KU LEUVEN

FACULTEIT GENEESKUNDE
BIOMEDISCHE WETENSCHAPPEN

Validatie van een kwantitatieve screeningsmethode voor 7 β-blokkers in bloed met behulp van LC-MS/MS

Validation of a quantitative screening method for 7 β-blockers in blood using LC-MS/MS

Masterproef voorgedragen tot het behalen van de graad van Master in de biomedische wetenschappen door

Flo Elsen

Promotor: Prof. dr. Eva Cuypers
Begeleidster: Sara Loix

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I. Preface

In this preface, I would like to take the opportunity to express my sincere thanks to a few persons who guided and supported me the past year during my work on this thesis.

First of all, I want to thank Prof. Eva Cuypers for giving me the opportunity to do my thesis in the laboratory of Toxicology and Pharmacology of the KU Leuven. This thesis-year really sparked my interest in toxicology and pharmacology. It helped me decide in which direction I would like to go in my professional life. I also want to thank Prof. Cuypers for assisting me and giving me the best guidance with all the problems that I encountered during my work. At certain moments it was really challenging but we could always find a solution.

I also want to thank Sara Loix for being my supervisor and especially for being so patient with me and with the LC-MS/MS. I wish her all the happiness in the world and a lot of success with her new job. This thesis would certainly not have been the same without her.

Then, a lot of gratitude goes to the Phd students Laura Van Hese and Michiel Vandenbosch for always answering my questions about method validation and for helping me with the tantrums of the LC-MS/MS.

Also, I want to thank Prof. Tytgat for giving me the opportunity to work here and for letting me be a member of his excellent team.

Last but not least, I want to thank all the staff members of the laboratory for creating such a nice working environment.

I am eternally grateful for my family and friends who are always there to support me and give me advise. They helped me grow into the person I am now. These five years of studying were sometimes challenging but I have learned so much about myself, about the world and other people. Now, I am closing a chapter of my life, but I am really looking forward to what this world still has to offer me and to all the challenges still to come.

II. Table of Contents

III. List of Abbreviations

Abbreviation	In Full
ACN	Acetonitrile
CE	Collision Energy
CEP	Collision Cell Entrance Potential
CID	Collision Induced Dissociation
CXP	Collision Cell Exit Potential
DP	Declustering Potential
EP	Entrance Potential
ESI	Electrospray Ionization
GC-MS	Gas Chromatography With A Mass Spectrometer
GC-NPD	Gas Chromatography With Nitrogen-Phosphorus Detection
GHB	Gamma-Hydroxybutyric Acid
HPLC-DAD	High Pressure Liquid Chromatography With A Diode Array Detector
IS	Internal Standard
LC	Liquid Chromatography
LC-MS/MS	Liquid Chromatography Tandem Mass Spectrometry
LLE	Liquid-Liquid Extraction
LOQ	Limit Of Quantification
ME	Matrix Effects
MS	Mass Spectrometer
MS_B	Mean Square Between Day
MS_w	Mean Square Within Day
n	Number Of Observations
NSAID	Non-Steroidal Anti-Inflammatory Drug
PP	Protein Precipitation
Q	Quadrupole
QC	Quality Control
R^2	Coefficient of determination Or R-Squared
RE	Recovery
RSD	Relative Standard Deviation
$RSD_{int\ pr}$	Relative Standard Deviation of Intermediate Precision
RSD_{ME}	Relative Standard Deviation of Matrix Effects
RSD_{RE}	Relative Standard Deviation of Recovery
RSD_{rep}	Relative Standard Deviation of Repeatability
RT	Retention Time
sMRM	Scheduled Multiple Reaction Monitoring
sMRM-IDA-EPI	Scheduled Multiple Reaction Monitoring-Information Dependent Acquisition-Enhanced Product Ion
SPE	Solid Phase Extraction
STDEV	Standard Deviation
WADA	World Anti-Doping Agency
WHO	World Health Organization
XB	Xenobiotic

IV. Abstract

Screening is the first step in the toxicological process. It is used to identify components that are present in the sample. Screening is a qualitative method, so it can only give an indication of which substances are present or absent, and needs confirmation by a confirmatory test. A disadvantage of these confirmatory tests is that they are time consuming, time that is sparse in some cases. Also these confirmatory tests are more laborious and expensive. In this research, we validated a quantitative screening method for seven β-blockers in blood using LC-MS/MS. A calibration curve with matrix-matched samples was constructed for each analyte covering the whole therapeutic range and low toxic range. Accuracy and precision results met the proposed criteria except for nebivolol and propranolol. When using the method in practice, it needs to be taken in to account that for these two analytes a certain margin of error on the result exists. Matrix-effects were within a range of 105%-143% and recoveries were within a range of 6%-15%. Stability tests concluded that the analytes stay stable during the whole analytical process which is important for quantification. After validation, five cases, with indication of β-blockers, were tested to determine the concentration of the β-blocker and to assess if the concentration was within the therapeutic range or at a toxic or lethal concentration.

1. LITERATURE STUDY

1.1. Introduction

The domain of toxicology studies the (adverse) effects of chemical substances on organisms. Forensic toxicology defined as 'the use of toxicology to help the medicolegal investigation of death, drug use and poisoning' (1)**,** is an important aspect of the forensic sciences and in the investigation of the cause of death.

Forensic toxicology can provide clarification in postmortem cases that are the result of homicidal, suicidal or accidental intoxications. It also provides clarification in antemortem cases: for victims of sexual assault who were administered gamma-hydroxybutyric acid (GHB) or flunitrazepam (Rohypnol®), for addicts who need to prove they are clean and for driving under the influence of alcohol or other psychotropic substances (2,3).

1.1.1. What is intoxication?

The world health organization (WHO) defines intoxication as 'a condition that follows the administration of a substance and results in disturbance in the level of consciousness, cognition, perception, judgement, or behavior.' Substances that cause intoxication are often called xenobiotics (XB's). These substances are not naturally produced by or expected to be present in a person. Paracelsus (1493–1541), also known as the father of toxicology, once wrote: 'Alle Dinge sind Gift, und nichts ist ohne Gift, allein die Dosis macht dass ein Ding kein Gift ist'. (4) Translated this means: 'All things are poison, and nothing is without poison, it is only the dose which makes a thing a poison'. This means that the dose-response relationship is very important in toxicology. The dose-response relationship describes the intoxication of a person caused by different concentration levels (doses) of the substance. Any XB can have a harmful effect but even seemingly harmless substances like water can be harmful, when administered in high amounts (5). The latter was illustrated at Tomorrowland 2018, a Belgian dance festival where two visitors died due to water intoxication after ecstasy use.

1.1.2. Biological samples used in forensic toxicology

Blood is always the preferred matrix, for postmortem as well as antemortem cases, because it corresponds best with the pharmacological effect (1) and gives an indication of recent drug-use. In antemortem cases, peripheral blood is the sole blood type that can be used. In post-mortem cases, peripheral blood samples are first choice because cardiac blood can be contaminated.

Urine is ideal for the screening of drugs because the analytes and their metabolites are present in much higher concentrations than in blood (1). Drugs can also be detected for a longer period in urine than in blood, weeks instead of days. Since urine is a relatively clean matrix, little sample preparation is needed which makes fast analyses possible. It's impossible to determine the degree of intoxication by analysis of a urine sample. Another disadvantage of urine is that it is an easy matrix to tamper with.

Hair is a matrix in which drugs can be detected during a long period, from months up to several years. In this way it provides a history of exposure to a substance (1). A drug can be incorporated into the hair in three ways; firstly from the blood during hair growth. Secondly from sweat and sebum after the hair is formed and thirdly form the environment. This can take up to three weeks. Incorporation is also dependent on the amount of melanin in the hair, the amount of sweat and sebum on the skin and the lipophilicity and basicity of the drug itself (4). Hair is especially used in cases where the subject needs to prove that he/she is clean from drug-use or alcohol-use. In urine the analyte can only be detected up to weeks, this would mean that the subject needs to donate a urine sample every week, which is not practical. Therefore hair is a more favorable matrix to use, although sample preparation is more time consuming and interpretation of the result is more difficult.

Alternative matrices for postmortem cases are organ biopts (liver, kidney), vitreous humor, bile, bone and bone marrow. These are not often analyzed and for this reason not discussed. An alternative matrix for antemortem cases is oral fluid, which is used for fast screening tests of drugs (cocaine, amphetamines, opiates …).

1.2. LC-MS/MS Screening

Forensic toxicology tries to answer the following questions related to poisoning (2):

- Has the person been poisoned?
- Which poison was administered? And how was it administered?
- Was the amount administered dangerous or lethal?

To answer these questions qualitative and quantitative analysis must be performed.

Screening of the sample is the first step in the toxicological process. Screening is used to identify the XB('s) that are present in the sample. Screening is a qualitive method, it can only give an indication of which substances are present or absent, and needs confirmation by a confirmatory test.

Liquid chromatography tandem mass spectrometry (LC-MS/MS) is the golden standard for screening.

1.2.1. Protein Precipitation

The first step in the analysis process is the cleanup of the sample by protein precipitation (PP), which is a fast and simple sample preparation technique. Additionally it also disrupts protein-drug binding which is important for measuring the total amount of drugs present in the sample (5). PP works by adding a precipitating solvent, for example acetonitrile (ACN), while continuously vortexing. The organic solvent decreases the dielectric constant of the plasma protein solution, which increases the electrostatic attraction between the charged proteins. The organic solvent additionally also displaces water molecules around the hydrophobic regions of the protein surface. In this way, the hydration layer and the hydrophobic interactions decrease. Eventually leading to aggregation and precipitation of the proteins (6).

PP is a non-selective technique, this can be a disadvantage because interfering components can be co-purified with the analytes. This can cause disturbance of the analyte ionization process and consequently ion suppression or enhancement (5). Also with PP you dilute the analyte instead of concentrating it. This makes the technique less sensitive. PP cannot be used for drugs that are strongly bound to proteins because they will co-precipitate. Nevertheless this sample preparation technique was maintained because PP is a low cost, easy and fast technique which is especially important in screening where a lot of samples need to be analyzed at once (high throughput sample preparation).

1.2.2. Liquid Chromatography

After sample preparation the compounds in the sample have to be separated. This is achieved with liquid chromatography (LC). The liquid chromatographer consists of a mobile phase, which contains the analyte of interest, and a stationary phase, the column. In this research we used reversed phase chromatography, here the stationary phase is hydrophobic. The sample is injected into the polar (aqueous) mobile phase and passes through the column. The analytes in the sample have a different affinity for the column, meaning that analytes that are more hydrophobic (less polar) have a higher affinity for the stationary phase and will therefore elute later than analytes that are hydrophilic and have a lower affinity for the column. In this way, the analytes in the sample are separated. The output of the LC is then directed to the mass spectrometer (MS) which will detect and identify the analytes.

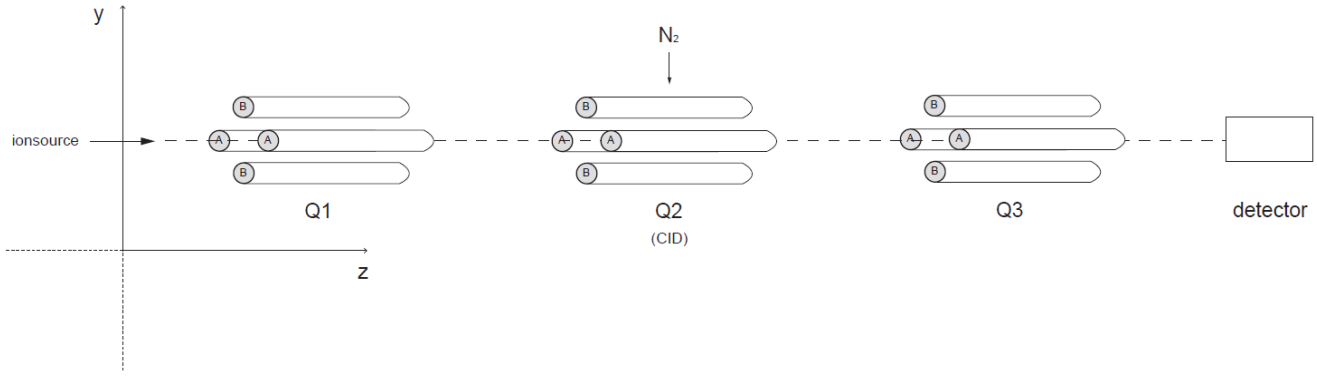
1.2.3. Electrospray Ionization

After eluting from the column but before going to the MS, the sample is ionized using electrospray ionization (ESI). A high voltage is applied to the liquid to create an aerosol. Due to the application of a voltage the solvent droplets become charged. The aerosol is then evaporated by the application of heat and dry nitrogen. The charges get closer and closer together until the droplet explodes into smaller, more stable droplets. At this moment the charges are transferred to the analyte which becomes ionized (7). The ionized analytes go to the mass spectrometer (MS).

1.2.4. Tandem Mass Spectrometry

The MS separates the ions based on mass-to-charge ratios (m/z). When the sample is evaporated and ionized, ions are removed from the source towards the mass analyzer. For this research the mass-analyzer was a triple quadrupole with a linear ion trap. The quadrupole consists of four parallel metal rods to which a constant voltage is applied (Figure 1). Rods A and B have an opposite charge but are kept under the same potential consisting of constant and varying voltages. Only the ions with a particular m/z can reach the detector. The other ions will collide with the rods and discharge, so they never reach the detector. Changing the voltages over time makes it possible to select ions of different masses to reach the detector (8). In tandem MS, two mass spectrometers are coupled (Figure 1). So the first quadrupole analyzer (Q1) filters the precursor ion (mother ion). This precursor ion is then fragmented by collisions with an inert gas, for example nitrogen. This process is called collision induced dissociation (CID) and happens in the collision cell (Q2) which is also a quadrupole. The difference between Q1, Q2 and Q3 is that Q2 is designed to maintain a low pressure of the collision gas and transmits most of the ions that are produced (9). The ions go to the second mass analyzer (Q3) which filters the product ions (daughter ions). The product ions are then detected with a pulse counting CEM detector and a mass spectrum is generated.

Figure 1: schematic overview of MS/MS



The use of tandem MS increases specificity. For example, analytes who are structural isomers (have the same structure) can be distinguished from each other because they generate different product ions. This distinction cannot be made with a single MS.
Another advantage of LC-MS/MS is that it has the possibility of multi-target screening, more specifically detection of multiple analytes with scheduled multiple reaction monitoring (sMRM). This method uses a survey scan where MRM transitions are monitored during the expected retention time window. So the operational modes for both precursor and product ions of the analytes that are included in the method are fixed in Q1 and Q3 respectively. In this way only these precursor- and product ions are detected, which makes MRM more selective for the individual analytes and more sensitive by decreasing the response to everything else. Then the MS/MS spectra that are obtained are compared with a library for identification of the analytes (10).

A disadvantage of the screening is that it only is a qualitative assessment, it can only show that the analyte is present or not present. For reliable interpretations of the result, so to say if the administered amount was indeed lethal or just in the therapeutic range, quantification is needed. In the laboratory of Toxicology and Pharmacology of the Catholic University of Leuven (KU Leuven) quantification is performed by additional analyses (depending on the analyte). These tests are also known as confirmatory tests. These include LC-MS/MS with another column, mobile phase or method, gas chromatography with a mass spectrometer (GC-MS) or a nitrogen-phosphorus detection (GC-NPD) and high pressure liquid chromatography with a diode array detector (HPLC-DAD). Most samples going through the confirmatory tests are prepared using Solid Phase Extraction (SPE), a minority are prepared using PP or Liquid-Liquid Extraction (LLE).

1.3. β-blockers

β-blockers decrease the cardiac activity by blocking the action of epinephrine and norepinephrine. β-blockers competitively antagonize (nor)epinephrine by binding the β-receptors in the central and peripheral nervous system. There are three types of β-receptors, namely the β1-receptor in the postsynaps, the β2-receptor in the pre- and postsynaps and the β3-receptor, in the adipose tissue. The β3-receptor is not relevant for this research and therefore not discussed. β-blockers are used for the treatment of angina, heart failure, high blood pressure, arrhythmias etc. and to protect the heart from a second myocardial infarction (11). In addition they can also be used in the management for disorders like migraine, glaucoma, tremor, hyperthyroidism etc. (12). β-blockers are not recreational drugs, like cocaine or amphetamines, because they don’t possess the ability to affect the cognitive competence.

However this does not mean that there is no misuse of them. Due to their heart rate lowering and tremor reducing properties β-blockers can be misused in sports that require steadiness and accuracy like archery, billiards, darts, shooting, gymnastics etc. In addition they suppress anxiety which is also beneficial in competition (13). For these reasons β-blockers are included to the prohibited list of the World Anti-Doping Agency (WADA) (14). The ability of β-blockers to suppress anxiety is also used as an off-label indication (for an unapproved indication) in situations where stress takes the upper hand for example with performance anxiety, exam nerves, public speaking etc (15).

Due to the increase of cardiovascular diseases, there is also an increase in β-blocker use. This may explain the prevalence of β-blockers in toxicology. Overdosing by β-blockers occurs rarely and is mostly associated by the prevalence of coexisting medical conditions treated with β-blockers and depression (12). Propranolol is mostly used in suicide attempts (16) because it is the most toxic β-blocker due to its non-selective mechanism (both β1- and β2 receptors are antagonized). Toxicity due to interactions with other drugs can be attributed to the change in response to the β-blocker or the co-ingested drug. Especially a potentiation of the effect is dangerous because unwanted effects will also be potentiated. β-blocker use needs to be carefully monitored with co-ingestion of cardioactive or psychotropic drugs like calcium channel inhibitors and tricyclic antidepressants respectively. But also with non-steroidal anti-inflammatory drug (NSAID) use and in patients taking insulin or oral antidiabetics. Symptoms of β-toxicity are proportional to the type of β-blocker and the amount that was ingested, generally toxicity will result in bradycardia, hypotension, arrhythmias, hypothermia, hypoglycemia, bronchospasm and seizures and in some cases death (17).

In the lab of Toxicology and Pharmacology of the KU Leuven β-blockers were found in approximately 13,6% of the cases coming from the Center of Forensic Medicine (CFG) of the University Hospital Leuven (UZ Leuven) in the last three years. Mostly found was bisoprolol (61,5% of the cases) and propranolol (18% of the cases). However in none of the cases a correlation with the pharmacological effect (therapeutic, toxic or lethal) could be made because there is no quantitative method for β-blockers available yet.

1.4. Validation

Method validation needs to be carried out according to the international guidelines (18,19). Validation is necessary to guarantee that the method is reliable, reproducible and to ensure the quality of the method. Unreliable data can lead to over- or underestimation of the analyte that will lead to a false interpretation and conclusions. This can be detrimental to the

investigation because the unreliable result might be contested in court and/or could cause unjustified legal consequences for the defendant or the victim (19). In this research selectivity, limit of quantification, linearity, accuracy, precision, matrix-effects and recovery are investigated.

2. OBJECTIVES

The first objective of this research is to develop a quantitative screening method for seven β-blockers in blood; acebutolol, atenolol, bisoprolol, labetalol, metoprolol, nebivolol and propranolol. This quantitative screening method would be less time consuming and less laborious than the confirmatory tests. The developed quantitative screening method is especially important in urgent cases when the lab is under pressure of the prosecutor's office. Also the screening uses only 100 µl of the sample instead of 1 ml, this is especially important when there is not enough sample and screening is the only test possible. The quantitative screening method will be less expensive than the confirmatory tests, which often makes use of the more expensive deuterated form of drugs. In addition the laboratory of Toxicology and Pharmacology of the KU Leuven does not yet have a method for quantification of β-blockers.

A second objective for this research is the re-evaluation of some of the β-blocker-related cases. When the quantitative screening method is validated, a correlation can be made with the pharmacological effect. Consequently it can be said if the dose was within the therapeutic range or at toxic or lethal concentration.

3. MATERIALS AND METHODS

3.1. Chemicals and reagents

Analytical reference standards of atenolol and propranolol were purchased from Cerilliant and bisoprolol was purchased from LGC. Acebutolol (Sectral 400 mg), nebivolol (Nebivolol EG 250mg), labetalol (Trandate 5mg/mL), metoprolol (Seloken I.V 1mg/mL) and propranolol (Inderal 10mg) were purchased from the pharmacy of UZ Leuven. All tablet and injection reference standards, except nebivolol and propranolol, were diluted in Milli Q water to obtain a concentration of 1 mg/mL. Nebivolol and propranolol tablets were diluted in methanol to obtain a concentration of 20 µg/mL and 100 µg/mL, respectively. All reference standards were stored at -20°C. The internal standard propranolol-d7 was purchased from LGC. For each calibrator level and quality control (QC)-samples a stock solution was made. The composition of these stock solutions can be found in the appendix. LC-MS grade acetonitrile was purchased from Biosolve (Valkenswaard, The Netherlands). Formic acid and ammonium formate (LC MS mobile phase additives) were purchased from Sigma Aldrich (Bornem, Belgium). Water was obtained from Milli Q Water Purification System (Millipore, Brussels, Belgium).

3.2. Samples

We obtained blank antemortem blood from the blood transfusion center (Gasthuisberg, Leuven, Belgium) and blank postmortem blood from the Centre of Forensic Medicine (CFG, Leuven, Belgium). All the samples were analyzed using LC-MS/MS before being used in the experiment in order to check if they were negative for β-blockers. In the experiments, we used samples spiked with the reference β-blocker standards.

3.3. LC-MS/MS

3.3.1. Method Optimization

During the research we made a switch from promazine to propranolol-d7 as internal standard (IS). Method optimization was performed by analyzing a IS-acetonitrile (ACN) solution with a concentration of 20ng/200µl, to see if a good peak could be observed. Secondly a solution of propranolol with the new internal standard (propranolol-d7) at low and high concentrations was analyzed, to see if the two peaks could be separated.

3.3.2. Sample Preparation

Sample preparation was done with protein precipitation. The IS, propranolol-d7, was spiked into ACN to obtain an IS-precipitating solution with a final concentration of 0.1 µg/ml. 200 µl of the IS-precipitating solution was added drop by drop to 100 µl of the spiked antemortem blood

while vortexing. Then the samples were centrifugated for 10 minutes at 2500 rpm and 6°C. After centrifugation the supernatans was diluted with 700 µl Milli Q water in a 1.5 ml vial.

3.3.3. LC-MS/MS

Verplaetse et al. already developed a method for the screening for the laboratory of Toxicology and Pharmacology (10). In this research the same operational settings were used for LC-MS/MS.

3.3.3.1. Liquid Chromatography

A UFLC Shimadzu system consisting of LC-20ADXR pumps, SIL-20ACXR autosampler, DGU-20A3 degasser, CTO-20A oven (Shimadzu Prominence, Antwerp, Belgium) in combination with a Allure PFP Propryl column (purchased from Restek, via Interscience, Louvain-la-Neuve, Belgium) was used to separate the compounds in time. The autosampler temperature was set at 15°C and the oven at 40°C. To avoid carry-over, the autosampler needle is rinsed before and after injection. Mobile phases consisted of (A) water with 2 mM ammonium formate and 0.2% formic acid and of (B) acetonitrile with 2 mM ammonium formate and 0.2% formic acid. Each run lasts 17,5 minutes under the following conditions: 0-10 min: 10-90%B and increase of flow rate from 0.5 mL/min to 1 mL/min; 10-15 min: 90%B at 1 mL/min; 15-15.5 min: 90-10%B; 15.5-17.5 min: 10%B at 0.5 mL/min. The injection volume was 30 µl.

3.3.3.2. Mass Spectrometry

A 3200 QTRAP (ABSciex, Halle, Belgium) system was used for analysis of the compounds separated by LC. The MS operated in a scheduled multiple reaction monitoring-information dependent acquisition-enhanced product ion (sMRM-IDA-EPI) multi-target screening approach. The screening method was only performed in the positive ionization mode. The electrospray conditions were as follows: gas 1: nitrogen, 40 psi; gas 2: nitrogen, 70 psi; ion-spray voltage: 4000 V; ion source temperature: 500°C; curtain gas: nitrogen, 20 psi; collision gas: high. The mass spectrum parameters can be found in Table 1. Each MRM is measured ± 90 s around the expected retention time of the compound.

3.3.3.2.1. MS Optimization

Sensitivity of the sMRM-MS depends on the tuning of the instrumental parameters such as collision energy (CE), declustering potential etc. For this reason MS optimization was performed. For each analyte, a 1 µg/ml methanolic solution was injected directly into the MS. The results of optimization can be found in Table 1. The underlined MRMs are used for quantification.

Table 1: Mass Spectrum Parameters: Q1 = m/z parent ion, Q3 = m/z product ion, DP = declustering potential, EP = entrance potential , CEP = collision cell entrance potential, CE = collision energy, CXP = collision cell exit potential

	Q1 (Da)	Q3(Da)	Time (msec)	DP (V)	EP (V)	CEP (V)	CE (V)	CXP (V)
Acebutolol	337.190	116.100	150.0	46.000	6.500	20.000	27.000	4.000
	337.190	319.300	150.0	46.000	6.500	20.000	19.000	8.000
	337.190	260.200	150.0	46.000	6.500	20.000	25.000	4.000
Atenolol	267.107	145.200	150.0	46.000	6.500	16.000	33.000	4.000
	267.107	190.100	150.0	46.000	6.500	16.000	25.000	4.000
	267.107	116.000	150.0	46.000	6.500	16.000	25.000	4.000
Bisoprolol	326.197	116.000	150.0	41.000	5.500	22.000	23.000	4.000
	326.197	106.900	150.0	41.000	5.500	22.000	51.000	4.000
	326.197	133.200	150.0	41.000	5.500	22.000	39.000	4.000
Labetalol	329.094	162.200	150.0	31.000	6.500	22.000	31.000	4.000
	329.094	311.300	150.0	31.001	6.500	22.000	17.000	4.000
	329.094	294.200	150.0	31.002	6.500	22.000	23.000	10.000
Metoprolol	268.165	116.000	150.0	41.000	8.500	14.000	23.000	4.000
	268.165	133.100	150.0	41.000	8.500	14.000	33.000	4.000
	268.165	121.200	150.0	41.000	8.500	14.000	29.000	4.000
Nebivolol	406.134	151.100	150.0	61.000	5.000	36.000	43.000	4.000
	406.134	103.200	150.0	61.000	5.000	36.000	71.000	4.000
	406.134	123.000	150.0	61.000	5.000	36.000	53.000	4.000
Propranolol	260.194	116.100	150.0	41.000	4.000	16.000	25.000	4.000
	260.194	183.200	150.0	41.000	4.000	16.000	23.000	4.000
	260.194	155.100	150.0	41.000	4.000	16.000	35.000	4.000

3.4. Method Validation

Method validation was performed only on antemortem samples and following the international guidelines (18,19). The following parameters were investigated: selectivity, limit of quantification, linearity, accuracy, precision, stability, matrix-effect and recovery.

3.4.1. Selectivity

Selectivity is the extent to which other substances, in the matrix, interfere with the determination of the analyte. Five antemortem and five postmortem samples were prepared using the sample preparation without IS. Two zero-samples, one antemortem and one postmortem, were prepared using the sample preparation with IS. These samples were extracted and analyzed to check for any interference. Methanolic standard solutions were injected on the LC-MS/MS for the seven β-blockers separately and together at the highest calibrator level to exclude mutual interference and interference with other compounds.

3.4.2. Limit of Quantification

Sensitivity is the ability of the method to detect the analyte at a particular concentration. It is often defined by detection limits; here it is defined as the limit of quantification (LOQ). For the assessment of the LOQ, five replicates of antemortem samples are spiked with the lowest therapeutic value of the seven β-blockers. Precision was calculated using analyte/IS ratios, as follows:

$$Precision = \%RSD = \frac{Mean\ analyte/IS\ ratio}{STDEV\ analyte\ /IS\ ratio} \times 100\% \quad (1)$$

The LOQ can then be defined as the minimum concentration that could be quantified with precision <20%.

3.4.3. Calibration Curve

The relationship between the analyte concentration and the response must be defined for reliable quantification. This is accomplished by setting up a calibration curve where the response of the analyte is plotted against the known concentration. The calibrators were matrix-based and six concentration levels were used. For each concentration level, we analyzed six different types of antemortem blood (replicates). Table 2 represents the concentrations for each calibrator used for the calibration curve. The underlined values are within the therapeutic range.

Table 2: Concentration levels calibration curve

	C1 (ng/mL)	**C2** (ng/mL)	**C3** (ng/mL)	**C4** (ng/mL)	**C5** (ng/mL)	**C6** (ng/mL)
Acebutolol	200	500	750	1000	1500	2000
Atenolol	75	100	500	800	1000	1500
Bisoprolol	10	25	75	100	150	200
Labetalol	80	200	300	500	750	1000
Metoprolol	20	75	150	300	750	1000
Nebivolol	5	7.5	10	20	50	100
Propranolol	20	100	200	300	500	1000

For each calibrator the %Recovery (%RE) and Precision (%RSD) are calculated, as follows:

$$\%RE = \frac{(mean\ predicted\ value - theoretical\ value)}{theoretical\ value} \times 100\% \quad (2)$$

$$\%RSD = \frac{STDEV\ between\ mean\ predicted\ value\ and\ theoretical\ value}{theoretical\ value} \times 100\% \quad (3)$$

The calibration curve is accepted if the coefficient of determination (R^2) is higher than or equal to 0.992 and precision (%RSD) is lower than 15% and 20% for the LOQ. In addition, the

variation between replicates for the same calibration level must not be big which is a concern when using PP.

3.4.4. Accuracy and Precision

Accuracy can be defined as the difference between the predicted test values and the theoretical value and is expressed as a percent deviation from the theoretical value. Precision can be defined as the closeness of agreement between a series of measurements and is expressed as the relative standard deviation (RSD). Accuracy and precision are assessed using quality control samples (low, medium, high) (Table 3) for each analyte and the calibration curve.

Table 3: Composition QC-samples

	LOW (ng/mL)	**MEDIUM** (ng/mL)	**HIGH** (ng/mL)
Acebutolol	500	1000	2000
Atenolol	100	750	1500
Bisoprolol	20	100	200
Labetalol	200	500	1000
Metoprolol	50	500	1000
Nebivolol	7,5	50	100
Propranolol	50	500	1000

Precision consists of three parts, namely repeatability, intermediate precision and reproducibility. Repeatability is defined as the precision under the same operating conditions and intermediate precision can be defined as within-laboratory variation. Reproducibility expresses the precision between laboratories and is not assessed during this research. Accuracy and precision are assessed on seven different days using two different donor samples of each QC concentration (low, high). Accuracy, repeatability and intermediate precision are calculated as follows (20):

$$Accuracy = bias(\%) = \frac{Mean\ calculated\ concentration - theoretical\ value}{theoretical\ value} \times 100 \qquad (4)$$

$$RSD_{rep}(\%) = \frac{\sqrt{MS_W}}{Mean\ calculated\ concentration} \times 100 \qquad (5)$$

With MS_w the mean square within day values.

$$RSD_{int\ pr}(\%) = \frac{\sqrt{\frac{MS_B + (n-1) \times MS_W}{n}}}{Mean\ calculated\ concentration} \times 100 \qquad (6)$$

With MS_B the mean square between day values and n the number of observations in each group.

A one-way ANOVA was performed on the predicted concentrations, using 'days' as the grouping variable. Accuracy and precision are accepted if bias%, RSD_{rep}(%) and $RSD_{in\ pr}$(%) are < 15% and < 20% for the LOQ.

To see if reliable quantification is still possible after dilution of samples containing very high concentrations, we tested a sample with a very high concentration of the analytes after dilution of the sample.

3.4.5. Stability

For reliable quantification, it is important that an analyte is stable during the whole sample preparation and analysis procedure. For this research processed sample stability, bench-top stability and freeze/thaw stability are tested. For the stability tests, three different antemortem donors were used.

Processed sample stability is assessed by analyzing QC samples at low and high concentration, after 0h, 4h, 8h and 12h of storage at 15°C in the autosampler. Afterwards, regression analysis is performed. Instability is indicated by a significantly negative slope ($p<0.05$), this shows a significant decline of the analyte concentration over time.

Bench-top stability is assessed by analyzing QC samples at low and high concentration after 4h of storage at room temperature on the bench-top. After analysis the concentration after 4h is compared with the concentration in the 0h control. Ratios between 90-110% are accepted, however ratios between 80-120% are also regarded as acceptable.

Freeze/thaw stability is assessed by analyzing QC samples at low and high concentration after three freeze/thaw cycles of 24h at -20°C and 1h at room temperature. After analysis, the concentration after 3 freeze/thaw cycles is compared with the concentration of the 0h control. Ratios between 90-110% are accepted, however ratios between 80-120% are also regarded as acceptable.

3.4.6. Matrix Effects and Recovery

Evaluation of ion enhancement or ion suppression due to components in the matrix is assessed by matrix-effects (ME) and extraction yields are assessed as recovery (RE). Five different types of donor blood at two concentration levels (low, high) for (A) methanolic standard, (B) post-extraction spiked sample and (C) pre-extraction spiked sample two concentration levels (low, high) are tested (5 replicates). ME and RE are calculated, using peak areas, as follows (20):

$$ME = \frac{B}{A} \times 100 \quad (7)$$

$$RE = \frac{C}{B} \times 100 \quad (8)$$

3.4.7. Case Study

After validation of the quantitative screening method, five cases with an indication of the presence of β-blockers, are re-analyzed to determine the concentration of the β-blocker that is present and to assess if it was within therapeutic range or at toxic or lethal concentrations. Consequently we can determine whether or not the beta-blocker contributed to the cause of death.

4. RESULTS

4.1. Selectivity

No interfering peaks were observed for both the blank and zero samples. In the methanolic standard solution of nebivolol there was interference with sulpiride and sulindac. A possible explanation for this is contamination during preparation of the nebivolol standard solution. A new nebivolol standard solution was made, here no interference was observed. Analysis of the methanolic standard solution of all the analytes together showed that all analytes could be separated and that there were no overlapping peaks.

4.2. Limit of Quantification

Results of the sensitivity analysis can be found in Table 4 together with the therapeutic range and retention time for each analyte. For each analyte the precision was less than 20%, this means that the lowest therapeutic values of each analyte could be used as LOQ.

Table 4: Therapeutic range, Rt = retention time, LOQ = limit of quantification, RSD = relative standard deviation

	Therapeutic range (ng/mL)	R_t (min)	LOQ (ng/mL)	RSD (%)
Acebutolol	200-1500	3.61	200	3,93
Atenolol	100-1000	1.57	75	10,37
Bisoprolol	10-100	4.86	10	14,63
Labetalol	80-650	4.94	80	6,35
Metoprolol	20-500	3.97	20	6,21
Nebivolol	5-20	7.59	5	6,54
Propranolol	20-300	5.85	20	13,32

The area under the curve (AUC) for the IS promazine was too variable, consequently the analyte/IS ratio was variable and the predicted values were not correct. Also no acceptable calibration curves could be obtained. Therefore we were obliged to change the IS to propranolol-d7. Propranolol-d7 is the deuterated form of propranolol which makes it a better IS because it has the same physiochemical characteristics as the analytes, therefore it will behave the same in sample preparation, separation and detection. The reason why propranolol-d7 was not immediately used as IS is because we didn't want to change the routine method of screening. Also deuterated forms are more expensive. Sensitivity analysis of nebivolol (lowest value) was performed again with the new IS, the result can be found in Table 5.

Table 5: Therapeutic range, Rt = retention time, LOQ = limit of quantification, %RSD

	Therapeutic range (ng/mL)	R_t (min)	LOQ (ng/mL)	RSD (%)
Nebivolol	5-20	7.59	5	14,29

The %RSD of nebivolol was less than 20%. Since nebivolol has the lowest LOQ of the seven β-blockers we tested, we can assume that if the re-analysis for nebivolol has a precision lower than 20%, the precision of the other analytes will as well be lower than 20%.

4.3. Calibration Curve

Calibration range, IS, calibration model, regression line and R-squared (R^2) can be found for each analyte in Table 6. The calibration curves for each analyte can be found in the appendix. Calibration ranges include therapeutic and (low) toxic plasma concentrations for each analyte. Toxic and lethal concentrations for each analyte can be found in the appendix.

Table 6: Calibration range, Internal Standard, Regression model, Regression line, R-squared

	Range (ng/mL)	IS	Model	Regression line	R^2
Acebutolol	200-2000	Propranolol-d7	Linear	y = 0,0486x - 1,8241	0,9959
Atenonol	75-1500	Propranolol-d7	Linear	y = 0,0141x - 0,7874	0,9938
Bisoprolol	10-200	Propranolol-d7	Linear	y = 0,0659x - 0,1949	0,999
Labetalol	80-1000	Propranolol-d7	Linear	y = 0,0073x - 0,3458	0,9973
Metoprolol	20-1000	Propranolol-d7	Linear	y = 0,0169x - 0,1414	0,9957
Nebivolol	5-100	Propranolol-d7	Linear	y = 0,0214x - 0,0285	0,9958
Propranolol	20-1000	Propranolol-d7	Linear	y = 0,0222x + 0,1167	0,9998

The acceptance criteria were fulfilled for the calibration curve of each analyte. For all analytes a linear regression by least squares method was used. The back calculated values of each donor separately show variation (supplementary tables in appendix). A possible explanation for this would be the sample preparation method. PP is not a very sensitive method because you dilute the compounds instead of concentrating them, like in SPE. The coefficient of determination or R^2 gives information on how well the predictions of the calibration curve approach the real data points. For each analyte R^2 was higher than 0,992 which indicates that the regression line fits the data and consequently the model can make a good prediction of the concentration found in the sample. In other words, >99% of the variation in Y can be explained by X. Homoscedastic datasets were observed for all the analytes meaning that the standard deviation on the error terms are constant and no weighing factor is needed. For all analytes the back calculated values were obtained within 87,8%-117,5%.

4.4. Accuracy and Precision

The results of the accuracy and precision analysis can be found in Table 7. All accuracy data (bias%) were within the acceptable range of <15% and <20% at the LOQ except for acebutolol (high), bisoprolol (high), labetalol (high), metoprolol (high), nebivolol (high) and propranolol (low, medium, high). An explanation for the deviating accuracy values at the QC_{high}-level could be an incorrect QC_{high} standard stock solution; all analyte standard solutions were methanolic based. When these analyte stock solutions are opened, while they are still cold, they will attract water which will result in a dilution of the concentration of the analyte in the analyte stock solution. This phenomenon possibly occurred with the QC_{high} stock solution, which could explain that only the predicted concentration of the QC_{high} samples variate largely from the theoretical concentration, while the predicted concentrations from the QC_{low} and QC_{medium} levels did not deviate largely (only for propranolol). For the precision data (RSD_{rep}%, $RSD_{int\ pr}$%) the same acceptance criteria were used. However for precision all analytes deviate from these proposed acceptance criteria. The largest deviating values for accuracy and precision were found for nebivolol and propranolol. A possible explanation for this could be the incomplete dissolution of the tablet, *Nebivolol EG*® and *Inderal 10 mg*® respectively, or the low solubility of nebivolol (0.0403 mg/mL) and propranolol (0.0617mg/L). Overall high accuracy and precision data can be explained by the sample preparation method, protein precipitation. With PP the components in the sample will be diluted instead of concentrated, as already explained. This results in a less sensitive sample preparation method. The accuracy and precision results can be improved by changing the sample preparation method to a more sensitive method like SPE.

Table 8 shows the predicted concentrations of the sample containing a very high concentration. Back calculated values are within a range of 82%-106% with deviating values for labetalol, nebivolol and propranolol. An explanation for the deviating values for nebivolol and propranolol could be the incomplete dissolution of the tablet, as explained earlier. Overall it can be stated that the method can make a good prediction of a very high concentration after dilution.

Table 7: Accuracy results shown as bias(%), precision results shown as relative standard deviation values of repeatability(RSD_{rep}) and intermediate precision ($RSD_{int.pr}$)

	LOW				MEDIUM				HIGH			
	Concentration (ng/mL)	Bias (%)	RSD_{rep} (%)	$RSD_{int\ pr}$ (%)	Concentration (ng/mL)	Bias (%)	RSD_{rep} (%)	$RSD_{int\ pr}$ (%)	Concentration (ng/mL)	Bias (%)	RSD_{rep} (%)	$RSD_{int\ pr}$ (%)
Acebutolol	500	-3,45	8,20	16,76	1000	-5,44	15,80	20,22	2000	-36,79	19,54	18,16
Atenonol	100	3,37	11,78	13,51	750	-7,70	12,87	25,25	1500	-19,57	12,81	14,64
Bisoprolol	20	3,82	15,28	14,88	100	-13,54	12,84	19,50	200	-32,65	21,73	27,96
Labetalol	200	-6,46	17,25	15,60	500	-5,88	19,84	20,40	1000	-25,42	22,34	37,42
Metoprolol	50	-2,58	11,38	14,53	500	-6,58	16,61	16,66	1000	-37,30	19,17	19,32
Nebivolol	7,5	-11,75	36,49	33,32	50	-9,30	24,09	36,14	100	-25,52	45,09	44,54
Propranolol	50	-22,10	35,05	26,71	500	-22,04	13,50	15,10	1000	-40,81	11,38	14,21

*underlined values deviate from the proposed acceptance criteria

Table 8: Results of analysis of a very high sample after dilution

	Nominal concentration (ng/mL)	Predicted concentration (ng/mL)	Back calculation (%)
Acebutolol	4000	3684,28	92,11
Atenolol	3000	3199,84	106,66
Bisoprolol	400	329,48	82,37
Labetalol	2000	1521,20	76,06
Metoprolol	2000	2055,60	102,78
Nebivolol	200	83,32	41,66
Propranolol	2000	1257,61	62,88

*underlined values deviate from the proposed acceptance criteria

4.5. Stability

Both stability of the analyte and the ratio analyte/IS were investigated (Table 9, Table 10). Processed sample stability results showed no significant decrease in concentration for all analytes, alone and during the analytical process, over a time from 0-12h. This indicates that the analytes are stable when kept in the autosampler. A negative slope was observed for labetalol (low) and nebivolol (low, high), however no significant p-value was observed. Bench-top stability test results for the analyte/IS ratio were in range of 78%-106% with small deviations detected for metoprolol (low) and nebivolol (low, high) (Table 9). Nebivolol has a very low therapeutic range (5-20 ng/mL), so small deviations in concentration can result in high procentual differences. This might explain the low bench-top stability results at QC_{low} (68,56%) for nebivolol. If we look at the bench-top stability of the analytes themselves, we see that atenolol (low), metoprolol (low) and nebivolol (low) deviate from the proposed criteria. Freeze/thaw stability results for the analyte/IS ratios were in range of 87%-119% with deviating values for acebutolol (high), atenolol (low, high), bisoprolol (low, high), labetalol (high), nebivolol (low, high) and propranolol (high) (Table 9). This means that there is loss of analyte/IS ratio after three freeze/thaw cycles for all analytes except for metoprolol in the method. For the analytes themselves freeze/thaw results range from 78%-102% with deviating values for atenolol (low, high), bisoprolol (high), labetalol (low, high) and nebivolol (low, high). Freeze/thaw instability needs to be taken into account when using the quantitative screening method in practice. Upon arrival at the lab, all samples are aliquoted into at least four separate tubes. Therefore, a separate tube can be used for each test and the freeze/thaw instability is no issue. The aliquotation prevents unnecessary freeze/thaw cycles. Overall it can be stated that the analytes stay relatively stable during the whole analytical process which is important for quantification.

Table 9: Bench-top, Freeze/thaw and processed sample stability results based on analyte/IS ratio's

	Bench-top (%)		**Freeze/thaw (%)**		**Processed sample stability (h)**	
	Low	High	Low	High	Low	High
Acebutolol	<u>78,75</u>	83,83	90,37	<u>71,92</u>	12	12
Atenonol	<u>78,26</u>	90,71	<u>75,85</u>	<u>63,24</u>	12	12
Bisoprolol	85,85	89,40	<u>73,79</u>	<u>63,87</u>	12	12
Labetalol	92,09	<u>79,23</u>	118,18	<u>68,37</u>	12	12
Metoprolol	<u>75,21</u>	95,22	92,92	87,40	12	12
Nebivolol	<u>67,37</u>	<u>74,33</u>	<u>60,72</u>	<u>62,77</u>	12	12
Propranolol	105,19	90,29	93,01	<u>68,57</u>	12	12

*underlined values deviated from the proposed acceptance criteria

Table 10: Bench-top, Freeze/thaw and processed sample stability results based on the analyte AUC

	Bench-top (%)		Freeze/thaw (%)		Processed sample stability (h)	
	Low	High	Low	High	Low	High
Acebutolol	83,23	90,88	98,43	83,26	12	12
Atenonol	68,44	98,44	66,75	72,68	12	12
Bisoprolol	88,73	98,56	78,51	73,98	12	12
Labetalol	97,81	87,55	132,72	73,81	12	12
Metoprolol	75,95	105,34	101,90	100,39	12	12
Nebivolol	68,56	83,19	66,92	73,05	12	12
Propranolol	112,25	99,03	105,88	79,09	12	12

*underlined values deviated from the proposed acceptance criteria

4.6. Matrix-effects and Recovery

Results from matrix-effects and recovery analysis can be found in Table 11. Matrix-effects for all analytes were within a range of 105,73%-142,61%, indicating ion enhancement. Matrix effects occur at the ion source, where the LC supersedes into the MS. Every molecule that co-elutes with the analyte can influence ionization of that analyte. Co-eluting analytes that belong to the same drug-class, as is the case here, can also influence ionization of the analyte (21). This might be a possible explanation for ion enhancement. Secondly, in this research ESI was used to generated ionized analytes. This technique is more susceptible to the generation of matrix-effects. However %RSD-values for ME were all within the acceptable ranges of <15% and <20% at the LOQ, meaning that ion enhancement is repeatable and can therefore be taken into account for quantification with matrix-matched calibration curves. Recoveries for analytes ranged from 6,19%-14,91%. Overall recovery is low, indicating that there is loss of analyte. This can be explained by PP, which is not a sensitive sample preparation method, as already been explained. PP is also strongly influenced by protein binding. Nebivolol and propranolol have high protein binding of 98% and >90% respectively. This is also an explanation for the low recovery. The highest recovery values were detected for acebutolol (RE_{high} = 14,91%), atenolol (RE_{high} = 12,55%) and metoprolol (RE_{high} = 11,58%) which have low protein binding. Recovery also showed acceptable %RSD values, except for atenolol ($\%RSD_{RE,low}$ = 25,54%), bisoprolol ($\%RSD_{RE,low}$ = 30,80%), metoprolol ($\%RSD_{RE,low}$ = 26,65%), nebivolol ($\%RSD_{RE,low}$ = 58,69%) and propranolol ($\%RSD_{RE,low}$ = 36,15%). Nebivolol is a very potent drug therefore low concentrations were used. This had a particular impact on the lowest QC level, where the AUC were manually integrated instead of automatically. Consequently, this resulted in a higher %RSD. With propranolol, there was also one deviating value, which might explain the high %RSD.

Table 11: matrix-effects (ME) and associated relative standard deviation (RSD_{ME}) and Recoveries (RE) and associated relative standard deviation (RSD_{RE})

	LOW				HIGH			
	ME (%)	RSD_{ME} (%)	RE (%)	RSD_{RE} (%)	ME (%)	RSD_{ME} (%)	RE (%)	RSD_{RE} (%)
Acebutolol	112,17	4,57	9,72	13,17	102,57	2,17	14,91	7,94
Atenolol	122,23	11,72	7,05	25,54	127,53	4,14	12,55	12,09
Bisoprolol	118,01	11,85	7,48	30,80	108,74	5,00	8,51	5,26
Labetalol	117,94	6,82	6,19	15,52	113,55	5,58	6,89	13,83
Metoprolol	113,46	8,40	8,25	26,65	109,05	3,12	11,58	6,29
Nebivolol	137,49	10,69	7,09	58,69	142,61	7,81	7,97	8,65
Propranolol	114,52	10,80	6,71	36,15	105,73	4,31	11,13	5,23

*underlined values deviate from the proposed acceptance criteria

4.7. Case Study

Table 12: Re-analysis of five cases with the validated quantitative screening method

	Analyte	**predicted concentration (ng/mL)**
CASE 1	atenolol	108,17
	acebutolol	153,59
CASE 2	atenolol	191,45
CASE 3	bisoprolol	13,70
CASE 4	metoprolol	80,75
CASE 5	bisoprolol	6,99
	propranolol	150,85

4.7.1. Case 1

Screening analysis showed the presence of atenolol and acebutolol. The concentration of atenolol was within the therapeutical range, while the concentration of acebutolol was sub-therapeutical. Acebutolol has intrinsic sympathomimetic activity, which means that acebutolol has a low-level agonist-activity at the β-receptor. Both β-blockers are β_1-selective antagonist, this means that there would be an increase in the pharmacological effect. Nonetheless, we can conclude that atenolol and acebutolol did not contribute to the cause of death.

4.7.2. Case 2

Screening analysis showed the presence of atenolol with a concentration within the therapeutical range. We can conclude that atenolol did not contribute to the cause of death.

4.7.3. Case 3

Screening analysis showed the presence of bisoprolol with a concentration within the therapeutical range. We can conclude that bisoprolol did not contribute to the cause of death.

4.7.4. Case 4

Screening analysis showed the presence of metoprolol with concentration within the therapeutical range. We can conclude that metoprolol did not contribute to the cause of death.

4.7.5. Case 5

Screening analysis showed the presence of bisoprolol and propranolol. The predicted concentration of bisoprolol was sub-therapeutic and the predicted concentration of propranolol was within the therapeutic range. Bisoprolol is a β_1-selective β-blocker, while propranolol is a non-selective ($\beta_{1/2}$) β-blocker. Propranolol has membrane stabilizing activity which means that it can reduce cardiac action potential. However this only happens at high concentrations (overdose), which is not the case in this case. Propranolol also has small intrinsic sympathomimetic activity. Co-ingestion of bisoprolol and propranolol will result in an increase in pharmacological effect. But overall it can be concluded that both β-blockers did not contribute to the cause of death.

5. GENERAL DISCUSSION AND CONCLUSION

In Table 13 an overview of other methods for the quantification of β-blockers can be found.
It can be stated that LC-MS/MS is preferred over GC-MS due to quicker and less extensive sample preparation and because it can identify a broader range of compounds. Disadvantages of LC-MS/MS are the sensitivity to matrix-effects, which can change the response of the analyte and the higher cost of the equipment. Also, the use of propranolol-d7 increases the cost of this method. The observed LOQ in this method was within the range of 5-200 ng/mL. When compared to the other methods in Table 13, this method is less sensitive, which is the major drawback. Limited sensitivity may be due to the sample preparation method, PP. As previously explained, PP has some limitations; low sensitivity, low analyte recovery, interference by co-elution of endogenous components or analyte co-precipitation. These limitations all influence the quantification of the analytes. More accurate results could be obtained by changing the sample preparation method from PP to a more sensitive sample preparation method, for example SPE. Although for screening purposes this is not possible due to a high amount of samples which would make it very labor-intensive. In total there are 23 β-blockers on the market, this method can detect and quantify seven. Important to note is that the β-blockers most important in toxicology, bisoprolol (most prescribed) and propranolol (most toxic), are included in this method. Despite these drawbacks the quantitative screenings method is relatively accurate, the analytes are stable and the method is able to make a good prediction of the concentration present in the sample. It is also a fast and easy method, which makes high throughput possible. This is especially important for the screening because it is used to analyze every case that enters the laboratory.

To conclude; in this research a new method for the quantification of β-blockers in blood using LC-MS/MS was fully validated for the following compounds: acebutolol, atenolol, bisoprolol, labetalol, metoprolol, nebivolol and propranolol. However, when using this method in practice, the following must be taken into account: for nebivolol and propranolol the acceptance criteria for accuracy and precision were not met. This will result in a certain margin of error on the result. Another remark that has to be made is that the screening is only used as an indication. Therefore high sensitivity is not so important. This method will only be used to indicate if the found concentration is within the therapeutic range or at toxic or lethal concentration. Afterwards confirmation is always needed by confirmatory tests which are more sensitive.

Table 13: Overview quantitative methods for β-blockers

Technique	Matrix	Sample preparation	Amount of β-blockers	LOD (ng/mL)	LOQ (ng/mL)	Reference
UHPLC-UHR-TOF MS	human serum	PP	6	0,5-10	0,3-33,3	Tomkova J et al. (2016)
GC/MS	urine	LLE	16		6,0-2565	Pujos E et al. (2009)
LC/MS	urine	LLE	16		0.53-2.23	Pujos E et al. (2009)
LC-APCI-MS	blood plasma	SPE	22	2,5-100		Maurer H.H et al. (2004)
LC-MS/MS	blood plasma	PP	4	1.0-3.0		Umezawa H et al. (2008)
LC-MS/MS	urine	SPE	7			Salem AA et al. (2017)
UHPLC-MS/MS	serum	PP	5	0,03-0,33	0,32-2,66	Gudersen et al. (2018)
UHPLC	urine	SPE	5	12,8-54,6	32,2-235,8	Baranowska I et al. (2011)
LC-MS/MS	Whole blood	PP	7	4,57-111,80	5-200	Flo Elsen (2019)

6. DUTCH SUMMARY

Introductie

Toxicologie is de studie van (ongewenste) effecten van chemische substanties op een organisme. Forensische toxicologie kan verheldering bieden in postmortem zaken die het resultaat zijn van homocidale, suïcidale of accidentele intoxicaties. Ook kan het opheldering geven in antemortem zaken zoals seksuele geweldpleging waarbij het slachtoffer gedrogeerd werd en bij het nagaan van abstinentie van alcohol- of drugsverslaafden (2,3).

De volgende vragen, gerelateerd aan intoxicatie, staan centraal in het toxicologisch onderzoek (2):

- Is het slachtoffer vergiftigd?
- Welk gif is er toegediend?
- En hoe is het toegediend?
- Was de toegediende hoeveelheid gevaarlijk of lethaal?

Om deze vragen te beantwoorden zijn er kwalitatieve en kwantitatieve analyses nodig.

<u>LC-MS/MS Screening</u>

Screening is de eerste stap in het toxicologisch proces. De screening wordt gebruikt om chemische substanties te identificeren die aanwezig zijn in het staal. Vloeistofchromatografie gekoppeld aan tandem massaspectrometrie (LC-MS/MS) is de gouden standaard voor screening. Vooraleer de analyse kan beginnen, zal er eerst een extractie van de componenten van interesse plaatsvinden. Dit gebeurt via proteïneprecipitatie (PP). Bij PP zal een organisch solvent, bijvoorbeeld acetonitril, worden toegevoegd aan het staal. Dit zal ervoor zorgen dat proteïnen in het staal neerslaan en de componenten van interesse in het supernatans terecht komen. Enkel het supernatans wordt gebruikt voor de analyses. Na extractie van de componenten zullen deze worden gescheiden. De scheiding gebeurt via vloeistofchromatografie (LC). In dit onderzoek wordt ‘reversed phase’ chromatografie gebruikt. Hierbij is de stationaire fase (kolom) apolair. Het staal wordt geïnjecteerd in de polaire mobiele fase, deze zal over de kolom passeren. De componenten hebben een verschillende affiniteit voor de kolom, dit zorgt ervoor dat deze op een verschillend tijdstip elueren van de kolom. Op deze manier worden de componenten van elkaar gescheiden. De output van de LC wordt naar de massa spectrometer (MS) geleid, die de componenten zal detecteren. Om gedetecteerd te kunnen worden door de MS moet het staal geïoniseerd zijn. Hiervoor wordt electrospray ionisatie (ESI) gebruikt. Er wordt een hoge spanning aangebracht op de vloeistof die uit de LC komt waardoor de analieten geïoniseerd raken en elkaar afstoten. De geïoniseerde

componenten gaan naar de MS. In tandem MS zijn er twee massaspectrometers aan elkaar gekoppeld. De eerste quadrupool (Q1) zal scannen voor het moederion. Dit moederion wordt dan gefragmenteerd in de tweede quadrupool (Q2) via botsingsggeïnduceerde dissociatie (CID). Daarna zal een derde quadrupool (Q3) filteren voor deze fragmenten of dochterionen. De dochterionen worden dan gedetecteerd door een puls-tellende CEM detector en er wordt een massaspectrum gegenereerd. Dit wordt vergeleken met een bibliotheek om zo de analieten te identificeren. Via 'scheduled multiple reaction monitoring (sMRM)' kunnen specifieke moeder- en dochterionen worden geselecteerd, dit maakt LC-MS/MS een zeer selectieve en sensitieve methode.

Kwantitatieve screeningsmethode

Een nadeel van screening is dat het enkel een kwalitatieve methode is. Voor een betrouwbare interpretatie is kwantificatie nodig. In het laboratorium voor Toxicologie en Farmacologie van de Katholieke Universiteit van Leuven (KU Leuven) gebeurt dit nu via additionele analyses. Deze worden ook wel conformatietesten genoemd. Deze testen nemen veel tijd in beslag, deze tijd is vaak schaars wanneer de zaak onder hoge druk staat van het openbaar ministerie. Ook zijn deze testen zeer arbeidsintensief en duur.

De doelstelling voor dit onderzoek is het ontwikkelen van een kwantitatieve screeningsmethode voor zeven β-blokkers: acebutolol, atenolol, bisoprolol, labetalol, metoprolol, nebivolol en propranolol. Deze kwantitatieve screening neemt minder tijd in beslag en is minder arbeidsintensief dan bevestigingstesten. Ook wordt er minder staal gebruikt, 100 µl in plaats van 1ml. Dit is vooral belangrijk wanneer er niet veel staal beschikbaar is. Daarenboven is er in het laboratorium voor Toxicologie en Farmacologie van de KU Leuven nog geen kwantitatieve methode voor β-blokkers beschikbaar.

Materiaal en Methode

Methodevalidatie

Methodevalidatie werd uitgevoerd volgens de internationale richtlijnen (18,19). Validatie is noodzakelijk om te garanderen dat de methode betrouwbaar en reproduceerbaar is en om de kwaliteit van de methode te waarborgen. In dit onderzoek werden selectiviteit, kwantificatielimiet, lineariteit, accuraatheid, precisie, matrix-effecten en opbrengst onderzocht.

Selectiviteit is de mate waarin andere stoffen in de matrix de bepaling van de componenten verstoren. Vijf antemortem en vijf postmortem stalen zonder IS en twee zero-stalen, één antemortem en één postmortem, met IS werden geëxtraheerd, geanalyseerd en nakeken op interferentie. Ook werden methanolische standaardoplossingen geïnjecteerd voor de zeven β-

blokkers apart en tezamen op het hoogste kalibratieniveau om te zien of er sprake is van onderlinge interferentie en interferentie met andere verbindingen.

De kwantificatielimiet (LOQ) van een methode geeft een idee van de gevoeligheid. Vijf verschillende typen antemortem bloed werden gespiked met de laagste therapeutische waarde voor de zeven β-blokkers. De LOQ kan dan worden gedefinieerd als de minimum concentratie die kan worden gekwantificeerd met een precisie van <20%.

Voor betrouwbare kwantificatie moet de relatie tussen de concentratie van de component en de respons van het toestel worden gedefinieerd. Hiervoor wordt er een kalibratiecurve opgesteld waarbij de respons van de component wordt uitgezet tegenover een gekende concentratie. Er werden zes kalibratieniveaus gebruikt, allen waren matrixgebaseerd. Voor elk kalibratieniveau werden er zes verschillende typen antemortem bloed gebruikt. In tabel 2 kan men de concentraties voor elk kalibratieniveau van de kalibratiecurve terug vinden. De onderlijnde waardes vallen binnen het therapeutisch bereik. De kalibratiecurve werd geaccepteerd wanneer de regressiecoëfficiënt (R^2) ≥ 0,992 en precisie < 15% en <20% voor LOQ.

Accuraatheid werd uitgevoerd om na te gaan of er een verschil is tussen de voorspelde waarde en de theoretische waarde. Ook werden herhaalbaarheid (RSD_{rep}) en 'intermediate precision' ($RSD_{int\ pr}$) bepaald. Over een tijdspanne van 7 dagen werden dagelijks twee verschillende typen antemortem bloed voor elke kwaliteitscontrole (QC) niveau (laag, medium, hoog) (tabel 3) geanalyseerd. Accuraatheid, herhaalbaarheid en 'intermediate precision' werden berekend zoals in vergelijking (4), (5) en (6) respectievelijk.

Voor betrouwbare kwantificatie is het belangrijk dat de component stabiel blijft over het gehele proces van staalvoorbereiding en analyse. Om dit na te gaan werden de stabiliteit van het verwerkte staal, bench-top stabiliteit en vries/dooi-stabiliteit getest. De stabiliteit van het verwerkte staal werd bepaald via analyse van QC-stalen (laag, hoog) na 0h, 4h, 8h en 12h bewaring op 15°C in de autosampler. Regressie-analyse werd uitgevoerd. Een significant negatieve richtingscoëfficiënt duidt op instabiliteit. Bench-top stabiliteit werd getest door analyse van QC-stalen (laag,hoog) (n=3) na 4h bewaring op kamertemperatuur. De concentratie na 4h werd vergeleken met de concentratie in de 0h controle. Vries/dooi-stabiliteit werd getest door analyse van QC-stalen (laag, hoog) (n=3) na drie vries/dooi-cyclussen van 24h op -20°C en 1h op kamertemperatuur. De concentratie na drie cyclussen werd vergeleken met de concentratie van de 0h controle. Voor bench-top- en vries/dooi-stabiliteit worden ratio's van 90%-110% geaccepteerd, echter ratio's tussen 80%-120% worden ook als aanvaardbaar beschouwd.

De evaluatie van ionensuppressie of ionenversterking als gevolg van matrixcomponenten wordt beoordeeld aan de hand van matrix-effecten (ME) en de extractieopbrengsten worden geëvalueerd als opbrengst (RE). Vijf verschillende soorten donorbloed op twee QC-niveaus (laag, hoog) voor (A) methanolstandaard, (B) post-extractie gespiked staal en (C) pre-extractie gespiked staal werden getest. ME en RE berekend zoals in vergelijking (7) en (8) respectievelijk.

Resultaten

Selectiviteit

Geen interfererende pieken werden gevonden voor de blanco- en zerostalen. Analyse van de methanolische standaard van de componenten toonde aan dat alle componenten konden gescheiden worden en dat er geen overlappende pieken waren.

Kwantificatielimiet

Resultaten van de sensitiviteitsanalyse kunnen worden terug gevonden in Table 4 samen met het therapeutisch bereik en de retentietijd voor elke component. Voor elke component kan de laagste therapeutische waarde gebruikt worden als LOQ, de precisie was telkens <20%.

Kalibratiecurve

Voor alle componenten werd een kalibratiecurve opgesteld die voldeed aan alle aanvaardbaarheidscriteria (tabel 6). Voor elke component werd er een lineaire regressierechte opgesteld met de methode van de kleinste kwadraten. De determinatiecoëfficiënt of R^2 geeft informatie over de mate waarin het model de werkelijkheid benadert. In dit onderzoek is R^2 > 0,992 voor elke component wat wil zeggen dat het model een goede predictie kan maken. Voor alle datasets werd homoscedasticiteit geobserveerd, dit betekent dat er geen gebruik moet worden gemaakt van een correctiefactor. De verkregen terug-berekende concentraties waren voor alle componenten in het bereik van 87,8%-117,5%.

Accuraatheid en Precisie

Alle data van accuraatheid (bias%) lagen in het geaccepteerde bereik van <15% en <20% bij de LOQ behalve voor acebutolol (hoog), bisoprolol (hoog), labetalol (hoog), metoprolol (hoog), nebivolol (hoog) en propranolol (hoog) (tabel 7). Een mogelijke verklaring hiervoor is het gebruik van een incorrecte QC_{hoog}-standaardoplossing. Doordat de standaardoplossingen op basis zijn van methanol trekken deze water aan wanneer ze worden geopend als ze nog koud zijn, hierdoor worden de componenten verdund. Voor de precisiedata (RSD_{rep}%, $RSD_{int\ pr}$%) werden dezelfde aanvaardbaarheidscriteria gebruikt. Echter voor precisie wijken alle

componenten af van deze criteria (tabel 7). De grootste afwijking voor accuraatheid en precisie werd geobserveerd voor nebivolol en propranolol. Een mogelijke verklaring is de incomplete oplossing van de tabletten, *Nebivolol EG*® en *Inderal*® respectievelijk, of de lage oplosbaarheid van nebivolol (0.0403 mg/mL) en propranolol (0.0617mg/L). Algemeen kunnen we de hoge accuraatheid- en precisiedata verklaren door de staalvoorbereiding, PP. Hierbij worden de componenten in het staal verdund, in plaats van geconcentreerd met als resultaat dat de staalvoorbereiding minder gevoelig is. Tabel 8 toont de voorspelde concentraties van de analyse van het “very high” staal na verdunning. De terug-berekende waarde langen in een bereik van 82%-106%. Dit toont aan dat het model, na verdunning nog een correcte voorspelling maakt.

Stabiliteit

Zowel de stabiliteit van de component als de component/IS werden getest. De resultaten van de stabiliteit van het verwerkte staal tonen aan dat de componenten stabiel zijn over een tijd van 0h-12h op 15°C (tabel 9, 10). Resultaten van bench-top stabiliteit waren in een bereik van 78%-106% met kleine afwijkingen voor atenolol (laag), metoprolol (laag) en nebivolol (laag) (tabel 9). Als we kijken naar de stabiliteit van de component zelf, zien we dat atenolol (laag), metoprolol (laag) en nebivolol (laag) afwijken van de voorgestelde criteria. Vries/dooi-stabiliteit toont aan dat er een bepaalde afbraak is van de componenten, behalve acebutolol en metoprolol, na drie vries/ontdooi-cyclussen. Hierbij lagen de resultaten in een bereik van 87%-119% voor component/IS met afwijkende waarden voor acebutololol (hoog), atenololol (laag, hoog), bisoprolol (laag, hoog), labetalol (hoog), nebivolol (laag, hoog) en propranolol (hoog) (tabel 9). Voor de component zelf lagen de resultaten in een bereik van 78%-102% met afwijkende waarden voor atenolol (laag, hoog), bisoprolol (hoog), labetalol (laag, hoog) en nebivololol (laag, hoog) (tabel 10). In de praktijk worden alle stalen meteen na aankomst in het labo gealiquoteerd, waardoor voor elke analyse een nieuw aliquot genomen kan worden en onnodige vries/dooi cyclussen worden voorkomen. Hierdoor vormt de vries/ontdooi-instabiliteit geen groot probleem voor kwantificatie. Algemeen kunnen we zeggen dat de componenten stabiel blijven gedurende het analytische proces wat belangrijk is voor kwantificatie.

Matrix-effecten en Opbrengst

Matrix-effecten waren voor alle componenten in het bereik van 105,73%-142,61%, dit wijst op ionenversterking (tabel 11). Echter, de %RSD-waarden voor ME lagen allemaal binnen het geaccepteerde bereik van <15% en <20% voor LOQ, wat betekent dat de ionenversterking herhaalbaar is en daardoor in rekening kan worden genomen voor kwantificatie met matrix-

gekoppelde kalibratiecurves. Opbrengst voor elke component lag in de range van 6,19%-14,91% (tabel 11), wat betekent dat er componenten verloren gaan. Een verklaring hiervoor is de staalvoorbereiding, PP. PP is heel gevoelig aan de binding tussen de plasmaproteïnen en de componenten. Nebivolol en propranolol hebben een hoge proteïnenbinding van 98% en >99% respectievelijk. Dit kan een verklaring zijn voor de lage opbrengst.

Discussie en Conclusie

Tabel 13 bevat een overzicht van methoden waarbij β-blokkers worden gekwantificeerd. LC-MS/MS wordt vaker gebruikt dan GC-MS vanwege een snellere, minder uitgebreide staalvoorbereiding en omdat het een groter aantal componenten kan identificeren. Nadelen aan het gebruik LC-MS/MS zijn de gevoeligheid aan matrix-effecten en dat het een hogere kost heeft. Het gebruik van propranolol-d7 zorgt ervoor dat deze methode ook duurder wordt. LOQ voor deze methode ligt binnen een bereik van 5-200 ng/mL. Wanneer je deze methode vergelijkt met de methoden in tabel 13 kan men vaststellen dat deze methode minder sensitief is, wat het grootste nadeel is. Een mogelijke verklaring voor de lagere sensitiviteit is de staalvoorbereidingsmethode, PP. PP heeft enkele beperkingen zoals; lage sensitiviteit, lage component opbrengst, co-elutie van interferende endogene componenten en co-precipitatie van de componenten. Accuraatheid en precisie van de methode kunnen verbeterd worden door een andere staalvoorbereidingsmethode te gebruiken, zoals bijvoorbeeld vaste fase extractie (SPE). Van de 23 β-blokkers die op de markt zijn, kan deze methode er zeven detecteren en kwantificeren. Een opmerking die hierbij moet gemaakt worden is dat de in toxicologische context meest relevante β-blokkers, nl bisoprolol en propranolol, wel in de methode zijn opgenomen. Ondanks deze nadelen is de methode relatief accuraat, blijven de componenten stabiel en kan het model een goed predictie maken van de concentratie aanwezig in het staal. Bijkomend is het ook een snelle en makkelijke methode waarbij “high throughput” mogelijk is.

Tot slot, in dit onderzoek werd er een nieuwe kwantificatiemethode voor β-blokkers in bloed volledig gevalideerd met gebruik van LC-MS/MS voor acebutolol, atenolol, bisoprolol, labetalol, metoprolol, nebivolol en propranolol. Bij gebruik van deze methode in de praktijk, moet men er rekening mee houden dat nebivolol en propranolol afwijkingen vertonen in accuraatheid en precisie. Dit zal resulteren in een bepaalde foutenmarge op het resultaat. Aangezien de screening enkel gebruikt wordt om een indicatie te krijgen of de gevonden concentratie binnen de therapeutische, toxische of lethale range valt, vormt de lagere gevoeligheid van de kwantificatiemethode geen beperking. Achteraf zal het resultaat steeds moeten worden bevestigd door bevestigingstesten.

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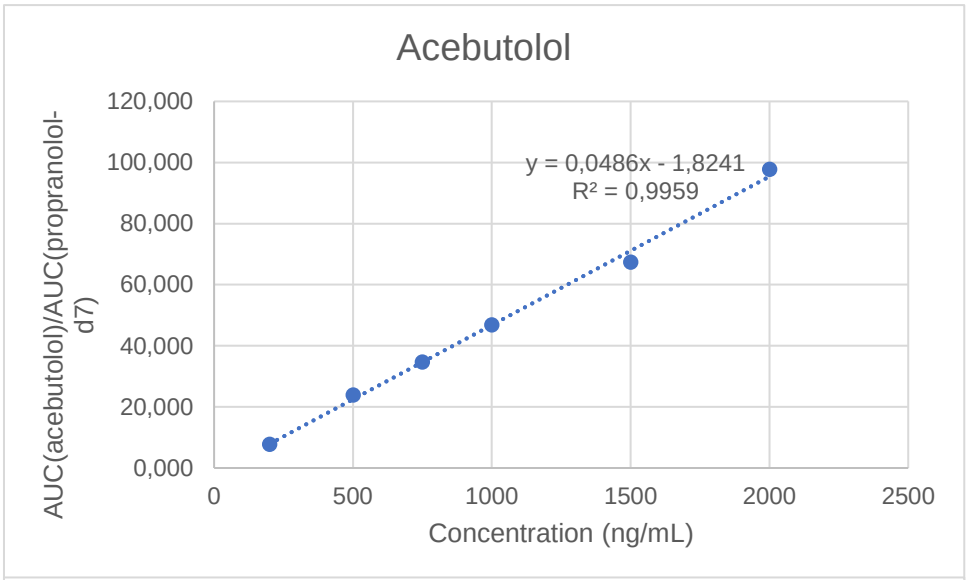
APPENDIX

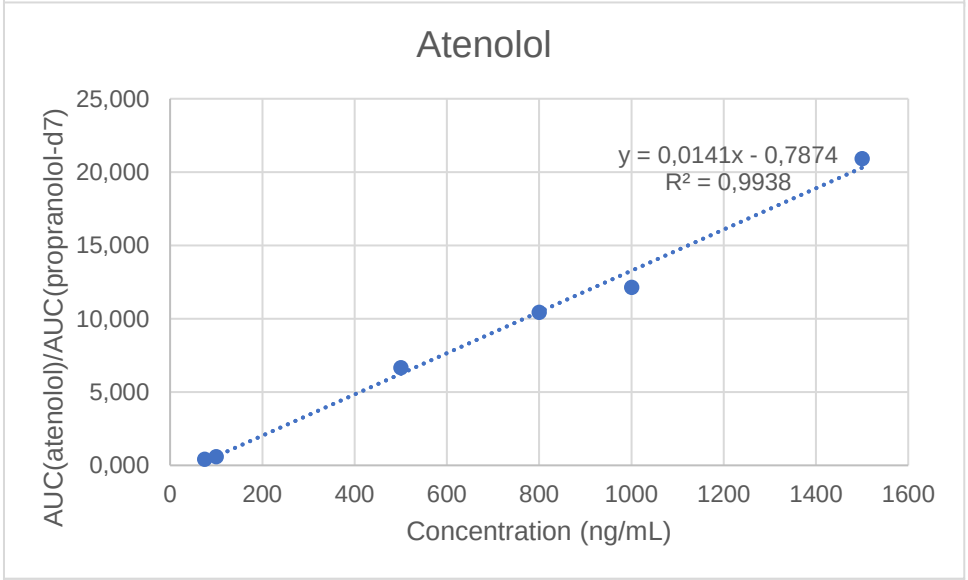
A. Stock solutions (Calibration curves/QC samples)

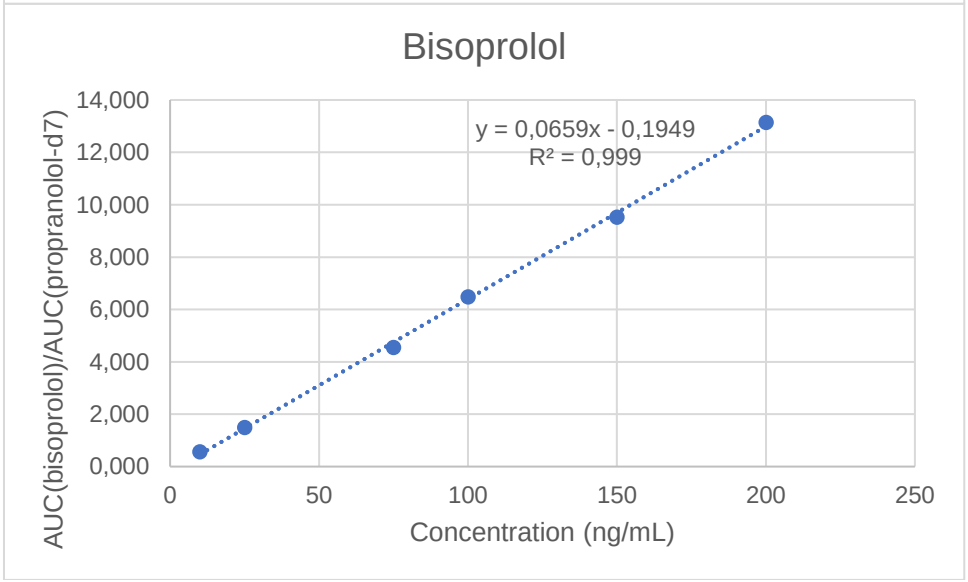
Analytes	(ng/ml)	(µg/ml)
C1 mix	C1	[STDmix]
acebutolol	200	20
atenolol	75	7,5
bisoprolol	10	1
labetalol	80	8
metoprolol	20	2
nebivolol	5	0,5
propranolol	20	2
Totaal		
Meoh		
C2 mix	C2	[STDmix]
acebutolol	500	50
atenolol	100	10
bisoprolol	25	2,5
labetalol	200	20
metoprolol	75	7,5
nebivolol	7,5	0,75
propranolol	100	10
Totaal		
Meoh		
C3 mix	C3	[STDmix]
acebutolol	750	75
atenolol	500	50
bisoprolol	75	7,5
labetalol	300	30
metoprolol	150	15
nebivolol	10	1
propranolol	200	20
Totaal		
Meoh		
C4 mix	C4	[STDmix]
acebutolol	1000	100
atenolol	800	80
bisoprolol	100	10
labetalol	500	50
metoprolol	300	30
nebivolol	20	2
propranolol	300	30
Totaal		
Meoh		
C5 mix	C5	[STDmix]
acebutolol	1500	150
atenolol	1000	100
bisoprolol	150	15
labetalol	750	75
metoprolol	750	75
nebivolol	50	5
Totaal		
Meoh		
C6 mix	C6	[STDmix]
acebutolol	2000	200
atenolol	1500	150
bisoprolol	200	20
labetalol	1000	100
metoprolol	1000	100
Totaal		
Meoh		

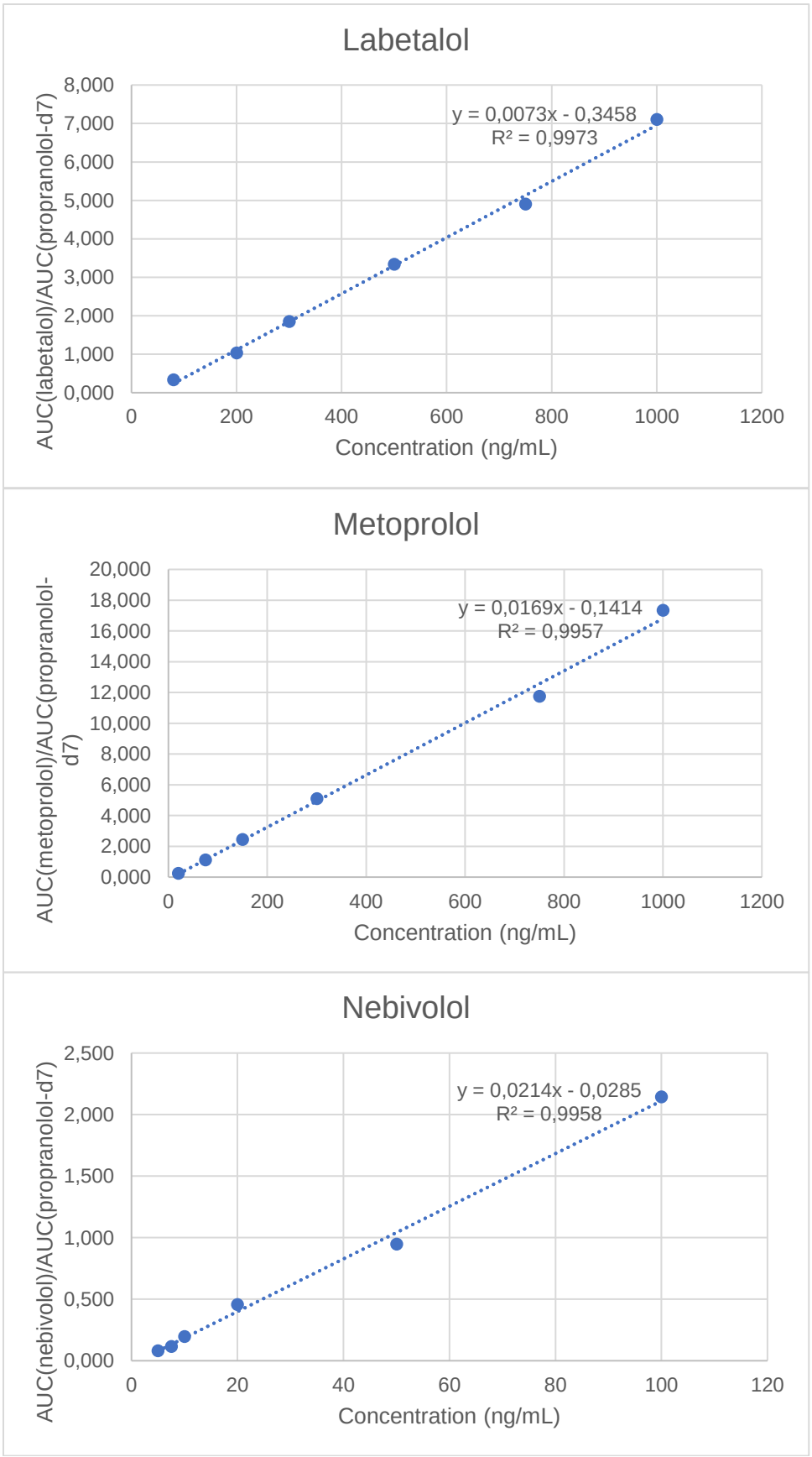
QC	**low**	[STDmix]
	(ng/ml)	µg/ml
Acebutolol	500	25
Atenolol	100	5
Bisoprolol	20	1
Labetalol	200	10
Metoprolol	50	2,5
Nebivolol	7,5	0,375
Propranolol	50	2,5
MEOH		
QC	**medium**	[STDmix]
	(ng/ml)	µg/ml
Acebutolol	1000	25
Atenolol	750	18,75
Bisoprolol	100	2,5
Labetalol	500	12,5
Metoprolol	500	12,5
Nebivolol	50	1,25
Propranolol	500	12,5
MEOH		
QC	**high**	[STDmix]
	(ng/ml)	µg/ml
Acebutolol	2000	50
Atenolol	1500	37,5
Bisoprolol	200	5
Labetalol	1000	25
Metoprolol	1000	25
Nebivolol	100	2,5
Propranolol	1000	25
MEOH		

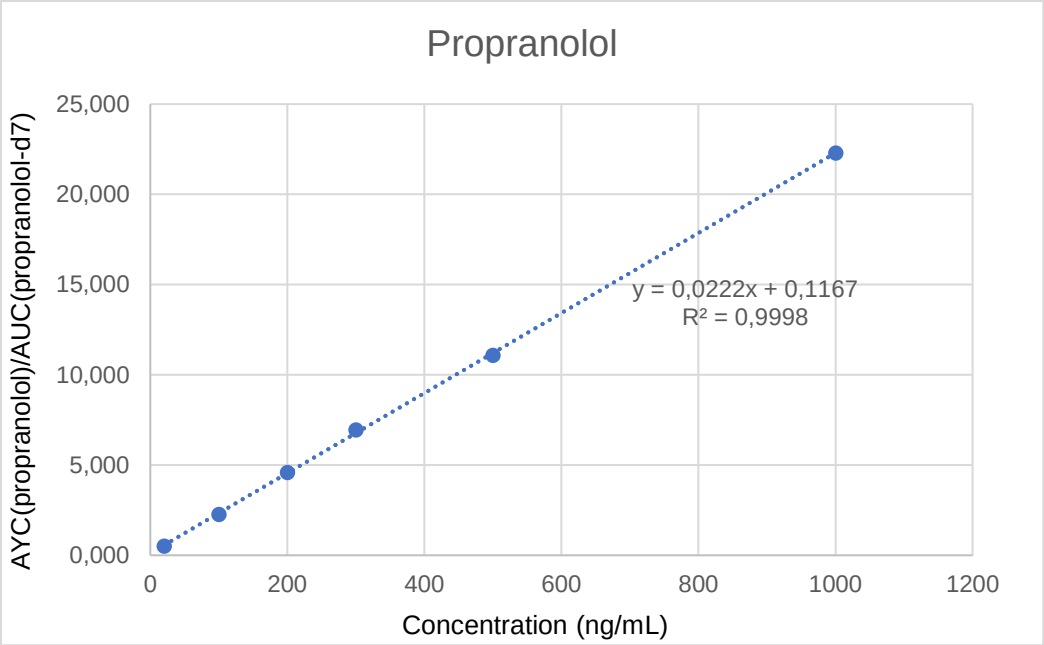
B. Calibration Curves








Labetalol
AUC(labetalol)/AUC(propranolol-d7)
8,000
7,000
6,000
5,000
4,000
3,000
2,000
1,000
0,000
y = 0,0073x - 0,3458
R² = 0,9973
0
200
400
600
800
1000
1200
Concentration (ng/mL)
Metoprolol
AUC(metoprolol)/AUC(propranolol-d7)
20,000
18,000
16,000
14,000
12,000
10,000
8,000
6,000
4,000
2,000
0,000
y = 0,0169x - 0,1414
R² = 0,9957
0
200
400
600
800
1000
1200
Concentration (ng/mL)
Nebivolol
AUC(nebivolol)/AUC(propranolol-d7)
2,500
2,000
1,500
1,000
0,500
0,000
y = 0,0214x - 0,0285
R² = 0,9958
0
20
40
60
80
100
120
Concentration (ng/mL)



C. Therapeutic, Toxic and Lethal plasma concentrations for the seven β-blockers

	Therapeutic (ng/mL)	Toxic (ng/mL)	Lethal (ng/mL)
Acebutolol	200-1500		15000-20000
Atenolol	100-1000	2000-3000	27000
Bisoprolol	10-100		
Labetalol	80-650	1000	
Metoprolol	20-500	1000	12000
Nebivolol	5-20		
Propranolol	20-300	1000	4000-10000

D. Back calculated values of each donor for each analyte

		Calibration concentration (ng/ml)	Donor	Predicted concentration (ng/mL)				Calibration concentration (ng/ml)	Donor	Predicted concentration (ng/mL)
Acebutolol	C1	5	donor 1	5,89		**Atenolol**	C1	75	donor 1	90,64
			donor 2	6,64					donor 2	80,31
			donor 3	4,79					donor 3	88,13
			donor 4	4,64					donor 4	81,36
			donor 5	4,44					donor 5	82,27
			donor 6	4,59					donor 6	89,02
	C2	7,5	donor 1	5,38			C2	100	donor 1	83,27
			donor 2	6,50					donor 2	103,99
			donor 3	8,76					donor 3	109,47
			donor 4	7,43					donor 4	91,81
			donor 5	5,94					donor 5	113,40
			donor 6	6,72					donor 6	80,34
	C3	10	donor 1	16,83			C3	500	donor 1	581,33
			donor 2	13,97					donor 2	482,04
			donor 3	7,44					donor 3	634,31
			donor 4	7,74					donor 4	498,66
			donor 5	8,98					donor 5	654,25
			donor 6	8,11					donor 6	320,88
	C4	20	donor 1	24,34			C4	800	donor 1	915,50
			donor 2	21,04					donor 2	622,10
			donor 3	26,30					donor 3	907,66
			donor 4	27,74					donor 4	869,13
			donor 5	20,23					donor 5	775,54
			donor 6	16,73					donor 6	686,58
	C5	50	donor 1	49,28			C5	1000	donor 1	845,24
			donor 2	43,11					donor 2	1012,10
			donor 3	40,34					donor 3	865,12
			donor 4	46,32					donor 4	827,73
			donor 5	44,06					donor 5	853,31
			donor 6	50,98					donor 6	1095,81
	C6	100	donor 1	122,99			C6	1500	donor 1	1566,61
			donor 2	91,45					donor 2	1402,52
			donor 3	124,17					donor 3	1631,42
			donor 4	72,80					donor 4	1217,55
			donor 5	105,03					donor 5	1447,27
			donor 6	93,02					donor 6	1972,53

		Calibration concentration (ng/ml)	Donor	Predicted concentration (ng/mL)				Calibration concentration (ng/ml)	Donor	Predicted concentration (ng/mL)
Bisoprolol	C1	10	donor 1	11,96		**Labetalol**	C1	80	donor 1	95,84
			donor 2	13,48					donor 2	99,42
			donor 3	10,92					donor 3	95,46
			donor 4	11,66					donor 4	86,46
			donor 5	12,22					donor 5	88,64
			donor 6	9,05					donor 6	98,08
	C2	25	donor 1	21,37			C2	200	donor 1	166,09
			donor 2	26,76					donor 2	180,59
			donor 3	30,20					donor 3	251,85
			donor 4	24,29					donor 4	189,97
			donor 5	26,77					donor 5	171,25
			donor 6	23,78					donor 6	176,41
	C3	75	donor 1	71,36			C3	300	donor 1	311,41
			donor 2	81,32					donor 2	276,97
			donor 3	75,84					donor 3	287,67
			donor 4	91,54					donor 4	281,01
			donor 5	69,53					donor 5	369,29
			donor 6	42,39					donor 6	281,49
	C4	100	donor 1	96,46			C4	500	donor 1	498,46
			donor 2	112,12					donor 2	534,51
			donor 3	125,80					donor 3	556,90
			donor 4	99,45					donor 4	577,45
			donor 5	94,15					donor 5	510,29
			donor 6	80,07					donor 6	351,12
	C5	150	donor 1	121,05			C5	750	donor 1	809,73
			donor 2	161,52					donor 2	702,29
			donor 3	182,29					donor 3	750,81
			donor 4	117,55					donor 4	666,93
			donor 5	175,10					donor 5	703,89
			donor 6	126,98					donor 6	686,20
	C6	200	donor 1	225,64			C6	1000	donor 1	1247,01
			donor 2	205,28					donor 2	968,77
			donor 3	201,95					donor 3	1080,92
			donor 4	165,00					donor 4	933,75
			donor 5	175,66					donor 5	817,10
			donor 6	241,14					donor 6	1079,10

		Calibration concentration (ng/ml)	Donor	Predicted concentration (ng/mL)				Calibration concentration (ng/ml)	Donor	Predicted concentration (ng/mL)
Metoprolol	C1	20	donor 1	21,35		**Nebivolol**	C1	5	donor 1	5,89
			donor 2	24,38					donor 2	6,64
			donor 3	26,90					donor 3	4,79
			donor 4	18,28					donor 4	4,64
			donor 5	22,40					donor 5	4,44
			donor 6	25,61					donor 6	4,59
	C2	75	donor 1	54,52			C2	7,5	donor 1	5,38
			donor 2	78,40					donor 2	6,50
			donor 3	82,26					donor 3	8,76
			donor 4	74,81					donor 4	7,43
			donor 5	73,48					donor 5	5,94
			donor 6	88,12					donor 6	6,72
	C3	150	donor 1	151,24			C3	10	donor 1	16,83
			donor 2	163,10					donor 2	13,97
			donor 3	122,41					donor 3	7,44
			donor 4	185,58					donor 4	7,74
			donor 5	184,03					donor 5	8,98
			donor 6	111,15					donor 6	8,11
	C4	300	donor 1	316,18			C4	20	donor 1	24,34
			donor 2	346,49					donor 2	21,04
			donor 3	346,49					donor 3	26,30
			donor 4	279,45					donor 4	27,74
			donor 5	272,65					donor 5	20,23
			donor 6	299,37					donor 6	16,73
	C5	750	donor 1	677,26			C5	50	donor 1	49,28
			donor 2	640,98					donor 2	43,11
			donor 3	673,74					donor 3	40,34
			donor 4	650,71					donor 4	46,32
			donor 5	812,83					donor 5	44,06
			donor 6	767,93					donor 6	50,98
	C6	1000	donor 1	974,72			C6	100	donor 1	122,99
			donor 2	1029,78					donor 2	91,45
			donor 3	1124,48					donor 3	124,17
			donor 4	854,44					donor 4	72,80
			donor 5	797,32					donor 5	105,03
			donor 6	1427,74					donor 6	93,02

		Calibration concentration (ng/ml)	Donor	Predicted concentration (ng/mL)
Propranolol	C1	20	donor 1	16,92
			donor 2	17,41
			donor 3	17,49
			donor 4	18,75
			donor 5	18,02
			donor 6	32,13
	C2	100	donor 1	101,21
			donor 2	98,45
			donor 3	97,25
			donor 4	110,09
			donor 5	107,11
			donor 6	85,96
	C3	200	donor 1	226,79
			donor 2	202,68
			donor 3	213,47
			donor 4	195,94
			donor 5	184,02
			donor 6	187,42
	C4	300	donor 1	324,27
			donor 2	250,36
			donor 3	355,62
			donor 4	332,58
			donor 5	342,46
			donor 6	242,49
	C5	500	donor 1	506,19
			donor 2	502,43
			donor 3	480,90
			donor 4	485,14
			donor 5	542,06
			donor 6	449,61
	C6	1000	donor 1	993,79
			donor 2	961,12
			donor 3	1146,19
			donor 4	1100,39
			donor 5	1006,59
			donor 6	785,73