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Metabolic Profiling of Metoprolol via HPLC Coupled with ESI-QqQLIT Mass Spectrometry

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Abstract

Metoprolol, a selective β_1-adrenergic antagonist, is widely prescribed for the treatment of hypertension and heart failure due to its proven mortality benefits. With the expected rise in cardiovascular diseases, understanding the metabolic fate of metoprolol is crucial for assessing its safety, efficacy, and pharmacokinetics. This study aimed to elucidate the in vitro metabolic pathways of metoprolol using High-Performance Liquid Chromatography coupled with Electrospray Ionization and hybrid Triple Quadrupole–Linear Ion Trap Mass Spectrometry (HPLC-ESI-QqQLIT-MS). Metabolism was simulated using in vitro systems, and metabolite identification was achieved through analysis of fragmentation patterns. The fragmentation pathway of metoprolol was first determined using Enhanced Product Ion (EPI) and MS^3 spectra, supported by high-resolution mass spectrometry (HRMS) data. Subsequent identification of unknown metabolites relied on comparison with the parent drug's fragmentation pattern, assuming structural similarity leads to analogous fragmentation behavior. Precursor Ion Scans (PI) and Neutral Loss Scans (NL), along with Data-Dependent Acquisition (DDA), enabled targeted detection and structural reconstruction of metabolites. A total of 29 product ions were identified in the collision-induced dissociation (CID) pathway of metoprolol. From these, 27 structures were proposed, revealing typical neutral losses of water, ammonia, propylene, and methanol. Identified metabolites included O-demethylmetoprolol (TP 1, m/z 254), three isomers of α-hydroxymetoprolol (TP 2a–2c, m/z 284), and metoprolol acid (TP 3, m/z 268). The findings demonstrate that HPLC-ESI-QqQLIT-MS, in combination with precursor and neutral loss scanning, is a robust and efficient technique for metabolite profiling and structural elucidation.

Keywords: Metoprolol, Metabolism phase 1 and 2, HPLC-ESI-QqQLIT-MS, Fragmentation Pathway, Mass Spectrometry, Precursor Ion Scan, Neutral Loss Scan, Structural Elucidation, β_1-Adrenergic Antagonist

Introduction

Metoprolol, 1-[4-(2-methoxyethyl)phenoxy]-3-(propan-2-ylamino)propan-2-ol, is a selective beta-1 adrenergic antagonist indicated for not only the management of hypertension but also for the management of heart failure due to its proven mortality benefits (Heidenreich & al., 2022). Due to the anticipated substantial rise in the global prevalence of hypertension and heart failure, there is an expected surge in healthcare expenditures on metoprolol formulations (Vaidya, & Patel,, 2012). Given its status as a xenobiotic, it is important to conduct experiments that delve into the mechanisms by which the body processes this vital drug for cardiovascular diseases. Metabolism studies serve as important means of understanding how the body handles drugs. Findings from such investigations further provide valuable safety information as drug metabolites could be inherently pharmacologic or toxic (Baillie & al., 2002). A method used for drug metabolism investigation at the pre-clinical stages involves the utilization of in vitro systems, such as microsomes, hepatocytes, and/or S9 fractions (Ball, Scatina, Sisenwine, & Fisher, 2015). This analytical process contributes to a comprehensive understanding of the metabolic pathway of the drug under scrutiny (Lee & Zhu, 2011). The introduction of analytical techniques like mass spectrometry (MS) and high-performance liquid chromatography (HPLC) has revolutionized the landscape of drug metabolism studies. Monitoring biotransformation processes, which result in changing the chemical structure of the parent drug with mass spectrometry becomes straightforward due to the predictability of enzymatic pathways in the body (Smith, Smith, Xia, & Ouyang, 2011). In profiling and identifying metabolites, analysts not only discern alterations in the molecular weights of the biotransformed drug in comparison to the parent drug but also examine the fragmented spectra of the metabolites against that of the parent drug (Brocker & al., 2020), (Arfwidsson, Hoffman, & and Research Laboratories, 1976). This approach of MS application facilitates structural elucidation (Wu & et al., 2008). For that matter, the aim of the current experiment was to investigate the in-vitro metabolism of metoprolol by HPLC – Electrospray Ionization (ESI) – MS that was equipped with a triple quadrupole (QqQ) and a Linear Ion Trap (LIT).

Theoretical part

The biochemical processes that involve both the constructive (anabolism) and degradative (catabolism) transformations of drugs taken into the body is called drug metabolism (Smith, Smith, Xia, & Ouyang, 2011). Facilitated by enzymatic actions, the intricate process of drug metabolism plays a pivotal role in the elimination of exogenous chemicals and substances within the body. The purpose is to convert one chemical moiety into another, ensuring the effective alteration of these substances within the physiological system. Although modifications of drugs can occur in the various parts of the body, for example hydrolysis of aspirin in the plasma, drugs are chemically modified predominantly in the liver, especially by

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the cytochrome P450 (CYP) system (Jambhekar & Breen, 2021). Drug metabolism comprises two distinct phases, namely phase 1 and phase 2 reactions (Shargel & Yu, 2016). These reactions typically unfold sequentially, collectively contributing to the reduction of lipid solubility. Enzymes play a crucial role in this process by either revealing the polar functional groups present in drugs or introducing polar chemical moieties to xenobiotics (Pearson & Wienkers, 2019). This orchestrated series of events ultimately enhances renal elimination. MS is a powerful analytical technique widely employed in drug metabolism studies (Arfwidsson, Hoffman, & and Research Laboratories, 1976). MS operates on the principle of separating ions based on their mass-to-charge ratio *(m/z)* (Burlingame, Whitney, & Russell, 1984). A typical mass spectrometer consists of an ionization source, a mass analyzer, and a detector. A triple quadrupole mass spectrometer (QqQ) is a type of mass spectrometer that consists of three quadrupole mass analysers (Q1, q2 and Q3) arranged in series Figure 1. Each quadrupole has a specific function in the analysis process. The working principle of QqQ has been extensively described (Graham Cooks & Kaiser, 2005).

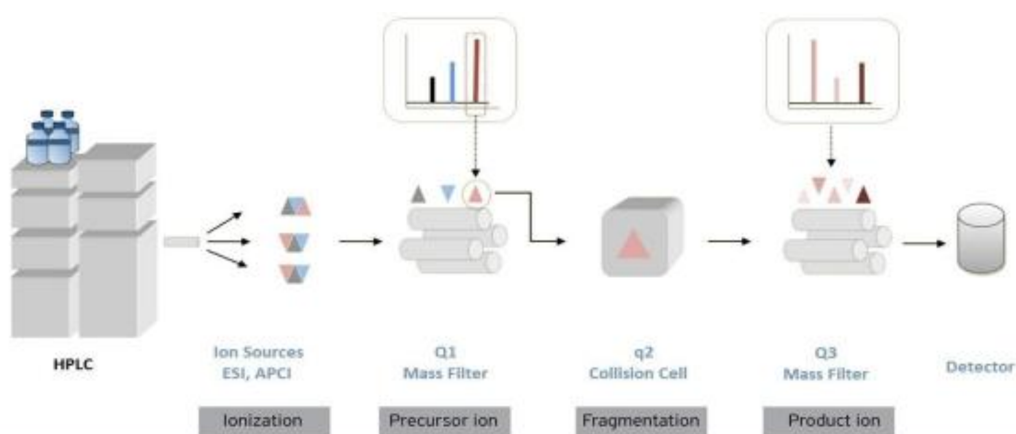

Figure 1: General Scheme of HPLC-ESI-QqQ (Proteomics, 2023)

Ion traps store ions for a considerable amount of time (msec to sec) and accumulate ions to form ion packages for other mass analyzer. Depending on the instrument there are two types of CID, ion beam CID and resonance induced CID. In linear ion traps (LIT), usually ion beam CID takes place by applying alternating current. LITs are used as mass analyzer with low resolution and are used for structural analysis and identification of fragments pathways. Mass accuracy is poor (ca. ± *m/z* 0.1) compared to other mass analyzer and resolution vary in the range from 102-103 (Burlingame, Whitney, & Russell, 1984). In QqQ instruments, a LIT is often implemented as the second stage of mass analysis (Q3), as shown in Figure 2.

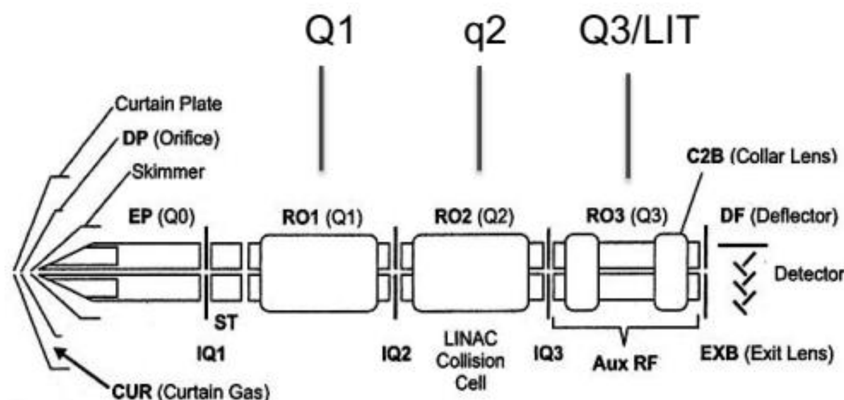

Figure 2: The scheme of QqQ - LIT used in structure elucidation of metoprolol metabolites (Burlingame, Whitney, & Russell, 1984)

Modes of Mass Spectrometry

The operational mode of a mass spectrometer is contingent upon the specific information sought during analysis. Various modes are operated to enable the acquisition of distinct data (Vatansever & et al., 2010), (March & Todd, 2009). The primary operating modes of a mass spectrometer includes Full Scan mode, Product Ion Scan (PIS), Precursor Ion Scanning (PrIS) and Neutral Loss Scan Mode (NLS) (Smith, Smith, Xia, & Ouyang, 2011).

In Full Scan mode, the first quadrupole (Q1) is operated in Scan mode, while the second quadrupole (q2) and third quadrupole (Q3) are operated in RF-only. PIS mode involves scanning for the product ions resulting from the fragmentation of a selected precursor ion (Lavagnini, Magno, Seraglia, & Traldi, 2006). This mode is valuable for elucidating the structural information of a compound by examining the characteristic fragment ions produced during collision-induced dissociation (CID). In this mode, the alternating current (AC) and DC of Q1 are fixed to one setting so that Q1 selects a specific precursor ion from the sample and allows it to pass through to q2. The q2 functions as a collision cell where the selected precursor ions are bombarded with neutral gas so that it undergoes CID, breaking them into fragment ions or product ions (PI). These PI are then allowed to pass through to Q3 and transmitted to the detector.

In the Enhanced Product Ion Scan (EPI), Q1 and q2 have the same functions as in PIS while for the scanning of product ions in Q3 the LIT is used.

PrIS facilitates the identification of the *m/z* of unknown ions (Lee & Zhu, 2011). In this technique, the instrument's AC and DC are systematically varied across a user-defined m/z range. The spectrum is captured within approximately one second, allowing all ions within the specified range to sequentially traverse the quadrupoles and be recorded. In this mode, Q1 is operated in Scan mode, q2 as a collision cell and Q3 acts as a mass filter, allowing specific product ions resulting from the fragmentation to reach the detector. NLS mode focuses on detecting ions that undergo a specific neutral loss during fragmentation. This mode is particularly beneficial for identifying compounds that share a common structural motif and lose a specific neutral fragment upon fragmentation (Lavagnini, Magno, Seraglia,

& Traldi, 2006). Like PrIS, Q1 is operated in Scan mode and q2 as a collision cell but Q3 is operated in Scan mode. Additionally, the difference between Q1 and Q3 in NLS is a constant *m/z* offset.

Results and Discussion

For the identification of metabolites of metoprolol, the fragmentation pathway of metoprolol was analysed first by recording an EPI of the test compound and MS^3 spectra of detected fragments. Additional high-resolution (HR) MS data was used to determine the fragmentation pathway. To identify the metabolites, it is assumed that similar compounds fragment in a similar way. Therefore, the fragmentation pathway of metoprolol and the fragments of the metabolites were compared to reconstruct the structures of the metabolites. To identify the fragments of the metabolites, either the *m/z* of the metabolites must be known, for example by using PrIS and NLS, or data-dependent acquisition (DDA) can be used to record an EPI after the intensity of a certain *m/z* in the PrIS exceeds the set threshold. The scheme for this approach is presented in Figure 3.

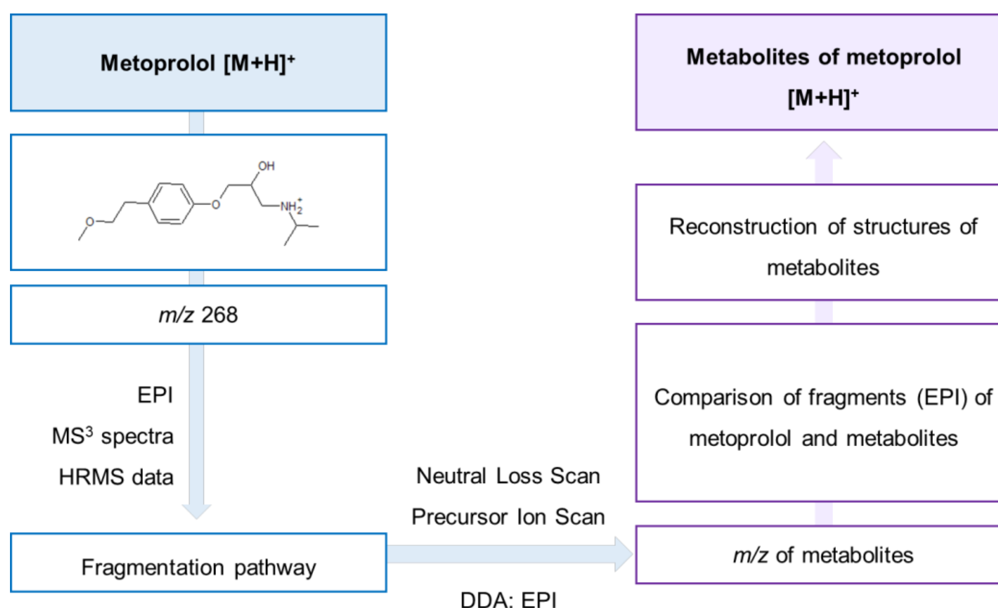

Figure 3: Scheme for the reconstructing the structures of the unknown metabolites of metoprolol

To examine the fragmentation pathway of metoprolol, a standard solution of metoprolol was infused into the MS via syringe pump and fragmented in an EPI while ramping the collision energy (CE) from 5 to 128 volts Figure 4.

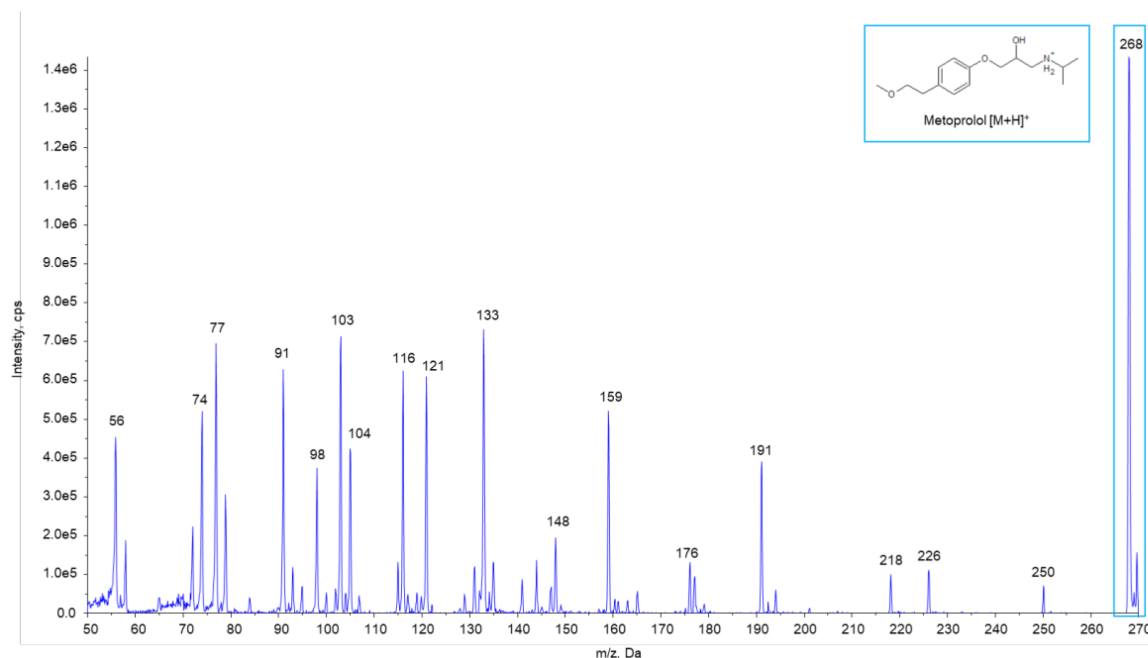

Figure 4: EPI of m/z 268, Metoprolol [M+H] +, measured as standard solution via syringe pump in positive ESI mode

The annotation of the PI is a combination of PI (for product ion) and *m/z* of the PI. At relatively low CE, PI 226, PI 250, PI 121, and PI 116 were obtained. The proposed structures of these PI are presented in Figure 5.

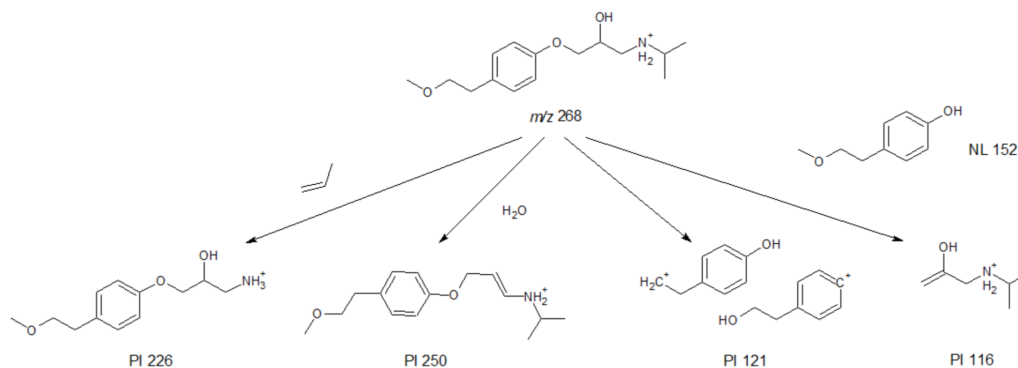

Figure 5: Proposed structures of PI of metoprolol (m/z 268)

PI 226, PI 250, and PI 116 were created by cleaving off propylene, water, and methoxyethylphenol (NL 152), respectively, and are most likely first-generation PI in the MS/MS measurement. The fragmentation pathway of metoprolol was separated into five parts. The first part (mostly first-generation PI and PI 121) and the second part includes the fragmentation of PI 226 and PI 250. PI 226 was fragmented to PI 194 by potentially cleaving off methanol, PI 176 by cleavage of ethanol and water, and PI 191 by elimination of ammonia.

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PI 194 was fragmented to PI 150 by cleaving off ethine and water. For proposing the structure of PI 147, HRMS data was used. The assigned sum formula of PI 147 was $C_{10}H_{11}O^{+}$. When comparing to the sum formula of PI 194, a loss of CH_5NO was observed which could potentially correspond to a loss of water and methylene imine. PI 191 was fragmented to PI 159 by cleaving off methanol. The same PI 159 can be formed by fragmentation of PI 176 by cleaving off ammonia. PI 176 was additionally fragmented to PI 147, PI 148, and PI 133 by cleaving off methylene imine, ethene, and ethine and ammonia, respectively.

To propose structures of the PI, HRMS data was used that is shown in Table 1 for PI 133 and Table 2 for PI 159 with the exact and theoretical *m/z*, the mass error in ppm, Ring Double Bond Equivalents (RDBE) and the assigned sum formula. For *m/z* with more than one assigned sum formula, the data for the *m/z* with higher intensity was chosen for Table 1 and Table 2.

Table 1: HRMS data for PI 133 and further fragments

m/z	exact m/z	theo. m/z	Δ (ppm)	RDBE	Sum formula
133	133.06477	133.06479	-0.13	5.5	$C_9H_9O^+$
105	105.06983	105.06988	-0.43	4.5	$C_8H_9^+$
103	103.05431	103.05423	0.77	5.5	$C_8H_7^+$
79	-				
102	-				
77	-				

Table 2: HRMS data for PI 159 and further fragments

m/z	exact m/z	theo. m/z	Δ (ppm)	RDBE	Sum formula
159	159.08046	159.08044	0.11	6.5	$C_{11}H_{11}O^+$
141	141.06969	141.06988	-1.35	7.5	$C_{11}H_9^+$
144	144.05696	144.05697	-0.04	7.0	$C_{10}H_8O^+$
131	131.08556	131.08553	0.22	5.5	$C_{10}H_{11}^+$
129	129.06979	129.06988	-0.64	6.5	$C_{10}H_9^+$
115	-				

PI 133 fragmented to PI 105 by cleavage of carbon monoxide. PI 105 fragmented to PI 103 by cleaving off molecular hydrogen and introducing a double bond at the ethyl group, producing

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a styrene cation as PI 103. This styrene cation fragmented further to PI 102 and PI 77. PI 77 is a phenyl cation that is a very typical fragment of aromatic compounds in ESI MS.

The last part of the fragmentation pathway of metoprolol includes the fragmentation of PI 121 and PI 116. PI 121 fragmented to PI 91 which is called the tropylium ion and PI 93. PI 116 was fragmented to PI 98 and PI 74 by cleaving off water, and propylene, respectively. PI 98 was further fragmented to PI 81 and PI 56 by cleavage of ammonia, and propylene, respectively.

Identification of metabolites of metoprolol

After identification of the fragmentation pathway of metoprolol, the oxidation sample, assay blank and reagent blank were analysed with reverse-phase (RP)-LC-MS/MS. To first identify the metabolites, the total ion chromatogram (TIC) of Full Scans of the samples and blanks are compared in Figure 6.

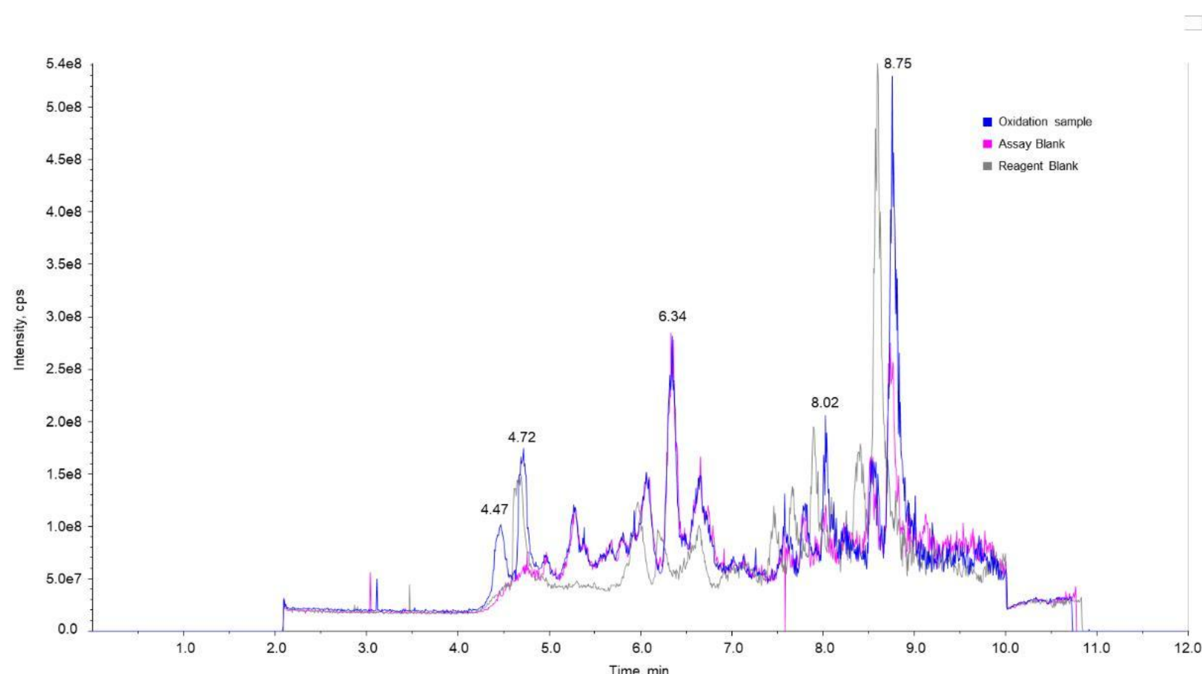

Figure 6: Overlay of TIC of oxidation sample, assay blank and reagent blank in full scan mode

The TIC of the different samples are slightly shifted because of changes between the measurements. Nonetheless, it is visible that the TIC of oxidation sample and assay blank share large similarities from a retention time (RT) of 5 min until the end of the measurement. In contrast to the oxidation sample, the signals at 4.47 min and 4.72 min are not visible in the TIC for the assay blank. The assay blank does not include metoprolol as a component and therefore does not create a signal for metoprolol (or its metabolites) but for potential metabolites of other compounds in the sample. In contrast to the assay blank, the reagent blank does include

metoprolol, but the metabolism was stopped immediately after adding the reagents. The reagent blank therefore shows signals for metoprolol and potential transformation products of metoprolol that were formed, for example during storage. After accounting for the signals, the signal of the metabolites of metoprolol should be distinguishable. The only remaining signal is the peak at 4.47 min. In this case, subtracting the peaks of oxidation sample, assay blank, and reagent blank, might have led to the correct signal. However, it could be possible that one peak does not only correspond to one metabolite or the signal of the metabolites might be of lower intensity than other components in the sample and cannot be found by looking at the TIC. By looking at the TIC there is no information about the *m/z* in the spectrum. To confirm if the peak at 4.47 min corresponds to the metabolites of metoprolol, different modes were used to identify the peak created by the metabolites, identify their *m/z* and finally reconstruct their structure.

To detect the metabolites of metoprolol, PrIS and NLS were utilized to find out the unknown *m/z* of the metabolites by deciding on a structural commonality of metoprolol and its metabolites. PI 116 and the corresponding NL 152 were chosen to create the methods for PrIS and NLS. The fragments were chosen because they are more specific than, for example PI 226 and NL 42 where the loss of propylene in NLS would most likely occur for a large number of matrix compounds in the sample. Choosing larger fragments, for example PI 226 in the PrIS, would limit the type of changes to changes at the amine group which would lead to missing a broad range of metabolites and leave a low mass neutral. By using PI 116 in the PrIS and the corresponding NL 152 in the NLS, changes in either side of the molecule can be detected. The only changes that cannot be detected are changes on both sides of the molecule. Then the *m/z* of the fragments will neither correspond to PI 116 nor NL 152. With the created methods in PrIS and NLS, oxidation sample, assay blank and reagent blank were measured, and the overlay of the TIC in PrIS is shown in Figure 7.

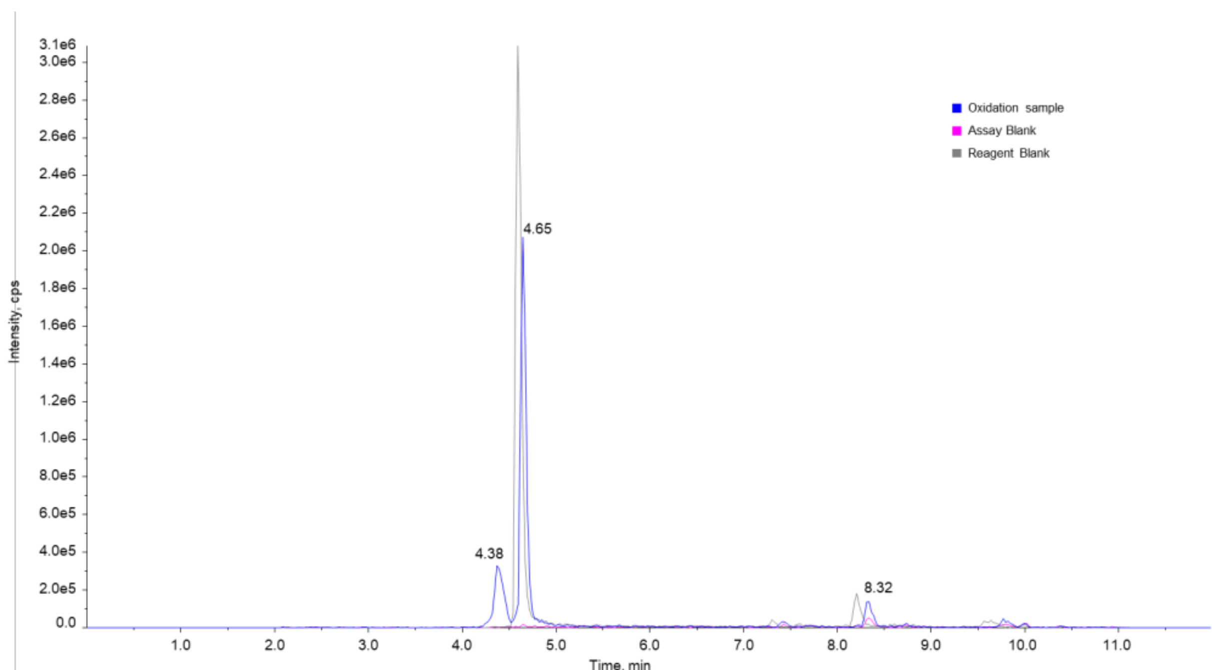

Figure 7: Overlay of chromatograms of oxidation sample, assay blank and reagent blank in precursor ion scan mode

Analogous to Figure 6, the peak of metoprolol should be visible by comparing oxidation sample and reagent blank. The peak at 4.65 min corresponds to metoprolol which makes the peak at 4.38 min correspond to the metabolites. In Figure 8 the TIC of the oxidation sample and the corresponding MS spectra at 4.38 min and 4.65 min are presented.

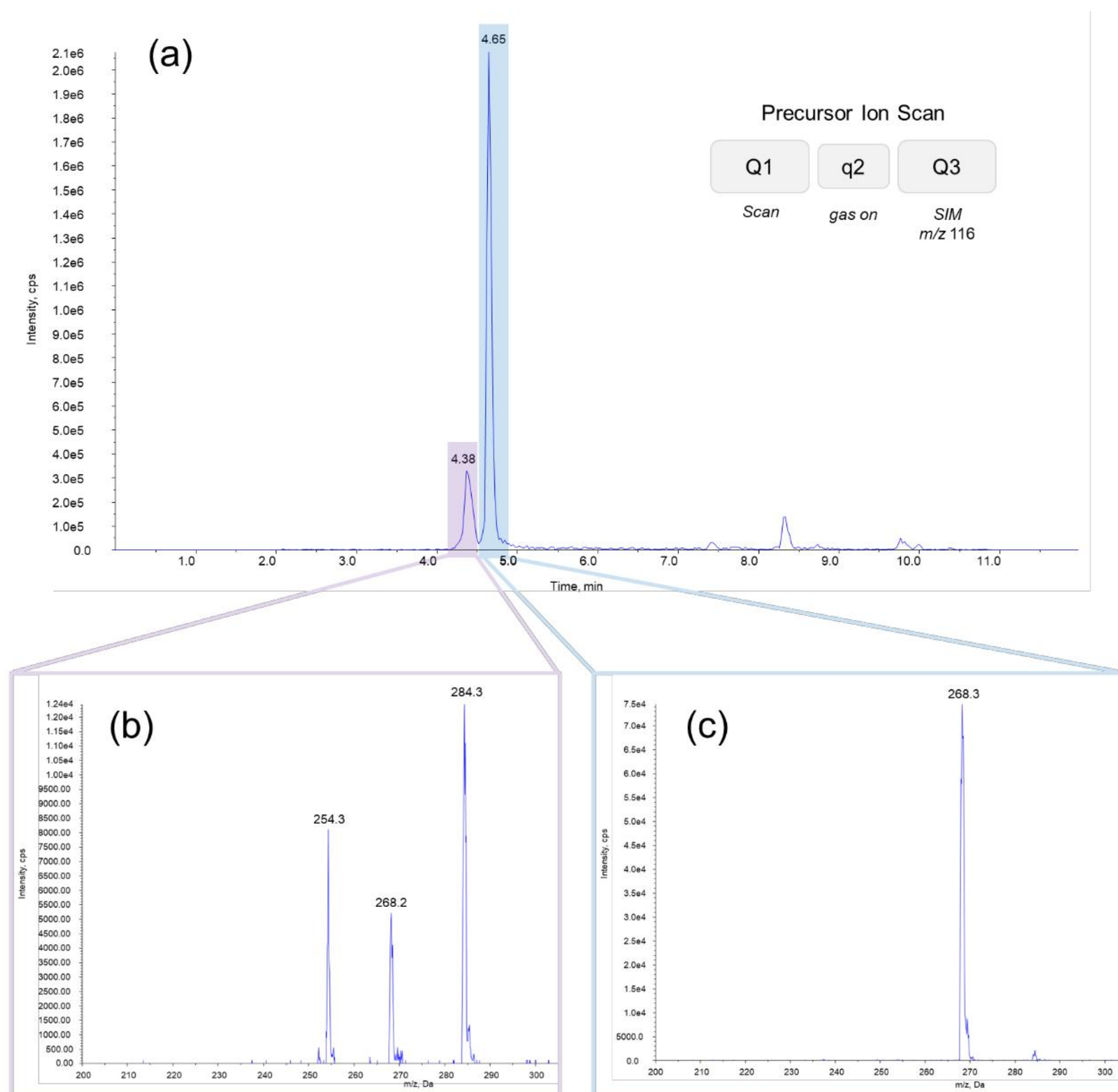

Figure 8: TIC of PrIS of oxidation sample with the modes in QqQ (a) and sections of MS spectra of peaks at RT of 4.38 min (b) and 4.65 min (c)

In Figure 8 it becomes apparent that the peak at 4.65 min corresponds to metoprolol which has *m/z* 268 for $[M+H]^+$. At 4.38 min there are three different *m/z* values (*m/z* 254, *m/z* 268, *m/z* 284*)* visible. In Figure 7 metoprolol eluted at 4.65 min which makes *m/z* 268 at 4.38 min a biotransformational product (TP) instead of the test compound metoprolol. In the MS spectra is becomes clear that one peak observed in the TIC in Full Scan and in PrIS does not only correspond to one metabolite. During the metabolism the test compound will undergo changes that make the molecule more polar. When applying RP-LC, the metabolites elute earlier that the test compound due to the changes in polarity.

Biotransformational Product 1 (*m/z* 254)

To reconstruct the structures of the metabolites, DDA was used to record EPI after a TP exceeded the threshold. The EPI of TP 1 in comparison to the EPI of metoprolol is presented in Figure 9.

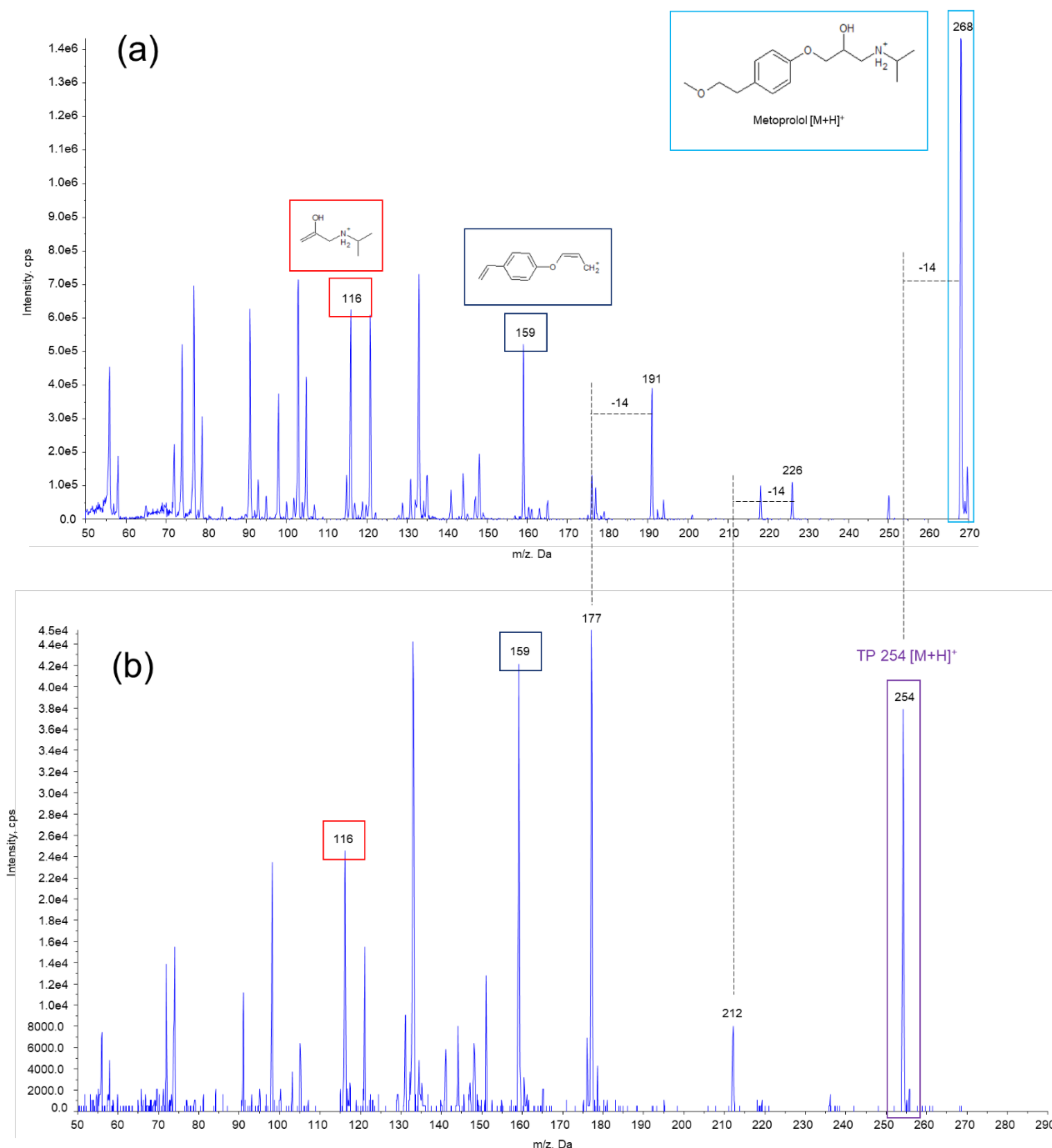

Figure 9: Comparison of EPI of metoprolol (a) and TP 1 m/z 254 (b), PI in common are PI 116 and PI 159

In Figure 9 the fragments that both metoprolol and TP 1 have in common are marked in boxes with the corresponding structures. PI 116 and PI 159 are also found in the EPI of TP 1, which means that the corresponding structures are still present in the TP. Metoprolol and the TP differ by 14 Da. In Figure 9 the fragments from metoprolol were matched to fragments of TP 1 that correspond to a loss of 14 Da. The structure of TP 1 does not differ in the part of the molecule that is not highlighted in Figure 10. The only part where TP 1 can differ is the highlighted part. A difference of 14 Da matches a loss of CH2 which is achieved by demethylation of the methoxy group to a hydroxyl group.

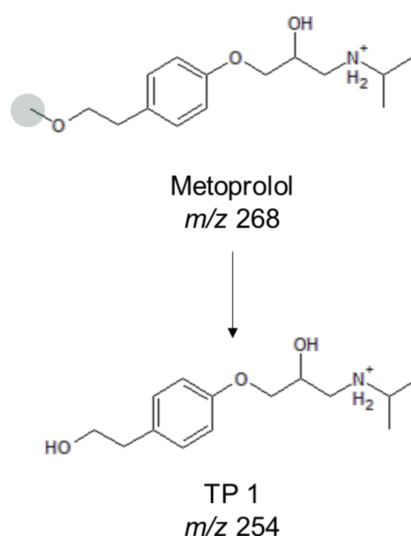

Figure 10: Proposed structure of TP 1 (m/z 254), O – demethylmetoprolol

Biotransformational Product 2 (*m/z* 284)

In the TIC of TP 2 in Figure 11 there are three peaks at RT 4.31 min (a), 4.42 min (b) and 4.51 min (c). In this case, TP 2 is not just one metabolite but multiple isomers that can partially be distinguished by looking at the PI in the EPI that are presented in Figure 12 to Figure 13.

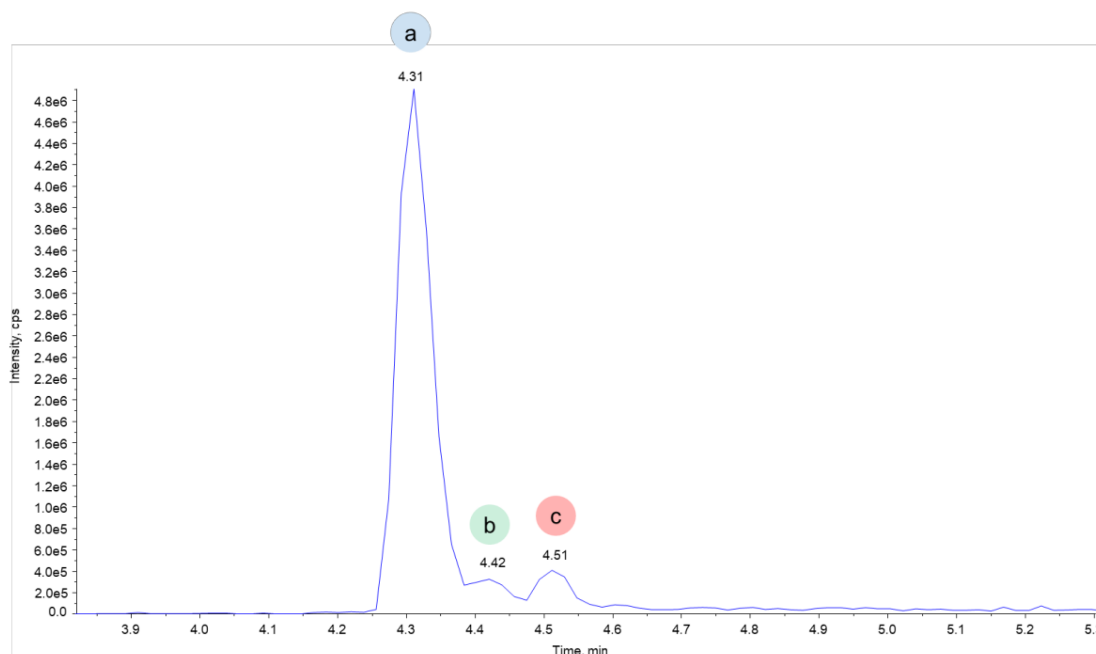

Figure 11: TIC of TP 2 in precursor ion scan mode with three isomers TP 2a, TP 2b, TP 2c

Three isomers, TP 2a, TP 2b and TP 2c, with different RT were identified. In Figure 12 the $[M+H]^+$ signal, the PI that are also formed by metoprolol and their structures (in colour), the PI that are exclusively formed by the TP (dashed boxes, PITP) and their corresponding signal in the fragmentation pathway of metoprolol and the mass difference were annotated.

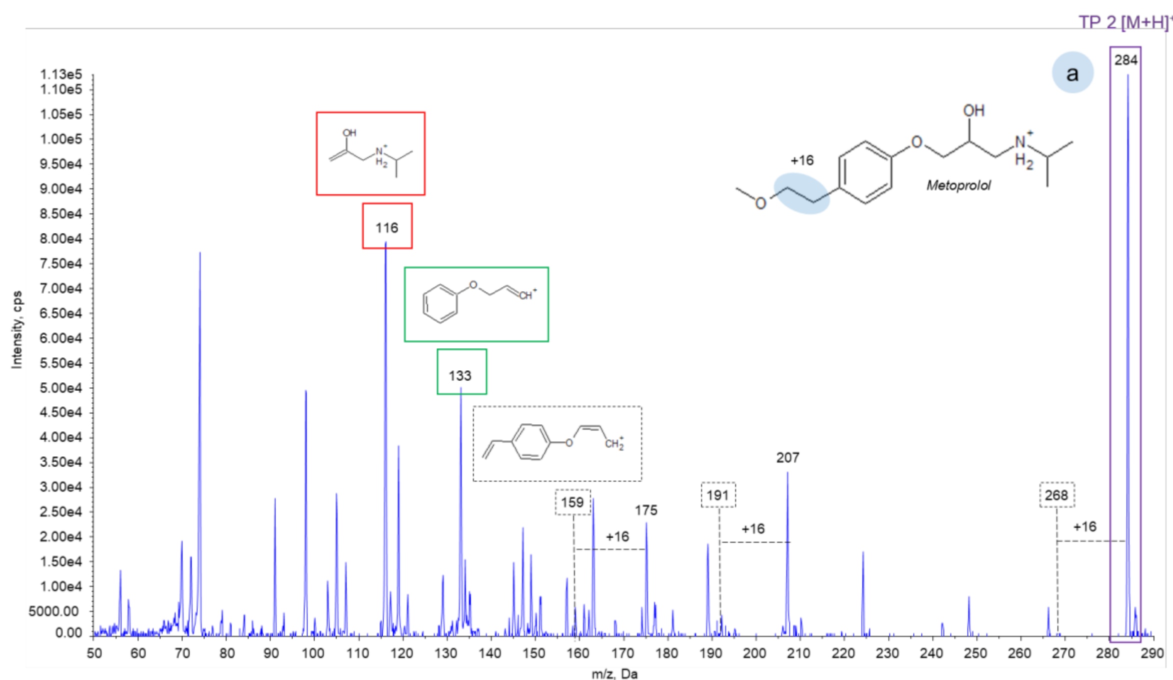

Figure 12: EPI of TP 2a with comparison to metoprolol (dashed boxes), identical fragments to metoprolol are PI 116 and PI 133, metoprolol must have been oxidized (+16) at highlighted carbon atoms

The difference between metoprolol and TP 2 is 16 Da which might correspond to an addition of oxygen. In case of TP 2a, the structures of PI 116 and PI 133 remain identical to that of metoprolol. When comparing PI 133 to PITP 175 which corresponds to PI 159 for metoprolol, the only places left where oxygen could have been added are the two carbons attached to the aromatic ring system. The oxygen must have been added there, creating a hydroxyl group. The annotated MS/MS spectrum for TP 2b is presented in Figure 13.

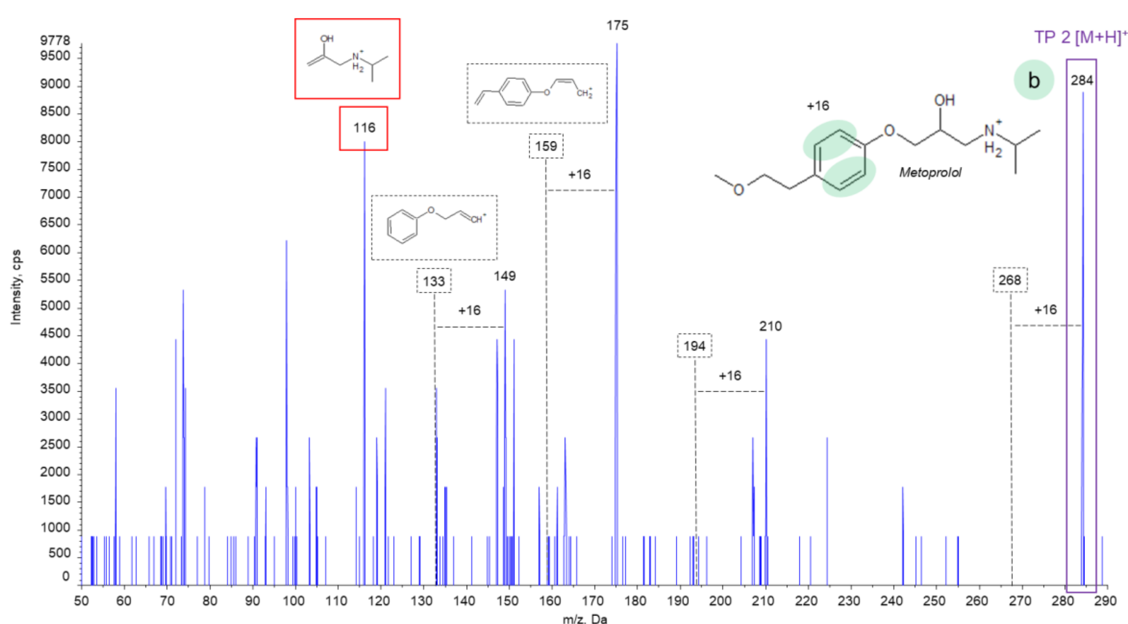

Figure 13: EPI of TP 2b with comparison to metoprolol (dashed boxes), identical to metoprolol is PI 116, metoprolol must have been oxidized (+16) at highlighted carbon atoms

For TP 2b, only PI 116 can be found in the MS/MS spectrum. In this case, comparison of PITP 149 (corresponding to PI 133) and PITP 175 (corresponding to PI 159) leads to the assumption that the oxygen must have been added at the aromatic ring system. The annotated MS/MS spectrum for TP 2c is presented in Figure 14.

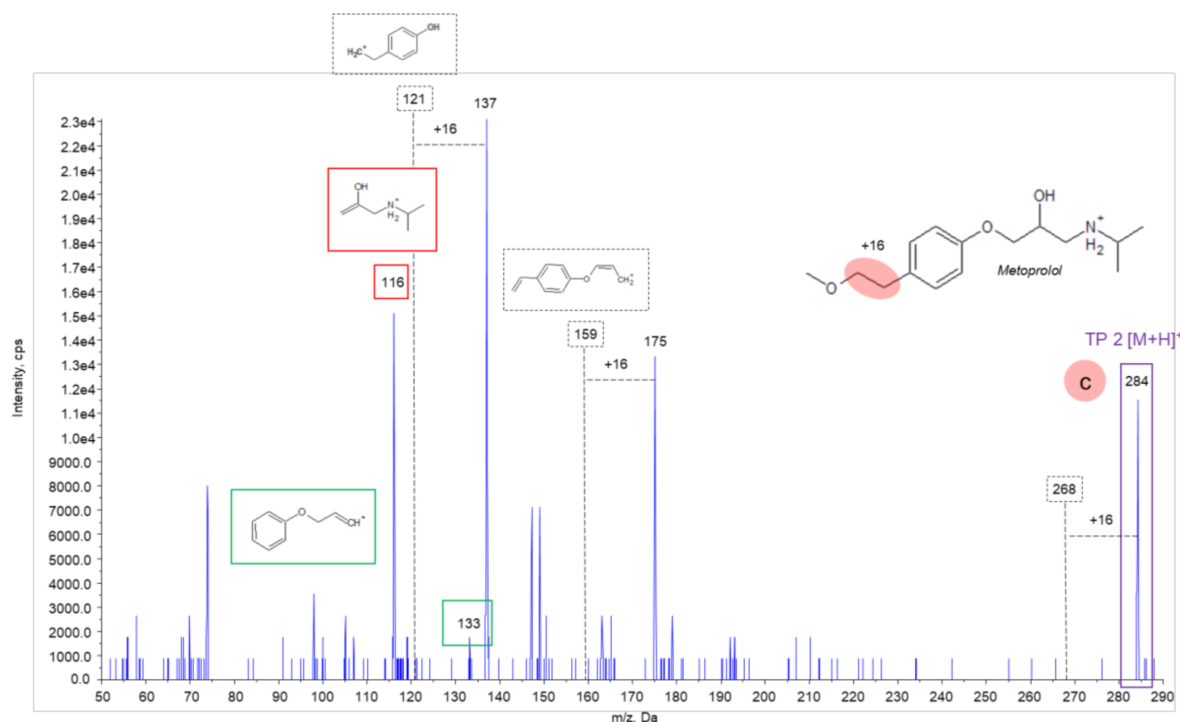

Figure 14: EPI of TP 2c with comparison to metoprolol (dashed boxes), identical fragments to metoprolol are PI 116 and PI 133, metoprolol must have been oxidized (+16) at highlighted carbon atoms

The analysis of the metabolites of metoprolol with HPLC-MS/MS was not suitable for differentiation of some of the isomeric TP. It was not possible to distinguish between the position at the aromatic ring systems or between the two positions at the carbon atoms adjacent to the ring system (Brocker & et al., 2020). For further analysis of the isomers, the compounds would need to be separated, collected, and measured by nuclear magnetic resonance (NMR) spectroscopy if there is enough material to perform NMR (Gathungu, Kautz, Kristal, Bird, & Vouros, 2020).

Biotransformational Product 3 (*m/z* 268)

For TP 3 to have the same *m/z* as the test compound metoprolol, there must be changes in the molecule that sum up to a net difference of 0. The changes must occur in the same part of the molecule, otherwise the TP would not have been detected utilizing PrIS and NLS. The EPI of TP 3 is presented in Figure 15. Some PI that could be found in the EPI of metoprolol are also visible in Figure 15. However, conducting a comparison between metoprolol and TP 3 is not possible due to the absence of discernible mass difference. In Figure 15 a new PITP 145 was formed that metoprolol did not fragment to.

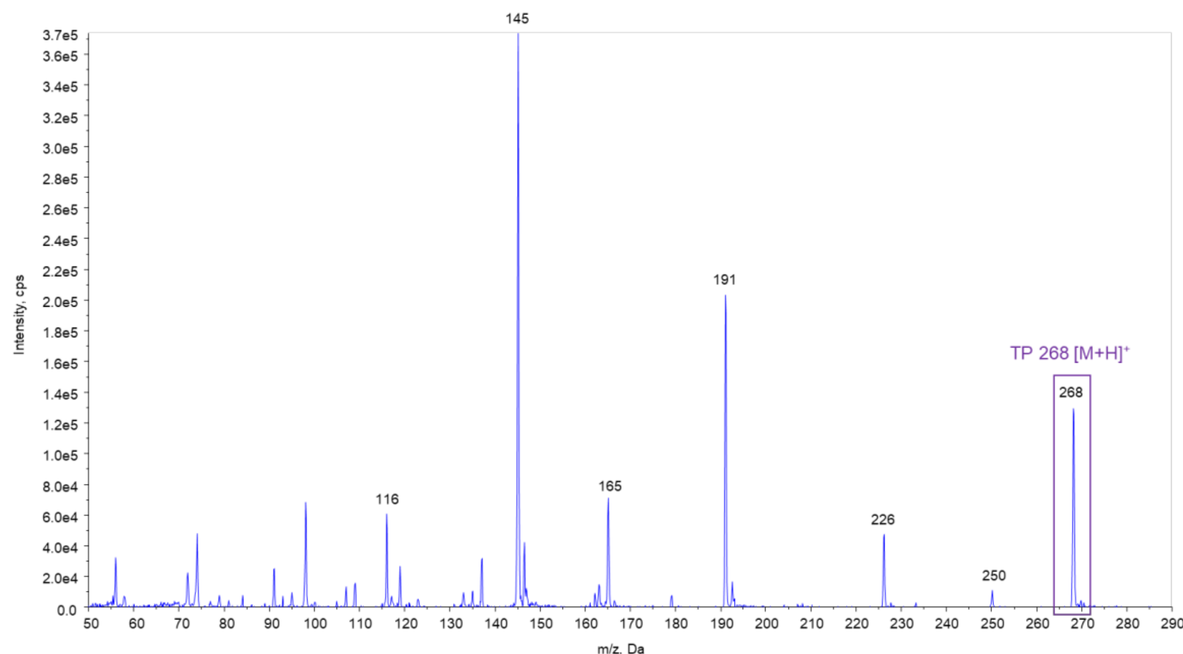

Figure 15: EPI of TP 3 without comparison to metoprolol due to the absence of mass difference, PITP 145 is a new PI

To be able to affirm the changes, different reactions that happen in phase 1 metabolism were lined up to create a net difference of 0. One of the reactions is the demethylation of the methoxy group to an alcohol. In the next step this alcohol is oxidized to an aldehyde that is then also oxidized to a carboxylic acid. 5 The demethylation causes a change of -14 Da, while the oxidation causes a change of +16 Da and -2 Da because two hydrogens are removed during oxidation to form the carboxylic acid. In total this equates to a net change of 0. The proposed structure of TP 3 is shown in Figure 16.

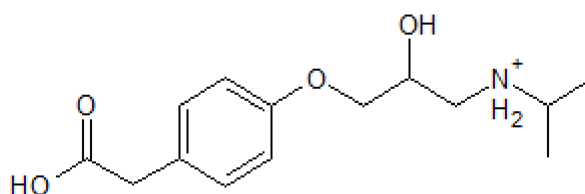

Figure 16: Proposed structure of TP3 metoprolol acid

The proposed structure of TP 3 fits the structure of metoprolol acid which is one of the main metabolites of metoprolol (Brocker & et al., 2020). However, without the literature reference, the structure of TP 3 could not have been identified confidently. To identify the structure more confidently, additional HRMS data of TP 3 and of the fragments could be used. In that case, the HRMS data could have been used to confirm that TP 3 has a sum formula of $[C_{14}H_{22}NO_4]^+$ that matches metoprolol acid.

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In conclusion, the TP of metoprolol in this experiment consist of O-demethylmetoprolol (TP 1), isomers of α-hydroxy metoprolol (TP 2a, TP 2b, TP 2c) and metoprolol acid (TP 3). The three main pathway for the metabolism of metoprolol lead to transformation to α-hydroxy metoprolol, N-deisopropylmetoprolol, and O-demethylmetoprolol which is rapidly oxidized to metoprolol acid (Brocker & al., 2020), (Gathungu, Kautz, Kristal, Bird, & Vouros, 2020), (Berger, Bachmann, Duthaler, Krähenbühl, & Haschke, 2018). N-desisopropyl was not detected in NLS and could not be detected in PrIS because the corresponding PI of N-desisopropyl does not match PI 116. With exception of N-deisopropylmetoprolol, the metabolites of metoprolol were identified correctly by reconstructing the structures.

Summary

Metoprolol is an important adrenergic antagonist that is not only administered for the management of hypertension but also for the management of heart failure. Due to its status as a xenobiotic and an expected surge in healthcare expenditures on metoprolol formulations, it is important to conduct research about the metabolic pathways of such a vital drug. Analytical techniques like HPLC-MS have revolutionized the landscape of drug metabolism studies and are used to monitor biotransformation processes to be able to provide information about toxic or pharmacological activity of developed drugs. For the identification of unknown metabolites of metoprolol, HPLC-ESI-QqQLIT-MS was used to identify the fragmentation pathway of metoprolol, and then Precursor Ion Scan and Neutral Loss Scan was utilized in combination with data-dependent acquisition to record Enhanced Product Ion Scans (EPI), leading to the reconstruction of the structures of the metabolites. In the CID fragmentation pathway of metoprolol, a total of 29 product ions were detected that were incorporated into the pathway.

For 27 product ions a structure could be proposed by using EPI, MS3 spectra and additional HRMS data. Typical losses of water, ammonia, propylene, and methanol were observed along the entire pathway. A combination of PI 116 and the corresponding NL 152 were used for the Precursor Ion Scan and Neutral Loss Scan. The fragments were chosen to ensure high specificity to avoid detecting a large number of matrix compounds while not limiting the metabolic changes to very narrow structural changes. For the reconstruction, the product ions of metoprolol and the metabolites were compared to determine the part of the structure where changes occurred during metabolism. The metabolites were identified as O-demethylmetoprolol as TP 1 (*m/z 254*), isomers of α-hydroxy metoprolol as TP 2a, TP 2b and TP 2c (*m/z* 284) and metoprolol acid as TP 3 (*m/z* 268). A summary of these results is presented in Figure 17. These metabolites are some of the main metabolites of metoprolol which confirms the suitability of HPLC-MS/MS to identify unknown metabolites by utilizing Precursor Ion Scan and Neutral Loss Scan and comparison of fragments from similar compounds

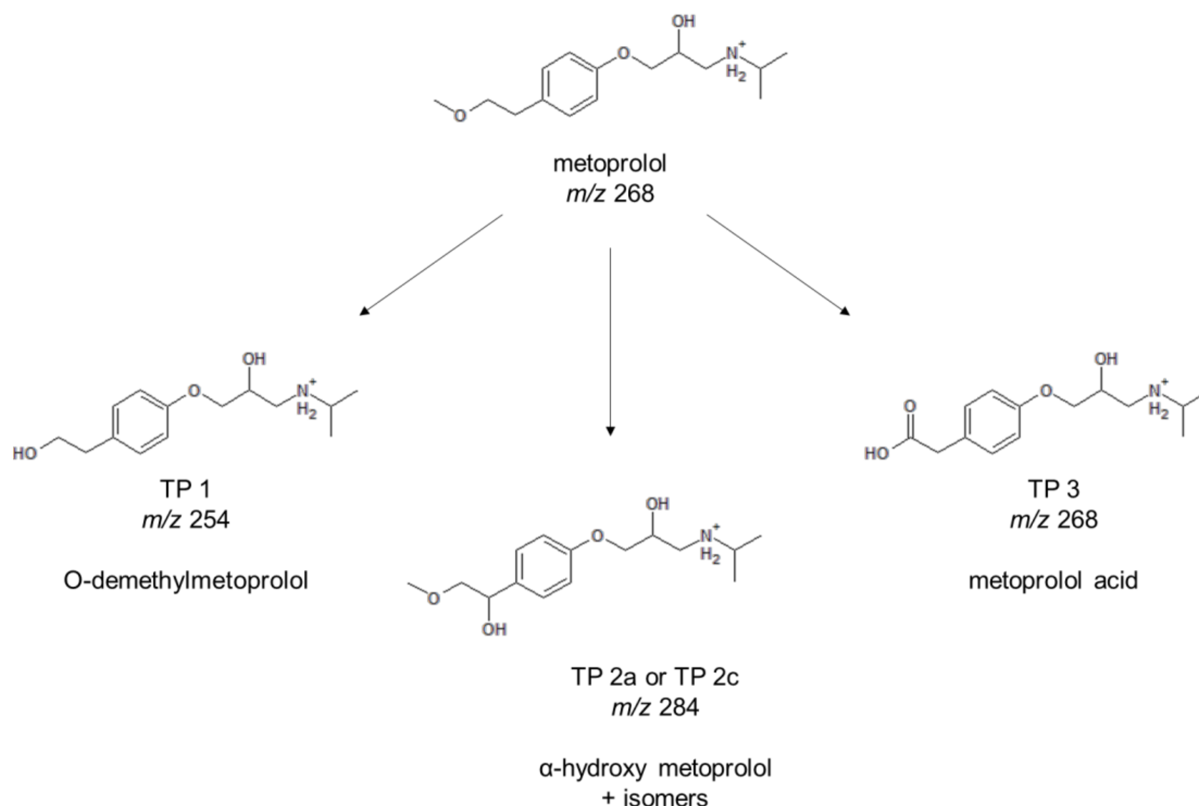

Figure 17: Proposed structure of the metabolic pathway of metoprolol, TP 1 (m/z 254) O - demethylmetoprolol, TP 2a, 2b, 2c (m/z 284) isomers of α- hydroxy metoprolol, TP 3 (m/z 268) metoprolol acid

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