DETECTION AND STEREOSELECTIVE ANALYSIS OF THREE METOPROLOL METABOLITES IN STP-EFFLUENT SAMPLES

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Abstract
The occurrence of betablockers in wastewaters is known for a long time. However knowledge about the presence of their metabolites is scarce. Therefore, water samples from the effluent of a German sewage treatment plant (STP) were taken with the aim of detecting transformation products of the betablocker metoprolol. Gas chromatography-mass spectrometry analysis revealed that not only the parent compound, but also three chiral metabolites were present in the samples, namely metoprolol acid, α-hydroxy-metoprolol and α-keto-metoprolol. The stereoisomers of the respective compounds were analysed using enantioselective GC-MS and enantio-selective HPLC. Metoprolol acid as well as the two pairs of enantiomers of α-hydroxy-metoprolol are present as almost racemic mixtures in the STP effluent, whereas for α-keto-metoprolol the second eluting enantiomer is more dominant than the first eluting one. These results imply that very different stereospecific preferences of the respective enzyme receptors are prevalent in the human body and in the microorganisms that are active in the STP.

Introduction
Among pharmaceuticals and personal care products, briefly PPCPs, as a "new" class of emerging pollutants, the group of betablockers has received considerable attention. These compounds are cardioselective ß-adrenergic blocking agents and are widely used in the treatment of hypertension and cardiac diseases[1]. In the last years several of them have been detected in sewage treatment plants as well as in surface waters, one of the most often detected compounds of which is metoprolol with concentration levels up to 2.3 µg/L in river water[2]. Metoprolol is a chiral compound with a stereogenic centre in the 2-propanolamin side chain. It is used as racemic mixture, where the $ß_1$-adrenergic receptor possesses a stronger affinity towards the *S*-form[3]. Metoprolol is extensively metabolized in the human body, where the metabolites amount to 85 % of the dose in man[4]. The main pathways are *O*-dealkylation with subsequent oxidation, aliphatic hydroxylation and oxidative desamination. The former pathway leading to the formation of metoprolol acid accounts thereby for 65 %. Like metoprolol many other pharmaceutical compounds are largely metabolized in the human body and the metabolites of some pharma-ceuticals have also been detected in the aquatic environment[5]. But thus far no reports were published about the occurrence of the metabolites of metoprolol in STP or surface water samples.

In the recent years emphasis was also placed on the analysis of single enantiomers, in order to get more detailed insight into the environmental fate and the transformation processes of chiral compounds. However, the analysis was mostly limited to the parent compounds. Therefore, it was the aim of the present study to identify metabolites of metoprolol in STP samples and to analyse the chiral transformation products by application of enantioselective chromatographic methods.

Materials and Methods
2 L water samples from the effluent of a German sewage treatment plant were taken and extracted by solid phase extraction (SPE) using an Oasis HLB-sorbent. Prior to GC-MS analysis the sample extracts were derivatised. The derivatisation was performed either with chloromethyl-dimethylchlorosilane or in a two step procedure with Methanol/H_2SO_4 and then with chloromethyldimethylchlorosilane. For the analyses of the stereoisomers on one hand side enantioselective GC-MS, on the other hand enantioselective fractionating HPLC with subsequent injection of the separated stereoisomers into the non-enantioselective GC-MS-system for the determination of their peak areas was applied. The following stationary phases were used: a) For non-enantioselective GC-MS: VF5-ms (Varian, Germany), 30 m, b) For enantioselective analysis: Hydrodex-β-6TBDM (Macherey-Nagel, Germany), 25 m for GC and Kromasil-CelluCoat (Akzo-Nobel, Sweden), 250 mm for HPLC analysis.

Verification of the method was carried out by application of reference compounds that were synthesised according to known procedures, including confirmation of their structures by NMR and GC-MS [6, 7].

Results and Discussion

The analysis of the STP effluent samples revealed that not only the parent compound metoprolol, but also three chiral metabolites were present, namely the metoprolol acid (**1**), α-hydroxy-metoprolol (**2**) and a corresponding keto-compound, α-keto-metoprolol (**3**). The former two transformation products are known to stem from human metabolism, but the latter compound is assumed to be formed before or during the STP-passage. The respective SIM-chromatograms of a STP-effluent sample extract after derivatisation with chloromethyldimethylchloro-silane for **(2)** and **(3**) are shown in **Figure 2**, while the corresponding SIM-chromatogram of a STP-effluent sample extract after derivatisation with methanol/H_2SO_4, followed by chloromethyldimethylchlorosilane for the metabolite **(1)** is given in **Figure 3**.

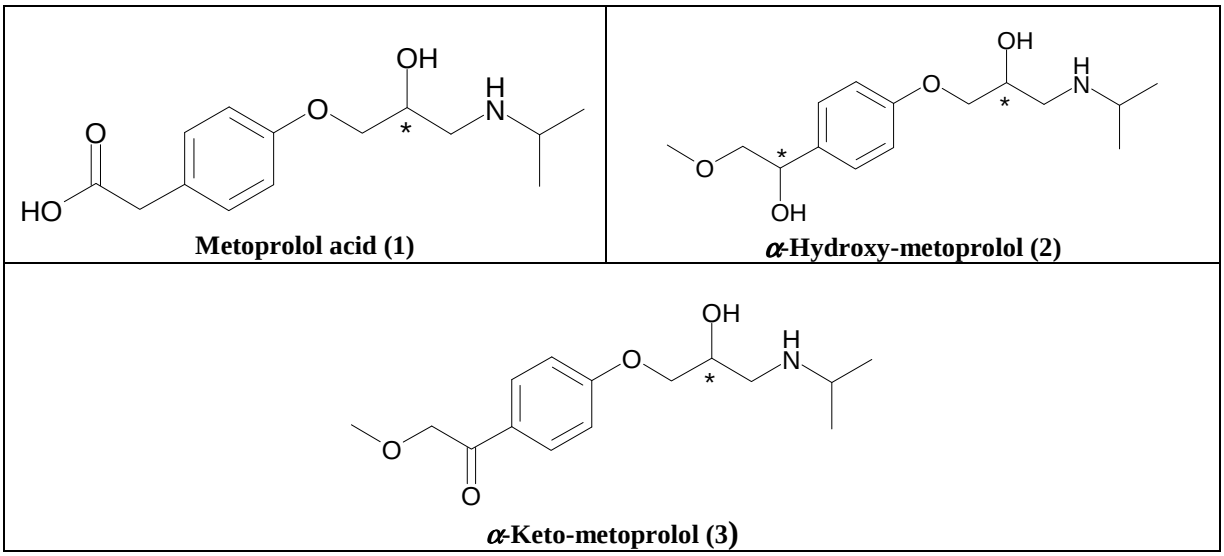

Figure 1: Structures of the three metoprolol metabolites identified in this work.

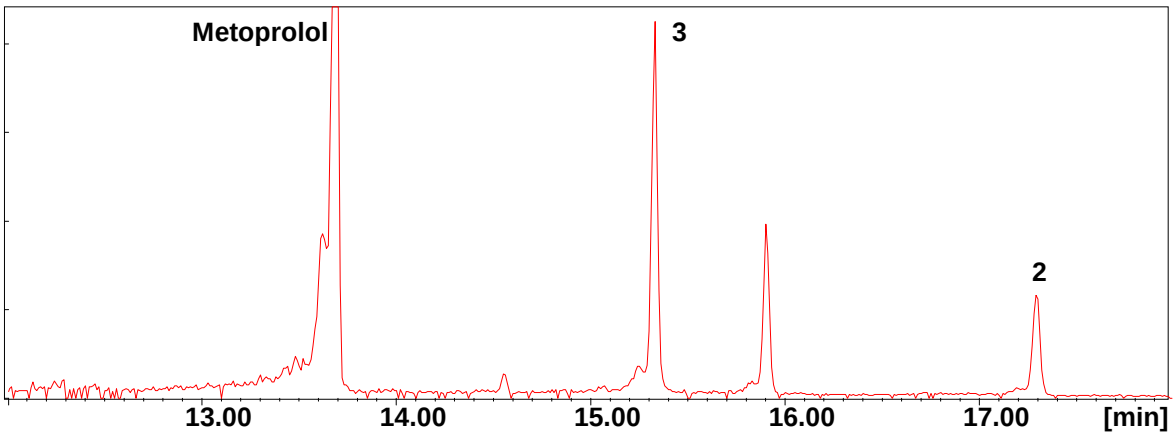

Figure 2: SIM-chromatogram of a STP-effluent sample extract after derivatisation with chloromethyldimethyl-chlorosilane (stationary phase: VF5-ms, 30 m, temperature program: 60 °C (2 min), 20 °C/min, 270 °C (2 min), 83 kPa; ion trace: 186); **(2)** ≡ α-hydroxy-metoprolol, **(3)** ≡ α-keto-metoprolol.

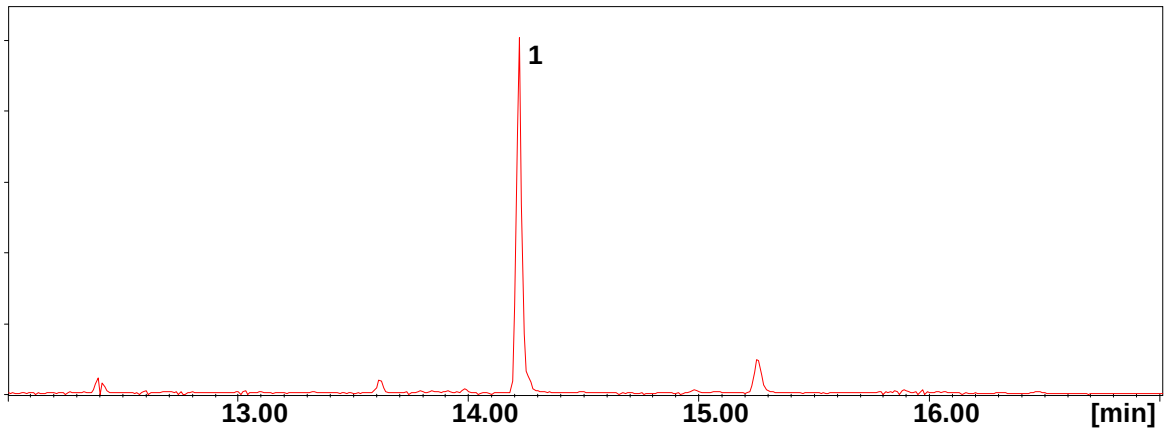

Figure 3: SIM-chromatogram of a STP-effluent sample extract after derivatisation with 1) methanol/H_2SO_4, 2) chloromethyl-dimethylchlorosilane (stationary phase: VF5-ms, 30 m, temperature program: 60 °C (2 min), 20 °C/min, 270 °C (2 min), 83 kPa; Ion trace: 186); **(1)** ≡ metoprolol acid.

For the determination of the stereoisomers of the three metoprolol transformation products enantioselective GC-MS and enantioselective HPLC were applied. Metoprolol acid **(1)** showed good separation of the enantiomers on the Hydrodex-β-6-TBDM-column, whereas for the two other compounds the chiral HPLC-phase (Kromasil-Cellucoat) was suitable. For **(3)** baseline separation of the enantiomers was obtained in its derivatised form, while for **(2)** the underivatised compound gave the best enantiomeric separation (see **Figure 4**). It has to be noted that for **(2)** four stereoisomers exist because of the presence of a second stereogenic centre in the 1'-position. The elution order on the applied chiral stationary phase is also marked in **Figure 4b**) [8].

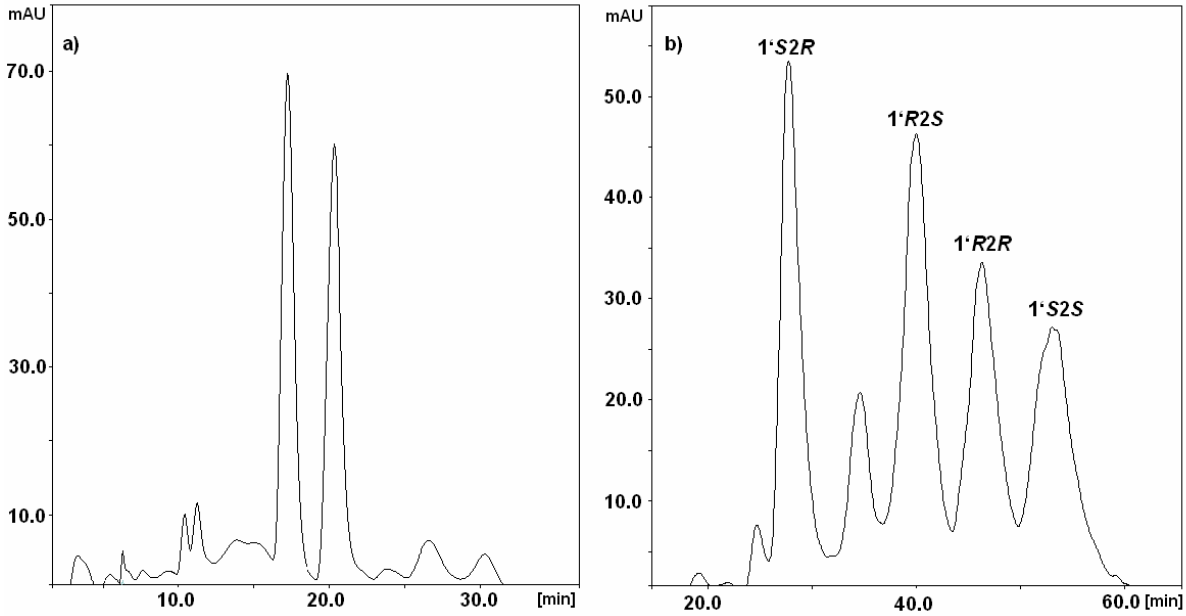

Figure 4: Enantioselective separation of the reference compounds on Kromasil-Cellucoat
a) α-Keto-metoprolol **(3)** (99/1/0.1 *n*-hexane, 2-propanol, diethylamine, 5 °C, 0.5 mL/min)
b) α-Hydroxy-metoprolol **(2)** (98/2/0.1 *n*-hexane, 2-propanol, diethylamine, 5 °C, 0.7 mL/min)

For the enantiomeric pairs of the respective compounds in the STP sample extracts the enantiomeric fractions (EF) were calculated:

EF = E1/(E1+E2)

where E1 and E2 are the peak areas of the first- and the second-eluting enantiomer on the respective columns. Thus, for the metabolites in the STP sample extracts EF values were calculated as follows: 0.5 for **(1)** (see **Figure 5**) reflecting a racemic mixture, while for **(3)** an EF of 0.36 can be inferred. The latter value implies that the formation of α-keto-metoprolol **(3)** in the STP shows an enantioselective preference. Enantioselective separations of (+)-(*R*)- and (−)-(*S*)-metoprolol acid **(1)** in extracts of human plasma and urine were reported by Cerqueira et al. who carried out a metabolism study of racemic metoprolol administered to a patient phenotyped as an extensive metabolizer of debrisoquine [9]. The enantiomeric ratio (+)-(*R*)/(−)-(*S*)-acid metabolite was 1.1 for plasma and 1.2 for urine. This implies that a change in the enantiomeric ratios of **(1)** occurs after the compound left the human body as to be inferred from the present STP sample values.

For compound **(2)** in the present investigation EF values of 0.5 were calculated for the first pair and 0.44 for the second pair of enantiomers. In **Figure 6** the relative concentrations of all four stereoisomers of **(2)** in the STP-samples are shown, in order to display also the diastereomeric relations of the compound. It can be inferred from this plot that the first pair of enantiomers, which exhibits a diastereomeric relation to the second pair, is dominating over the second one by a factor of almost two.

It is interesting to note that Cerqueira et al. developed a direct stereoselective HPLC separation of the four stereoisomers of α-hydroxymetoprolol **(2)** in human plasma and urine extracts on a Chiralpak® AD column[10]. The method was employed to determine the concentrations of α-hydroxymetoprolol stereoisomers in the metabolism study with hypertensive patients mentioned above. The authors observed stereo-selectivity in the α-hydroxymetoprolol formation favouring the new 1'*R* chiral centre from both metoprolol enantiomeric pairs. The similar renal clearances (Cl_R) of the four stereoisomers demonstrated absence of stereoselectivity in their renal excretion. (-)-(*S*)-metoprolol was slightly more α-hydroxylated than its antipode, suggesting that this pathway is not responsible for plasma accumulation of this enantiomer in humans.

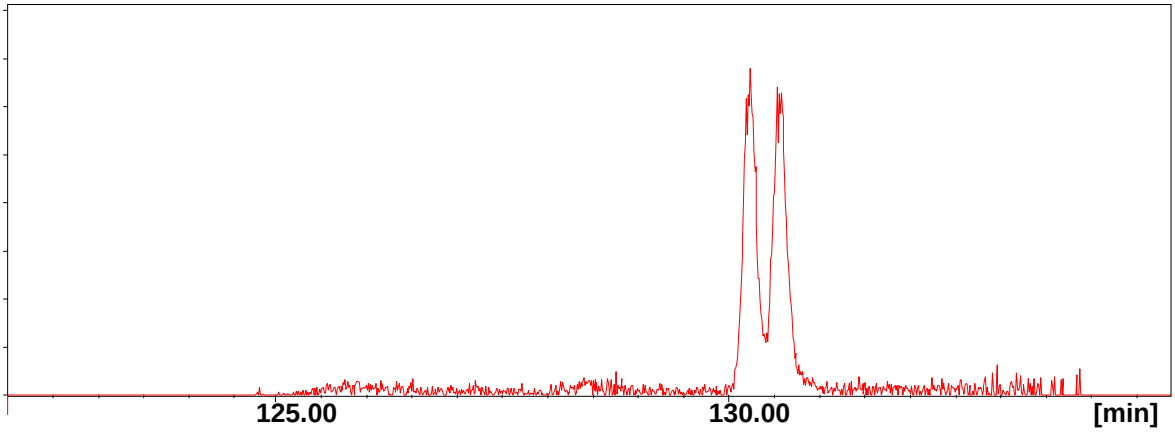

Figure 5: Enantioselective analysis of metoprolol acid **(1)** in the STP-effluent sample extracts (stationary phase: Hydrodex-ß-6TBDM, 25 m; temperature program: 60 °C (15 min), 5 °C/min, 180 °C (80 min), 5 °C/min, 220 °C (13 min), 55 kPa)

Comparison of the results obtained in the present work with those reported by Cerqueira et al. suggests that in the human body α-hydroxymetoprolol formation is favouring the new 1'*R* chiral centre from both metoprolol enantiomers, renal clearances largely show racemic mixtures and no stereoselective preference, while in the STP sample extracts the first pair of enantiomers (1'*S*2*R* ;1'*R*2*S*) is dominating over the second pair (1'*R*2*R* ; 1'*S*2*S*)

with a factor of almost two. Furthermore, no preference of the 1'*R* centre is encountered. Instead, the former enantiomeric pair is racemic, while in the latter pair the 1'*S* centre is dominating. In conclusion, very different stereospecific preferences of the respective enzyme receptors in human bodies and in microorganisms that are active in STPs can be revealed by enantioselective chromatographic methods.

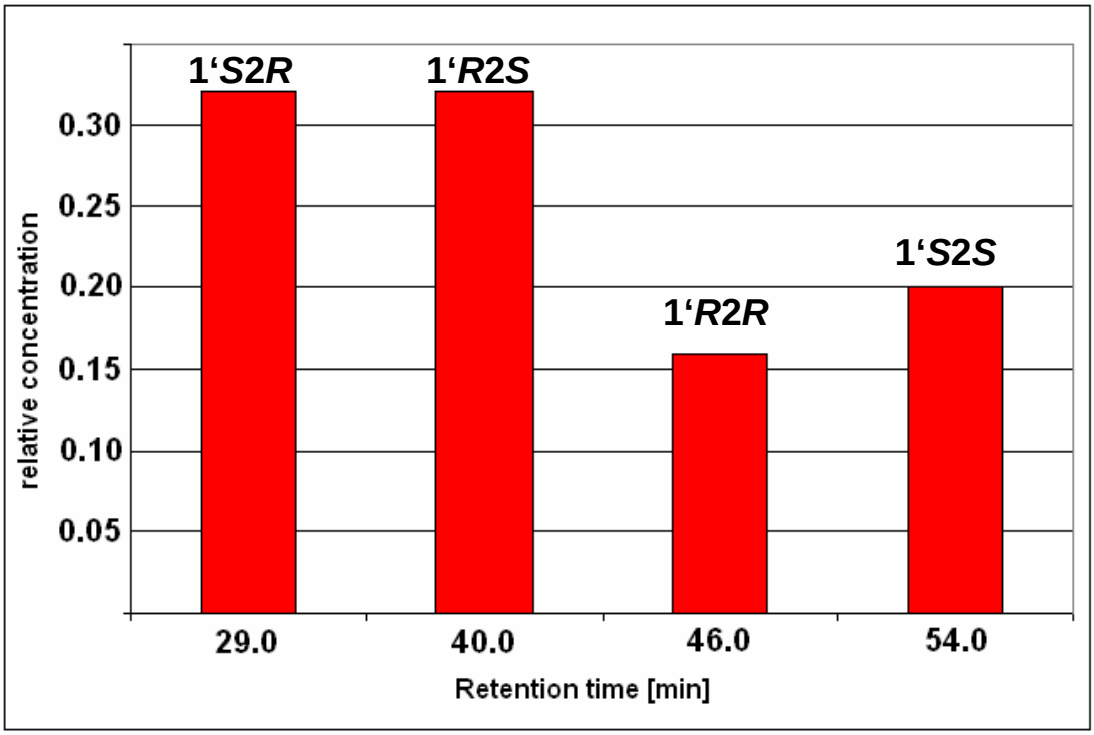

Figure 6: Relative concentrations of all four stereoisomers of α-hydroxy-metoprolol **(2)** in the STP samples.

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