Buser et al. [45] published the first study on the enantioselective analysis of an API in wastewater and surface water. The enantiomers of ibuprofen were degraded in both wastewater and surface water. The authors also found that the concentrations of the pharmacologically active S-enantiomer of the NSAID ibuprofen were higher than the inactive R-enantiomer in raw wastewater (i.e. $S >> R$). However, the transformation process was more rapid for S-ibuprofen than for the R-enantiomer, resulting in the reversal of the enantiomeric composition of ibuprofen in surface water (i.e. $R > S$) [45]. Fono and Sedlak [46] were able to identify discharges of untreated wastewater in surface water by the enantiomeric composition of the β-blocker propranolol. Racemic propranolol in surface waters indicated that
its source in the water was raw sewage, whereas an altered enantiomeric composition showed that the source of the water was treated sewage [46].

The enantiomeric fate of APIs and metabolites is of importance for more than gaining knowledge about the source and fate of pharmaceutical residues. The enantiomeric composition of a chiral compound in environmental matrices might be of significance to water living organisms [47, 48]. For example, the water flea *Daphnia magna* is more sensitive to the *S*-enantiomer of the β-blocker atenolol than to the *R*-enantiomer [47]. However, (*R*)-atenolol shows a higher level of toxicity to the ciliate *Tetrahymena thermophila* than the *S*-enantiomer [47]. In aqueous environmental matrices, atenolol has been shown to be racemic in untreated wastewater [49], however, in another study (*R*)-atenolol was the most prevalent enantiomer found in untreated wastewater [50]. In treated wastewater, the enantiomeric composition of atenolol has been determined to be racemic [50] or non-racemic [49]. This example illustrates the complexity of chiral contaminants and the complexity of prediction of their ecotoxicological effects. Furthermore, environmental risk assessments must be based on reliable data about the actual concentrations of APIs [51] and metabolites, and their enantiomers in the aquatic environment. Thus, the development of analytical methods for trace level analysis of these compounds is of considerable importance.
2. Aims

The overall aim of this thesis was to develop LC-MS/MS methods for the trace analysis of active pharmaceutical ingredients and their metabolites in aqueous environmental matrices. In addition, for the chiral APIs and metabolites, the objective was to develop enantioselective LC-MS/MS methods. The target compounds were extracted by solid phase extraction from the aqueous environmental matrices. More detailed aims were determined in conjunction with Papers I–IV to ensure that these general goals could be attained. These aims were to:

- Develop an enantioselective LC-MS/MS method for the quantification of fluoxetine and its metabolite norfluoxetine in raw and treated wastewater samples (I, II).
- Develop an enantioselective LC-MS/MS method for the analysis of metoprolol and two of its chiral metabolites (i.e., both basic and acidic analytes) in treated wastewater samples (III).
- Validate the developed methods at trace level concentrations in the actual matrix in which the compounds were detected by using the isotope-labeled compounds of the analytes (I–IV).
- Study the matrix effects of raw and treated wastewater on the enantiomers of fluoxetine and norfluoxetine in the mass spectrometer interface (II).
- Investigate the impact of a chemical equilibrium between nordiazepam and a transformation product on the solid phase extraction recoveries for nordiazepam (IV).
- Identify an unknown compound detected in treated wastewater (IV).
- Employ the developed analytical methods for trace level quantifications of the selected APIs and metabolites, as well as their enantiomers, in the environmental samples studied (II–IV).
3. The analytical method

Environmental water samples, such as raw and treated wastewater and surface water, are complex matrices that contain high amounts of matrix compounds and generally only trace amounts of the target APIs and their metabolites. The APIs and metabolites are usually present at concentrations down to the ng L\(^{-1}\) range [51, 52]. The matrix compounds are a mix of different substances such as dissolved organic carbons, e.g., bacteria, viruses and colloidal organic matter [53, 54]. Owing to the presence of these compounds, environmental water samples are often considered to be more complex than biological fluids such as blood, plasma and urine [42].

A variety of different extraction techniques have been applied for the cleanup and preconcentration of the target compounds in water samples. Molecular imprinted polymers for solid phase extraction [55], hollow fiber liquid-phase microextraction with [56] or without organic solvents [57], stir bar sorptive extraction [58, 59], polar organic chemical integrative samplers [60] and in-line solid phase extraction integrated in the separation step [61] are some of the extraction techniques used for environmental matrices. Liquid-liquid extraction has become a less prominent technique for the preparation of environmental water samples [51]. At present, the most commonly used extraction technique for emerging contaminants in water analysis is off-line solid phase extraction (SPE) [6, 31]. On some occasions, the SPE extracts are cleaned up by introducing an extraction step to further reduce the quantity of undesirable compounds in the matrix [6].

High performance liquid chromatography (HPLC) is commonly used for the further separation of pharmaceuticals and their metabolites in environmental matrices [62]. However, the number of publications currently employing ultra performance liquid chromatography (UPLC) is increasing [15]. Furthermore, reversed phase (RP) liquid chromatography (LC) with standard C18 and C8 columns and mobile phases with volatile buffers and methanol or acetonitrile have been widely applied for the separations. Mobile phase additives such as ammonium acetate, acetic and formic acid are used to enhance the efficiency of the ionization in the MS interface [62], as well as to regulate the chromatographic separation. Gas chromatography (GC) is applicable for the separation of APIs in environmental samples, with some of the advantages of this technique being the high selectivity and sensitivity [63]. However, a major drawback with GC is the general need for
derivatization of the analytes [63, 64]. Other techniques, such as capillary electrophoresis (CE), have also been applied for the separation of APIs in environmental samples [65].

Since the development of the atmospheric pressure ionization sources e.g. electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI), the number of applications using LC-MS techniques have progressed [66]. At present, liquid chromatography coupled to mass spectrometry equipped with an ESI source is the key technique for environmental analysis [66, 67]. However, one of the main problems in LC-ESI-MS analysis is the co-elution of matrix compounds and the analytes into the MS interface, which gives rise to matrix effects, in general in the form of ion suppression [68]. Different approaches have been employed in order of minimizing these effects. The standard addition calibration method and the use of isotope-labeled internal standards are most often used to correct for matrix effects during quantification [68]. However, ion suppression decreases the overall sensitivity for the analytes in the analytical method. To maintain the sensitivity, a selective extraction method [6] or an efficient separation step conducted prior to the MS detection is required.

The tandem MS mass analyzers (MS/MS), operating in selected reaction monitoring (SRM) mode, offer high selectivity and sensitivity for trace level analytes in the aquatic environment [69]. MS/MS instruments, such as the tandem quadrupole mass analyzers (QqQ) are, therefore, the most commonly used instruments [67]. Ion trap mass analyzers are also widely employed in environmental analysis [67, 69]. However, for the structure elucidation and the identification of unknown compounds in the aquatic environment, high resolution MS is required. Orbitrap mass analyzers and the so-called hybrid instruments have been applied for identification of e.g. transformation products. The confirmation of positive findings in environmental matrices has also been performed using these mass analyzers owing to the high resolution and the mass accuracy obtained [31, 67, 70].
4. Development of methods for the trace analysis of APIs and metabolites in aqueous environmental samples

This chapter summarizes the analytical methods developed in Papers I–IV for the trace analysis of some selected APIs and their metabolites in aqueous environmental matrices. In this thesis, the environmental water matrices were comprised of samples of raw and treated wastewater obtained from one WWTP as well as surface water from River Fyris in Uppsala, Sweden. The Papers I–IV, are not presented individually in separate chapters, instead a chronological view is taken to demonstrate the performance of the analytical methods. For more detailed information about a specific section, the reader is referred to Papers I–IV. The chemical structures of the APIs and the metabolites studied in this thesis are given in Table 1 together with their \( pK_a \) values [71].

Table 1. Chemical structures and \( pK_a \) values [71] for the active pharmaceutical ingredients and metabolites studied. The asterisks denote chiral centers.

<table>
<thead>
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<th>Structure</th>
<th>Name</th>
<th>( pK_a )</th>
<th>Paper</th>
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<tr>
<td><img src="image" alt="Fluoxetine" /></td>
<td>Fluoxetine</td>
<td>10.1</td>
<td>I and II</td>
</tr>
<tr>
<td><img src="image" alt="Norfluoxetine" /></td>
<td>Norfluoxetine</td>
<td>9.1</td>
<td>I and II</td>
</tr>
<tr>
<td><img src="image" alt="Metoprolol" /></td>
<td>Metoprolol</td>
<td>9.4</td>
<td>III</td>
</tr>
<tr>
<td><img src="image" alt="(\alpha)-Hydroxymetoprolol" /></td>
<td>(\alpha)-Hydroxymetoprolol</td>
<td>9.4</td>
<td>III</td>
</tr>
<tr>
<td><img src="image" alt="Deaminated metoprolol" /></td>
<td>Deaminated metoprolol</td>
<td>3.4</td>
<td>III</td>
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Table 1 (continued).

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<th>Name</th>
<th>pKₐ</th>
<th>Paper</th>
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<tbody>
<tr>
<td></td>
<td>Diazepam</td>
<td>3.4</td>
<td>IV</td>
</tr>
<tr>
<td></td>
<td>Nordiazepam</td>
<td>3.2 and 11.7</td>
<td>IV</td>
</tr>
</tbody>
</table>

4.1. Sample collection and storage conditions

The matrices studied in this thesis were comprised of three sets of samples, those obtained from wastewater influents (raw) and effluents (treated), and other obtained from surface water. The raw (I, II) and treated (I–IV) wastewater samples were collected from the wastewater treatment plant, Kungsängsverket, in Uppsala, Sweden. Kungsängsverket is the biggest WWTP in the municipality of Uppsala and receives water from approximately 160,000 inhabitants from the city of Uppsala and the communities of Bälinge and Lövstalöt. The wastewater is treated by physical, biological and chemical treatment before being discharged into the River Fyris, Fig. 2 [72]. The raw sewage discharges from the eastern parts of Uppsala are treated in section C, Fig. 2. The discharge from the remaining parts of the city is treated in section A, B or C [72]. In this thesis, the raw wastewater samples were collected at the inlet of section C, behind the bar screens, but prior to the grit chambers and the addition of ferric chloride, Fig. 2. The treated wastewater samples were collected just prior to the point at which they are released into the River Fyris. The surface water was collected from the River Fyris in Uppsala, Sweden, approximately 3 km upstream from the WWTP Kungsängsverket.

All of the environmental water samples analyzed were collected as grab samples in amber glass bottles. Grab samples of environmental water are considered to be adequate for the development of analytical methods [73], however, when the aim of a study is to screen for or assess the fate of APIs and metabolites in aquatic systems, continuous sampling or flow-proportional sampling modes are recommended [73]. The collected water samples from Kungsängsverket and River Fyris were transported for
approximately 20 min and stored at –2 °C in the dark before sample preparation and solid phase extraction (I–IV).

Figure 2. Schematic overview of the wastewater treatment plant Kungsängsverket in Uppsala, Sweden. The arrows illustrate the locations at which samples were collected (I–IV).

4.1.2. Storage conditions for diazepam and nordiazepam

Wastewater samples are often stored under acidic conditions to slow down the microbial degradation and to preserve the collected samples before the solid phase extraction [74]. Environmental water samples are, therefore, often acidified during storage [75-78] e.g., to preserve the sample or to adjust the pH to the optimal value prior to performing solid phase extraction. Environmental water samples are often not stored for longer than 20 h [76] or are extracted within a week [78] or 14 days [79] from the time of collection.

However, benzodiazepines, such as diazepam and nordiazepam, are known to undergo hydrolysis in acidic aqueous samples [80]. Archontaki et al. [80] found that the degradation of nordiazepam occurred in two steps in acidic conditions. The degradation process included a reversible first step from nordiazepam (a) to a transformation intermediate of nordiazepam (b) and then an irreversible second step to the final degradation product (c) Fig. 3.
In Paper IV, the stability of diazepam and nordiazepam were studied for twelve days at pH 3.1 in a mixture of acetonitrile/10 mM formic acid in Millipore water (10/90, v/v) at 4 °C and at room temperature. The stability of the compounds was not studied in wastewater, but in a simple matrix to make it possible to evaluate the stability without prior extraction. Diazepam was found to be degraded by 10 and 12 % at 4 °C and at room temperature, respectively, Fig. 4. In contrast, the degradation of nordiazepam was more severe: within the twelve days, 26 and 69 % of the initial concentration of nordiazepam had been degraded at 4 °C and at room temperature, respectively, Fig. 4.

The transformed intermediate (b) of nordiazepam that was identified by Archontaki et al. [80], Fig. 3, was found to be formed during the storage of the nordiazepam solutions. A peak with a mass-to-charge ratio (m/z) corresponding to the mass of the transformed intermediate (b) was detected with LC and tandem quadrupole mass spectrometry in the stored nordiazepam solutions. Furthermore, the isotopic pattern for the intermediate in the MS spectra indicated that the compound contained chlorine. Thus, it was concluded that the transformation intermediate of nordiazepam was
present in the stored acidic solutions of nordiazepam, however, the final degradation product \((c)\) was not detected in the samples.

Improper sample preservation or storage conditions are some of the factors that might result in inaccurate data, giving rise in inappropriate conclusions about the analytes in the environmental samples \([73]\). In Paper IV, it was concluded that nordiazepam was readily degraded in the acidic aqueous solutions. Thus, it is of great importance to determine the stability of the analytes studied to make it possible to select the proper storage conditions for the wastewater samples.

4.2. The solid phase extraction methods

As discussed above, solid phase extraction is most commonly used technique for the extraction of pharmaceutical residues in the aquatic environment \([6, 31]\). Commonly used SPE sorbents are octadecylsilica, polymeric hydrophilic-lipophilic balanced sorbents and mixed-mode sorbents with both reversed phase and cationic exchange properties \([6, 51]\). Polymer based sorbents give better recoveries when both polar and non-polar compounds are to be analyzed from the same water samples, and therefore they are often the sorbents of choice for investigation of APIs and metabolites in the environment. In addition, these sorbents are considered to have a greater capacity than alkyl bonded ones \([6]\). Nevertheless, it can still be a matter of trial-and-error to predict which SPE sorbent is the best choice for a certain class of APIs since the structure of the sorbents is not always sufficiently well specified by the manufacturers \([51]\).

One of the most widely employed sorbents is that compromised of the copolymers of divinylbenzene and vinylpyrrolidone \([51]\) (commercialized as Oasis HLB by Waters \([81]\)). In this thesis, four different polymer based SPE sorbents (I–III) and one silica based octadecyl column (IV) were evaluated for the extraction of the target compounds and their metabolites (Table 1). The SPE sorbents used are given in Table 2.

Table 2. The SPE sorbents evaluated for the extraction of the APIs and metabolites in this thesis.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sorbent</th>
<th>Paper</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oasis HLB</td>
<td>Polyvinylpyrrolidone-divinylbenzene</td>
<td>III</td>
</tr>
<tr>
<td>Strata X</td>
<td>Polymer based with hydrophobic and hydrophilic interactions</td>
<td>III</td>
</tr>
<tr>
<td>Oasis MCX</td>
<td>Polyvinylpyrrolidone-divinylbenzene with sulphonic groups</td>
<td>I, III</td>
</tr>
<tr>
<td>Evolute CX-50</td>
<td>Mixed mode resin based sorbent with sulphonic groups</td>
<td>I, II</td>
</tr>
<tr>
<td>SPEC C18 AR</td>
<td>End-capped octadecyl modified silica</td>
<td>IV</td>
</tr>
</tbody>
</table>
The extraction recovery is an important parameter to determine during method development and validation. However, by the utilization of LC-MS/MS the sample matrix might give rise to ion suppression or ion enhancement in the MS interface. During the extraction recovery determinations it is, therefore, necessary to eliminate the uncertainty associated with the matrix effects on the recoveries calculated. In this thesis, the extraction recoveries were determined in accordance with the guidelines specified by Matuszewski et al. [82] (I, III, IV). The authors provided a guideline for the determination of extraction recovery and for assessments of matrix effects in biological matrices by the use of LC-MS/MS [82]. Blank matrices are required for these assessments, which can be difficult or even impossible to obtain during method development for the analysis of APIs in wastewater. However, by the use of isotope-labeled standards, which are not expected to be found in environmental matrices, the extraction recovery and matrix effects can be determined for the isotope-labeled standards in the actual matrix in which the target analytes are detected.

4.2.1. Solid phase extraction of fluoxetine and norfluoxetine in raw and treated wastewater

In Paper I, a solid phase extraction method using Evolute CX-50 cartridges (200 mg, 6 mL) was developed for fluoxetine and norfluoxetine in raw and treated wastewater. In initial assessments, it was concluded that the extraction recoveries obtained for the analytes in treated wastewater were higher with Evolute CX-50 than with Oasis MCX, with the exception of (R)-norfluoxetine-d₅. Thus, Evolute CX-50 was chosen for further optimization.

Strong cationic exchange mixed-mode polymeric sorbents can be used for the simultaneous extraction of basic, acidic and neutral analytes at low sample pH [83], i.e., in so called multi-residue analytical methods. However, in Paper I, a method was developed for basic compounds by the use of Evolute CX-50. One advantage of using a polymer based sorbent with strong cationic interactions, though, is that the cartridges can be washed with a pure organic solvent, such as methanol, after the sample application to remove matrix compounds present in the wastewater. In Paper I, the water samples were filtered through 0.7 µm glass fiber filters and volumes of 200 mL raw or 500 mL treated wastewater were extracted at pH 4 by the Evolute CX-50 sorbents.
Interestingly, during the SPE method development, the enantiomers of norfluoxetine were not detectable in treated wastewater, Fig. 5A. However, when a washing step of 6 mL methanol was included to the SPE procedure, the enantiomers of norfluoxetine became detectable Figs. 5B and C. Thus, one can deduce that the sensitivity for the enantiomers of norfluoxetine increased when 6 mL methanol was incorporated in the SPE method. The sensitivity probably increased because interfering matrix compounds that give rise to ion suppression in the ESI MS interface were more effectively eliminated with the methanol wash. It must be emphasized that the treated wastewater, extracted by the two methods, was obtained from the same grab sample. These findings clearly demonstrate the importance of using reliable analytical methods to properly assess the presence or absence of a certain compound in the aquatic environment.

The extraction recoveries for the final method were determined for the enantiomers of fluoxetine-d₅ and norfluoxetine-d₅ at one high (500 pM) and one low concentration (50 pM) using Evolute CX-50, Fig. 6.
Figure 6. SPE recoveries for the enantiomers of fluoxetine-d$_5$ and norfluoxetine-d$_5$ in raw and treated wastewater at the enantiomeric concentrations of 500 or 50 pM. The value for the RSD (%) for the determined recovery is given above its respective bar ($n = 3–5$). The samples were extracted by Evolute CX-50 SPE cartridges.

The extraction recoveries obtained in raw and treated wastewater were higher at 500 pM than at 50 pM, except for norfluoxetine-d$_5$ in treated wastewater, Fig. 6. The reduced recoveries at the lower concentration might be a result of an increased effect of adsorption of the basic analytes e.g., to the silanol groups of the used glassware [84] or to the pieces of plastic tubing [85]. Silanised glassware was recently demonstrated to reduce the adsorption of fluoxetine onto glassware [86]. These findings were published after our experiments had been conducted, nevertheless, during quantification of fluoxetine and norfluoxetine such non specific adsorption effects can be compensated for by the use of fluoxetine-d$_5$ and norfluoxetine-d$_5$ as internal standards.

To summarize, in Paper I, it was demonstrated that the utilization of the isotope-labeled standards, fluoxetine-d$_5$ and norfluoxetine-d$_5$, could be extended to develop the solid phase extraction method for fluoxetine and norfluoxetine. Furthermore, the extraction recoveries could be determined at trace level concentrations in the actual wastewater in which the target compounds were detected.
4.2.2. Solid phase extraction of metoprolol and its metabolites α-OH-met and COOH-met

In Paper III, a solid phase extraction method was developed for the two basic compounds, metoprolol and α-hydroxymetoprolol (α-OH-met) as well as the acidic metabolite deaminated metoprolol (COOH-met), Table 1. The compounds were extracted simultaneously at pH 7 by the hydrophilic-lipophilic polymeric SPE sorbents Oasis HLB and Strata X (Table 2). The mixed mode sorbent Oasis MCX (Table 2) was also investigated at a sample pH of 1.3.

In the initial experiments (analytes dissolved in phosphate buffer), the recovery for COOH-met was only 28 ± 11 % by employing the Oasis MCX sorbent, Table 3. This sorbent was therefore not further considered in the method development. However, the extraction recoveries were found to be higher and the RSD % values lower for Oasis HLB in comparison to Strata X. Thus, Oasis HLB was chosen for the extraction of the analytes from environmental water. Interestingly, the extraction recovery for α-OH-met in surface water from River Fyris was higher than the recovery obtained for α-OH-met in phosphate buffer, Table 3. In contrast, for metoprolol and COOH-met, the extraction recoveries were decreased by the more complex matrix.

Table 3. SPE extraction recoveries (%) and RSD % values for metoprolol, α-OH-Met and COOH-met in buffer and surface water from River Fyris at a concentration of 500 pM, n = 3–4.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Oasis MCX Buffer pH 1</th>
<th>Strata X Buffer pH 7</th>
<th>Oasis HLB Buffer pH 7</th>
<th>Oasis HLB Surface water pH 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metoprolol</td>
<td>82 ± 15</td>
<td>75 ± 5.3</td>
<td>83 ± 2.0</td>
<td>73 ± 2.8</td>
</tr>
<tr>
<td>α-OH-Met</td>
<td>57 ± 16</td>
<td>44 ± 4.3</td>
<td>57 ± 1.9</td>
<td>74 ± 3.5</td>
</tr>
<tr>
<td>COOH-Met</td>
<td>28 ± 11</td>
<td>102 ± 12</td>
<td>117 ± 3.2</td>
<td>86 ± 12</td>
</tr>
</tbody>
</table>

Oasis HLB has been used previously for the extraction of these three compounds from dog plasma [87]. In Paper III it was demonstrated that Oasis HLB could be employed for the simultaneous extraction of metoprolol, α-OH-met and COOH-met from environmental water samples at low sample concentrations (500 pM).

4.2.3. Solid phase extraction of diazepam and nordiazepam

In Paper IV, a solid phase extraction method was developed for diazepam and nordiazepam involving the use of SPEC C18 AR discs at neutral sample pH. In that study, it was also demonstrated that nordiazepam was readily degraded in an acidic aqueous solution (Section 4.1.2.). Within twelve days, 69 % of the initial amount of nordiazepam had been degraded in a water
solution at pH 3. However, in a recent publication by Baker et al. [86], diazepam and nordiazepam were shown to be more stable at acidic pH than at neutral pH in wastewater samples.

Interestingly, when a twelve day old solution of nordiazepam (i.e. a solution now containing 31% of the initial concentration) was added to a sample of phosphate buffer at pH 7 and extracted by the use of the SPEC C18 AR discs, the degraded nordiazepam was found to be regenerated (IV). The regenerated nordiazepam accounted for 77% of the freshly prepared solution of nordiazepam. Thus, the chemical equilibrium (Fig. 3) of nordiazepam (a) and of the transformed intermediate (b) was found to have shifted towards nordiazepam during the solid phase extraction. The ratio of nordiazepam (a) to the transformed intermediate (b) in a stored solution was determined to be 0.75 (n = 2). However, when this solution was added to 4 mL methanol and evaporated to dryness at 60 °C under a gentle stream of nitrogen (to simulate the evaporation step of the SPE extracts), the ratio of (a) to (b) was shifted to 1.9 (6.8 RSD %, n = 4). Thus, it was concluded that the chemical equilibrium was shifted towards nordiazepam during the evaporation step.

These findings, i.e. (i) the degradation of nordiazepam under acidic conditions and (ii) the regeneration of nordiazepam during the SPE extraction, might influence the results obtained by the analytical method. Firstly, it is recommended that nordiazepam is stored at low pH by Baker at al. [86], however, the authors of that publication determined the stability of the compounds in wastewater and therefore extracted the samples by SPE prior to analysis. The true stability of nordiazepam might, therefore, never have been discovered because nordiazepam might have been regenerated in their extraction method. Moreover, when extraction recoveries are determined according to guidelines by Matuszewski et al. [82] the obtained results might be overestimated, as was shown in Paper IV. This overestimation arises because the extraction recovery is determined by the ratio of an extracted sample to a non-extracted one. Finally, the overall accuracy of the method is impaired if nordiazepam is degraded in either the wastewater sample or in the solvent used i.e., for injection into the analytical system. The effect of any shift in the chemical equilibrium of nordiazepam and the transformation intermediate on the overall accuracy might, however, be compensated for by the use of an isotope-labeled internal standard.

4.3. The liquid chromatographic separation systems

As discussed above, liquid chromatography is the most commonly used separation technique for APIs in the aquatic environment [62]. Numerous
achiral separation methods have been developed for the analysis of pharmaceutical residues in environmental matrices, however, most of these methods focus on the parent compounds of the APIs, with the metabolites being included less frequently [68]. Furthermore, enantioselective separation methods have only been developed for a small number of the chiral APIs on the market [88]. According to Hashim et al. [88], only a handful of publications on chiral separations of APIs in wastewater had been reported up until the year of 2010. In this thesis, separation methods were developed for APIs as well as for their metabolites (I–IV) and, furthermore, for the chiral APIs and their metabolites that were studied, enantioselective separation methods were developed (I–III).

4.3.1. Chiral separation methods

Chiral separation methods have previously been developed for APIs in the aquatic environment [88]. These separations were performed in GC by indirect [46, 89] and direct [45, 90] separation methods after derivatization of the analytes by the use of chiral and achiral derivatization reagents, respectively. Direct LC separation methods have also been employed. For example, Shao et al. [91] developed a LC method that utilized fluorescence detection. The enantiomers of the analyte were separated on an octadecylsilica column by the use of chiral mobile phase additives [91]. However, HPLC methods utilizing chiral stationary phases (CSPs) are more commonly applied. For example, the protein based CSP Chiral CBH was employed for the enantiomeric analysis of some compounds of potential abuse in reversed phase mode [92]. MacLeod et al. [50] used the vancomycin-based CSP Chirobiotic V in reversed phase mode for the separation of β-blockers, selective serotonin reuptake inhibitors and one β2-agonist [50]. Two dimensional LC systems have also been developed using non-commercialized polysaccharide based CSPs for enantiomeric separation [93, 94], however, the metabolites of the selected APIs were not included in any of the above developed chiral methods. In this thesis, chiral separation methods were developed for fluoxetine and its main metabolite, norfluoxetine (I, II), as well as for metoprolol and two of its metabolites (III).

In Paper I, a chiral LC method was developed for fluoxetine and norfluoxetine based on chiral α1-acid glycoprotein (AGP). The enantiomers were separated with acetonitrile/10 mM ammonium acetate buffer pH 4.4 (3/97, v/v), Fig 7.
AGP is a protein with an isoelectric point of 2.7 that is extracted from plasma [95]. Silica immobilized AGP has a broad application range and can be used for the separation of basic [96] as well as acidic compounds [97] under reversed phase conditions, but many of the AGP methods developed involve the use of phosphate buffers [95]. As a result of this, Michishita et al. [95] recently published a guideline for the development of MS compatible AGP methods. In Paper I, the enantioselective interaction of fluoxetine and norfluoxetine with Chiral AGP using a MS compatible mobile phase was investigated. The pH, amount of organic modifier, and the total concentration of ammonium acetate added were investigated as a function of the enantiomeric peak resolution ($R_s$) (Fig. 8), retention factors ($k$), separation factors ($\alpha$) and plate number ($N$). The pH of the ammonium acetate buffer seemed to have the most drastic effect on the enantiomeric separation, Fig. 8A, but the amount of organic modifier and the concentration of ammonium acetate also had an impact the enantioselectivity, Figs. 8B and C.
Figure 8. The effect of the (A) ammonium acetate buffer pH, (B) amount of organic modifier and (C) total concentration of added ammonium acetate in the mobile phase on the enantiomeric peak resolution of fluoxetine and norfluoxetine using Chiral AGP.

By increasing the mobile pH the conformation of the AGP might be changed as well as the overall negative net charge of the protein. Thus, the retention and the separation factors of the positively charged analytes were increased as the pH was raised. The ionic strength of the mobile phase can also be used to regulate the hydrophobic as well as the ionic interactions between the analyte and the mobile phase. As the ionic strength of the mobile phase is increased, a general characteristic of the hydrophobic interactions is that the interactions increase, at the same time the ionic interactions are decreased [98-100]. With buffer concentrations up to 50 mM, the retention factors rose for (S) and (R)-fluoxetine. The increase in retention factor for the first eluting enantiomer was, however, higher than for the second eluting enantiomer, and, as a result, the enantiomeric resolution for fluoxetine decreased (from 2.67 to 2.14) as a result of the increased buffer concentration (Fig. 8C). At concentrations higher than 50 mM, the retention factors the enantiomers of fluoxetine slightly decreased. The overall increase in the retention indicates that the hydrophobic interactions between fluoxetine and the Chiral AGP are more influenced by the ionic strength than the decrease in ionic interactions.

Small amounts of organic modifiers in the mobile phase decrease the hydrophobic interactions between the analytes and the protein based CSP [98, 101]. As a general directive, up to 15 % of organic modifier in the liquid phase is recommended [95]. In Paper I, the content of acetonitrile in the mobile phase was a compromise between the retention times and
enantiomeric resolution. 3% of acetonitrile was chosen in the mobile phase as a satisfactory enantiomeric resolution (Fig. 8B) and acceptable retention times were achieved (Fig. 7) for both fluoxetine and norfluoxetine.

Four different CSPs were evaluated for the chiral separation of metoprolol, \( \alpha \)-OH-met and COOH-met in this thesis, the polysaccharide based Chiralcel OD-H, the vancomycin based Chirobiotic V, and the protein based CSPs Chiral AGP and Chiral CBH (III). In the final method, the enantiomers of the acidic metabolite COOH-met were separated by Chiral AGP, Fig. 9. The first eluted enantiomer was denoted by E1 and the second eluted enantiomer by E2. The retention times for COOH-met E1 and E2 were 7.1 min (1.6 RSD%, \( n = 6 \)) and 12.3 min (1.7 RSD% \( n = 6 \)), respectively, and the enantiomeric \( R_s \) was 2.

![Figure 9. Enantiomeric separation of COOH-met by the use of methanol/10 mM ammonium acetate buffer pH 5.0 (5/95, v/v) and Chiral AGP. The flow rate was set to 0.22 ml min\(^{-1}\).](image)

As expected, the retention times for the enantiomers of COOH-met decreased when the mobile phase pH was increased from 4.0 to 5.0, Table 4. The significant decrease observed (from 74 to 12 min for COOH-met E2) might be caused by the higher pH giving rise to an increase in the negative net charge of the AGP as well as an increase in the fraction of the negative charged COOH-met molecules. These results are in accordance with results by Michishita et al. [95].

Table 4. Enantiomeric separation of \( \alpha \)-OH-met, metoprolol and COOH-met using Chiral AGP and ammonium acetate at pH 4, 5 or phosphate buffer at pH 7. The retention times (\( t_R \)) and enantiomeric peak resolution are given. The flow rate was set to 0.22 ml min\(^{-1}\). The first and second eluted enantiomers are denoted by E1 and E2, respectively, n.i. = not injected.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Ammonium acetate pH 4</th>
<th>Ammonium acetate pH 5</th>
<th>Phosphate pH 7</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( t_R ) [min]</td>
<td>( R_s )</td>
<td>( t_R ) [min]</td>
</tr>
<tr>
<td>( \alpha )-OH-Met</td>
<td>1.1</td>
<td>-</td>
<td>1.9</td>
</tr>
<tr>
<td>Metoprolol E1</td>
<td>1.6</td>
<td>-</td>
<td>2.4</td>
</tr>
<tr>
<td>Metoprolol E2</td>
<td>1.6</td>
<td>-</td>
<td>3.0</td>
</tr>
<tr>
<td>COOH-Met E1</td>
<td>41</td>
<td>2.4</td>
<td>7.1</td>
</tr>
<tr>
<td>COOH-Met E2</td>
<td>74</td>
<td>12</td>
<td></td>
</tr>
</tbody>
</table>
The enantiomers of metoprolol were separated at pH 7 by the use of the AGP column, however, the enantiomers of α-OH-met were not separated at any of the pH values investigated, Table 4. The recommended pH interval for Chiral AGP is 4.0–7.0 [95], therefore the pH was not further increased. Furthermore, pH gradient elution have been performed for metoprolol on Chiral AGP by Balmér et al. [102]. Elution through the use of a pH gradient was, however, not investigated in this study as the enantiomers of α-OH-met were not separated within the recommended pH interval.

For the simultaneous enantiomeric separation of α-OH-met and metoprolol, the chiral cellobiohydrolase (CBH) column was employed, as can be seen from Fig. 10. Chiral CBH have been used before for the enantiomeric separation of basic APIs, e.g. β-blockers [103], as well as some acidic APIs [104]. Unfortunately, the enantiomers of the acidic metabolite COOH-met were not separated in this chromatographic system.

![Figure 10](image)

**Figure 10.** Chiral CBH was used for the stereoisomeric separation of α-OH-met and metoprolol in treated wastewater. The mobile phase was isopropanol / hydroxylamine:acetic acid buffer pH 7.0 (3.6/94.6, v/v).

The enantiomers of α-OH-met were initially resolved by Chiral CBH and phosphate buffer at pH 7 and detected at 230 nm, but the phosphate buffer was exchanged for a hydroxylamine/acetic acid buffer at pH 7.0 [105] to make the method MS compatible. The effect of the type of organic modifier on the enantiomeric separation was determined for α-OH-met by using 0.47 M methanol, ethanol, acetonitrile or isopropanol in the mobile phase. The four stereoisomers of α-OH-met were separated in all mobile phases. The highest signal-to-noise ratio was, however, achieved by using 0.47 M isopropanol (equals 3.6 % isopropanol, v/v) in the mobile phase.
4.3.2. Achiral separation methods

Diazepam and nordiazepam were separated by RPLC using a C18 column (50×2.1 mm, 5 µm) and an isocratic as well as a gradient elution system. The mobile phases, A and B, consisted of 5 mM formic acid in Millipore water and 5 mM formic acid in acetonitrile, respectively. In the isocratic system, the proportion of A and B in the mobile phase was 40/60 (v/v). The gradient for the elution system was programmed as follows for mobile phase B: initial – 30 %, 2 min – 65 %, 2.10 min – 100 %, 2.80 min – 100 %, and 2.81 min – 30 %. The precision of the retention times in the two systems, given as the RSD, was < 0.9 % for both systems, Table 5. The peak resolution was $R_s \geq 3.1$.

Table 5. Retention times for diazepam-d$_5$ and nordiazepam-d$_5$ in the isocratic chromatographic system and for diazepam and nordiazepam in the gradient elution system ($n = 6$). The flow rate was set to 0.25 ml min$^{-1}$. RSD % values are given in brackets.

<table>
<thead>
<tr>
<th>Sample matrix</th>
<th>Nordiazepam-d$_5$</th>
<th>Diazepam-d$_5$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$t_R$ [min]</td>
<td>$t_R$ [min]</td>
</tr>
<tr>
<td>Mobile phase</td>
<td>2.6 (0.54)</td>
<td>4.1 (0.27)</td>
</tr>
<tr>
<td>Treated wastewater</td>
<td>2.6 (0.62)</td>
<td>4.1 (0.30)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sample matrix</th>
<th>Nordiazepam</th>
<th>Diazepam</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$t_R$ [min]</td>
<td>$t_R$ [min]</td>
</tr>
<tr>
<td>Mobile phase</td>
<td>2.4 (0.90)</td>
<td>2.9 (0.70)</td>
</tr>
<tr>
<td>Treated wastewater</td>
<td>2.4 (0.35)</td>
<td>2.8 (0.29)</td>
</tr>
</tbody>
</table>

4.4. Mass spectrometry detection methods

Tandem quadrupole mass analyzers are the most commonly used detection technique for targeted trace level analysis of organic compounds such as APIs and pesticides in environmental matrices [4, 67]. In the selected reaction monitoring (SRM) mode, the first and second analyzers are focused on the selected precursor and product ions, respectively. The product ion is formed by collision induced dissociation with an inert gas in the collision cell [106]. The SRM mode is associated with high sensitivity as the mass analyzer is not operating in the scanning mode, thereby increasing the duty cycle of the instrument. Furthermore, in comparison with the selected ion monitoring (SIM) mode, SRM is also associated with a high increase in selectivity [106]. However, the tandem quadrupole mass analyzers operate at unit resolution and provide low sensitivity in the full-scan mode [67]. For the identification of unknowns in the environmental samples, high resolution mass spectrometry (HRMS) is required. Thus, hybrid instruments, such as
the quadrupole time-of-flight (QTOF) instruments, have shown to be useful for the identification and structure elucidation of unknown compounds at trace level concentration in different matrices [4, 67]. The hybrid TOF analyzers commonly include a quadrupole mass analyzer (Q1) and a collision cell (q) followed by a time-of-flight mass analyzer, giving the instrument the QqTOF configuration. The advantages of the QqTOF instrument are the high sensitivity and the high mass accuracy in both MS and MS/MS modes [106].

In this thesis, the target analytes in the wastewater samples were detected using tandem quadrupole mass spectrometers operating in the SRM mode (I–IV). In Paper IV, a QqTOF instrument was employed for the identification of an unknown compound that was detected in treated wastewater samples.

4.4.1. The tandem quadrupole MS detection methods

The APIs and metabolites were detected in positive or negative ESI mode in the environmental water samples (I–IV) and the peaks were recorded in SRM mode. In Papers I and II, the enantiomers of fluoxetine and norfluoxetine were separated by the use of the Chiral AGP but (S)-fluoxetine, (S)-fluoxetine-d5, (S)-norfluoxetine and (S)-norfluoxetine-d5 were co-eluting, Fig. 7. The last eluting enantiomers, (R)-fluoxetine, (R)-fluoxetine-d5, (R)-norfluoxetine and (R)-norfluoxetine-d5 were also found to be co-eluting in the chromatographic system, Fig. 7. Fluoxetine, norfluoxetine and the isotope-labeled standards were, therefore, separated by their respective mass-to-charge ratio in the QqQ mass spectrometer. Thus, the tandem quadrupole MS detector was set to monitor the four compounds in the same time window. For the identification of the trace level compounds in the complex matrices, two SRM transitions are often used for each analyte [67]. But to make it possible to achieve sufficient acquisition time for each SRM transition, only one SRM transition was monitored for fluoxetine and norfluoxetine, respectively, Table 6. The exclusion of the identifier ions was considered to be acceptable since the retention times of the isotope-labeled standards could be used for the identification of the enantiomers of fluoxetine and norfluoxetine in the wastewater samples.

Two SRM transitions were used for diazepam and nordiazepam (IV) as well as the stereoisomers of metoprolol, α-OH-met and COOH-met (III) during the analysis of the compounds in the environmental water samples. The SRM transitions are given in Table 6. The ratio of product ion 1 to product ion 2 for each analyte in the environmental samples was compared with the ratio obtained from standard solutions.
Table 6. The SRM transitions used in the LC-ESI-MS/MS methods.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Precursor ion m/z</th>
<th>Product ion 1 m/z</th>
<th>Product ion 2 m/z</th>
<th>Paper</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluoxetine</td>
<td>310</td>
<td>44</td>
<td>-</td>
<td>I, II</td>
</tr>
<tr>
<td>Fluoxetine-d₅</td>
<td>315</td>
<td>44</td>
<td>-</td>
<td>I, II</td>
</tr>
<tr>
<td>Norfluoxetine</td>
<td>296</td>
<td>134</td>
<td>-</td>
<td>I, II</td>
</tr>
<tr>
<td>Norfluoxetine-d₅</td>
<td>301</td>
<td>139</td>
<td>-</td>
<td>I, II</td>
</tr>
<tr>
<td>Metoprolol</td>
<td>268</td>
<td>116</td>
<td>74</td>
<td>III</td>
</tr>
<tr>
<td>Metoprolol-d₅</td>
<td>275</td>
<td>123</td>
<td>-</td>
<td>III</td>
</tr>
<tr>
<td>α-OH-Met</td>
<td>284</td>
<td>116</td>
<td>74</td>
<td>III</td>
</tr>
<tr>
<td>α-OH-Met-d₅</td>
<td>289</td>
<td>121</td>
<td>-</td>
<td>III</td>
</tr>
<tr>
<td>COOH-Met</td>
<td>239</td>
<td>151</td>
<td>119</td>
<td>III</td>
</tr>
<tr>
<td>Diazepam</td>
<td>285</td>
<td>193</td>
<td>154</td>
<td>IV</td>
</tr>
<tr>
<td>Diazepam-d₅</td>
<td>290</td>
<td>198</td>
<td>-</td>
<td>IV</td>
</tr>
<tr>
<td>Nordiazepam</td>
<td>271</td>
<td>208</td>
<td>140</td>
<td>IV</td>
</tr>
<tr>
<td>Nordiazepam-d₅</td>
<td>276</td>
<td>213</td>
<td>-</td>
<td>IV</td>
</tr>
</tbody>
</table>

4.4.2. Identification of an unknown compound in treated wastewater

As discussed above, the tandem quadrupole mass spectrometer operating in SRM mode is a highly selective detection method [106]. However in Paper IV, when treated wastewater samples were analyzed by the RP gradient LC system, an unknown compound was detected in the SRM channel for nordiazepam, m/z 271 → 140, Fig. 11. Interestingly, the isobaric interference was detected in all of the extracted wastewater samples but was not detected in any of the standard solutions.

![SRM chromatogram for nordiazepam (2.40 min) and the unknown compound (2.14 min) in treated wastewater. The samples were extracted and analyzed using an LC-QqQ system (IV).](Image)

*Figure 11.* SRM chromatogram for nordiazepam (2.40 min) and the unknown compound (2.14 min) in treated wastewater. The samples were extracted and analyzed using an LC-QqQ system (IV).
An LC-QqTOF MS system was employed for HRMS determinations of the unknown compound in the wastewater samples. The two most abundant ions in the MS spectrum of the isobaric interference had mass-to-charge ratios of 397.2 and 287.1. Extracted treated wastewater samples contain high amounts of matrix compounds, even after extraction, which complicates the determination of the actual precursor ion. The ion with an \( m/z \) at 287.1 (accurate \( m/z \) 287.0569) was the only ion of the two that had the same retention time as the unknown compound. These findings indicate that the ion with an \( m/z \) of 271.1 (used in the SRM transition for nordiazepam) could be formed by in-source fragmentation of the ion with a mass-to-charge ratio of 287.0569.

The ion at \( m/z \) 287.0569, Fig. 12 A, was used to calculate the sum formula of \( \text{C}_{15}\text{H}_{11}\text{ClN}_2\text{O}_2 \) that had a mass difference of 4.6 ppm from the observed ion. The calculated isotopic pattern of the sum formula corresponded well with the isotopic pattern of the unknown compound. A data base search for 287.0569 within an in-house data base at the Swedish Medical Products Agency containing the exact masses for approximately 4 000 APIs gave two hits. The two hits, oxazepam and demoxepam, had the exact same masses. Oxazepam was, however, considered to be more likely to be found in wastewater samples since demoxepam is a metabolite formed from chlordiazepoxide [107], which was withdrawn from the Swedish market in 1996.

The ion with a mass-to-charge ratio of 269.0466 in the MS spectrum of the unknown compound corresponds to an in-source fragmentation of oxazepam found by Valavani et al. [108], Fig. 12A. The \( ^{37}\text{Cl} \) isotope of the in-source fragmentation ion gave rise to an ion at \( m/z \) 271.0447, Fig. 12B. Consequently, the \( ^{35}\text{Cl} \) isotopic ion of the in-source fragment had a
mass-to-charge ratio close to the one for the \([\text{M+H}]^+\) of nordiazepam, which was used as a precursor ion in the SRM transition for nordiazepam.

To confirm that the peak of the unknown compound belonged to oxazepam a standard solution of oxazepam was analyzed in the LC-QqTOF system. The retention time of the standard and the unknown compound was the same in the LC-QqTOF system, i.e. 3.2 min. Furthermore, product ion scans were acquired for nordiazepam and oxazepam to determine if the two compounds were fragmented into the same product ions, Fig. 13. It was concluded that three of the product ions produced by collision induced dissociation of oxazepam and nordiazepam had the same mass-to-charge ratios with a difference of \(\leq 0.022\) Da. The ion used in the SRM transition for nordiazepam, \(m/z\) 140.0, was present in both spectra.

![Figure 13. LC-QqTOF MS/MS spectra of working standards of (A) oxazepam (\(m/z\) \([\text{M-H}_2\text{O}]^+\) = 271.0) and (B) nordiazepam (\(m/z\) \([\text{M+H}]^+\) = 271.0).](image)

The peak of oxazepam was eliminated from the SRM channel for nordiazepam by reducing the cone voltage in the QqQ system from 50 to 25 V. Thus, the in-source fragmentation of oxazepam to the ion with a mass-to-charge of 271.0 was decreased.

### 4.5. Method validation

At present, there are no harmonized validation guidelines available for analytical methods developed for the analysis of APIs and metabolites in the aquatic environment. In this thesis, isotope-labeled compounds were used for method validation at trace level concentrations in the actual matrix in which the analytes were detected (I–IV).
4.5.1. Method validation by the use of isotope-labeled standards

Isotope-labeled compounds have almost the same physical and chemical properties as their unlabeled compounds [109] and are, therefore, suitable for validation purposes. The advantages of using isotope-labeled compounds are that these compounds would not be expected to be present in environmental matrices. Thus, the validation can be assessed at low concentrations in the actual matrix. In this thesis (I–IV), isotope-labeled compounds were used for the determination of: solid phase extraction recoveries, the accuracy of the method, precision of the chromatographic system, method quantification limit (MQL), method detection limit (MDL) and matrix effects in the MS interface. In addition, the linearity of the calibration curves (expressed as the correlation coefficient, $R^2$) was determined by the use of the isotope-labeled compounds as internal standards (II, III).

In Paper II, the matrix effects in the electrospray ionization source were studied. These effects were caused by i) the co-elution of the analytes and the isotope-labeled compounds (Fig. 7) and ii) the co-elution of the analytes and matrix compounds from the wastewater samples. The response for norfluoxetine in the MS detector was suppressed by increased concentrations of fluoxetine-d5. However, the suppression of ionization for norfluoxetine was compensated for by the use of nordiazepam-d5 as the internal standard (II). The observed suppression of norfluoxetine by fluoxetine-d5 would, therefore, not affect quantification. The matrix effects were also determined by post-column infusion of fluoxetine-d5 and norfluoxetine-d5 into the MS interface [110] and by the matrix factors (MF) as described by Matuszewski et al. [82]. During the time that the matrix compounds were eluting from the Chiral AGP column, the ionization of fluoxetine-d5 and norfluoxetine-d5 were suppressed for approximately 15 min, Fig. 14. There was no difference in the trends associated with ion suppression caused by the raw and treated wastewater extracts. This observation might be attributable to the smaller volume of the more complex raw wastewater that was extracted than the volume of the treated wastewater samples. Moreover, the more polar compound, norfluoxetine-d5, was found to be more suppressed by the matrix compounds than the less polar compound fluoxetine-d5. These results are in accordance with results from other studies where polar compounds were more attributed to ion suppression than less polar ones [110]. Furthermore, analytes are subjected to ion suppression if MF $< 100\%$ and, moreover, they are subjected to ion enhancement if MF $> 100\%$ [82]. The stereoisomers of the metabolite $\alpha$-OH-met were also subjected to more ion suppression (MF ranging from 32–37%) than the enantiomers of metoprolol. The MF values were determined to be 51 and 76% for the respective metoprolol enantiomers (III). This difference in response for the metoprolol enantiomers might be explained to some extent by the shorter retention time.
for metoprolol E1 than for metoprolol E2, Fig. 10. As shown by the post-column infusion chromatograms displayed in Fig. 14, the most pronounced ion suppression was observed at shorter retention times (II).

![Figure 14](image)

*Figure 14. LC-MS/MS post-column infusion chromatograms for A) fluoxetine-d₅ and B) norfluoxetine-d₅ when samples of mobile phase, or extracted raw or treated wastewater samples were injected in the LC-system.*

### 4.5.2. Sample and instrumental contamination

According to Richardson and Ternes [31], one of the major analytical trends in the development of methods for the analysis of emerging contaminants in water matrices is the use of isotope-labeled standards. Such standards permit, for example, more accurate quantifications and are valuable for the compensation of such effects as ion suppression [31]. However, when isotope-labeled standards are used in the analytical method, whether in an extended way or as internal standards, it is of great importance to assess the isotopic purity of the compounds to avoid the risk of self-contamination. In Paper I, Millipore water was spiked with fluoxetine-d₅ and norfluoxetine-d₅, and extracted according to the method. The samples were injected into the
LC-MS/MS system and the SRM channels for fluoxetine and norfluoxetine were monitored for peaks with a signal-to-noise ratio $\geq 3$. No peaks for fluoxetine and norfluoxetine were detected, thus, the labeled standards were considered to be sufficiently pure to be used in the method.

The high sensitivity obtained in the SRM mode in the MS detector, in combination with the large concentration factors in the solid phase extraction step, increase the risk of carryover and cross-contamination. Together with cross-talk effects in the mass spectrometer, these parameters can influence the overall accuracy and precision of the method, i.e., the overall reliability. Therefore assessment of cross-contamination, carryover and cross-talk effects is of great importance during the analysis of trace level compounds. The possible cross-contamination during the solid phase extraction was determined by the extraction of Millipore water in parallel with all extracted standards and environmental water samples (I–IV). Possible carryover in the LC-MS system was assessed by the injection of mobile phase or Millipore water at regular intervals between the samples (I–IV). In the tandem quadrupole mass spectrometer, cross-talk effects were determined by injecting one of the analytes into the LC-MS/MS system and monitoring the other SRM channels for peaks with a signal-to-noise $\geq 3$ (I, III).

To summarize, the risk of contamination during the sample handling and the analysis of the environmental water samples was minimized in the methods developed in this thesis. Thus, the positive findings in the wastewater and surface water samples were determined to originate from the environmental water samples and not from the laboratory.

4.6. Analysis of the target compounds in environmental water matrices
The analytes in the wastewater samples were identified by comparing the retention times for the “naturally” occurring analytes with the retention times for the isotope-labeled compounds from the same injection (I–IV). Furthermore, for identification purposes, the ratio of the two product ions in the SRM transitions (Table 6) was compared with those ratios obtained from standard solutions (III, IV).

4.6.1. Chiral analysis of fluoxetine and norfluoxetine
The method developed for fluoxetine and norfluoxetine was applied to raw and treated wastewater, Fig. 15 A. The enantiomers were quantified by the extended use of the isotope-labeled internal standards in a one-point
calibration method (II). The concentration of \((S)\)-fluoxetine was found to be higher than the concentration of \((R)\)-fluoxetine in both raw and treated wastewater, Table 7. The fluoxetine enantiomers have previously been analyzed in wastewater samples from a Canadian wastewater treatment plant [50]. In that study, it was found that the raw wastewater was more enriched in \((R)\)-fluoxetine than the treated wastewater [50]. These differences in concentrations between the wastewater samples might be of significance for water-living organisms. For example, \((S)\)-fluoxetine has been shown to be more toxic for a teleost fish than \((R)\)-fluoxetine [48]; on the other hand, the \(R\)-enantiomer of fluoxetine is more toxic for \textit{Daphnia magna} than for the protozoan \textit{Tetrahymena thermophila} [47].

![Figure 15. Schematic overview of the analytical methods for (A) fluoxetine and norfluoxetine in wastewater (I, II) and for (B) diazepam and nordiazepam in treated wastewater and surface water (IV).](image)

To the best of our knowledge, these experiments were the first time the enantiomers of the metabolite norfluoxetine were quantified in environmental water samples. The concentrations of \((S)\)-norfluoxetine were higher than the concentrations of \((R)\)-norfluoxetine in both raw and treated wastewater samples, Table 7. The enantiomeric fraction (EF) was determined by dividing the concentration of the first eluting enantiomer by the total concentrations of the first and second eluting enantiomers [111]. For APIs in environmental matrices a shift in EF is often used as an indicator of biologically mediated degradation processes [88]. Furthermore, neither the EF values for fluoxetine, nor those for norfluoxetine were significantly different in the raw wastewater compared to the treated wastewater, i.e. there was no indication of microbial degradation of the compounds in the WWTP. Nevertheless, the water samples were collected as grab samples and the raw and treated water samples did not represent the same plug of water.
Table 7. The “naturally” occurring concentrations of fluoxetine and norfluoxetine in raw and treated wastewater samples. RSD % values are given in brackets, n = 6.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Raw</th>
<th>Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>(S)-Fluoxetine</td>
<td>52 (13)</td>
<td>48 (5)</td>
</tr>
<tr>
<td>(R)-Fluoxetine</td>
<td>21 (18)</td>
<td>19 (10)</td>
</tr>
<tr>
<td>(S)-Norfluoxetine</td>
<td>27 (13)</td>
<td>9 (14)</td>
</tr>
<tr>
<td>(R)-Norfluoxetine</td>
<td>12 (11)</td>
<td>4 (12)</td>
</tr>
</tbody>
</table>

4.6.2. Chiral analysis of metoprolol and its metabolites in treated wastewater

The stereoisomers of metoprolol and α-OH-met were quantified in treated wastewater by the use of one-point calibration as described in Paper II and III. An overview of the analytical methods is given in Fig. 16.

![Figure 16. Schematic overview of the analytical methods for the stereoisomers of metoprolol, α-OH-met and COOH-met in treated wastewater (III).](image)

The concentration of metoprolol was found to be higher than the concentration of the metabolite α-OH-met, Table 8. Furthermore, the enantiomeric fraction of metoprolol in treated wastewater was close to racemic, Table 8. Metoprolol has been shown to be racemic in treated wastewater in the aforementioned studies from Canada [49, 50]. The concentrations of the stereoisomers of α-OH-met were quantified in the range 54–151 pM in the treated wastewater. No reports have appeared in the literature about the chiral separation of α-OH-met in environmental matrices. As the separate stereoisomers of α-OH-met were not available in Paper III, the peaks of the two pairs of enantiomers could not be identified. The elution order of the four stereoisomers of α-OH-met was therefore denoted S1, S2, S3 and S4. However, the enantiomeric fractions for the four
peaks of $\alpha$-OH-met were compared to investigate which pair of peaks had an EF value closest to 0.50, the value for a racemic mixture. The EF value of $\alpha$-OH-Met S2 and S3 was determined to be 0.50 (0.01 RSD %), and for $\alpha$-OH-Met S1 and S4 the EF was determined to be 0.50 (0.020 RSD %). Therefore it might be possible that the first and the last eluted peaks were one pair of enantiomers and the second and third eluting peaks corresponded to the other pair of enantiomers. This was, however, not confirmed. Interestingly, the first three stereoisomers of $\alpha$-OH-met were determined to range from 54–61 pM in the treated wastewater samples but the concentration of the fourth eluting stereoisomer was higher, 151 pM. This indicates that $\alpha$-OH-met might have been subjected to e.g., a microbial process in the water matrix or a stereoselective process in the human body.

Table 8. The “naturally” occurring concentrations of metoprolol and $\alpha$-OH-met in treated wastewater samples presented with the enantiomeric fractions (EF). RSD % are given in brackets, $n = 3$.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Treated wastewater [pM]</th>
<th>EF</th>
</tr>
</thead>
<tbody>
<tr>
<td>(R)-Metoprolol</td>
<td>1770 (5.9)</td>
<td>0.51 (0.49)</td>
</tr>
<tr>
<td>(S)-Metoprolol</td>
<td>1860 (6.1)</td>
<td></td>
</tr>
<tr>
<td>$\alpha$-OH-met S1</td>
<td>61 (2.4)</td>
<td>$S1 &amp; S4$</td>
</tr>
<tr>
<td>$\alpha$-OH-met S2</td>
<td>54 (2.6)</td>
<td>0.29 (2.7)</td>
</tr>
<tr>
<td>$\alpha$-OH-met S3</td>
<td>59 (1.0)</td>
<td>$S2 &amp; S3$</td>
</tr>
<tr>
<td>$\alpha$-OH-met S4</td>
<td>151 (2.0)</td>
<td>0.48 (1.3)</td>
</tr>
</tbody>
</table>

The extracted wastewater samples were further analyzed in the Chiral AGP system, Fig 16. However, the signal-to-noise ratios for the peaks of COOH-met were found to be close to three in the treated wastewater. The concentrations of the COOH-met enantiomers might have been low in the raw wastewater or the attenuation of the enantiomers in the wastewater treatment plant might have been adequate. However, the retention times for the COOH-met enantiomers were found to be shortened by the injected matrix. Obviously, the sample extraction needs to be further optimized before any conclusions can be drawn about the occurrence of these compounds in the wastewater matrix.

4.6.3. Analysis of diazepam and nordiazepam in treated wastewater and surface water

In Paper IV, diazepam and nordiazepam were quantified by the standard addition method. A schematic overview of the method is given in Fig. 15 B. The isotope-labeled compounds, diazepam-$d_5$ and nordiazepam-$d_5$, were used as internal standards to compensate for particular potential sources of error, such as shifts in the chemical equilibrium, extraction loss and any variations that might occur throughout the entire analytical method.
The concentration of nordiazepam was found to be higher than the concentration of diazepam in treated wastewater samples. The concentrations obtained from two different sampling occasions, were 66 and 75 pM for nordiazepam and 8.5 and 7.5 pM for diazepam. These results are in accordance with results from other studies where nordiazepam was detected at higher concentrations than diazepam [76]. Furthermore, in other studies, nordiazepam was detected in the water samples whereas diazepam was not [77, 112]. In the surface water samples, collected 3 km upstream from the WWTP, neither diazepam nor nordiazepam were detected. These results indicate that diazepam and nordiazepam enter the aquatic environment by the discharges from the WWTP.
5. Conclusions

In this thesis, chiral and achiral LC-MS/MS methods have been developed for the determination of APIs and their metabolites in aqueous environmental matrices. The analytes were extracted from the water samples by solid phase extraction. It was demonstrated that isotope-labeled standards were suitable for method validation. As the presence of isotope-labeled standards was neither expected, nor detected, in the environmental matrices studied, the method validation could be conducted at trace level concentration in the actual matrix in which the target compounds were present.

In Papers I and II, a method was developed for the analysis of fluoxetine and norfluoxetine in wastewater samples. The enantiomers of the respective compounds were separated by LC employing the CSP Chiral AGP. The target compounds were extracted from the matrices using Evolute CX-50. The importance of optimizing the SPE method to obtain accurate results was demonstrated. The suppression of ionization in the ESI source by the extracted raw and treated wastewater could be studied as a function of time by the direct infusion of fluoxetine-d₅ and norfluoxetine-d₅ into the MS interface. It was found that the responses for fluoxetine-d₅ and norfluoxetine-d₅ were suppressed for about 15 min by the wastewater matrices. Furthermore, the more polar compound, norfluoxetine-d₅, was subjected to more ion suppression than the less polar one, fluoxetine-d₅. The concentrations of the enantiomers of fluoxetine in the wastewater samples were determined to be in the range 19–52 pM by applying a one-point quantification method. For norfluoxetine, the concentrations ranged from 4 to 27 pM. Moreover, the concentrations of (S)-fluoxetine and (S)-norfluoxetine were found to be higher than those for the R-enantiomers in both raw and treated wastewater. This was the first time the concentrations of the norfluoxetine enantiomers were reported in environmental water samples.

In Paper III, metoprolol, α-OH-met and COOH-met were simultaneously extracted from the environmental water samples with Oasis HLB. It was demonstrated that the stereoisomers of the bases, metoprolol and α-OH-met, could be separated by the CSP Chiral CBH in the same separation system. The enantiomers of the acidic analyte, COOH-met, were separated by Chiral
AGP. The chiral separations were conducted by RPLC and using MS compatible mobile phases. It was concluded that (S)-metoprolol and (R)-metoprolol were present in the same concentration range in treated wastewater, 1860 and 1770 pM, respectively. The concentrations of the first three eluting stereoisomers of α-OH-met were 54–61 pM. However, the fourth eluting stereoisomer was present at the higher concentration of 151 pM. In this thesis, the concentrations of the stereoisomers of a metabolite to a β-blocker are reported in wastewater samples for the first time.

In Paper IV, it was demonstrated that a chemical equilibrium between nordiazepam and a transformed intermediate of nordiazepam was shifted towards the intermediate in acidic water solutions, but towards nordiazepam during solid phase extraction. These shifts in the chemical equilibrium might result in an overestimation of the extraction recoveries for nordiazepam. Furthermore, an isotope-labeled internal standard of nordiazepam needs to be added to the wastewater samples immediately after sample collection to compensate for any shift in the chemical equilibrium. Unless this is carried out, one cannot guarantee the accuracy of the method.

In the LC-MS/MS system developed for diazepam and nordiazepam, an isobaric interference was observed with the same precursor and product ion as nordiazepam; the compound was determined to be oxazepam. Prior to its identification, the unknown compound was studied in the treated wastewater samples using a combination of LC and HRMS, employing a QqTOF MS system and an in-house developed database containing about 4000 APIs. Finally, the concentrations of nordiazepam were determined to be one order of magnitude higher than the concentrations of diazepam in the wastewater effluents. None of these compounds was detected in the River Fyris. These results indicate that diazepam and nordiazepam reach the aquatic environment through the discharges from WWTPs.

The development of analytical methods for the trace analysis of APIs and their metabolites is not always straightforward. In this thesis, some of the important aspects to consider during method development and method validation have been highlighted. The exchange of HPLC for UPLC ought to improve the efficiency of the separation system as this result in higher selectivity and less dilution of the analytes, i.e. increased sensitivity. Unfortunately, at present, there are very few CSPs available for UPLC. Hopefully, the analytical methods developed will be applied in future monitoring programs to gain knowledge about the occurrence and fate of the selected APIs and their metabolites in the aquatic environment. It would also be of interest to modify and apply the chiral and achiral methods developed and presented in this thesis to other environmental samples, such as terrestrial matrices.
6. Summary in Swedish / Populärvetenskaplig sammanfattning

Läkemedel är substanser som är avsedda att förebygga, lindra eller bota sjukdomar och sjukdomssymtom hos människor och djur. Det främsta syftet med all tillverkning av läkemedel är att upprätthålla hälsa hos människa och djur. Men under de senaste årtiondena har läkemedelsanvändningen ökat vilket har resulterat i att läkemedelsrester har uppmätts i den akvatiska miljön; i sjöar, floder, havsvatten, grundvatten och till och med i dricksvatten. Konsekvenserna av att dessa substanser sprids till naturen är fortfarande till stor del okända.


Den potentiella faran med att läkemedel sprids till miljön är att substanserna kan vara svårnedbrytbara och ekotoxiska men även att de kan lagras i vattenlevande växter och djur. De potentiella ekotoxikologiska effekterna är dock till stor del fortfarande okända. För att bedöma dessa potentiella risker behövs information om vilka läkemedel som finns i miljön och i vilken koncentration de förekommer. Syftet med denna avhandling var att utveckla analysmetoder för att mäta läkemedel och dess metaboliter samt bestämma dess koncentrationer i avloppsvatten och i ytvatten. Koncentrationerna av läkemedelsresterna i vattenmiljön är låga, ofta förekommer endast några miljarddels gram av substansen per liter vatten. Detta ställer höga krav på de analysmetoder som används för att analysera vattnet. Avhandlingen består av fyra delarbeten där vattenprov från Uppsalas största avloppsreningsverk (Kungsängsverket) har analyserats. Även ytvatten, hämtat från Fyrisån i Uppsala, analyserades med samma frågeställning.
De analysmetoder som utvecklades är uppdelade i tre delsteg. I första steget renas vattenproven från oönskade ämnen som annars skulle störa mätningen av läkemedelsresterna. Detta stege leder också till att läkemedlen och metaboliterna uppkoncentreras. I det andra steget separeras läkemedlen från varandra så att de kan mätas enskilt i det tredje steget.

Nedan ges en kort sammanfattning av avhandlingens ingående delarbeten.

I delarbete I och II utvecklades en analysmetod för läkemedelssubstansen fluoxetin och dess metabolit norfluoxetin. Fluoxetin säljs bl.a. under produktetnamnet Fontex i Sverige men är kanske mer känt som Prozac. I människans omvandlas fluoxetin delvis till metaboliten norfluoxetin. Fluoxetin är en så kallad kilar molekyl, d.v.s. substansen förekommer i två former. Dessa två former förhåller sig till varandra som spegelbilder på motsvarande sätt som högerhand och vänsterhand förhåller sig till varandra. De två formerna kallas (R)-fluoxetin och (S)-fluoxetin och är varandras spegelbilder. I läkemedlet Fontex föreligger fluoxetin i dessa två spegelbildsformer. I naturen har det visat sig att (S)-fluoxetin är mer toxisk för en specifik mört än vad (R)-fluoxetin är. Vår studie visade att koncentrationerna av (S)-fluoxetin var högre än koncentrationerna av (R)-fluoxetin i det vatten som släpps ut i Fyrisån från Kungsängsverket. På motsvarande sätt var koncentrationerna högre för metaboliten (S)-norfluoxetin än (R)-norfluoxetin. Dessa skillnader i koncentrationer mellan spegelbilderna kan vara av betydelse för vattenlevande organismer.


I den sista studien, delarbete IV, analyserades vatten på förekomst av den lugnande och ångestdämpande läkemedelssubstansen diazepam som säljs under produktetnamnet Stesolid. I vatten som togs från Fyrisån, uppströms om Kungsängsverket utsläppspunkt, uppmättes inget diazepam. Nordiazepam, en metabolit till diazepam, återfanns inte heller. I det renade vattnet från Kungsängsverket detekterades däremot både diazepam och nordiazepam. Detta tyder på att diazepam och nordiazepam sprids till miljön via utsläppen från reningsverken. Intressant nog var koncentrationerna av metaboliten nordiazepam högre än koncentrationerna av diazepam. Detta visar att det
inte bara är av yttersta vikt att utveckla analysmetoder för läkemedelssubstanserna som finns i de registrerade läkemedlen på apoteken, utan även för nedbrytningsprodukterna som bildas i människa. I avloppsvattnet från Kungsängsverket detekterades även en tredje okänd substans som den utvecklade metoden inte var avsedd att mäta. Med analytiska instrument, som kan bestämma substansers vikt med hög noggrannhet, kunde den okända substansen identifieras som det lugnande läkemedlet oxazepam, Sobril. Oxazepam är nära besläktat med diazepam.

De analysmetoder som utvecklades i detta avhandlingsarbete kan användas för att öka kunskapen om riskerna som är förknippade med läkemedelsrester i miljön. De analytiska metoderna är även ett viktigt redskap i utvecklingsarbetet av avloppsverkens reningsprocess. Målet bör vara att, i framtiden, rena vattnet från läkemedel och metaboliter innan det släpps ut i våra vattendrag.
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