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Method development and validation to determine the antidiabetic drug metformin in human lithium heparin plasma with RP-HPLC analysis

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"I don't want to believe, I want to know."

(Carl Sagan)

Abbreviations

ACN	Acetonitrile
conc.	Concentration
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
IR	Insulin resistance
MF	Metformin
MS	Mass spectroscopy
rpm	Revolutions per minute
RP - HPLC	Reversed phase high-pressure liquid chromatography
SNP	Single nucleotide polymorphism
SPO	Standard operating procedure
T2DM	Type 2 diabetes mellitus
THF	Tetrahydrofuran
Tris	Tris(hydroxymethyl)-aminomethane
UV-Vis	Ultra-violet visible spectrophotometry
WHO	World health organization

Abstract

This thesis focusses on the establishment of a suitable and reliable method for the determination of the antidiabetic drug metformin in human lithium heparin plasma. Samples (n=44) from patients affected by T2DM and PCOS were obtained from the Medical University of Graz. Samples were deproteinized with acetonitrile and MF concentrations were determined with RP-HPLC with pre-column fluorescence derivatization. Analysis was run with a linear gradient elution with increasing concentrations of acetonitrile from 5% up to 65%. MF fluorescence maxima were detected at 279 nm for excitation and at 440 nm for emission. The formed metformin fluorophore was stable over 24 h with a retention time of 8.8 minutes, no endogenous plasma substances interfered with the detection. The calibration curve of metformin in human plasma was linear in the range of 0.95 - 4.76 µg/ml with a correlation coefficient of 0.997. Recovery was calculated to be 109%. LOD and LOQ were determined to be 190 ng/ml and 650 ng/ml, respectively. Mean MF concentration in patient samples was determined to be 3.18 ± 1.41 µg/ml. The majority of the obtained results was within the expected range of 1-4 µg/ml. The applied method has proven to be simple and suitable for metformin quantification.

Zusammenfassung

Diese Arbeit beschäftigt sich mit der Entwicklung einer zuverlässigen Methode für die Bestimmung des Antidiabetikums Metformin in humanem LiHep Plasma. Proben (n=44) von an T2DM oder PCOS erkrankten PatientInnen wurden von der Medizinischen Universität Graz bereitgestellt. Die Bestimmung von Metformin erfolgte mit einer Umkehrphasen-HPLC mit Fluoreszenz Detektion, bei der die Proteine im Plasma zuerst mit eiskaltem ACN gefällt wurden. Eine lineare Gradientenelution mit steigenden ACN Konzentrationen von 5-65% wurde durchgeführt. Für MF konnten Fluoreszenz Maxima von 279 nm für Anregung, sowie 440 nm für Emission ermittelt werden. Der gebildete MF-Fluorophor mit einer Retentionszeit von 8.8 min war stabil über 24 h, keine störenden endogenen Plasma Peaks wurden gefunden. Die Kalibrationsgerade für mit Metformin versetztem Plasma war linear im Bereich von 0.95 bis 4.76 µg/ml, mit einem R^{2}-Wert von 0.997. Die Wiederfindungsrate wurde mit 109% bestimmt, für LOD und LOQ konnten folgende Werte ermittelt werden: 190 ng/ml sowie 650 ng/ml. In den Patientenproben konnte eine mittlere Metformin Konzentration von 3.18 ± 1.41 µg/ml nachgewiesen werden, dabei liegt der Großteil der erhaltenen Resultate im erwarteten Konzentrationsbereich von 1-4 µg/ml. Die verwendete Methode erwies sich als verlässlich und einfach anwendbar für die Bestimmung von Metformin.

1. Introduction

This thesis focuses of the establishment of a reliable and easy feasible RP-HPLC method, with pre-column fluorescence derivatization for the determination of metformin in human lithium heparin plasma. Samples (n=44) were provided from the Medical University of Graz from patients affected by T2DM and PCOS.

1.1 Type 2 diabetes mellitus

Diabetes mellitus is on the rise. It is a series of metabolic disorders characterized by increased blood glucose concentrations. According to the WHO in 2017, an elevated blood glucose level is the third highest risk factor for premature mortality besides tobacco use and high blood pressure. In 2015 approximately 415 million people worldwide were affected by diabetes and it is estimated, that diabetes will affect more than 642 million people across the globe in 2040 (**Figure 1**). It is also supposed, that one in two adults with diabetes still remains undiagnosed. There are three main types of diabetes. Type 1, where not enough insulin is produced due to an autoimmune disease, where the insulin producing beta cells in the pancreas are attacked. Type 2, where enough insulin is produced, but the cells become resistant over time therefore insulin becomes ineffective, which leads to elevated blood glucose levels. And gestational diabetes, the third type, where women without prior illness develop high blood sugar levels during pregnancy (IDF Diabetes Atlas, 2015).

Type 2 diabetes mellitus is the most common form of diabetes. It accounts for approximately 90% of diabetes cases. (Rubino, 2008) It is primarily due to genetic factors and lifestyle properties. Obesity, sedentary and inactive lifestyle as well as tobacco and excessive consumption of alcohol play a major in the development of this disease.

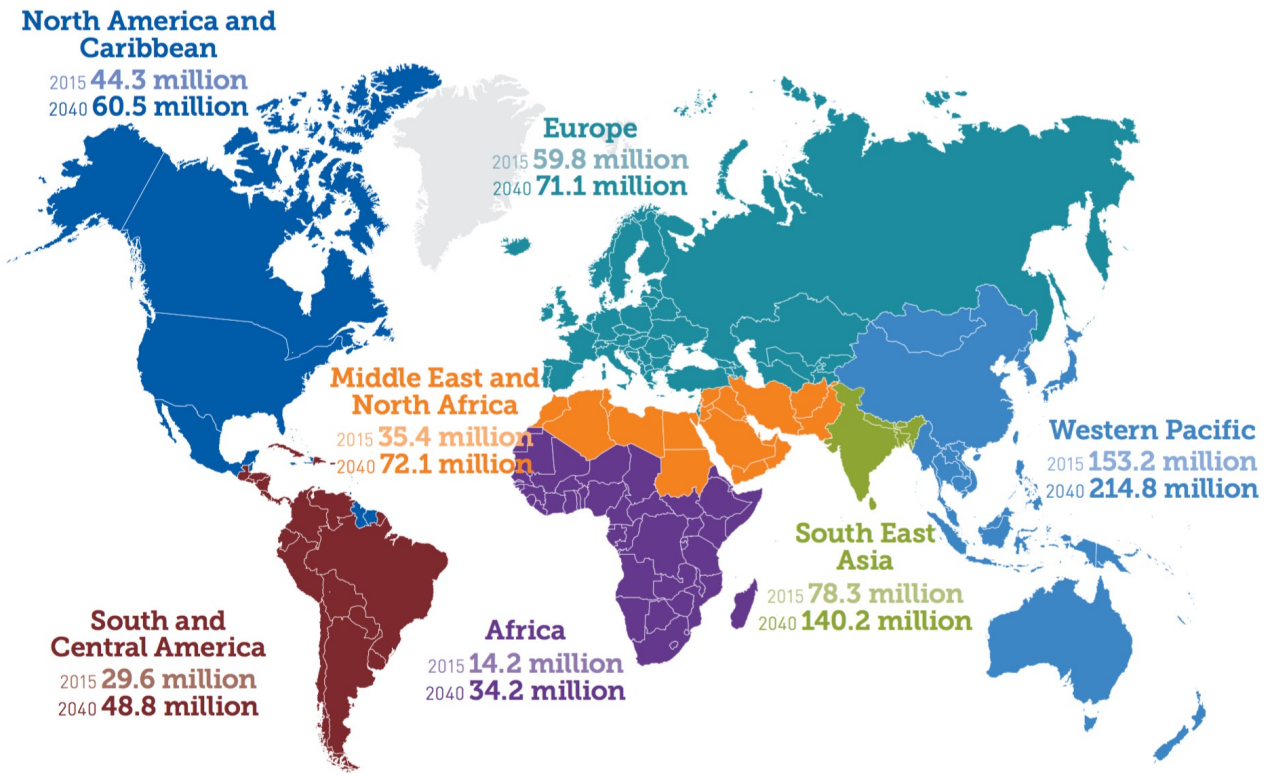


Figure 1. Prevalence of diabetes mellitus worldwide. Depicted are people affected by this disease in 2015 and the estimated distribution of DM globally in 2040. (IDF, Diabetes Atlas 2015)

T2DM is characterized by elevated blood glucose concentration, insulin resistance and lack of insulin. (Olokoba et al., 2012)

Managing the continuous rise of T2DM is crucial as it places a severe burden on healthcare systems worldwide. Over the last decades, metformin has become the first line medication in the treatment of T2DM. Intensive research on metformin and its pharmacokinetics could contribute in battling the impacts of this disease.

1.2 Polycystic ovarian syndrome

Polycystic ovarian syndrome is the most common endocrine disorder prevalent in women of reproductive age. About 6 to 10% of females are affected. (Karakas, 2017) PCOS is a series of conditions due to elevated androgens in women, where the ovaries are covered in cysts (**Figure 2**). The disorder is characterized by at least two out of the three following features: olio/ anovulation, hyperandrogenism and polycystic ovaries. (Lashen, 2012) Symptoms of PCOS include infertility, hirsutism, obesity and insulin resistance. Insulin resistance is characterized through cells which fail to respond to insulin on a normal level.

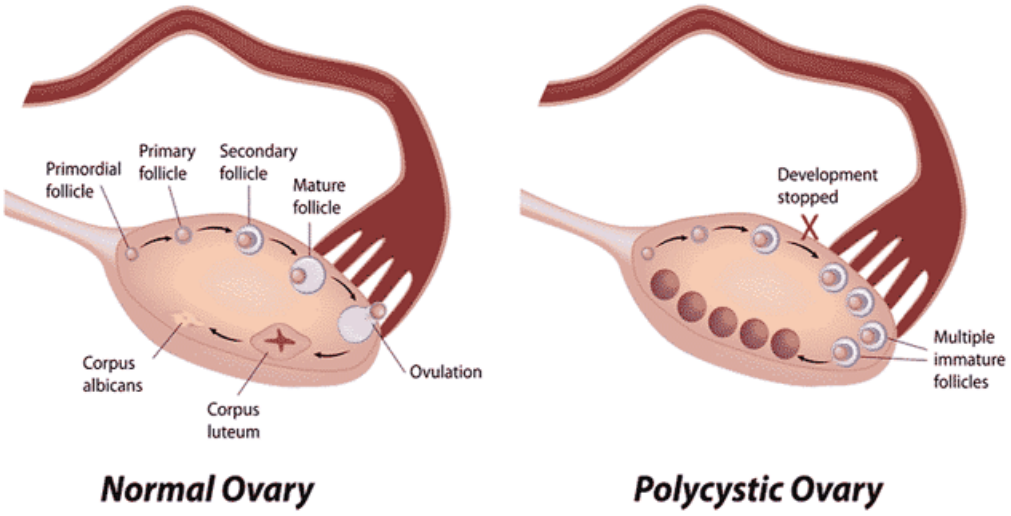


Figure 2. Polycystic ovarian syndrome. The left side shows a normal developed ovary, the right side depicts a polycystic ovary. (Alila Medical Media / Shutterstock)

It is frequently associated with women affected by PCOS and although IR affects non obese women as well, it is far more common in obese women, with severity ranging from impaired glucose tolerance to T2DM. (Haas & Bentov, 2017)

The role of metformin in infertile females is still controversial. Previous studies indicate, that metformin may be beneficial in the treatment of infertility associated with PCOS. In clinical use, metformin was shown to be able to suppress lipogenesis and increase the synthesis of sex-hormone-binding globulin (SHBG) in the liver, which resulted in a reduced level of free testosterone. It has also been shown, that menstrual frequency, ovulation and live birth rates were improved. (Morin-Papunen et al., 2012)

However more recent studies do not find any significant improvement in fertility treatments when metformin was administered. (Haas & Bentov, 2017) Although metformin may not have proven to be beneficial for women whose infertility is caused by PCOS, it aids in PCOS acquired insulin resistance.

1.3 Metformin

The antidiabetic drug metformin has become the preferred first line medication for the treatment of type 2 diabetes mellitus (former: non-insulin-dependent diabetes mellitus). Metformin is a hydrophilic base and belongs to the chemical group of biguanides. It is a guanidine derivative (1,1-dimethylguanidine, **Figure 3**) and its history can be traced back to *Galega officinalis,* a traditional medicinal plant in Europe, rich in guanidine, which was

associated with the ability to lower blood glucose. Guanidine itself has proven to be too toxic for clinical use. (Bailey, 2017)

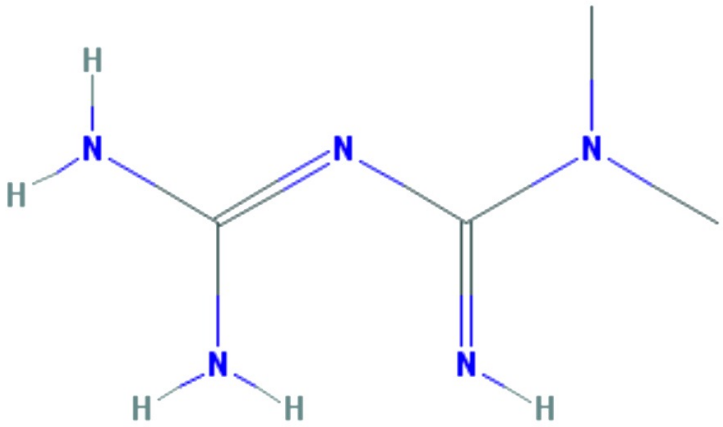


Figure 3. Chemical structure of metformin. (PubChem CID: 4091)

Other glucose-lowering biguanides, among them phenformin and buformin, were examined regarding their antidiabetic properties. Phenformin and buformin turned out be more potent than metformin, but they were highly associated with lactic acidosis and their use became negligible. (Bailey, 2004) Lactic acidosis with metformin occurs rarely, mostly in patients with existing renal disorder, however there are several side effects to metformin intake. Amongst other things it can lead to nausea, diarrhea, abdominal pain and malabsorption of vitamin B12, depending on dose and duration of treatment. (Lashen, 2010)

After oral administration, metformin is absorbed in the intestines via the plasma monoamine transporter (PMAT) and organic cation transporter 3 (OCT3). Metformin is then transported via the portal vein into the liver. The uptake into hepatocytes occurs through OCT1/3 and excretion from the liver takes place through the multidrug and toxin extrusion 1 (MATE1) transporter. Metformin is then absorbed from the circulation into renal epithelial cells and is then excreted unchanged through the urine (**Figure 4**).

Although the exact mechanisms of action of metformin remain unclear (Madiraju et. al, 2014), it primarily acts to suppress hepatic glucose production. It is considered to increase insulin sensitivity in vivo which results in reduced plasma glucose concentrations due to reduced glucose absorption in the gastrointestinal tract, it enhances glucose uptake in muscle and liver cells, and it reduces hepatic gluconeogenesis by inhibiting the mitochondrial respiratory-chain complex. (El-Mir et al., 2000) Metformin can be administered as monotherapy, or in combination with other antidiabetics, such as sulfonylureas and

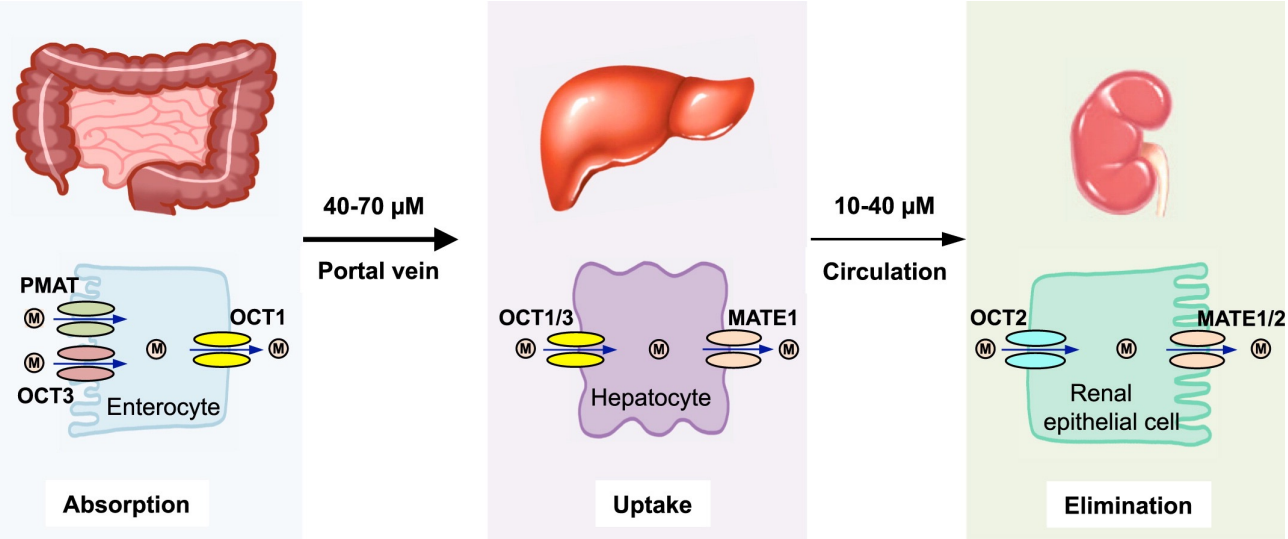


Figure 4. Pharmacokinetics of metformin. Metformin is absorbed from the intestine by enterocytes through OCT3 and transported via the portal vein into the liver. It is not metabolized in the hepatocytes and is eliminated by renal excretion. (He and Wondisford, 2016)

thiazolidinediones. It is available as direct-or expanded release formulation. (Lewin, 2007) Compared to sulfonylureas, metformin has major clinical benefits since it does not cause hypoglycemia and it doesn't decrease blood glucose levels in non-diabetic individuals. (Klip & Leiter, 1990)

Metformin is absorbed in the proximal part of the small intestine and its absorption is completed within 6 h, its oral bioavailability is 50 to 60%. No metabolites or conjugates of metformin have been identified. (Basak, 2007)

Metformin in plasma was found to be stable up to 2 years when stored at -80°C. Its plasma concentration should reach its peak approximately 2 to 2.5 h after ingestion (**Figure 5**) and concentrations are expected between 1 and 2 µg/ml after an oral dose of 500 or 1000 mg. Metformin is removed rapidly from the body, more than 80% are removed unchanged by renal excretion. (Cheng & Chou, 2001)

To adjust treatment correctly and prescribe adequate dosage of metformin, knowledge of metformin concentrations in plasma is crucial.

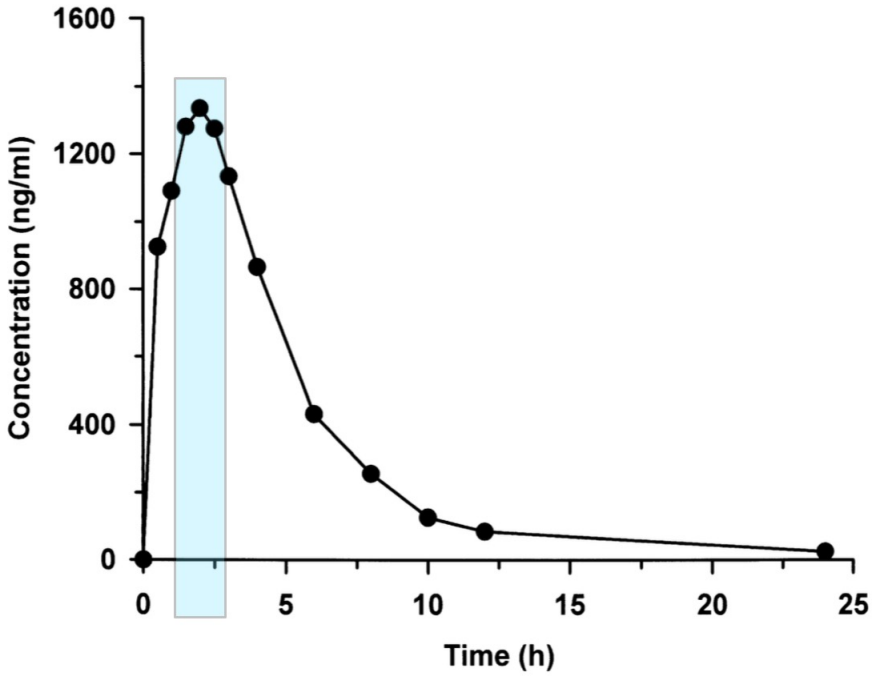


Figure 5: Plasma concentration of metformin over a period of 25 h. Peak concentration of 1.3 ~1.4 µg/ml is reached after 2 - 2.5 h after administration of a 500 mg tablet. (Cheng & Chou, 2001, own design)

1.4 Lithium heparin plasma

Blood is a connective tissue composed of liquid plasma and solid components as erythrocytes, leukocytes and thrombocytes. Plasma is obtained by centrifugation, where the solid components precipitate at the bottom of the centrifugation tube and the liquid plasma fraction is present at the top. The cellular part accounts for approximately 46% and consists primarily of erythrocytes (also called hematocrit). A small layer of white blood cells and platelets (thrombocytes) is formed on top of the red blood cell fraction (**Figure 6**). Plasma is a complex biological fluid, which consists mainly of water (90%), numerous proteins, of which albumin is the most abundant, inorganic salts, sugars, lipids and hormones. The total protein content is approximately 60-80 g/l. (Campbell, 2003)

Heparin is a commonly used anticoagulant, which prevents blood from clotting. In plasma sampling, it is sometimes favored over other anticoagulants like EDTA, because it has little chelating properties, it has a low cation concentration and its interference with water is negligible. Besides lithium heparin, there are two other heparin salts which are commonly used for blood sampling: sodium heparin and ammonium heparin. (Kulkarni et al., 2016)

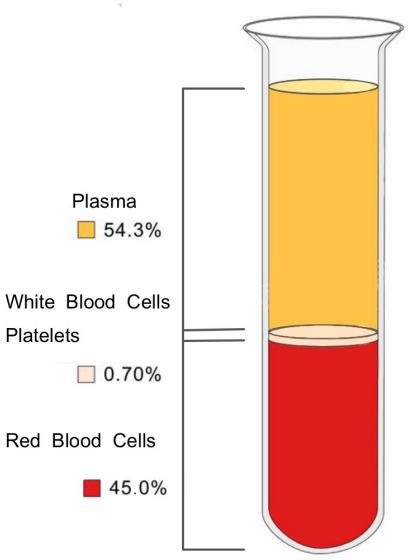


Figure 6. Human blood composition. Own design.

1.5 Protein precipitation extraction

When it comes to analytical testing biological samples are often difficult to analyze due to their complex matrices, as endogenous substances may interfere with the correct detection of the substance of interest. Induced protein precipitation often facilitates accurate analysis. For MS analysis of biological matrices, liquid—liquid extraction as well as solid phase extraction represent the main sample preparations methods. Protein precipitation extraction is however the most common sample preparation technique when it comes to drug discovery and pharmacokinetics. Acetonitrile has proven to be effective regarding its protein precipitation properties. It is, besides methanol, usually the solvent of choice, and is able to remove the majority of proteins from the plasma sample. Major benefits alongside protein removal are the low cost of acetonitrile and the simple feasibility of the sample preparation method. (Alshammari et al., 2015)

Since metformin does not bind to plasma proteins it should remain unaffected in the supernatant after PPE with acetonitrile and subsequent centrifugation (Hardman, 2001).

1.6 RP-HPLC analysis

The determination of metformin in human lithium heparin plasma was carried out with RP-HPLC with pre-column fluorescence derivatization. Separation of analytes in HPLC is based on the relative affinity of molecules for the stationary and the mobile phase. Here, in RP-HPLC, the column packing is non polar whereas the mobile phase is slightly polar. The major

advantage over normal phase HPLC is, that there is a much vaster option in choosing the stationary phase and therefore enhanced analytical options. It can be used with hydrophilic compounds (polar), hydrophobic compounds (non—polar), compounds and ionic compounds. In this analysis a fluorescence detector was used. Although fluorescence detection is less common than UV-Vis detection, it is sometimes favored due to its increased selectivity and sensitivity. Fluorescence is a type of luminescence. It occurs, when a molecule absorbs light or other electromagnetic radiation in the singlet ground state and is promoted to a singlet excited stage. The emitted light has lower wavelength and lower energy than the excitation wavelength. For a given molecule, both the excitation and the emission wavelengths are specific. This could allow the detection of a molecule which would not be possible by UV-Vis detection.

1.7 Derivatization of metformin

Since metformin is not fluorescent by itself the plasma sample needs to be derivatized in order to produce a quantifiable signal. Derivatization is a chemical process, where a compound is transformed into a product of similar chemical structure but with new desired chemical properties. Metformin belongs to the chemical group of biguanides, characterized by the presence of guanidine functional groups. Regarding the derivatization process, the proposed mechanism of action is that the hydrogen atom of the amino function may be substituted. (Marescan et al., 1992) For the derivatization process benzoin was chosen to be the fluorogenic reagent (**Figure 7**).

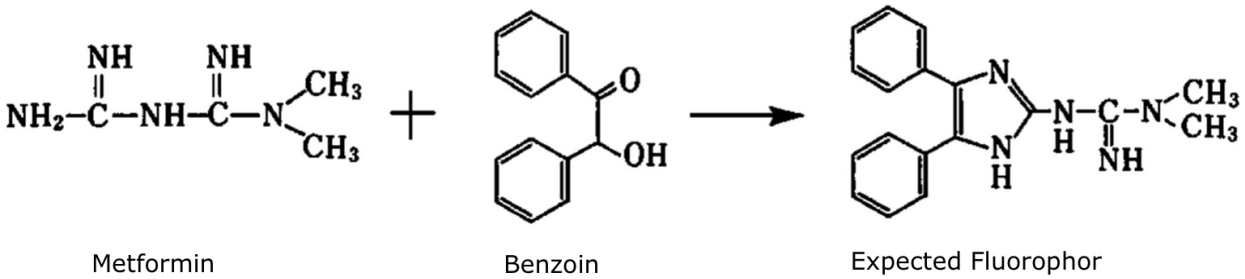


Figure 7. Derivatization reaction of metformin. Depicted are metformin, the fluorogenic reagent benzoin and the expected fluorophore. (Ohta et al., 1993)

1.8 L-arginine and urea

L-arginine and urea are chemical substances which are present in human plasma. Both L-arginine and urea possess amino groups which may react with the derivatization reagent benzoin when subjected to the derivatization process (**Figure 8**). L-arginine is a semi essential α-amino acid used in the biosynthesis of proteins and a precursor of NO-synthesis, an important cellular signaling molecule. L-arginine plasma levels are usually in the range of 40-114 µmol/l (13.9 µg/ml). (Lüneburg et al., 2011)

Urea is an organic compound and plays an important role in many biological processes. It is generated from the breakdown of proteins and is the principal nitrogenous waste product of metabolism. The normal range for urea levels in plasma is 2.5-7.8 mmol/l (150 µg/ml - 469 µg/ml). (Higgins, 2016)

Endogenous plasma substances may present a hurdle when human plasma samples are analyzed. Both substances are investigated if a possible derivatization product is formed which may interfere with metformin determination during HPLC analysis.

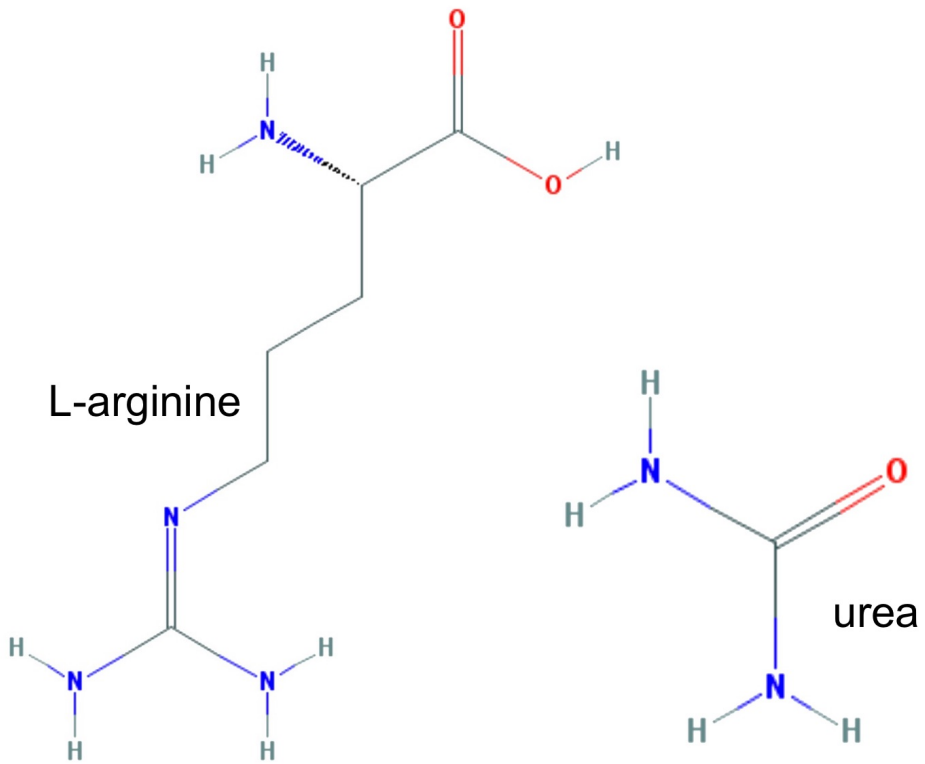


Figure 8. Chemical structures of L-arginine and urea. Source: PubChem CID 6322 & CID 1176

1.9 Single nucleotide polymorphisms (SNPs)

Single nucleotide polymorphisms are the most common form of genetic variations. A single nucleotide polymorphism is a variation at a single position in a DNA sequence, which occurs in ≥ 1% of the population. SNPs are a natural phenomenon and occur on average once in every 300 nucleotides, whereupon the majority of these variations are found in the DNA between genes. When SNPs occur within a gene or in a regulatory region near a gene, they may play a more direct role in disease development by affecting the genes function. (Pratt, 2014)

1.10 Aim of thesis

As metformin is the first line therapy for T2DM, intensive research on metformin becomes more and more important. This work is part of a clinical study conducted by the Medical University of Graz. In the clinical study the SNPs associated with metformin resistance are examined. There is a great variability in clinical response to metformin and it seems that the variability is caused mainly by genetic factors. Through intensive research on metformin in the past, several SNPs were identified as modifiers of metformin response. The main focus of the study is to examine if SNPs in introns of carrier proteins have any beneficial or harmful effect on metformin uptake or clearance. 300 patients are included in this clinical trial, whereupon 100 patients are affected by T2DM, 100 are affected by PCOS and 100 patients, who changed their therapy to an other antidiabetic drug due to an ineffectiveness of metformin.
The focus of this study is the establishment of a reliable HPLC method for the quantification of metformin in human lithium heparin plasma. Metformin plasma concentrations provide useful information if certain SNPs have any impact on metformin pharmacokinetics. Metformin concentration in plasma samples of 44 patients has been examined. A second goal was to set up a Standard Operating Procedure for metformin determination, to allow routine analysis of further plasma samples.

2. Material & Methods

2.1 Laboratory equipment

Pipettes used throughout the analysis where from Pipetman neo P2000 N, in the range of 20 - 200 µl and 100 - 1000 µl. A Siemens Ultra Clear Water Purification System was used to obtain

highly purified water which was used throughout all experiments. All weighing was conducted on a Mettler Toledo Analytical Scale AG245. Degassing of mobile phases occurred with an Elma Transsonic T460. The pH of mobile phases was adjusted with a pH meter from WTW pH 3110. For every centrifugation an Eppendorf 5804 R centrifuge was used.

2.2 List of Chemicals

Hydrochloric acid fuming 37%, sodium hydroxide and tris(hydroxymethyl)aminomethane were bought from ROTH, Darmstadt Germany. Benzoin, 2-mercaptoethanol and L-arginine were purchased from Sigma Aldrich, Austria. Potassium hydroxide and sodium thiosulfate were obtained from J.T. Baker, Germany. Urea was obtained from Merck, Germany. Tetrahydrofuran was purchased from Promochem, Germany. Acetonitrile, acetone and ethanol were bought from Chem-Lab-NV, Belgium. All chemicals were of analytical grade.

2.3 Preparation of metformin stock solution

One 850 mg metformin Hexal tablet, gratefully received from the Medical University of Graz, (active pharmaceutical ingredient: metforminhydrochlorid, **Figure 9**) tablet was powdered and dissolved in ethanol (abs. 100%) and the final volume was made to 85 ml of the same solvent. The solution was filtered through a folded filter (MN 615 1/4 150 mm - Macherey-Nagel, Made in Germany) and the clear stock solution with a resulting concentration of 10 mg/ml stored in 1.5 ml Eppendorf tubes in a deep freezer at - 70 °C up until further use. Before use, the stock solution was put in an ultrasonic bath to dissolve possible formed metformin crystals. All dilutions of metformin standards used throughout the experiment were carried out with ethanol.

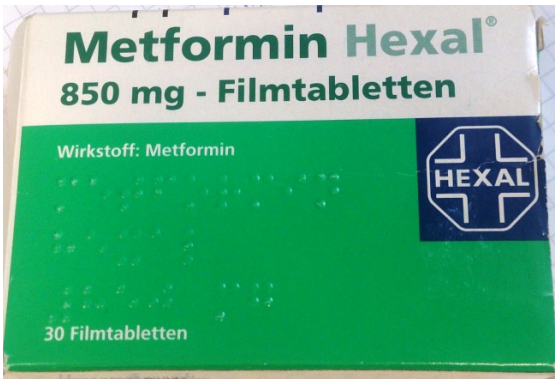


Figure 9. Metformin Hexal tablets.

2.4 Plasma sample preparations

2.4.1 Standard

Pooled drug-free plasma was obtained from the Medical University of Graz and stored at a deep freezer at -70 °C. Plasma was thawed at room temperature and partitioned into 1 ml samples in 2 ml Eppendorf tubes. For calibration each 1 ml plasma sample was spiked with 100 µl metformin stock solution to obtain end concentrations of 4.76, 3.81, 2.86, 1.9 and 0.95 µg/ml, respectively. To determine the baseline of the plasma sample one plasma sample was left blank, 100 µl pure ethanol without any additional metformin was added. For plasma sample clean up, 1 ml ice cold ACN was added, shaken by hand and incubated for 5 minutes. Afterwards the samples were centrifuged at 14000 rpm for 5 minutes. Each 300 µl clear supernatant was then used for the following derivatization procedure.

2.4.2 Patient samples

44 patients were evaluated regarding their plasma metformin concentration. The samples were obtained from the Medical University of Graz. There the plasma samples were collected from the patients at two different time points and stored at a deep freezer at -70 °C until transport to our laboratory. They drew the first sample at sampling point to, when the patients were in a fasting state, the second sample was collected 2 to 2.5 h after metformin administration (t=2). So in total, 88 samples (2 time points per patient) were analyzed (**Figure 10**). Sample clean up was identical with previously described standard procedure. No additional metformin was added.

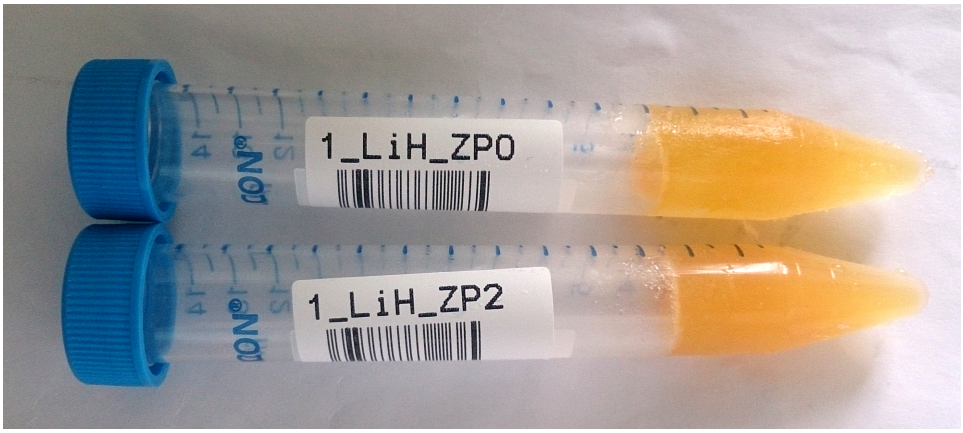


Figure 10. Lithium heparin plasma samples. Exemplary picture of plasma samples from patient 1. Samples from time point zero and time point 2 are depicted.

2.5 Derivatization of metformin

300 µl clear supernatant obtained from the preceded centrifugation step were transferred to a 1.5 ml Eppendorf tube and used for the derivatization reaction. To the supernatant 150 µl 10 mM benzoin and 150 µl 0.2 M sodium thiosulfate / 0.2 M 2-mercaptoethanol (1:1 v/v) were added. Then 300 µl of 1 M potassium hydroxide were added and the solution was put on a heating block at 99 °C for 7 minutes.

After 7 minutes, the reaction was stopped and the mixture was put on ice, to reach a temperature of roughly 15 ± 5 °C. Then 300 µl 1 M tris and 2 M hydrochloric acid (1:1 v/v) were added to adjust the pH of the solution between 9 and 10.5.

For HPLC analysis the whole sample was then transferred to 1.5 ml HPLC vials.

Table 1. Summarized chemical agents for metformin derivatization.

300 µl supernatant (or MF stock solution)
150 µl benzoin (10 mM)
150 µl sodiumthiosulfate (0.2 M) + 2-mercaptoethanol (0.2 M)
300 µl KOH (1 M)
300 µl Tris (1 M) + HCL (2 M)

2.6 RP-HPLC

The quantification of metformin in human lithium heparin plasma occurred with RP-HPLC analysis coupled with pre-column fluorescence derivatization. The separation was carried out on a Phenomenex Kinetex 5 µm EVO C18 column (150 x 30 mm; 100 Å). Determination of metformin occurred with fluorescence detection. For excitation the wavelength was set to 279 nm and for emission to 440 nm. The sensitivity of the fluorescence detector, the PMT gain was set to 15. For eluting the column bound metformin, a linear gradient elution with 2 different mobile phases was applied, with concentrations of acetonitrile increasing from 5% up to 65% (**Figure 11**). The flow rate was maintained at 0.6 ml/min. Mobile phase A contained 5% acetonitrile, 21% tris buffer (0.5 M) and 74% aqueous phase. Mobile phase B contained 65% acetonitrile, 15% tris buffer (0.5 M), 10% THF and 10% aqueous phase. The applied method was adapted from Ohta et al.,(1993). The exact composition of the two mobile phases and the applied gradient were tested experimentally and developed by a member of the same

research group (Verena Buchgraber). The pH of the aqueous phase was adjusted to 8 by using sodium hydroxide (2 M). Mobile phases were degasses by using an ultrasonic bath. Injection volumes were 20 µl. The exact composition of the HPLC system is listed in **Table 1**.

Table 1. HPLC settings

HPLC Device	hp Hewlett-Packard series 1000		
Column	Phenomenex Kinetex 5 μm EVO C18 100 Å 150 x 30 mm		
Software	Chemstation for LC 3D Rev.A 10.02 (1757) Agilent Technologies 1990 - 2003		
Mobile Phases	**A**: 5% ACN, 21% tris buffer (pH 8), 74% ultrapure H_2O **B**: 65% ACN, 15% tris buffer (pH 8), 10% THF, 10% ultrapure H_2O		
Gradient	0 min: 100 % A 2 min: 100 % A 7 min: 100 % B		
Stop Time	12 min	**Injection Volume**	20 μl
Flow Rate	0.6 ml / min	**Column Temperature**	25 °C
Excitation Wavelength	279 nm		
Emission Wavelength	440 nm		
PMT Gain	15		

The applied gradient is visible in **Figure 11**.

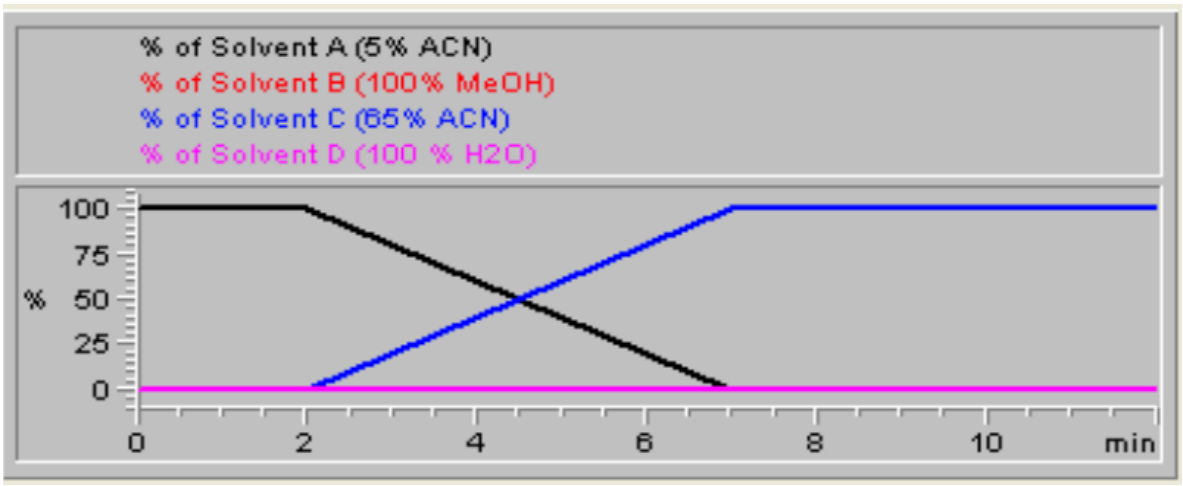


Figure 11. Time profile of applied gradient visualized.

2.7 Stability of derivatized metformin

Metformin stock solution (conc. 10 mg/ml) was diluted with ethanol 1:10 to obtain a concentration of 10 µg/ml. The sample was derivatized and subjected to RP-HPLC analysis to check the stability of the formed metformin fluorophore over time. Per sample, the analysis, including stop time and post time, takes approximately 16 minutes. The same sample was measured for 5 hours (19 measurements) and then again after 24 hours. The metformin sample was stored overnight in the HPLC auto sampler at room temperature.

2.8 Fluorescence formation over time

Fluorescence development rates of the forming metformin fluorophore were investigated. The kinetics of the metformin fluorophore stability of a metformin standard sample were tested. 5 samples with the same concentration were prepared as follows: To a 300 µl (conc. 5 µg/ml) metformin standard sample 150 µl benzoin, 150 µl 0.2 M sodium thiosulfate and 0.2 M 2-mercaptoethanol and 300 µl 1 M KOH were added. The samples were put on a heating block at 99 °C with varied exposure time, whereupon 5 different times were tested, 2, 4, 6, 8 and 10 minutes, respectively. Every sample was removed from the heating block at its distinct time point and then put on ice. After cooling down to approximately 10 °C, 300 µl 1 M tris and 2 M hydrochloric acid were added, and the samples were then analyzed by HPLC analysis.
To detect the kinetics of fluorescence formation in metformin spiked plasma, the same procedure was carried out. The plasma samples were mixed 1:1 with ice cold ACN and spiked with 100 µl metformin (conc. 100 µg/ml).

2.9 Derivatization of L-arginine and urea

L-arginine and urea were derivatized in the same manner as metformin to see if their amino groups react as well with the derivatization reagent benzoin and produce a detectable signal when subjected to HPLC analysis. For that, 100 mg L-arginine and 100 mg urea were dissolved in each 10 ml ultrapure H_2O. Concentrations of 1 mg/ml of L-arginine and 1 mg/ml urea were tested.

2.10 Evaluation of excitation and emission wavelength maximum

To detect the excitation and emission fluorescence maximum of the metformin fluorophore a 10 µg/ml metformin standard sample was prepared. It was then pre-column derivatized and the fluorescence spectra for the metformin fluorophore was recorded from 220 to 425 nm for excitation and 340 nm to 550 nm for emission. HPLC settings were identical with standard measurements, except for wavelengths. Steps in spectra determination were 5 nm. The resulting spectra were then investigated for the maximum response wavelength.

2.11 Uniformity of metformin tablets

10 metformin Hexal tablets were pre-weighed and their distinct weight noted. Each tablet was pulverized separately and transferred to a 100 ml volumetric flask. 85 ml ethanol were added to dissolve the metformin. The porcelain mortar and pestle were rinsed carefully with ethanol to capture possible metformin residues and the ethanol was then added to the flask. The flasks were filled up with ethanol to a total volume of each 100 ml. Each flask was shaken rigorously and then sonicated. From each flask, 3 times 1 ml metformin-solution (conc. 8.5 mg/ml) was withdrawn and diluted with ethanol to reach a concentration of 8.5 µg/ml.
The 30 samples in total where then derivatized and analyzed by HPLC analysis.

2.12 Signal to noise ratio

A 2 µg/ml metformin sample was analyzed by HPLC. The same sample was measured 5 times with varying PMT gain values of 11, 12, 13, 14 and 15. All other HPLC settings were not altered.

2.13 Method validation

2.13.1 Linearity and calibration curve

- Metformin standard

The stock solution of metformin was thawed at room temperature followed by mixing to ensure homogeneity. It was then diluted with ethanol to obtain working solutions with concentrations of 5, 4, 3, 2, and 1 µg/ml respectively. Pure ethanol without any metformin added was used for the detection of the baseline signal. Every sample was prepared as triplicate and subjected to the subsequent derivatization reaction and analyzed by RP-HPLC.

- Metformin spiked plasma

To determine the linear range of metformin in the expected metformin plasma concentrations of the patient samples, 1 ml plasma samples were spiked with different dilutions of 100 µl metformin stock solution to reach concentrations of 4.76, 3.81, 2.86, 1.90 and 0.95 µg/ml. To detect the baseline of pure plasma, 100 µl pure ethanol were added to 1 ml plasma. After protein precipitation, the samples were derivatized and analyzed by RP-HPLC.

To calculate the LOD and the LOQ, the lowest (1 mg/ml MF standard, 0.95 µg/ml MF plasma) and the highest (5 µg/ml MF standard, 4.76 µg/ml MF plasma) concentration were measured three times, the other concentrations were measured only once. Linearity and LOD/ LOQ were verified by Validata 3.02, a macro designed for data validation from Microsoft Excel.
Recovery of metformin was calculated with the aid of MF standard and MF plasma concentrations. Both were tested in the linear range of 1 to 5 and 0.95 to 4.96 µg/ml, respectively. The slopes of the calibration curves were used to determine the recovery rate. The following formula was used for its calculation.

$$\%\ \text{recovery} = \frac{\text{slope MF spiked plasma}}{\text{slope MF standard}} * 100$$

Figure 12. Formula for recovery calculation

2.13.2 Robustness

As a part of method validation, the robustness of the system was tested. The applied HPLC method was subjected to changes in system parameters. Changes included pH variation, change in column temperature and change of temperature in the heating block during the derivatization reaction. For every analysis a 10 µg/ml metformin standard sample and a metformin spiked plasma sample (conc. 9.8 µg/ml) were prepared. Measurements were conducted as triplicates.

- pH

The pH value of the tris buffer was varied and tested at pH 7, pH 8 and pH 9. PH values were adjusted with 1 M NaOH and 2 M HCl. The mobile phases with varying pH concentrations were prepared freshly before every analysis.

- Column Temperature

To see if the column temperature has any influence on the peak height, respectively metformin concentration, the temperature of the column was set to 20 °C, 25 °C and 30°C. The samples were analyzed consecutively.

- Heating block

The temperature in the heating block used for the derivatization procedure was changed from 99 to 95 and 90 °C. All samples were exposed for 7 minutes.

3. Results & Discussion

3.1 Metformin standard solution

Benmessaoud et al., 2016 described that metforminhydrochlorid is equally soluble in ethanol and in water (**Figure 13**). In this experiment, ethanol was used for the dissolution of metformin tablets. Formulations of metformin tablets contain, beside metformin-hydrochlorid as pharmacologically active ingredient, several additives for coating or as excipients like hypromellose and magnesium stearate. Umapathi et al., 2012 showed, that the recovery of metforminhydrochlorid whilst using ethanol or methanol as solvents was significantly higher than in water. This is due to the fact, that the additive, in that case croscarmellose sodium is very poorly soluble in ethanol/methanol hence metformin was not retained in solution due to charge interaction. Additives in the formulation of metformin Hexal tablets, like hypromellose and magnesium stearate are practically insoluble in ethanol as well. (Eyjolfsson, 2015) Due to these findings, ethanol was chosen for metformin stock solution preparation.

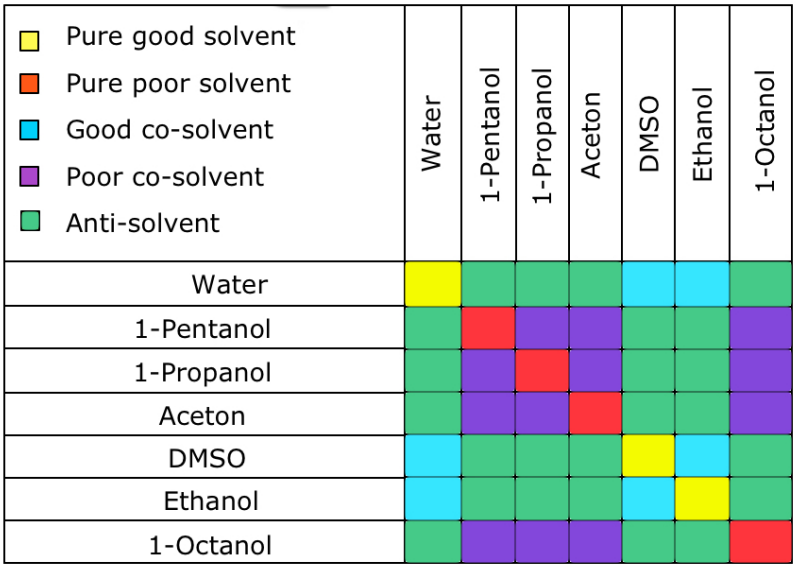


Figure 13. Solubility of metforminhydrochlorid in different solvents. Water, DMSO and ethanol have proven to be good pure solvents (marked in yellow). (Benmessaoud et al., 2016)

Metformin standard solution produced a peak at a retention time of 8.8 minutes. The peak was clearly distinguishable from the signal of the blank value, no interfering peaks in the baseline signal, solvents and blank value, were observed (**Figure 14**). The huge peak at a retention time of 1.8 minutes derives from solvents and derivatization reagents which are poorly retained on the C18 column and therefore elute right at the beginning of the analysis. The peak at a retention time of 5.5 minutes originates solely from the two mobile phases. When just the gradient with the two mobile phases was run, without any injection and no other chemical added, this peak appeared as well. Interestingly, the peak only appeared when the gradient elution was run and was not visible with linear elution. Although ultrapure H_2O was obtained from an Ultra clear purification system, the most likely reason for this peak would be, that it derives from substances dissolved in H_2O which was used for mobile phase preparation.

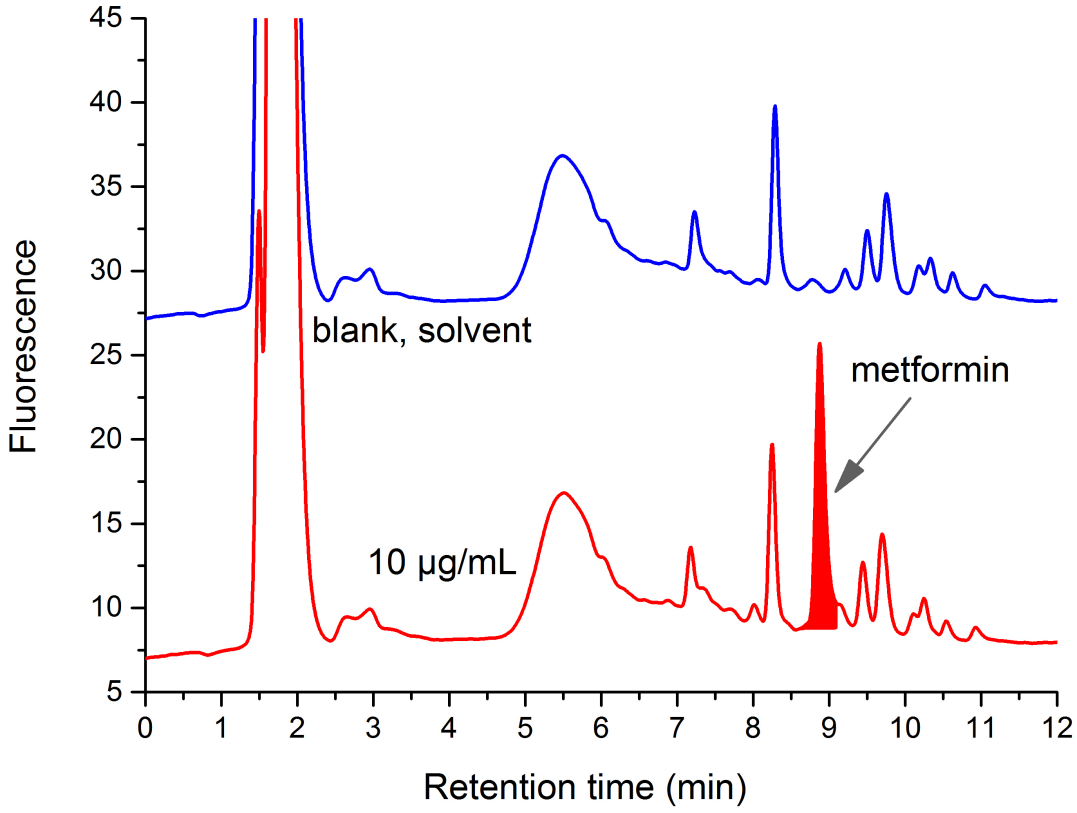


Figure 14. Chromatogram of metformin standard. The x-axis shows the retention time in minutes and the y-axis shows the fluorescent response signal. The blue line represents the blank value without any metformin, the red line represents 10 µg/ml metformin added. A retention time of 8.8 minutes was obtained for metformin.

3.2 Metformin in human plasma

In **Figure 15**, the chromatogram of metformin spiked plasma (10 µg/ml) is depicted. The metformin peak was identified at a retention time of 8.8 minutes and was clearly differentiable from the baseline signal. No endogenous plasma compounds interfered with metformin measurements. The enormous peak at a retention time of 7.6 minutes, detected in all analyzed plasma samples with varying intensities, originates from substances present in plasma which produce a fluorescent signal when subjected to the derivatization reaction. Since the peak does not interfere with metformin concentration determination, it can be considered insignificant. The characteristics of L-arginine and urea were analyzed, to see if they produce a signal which is part of the major peak at this particular retention time.

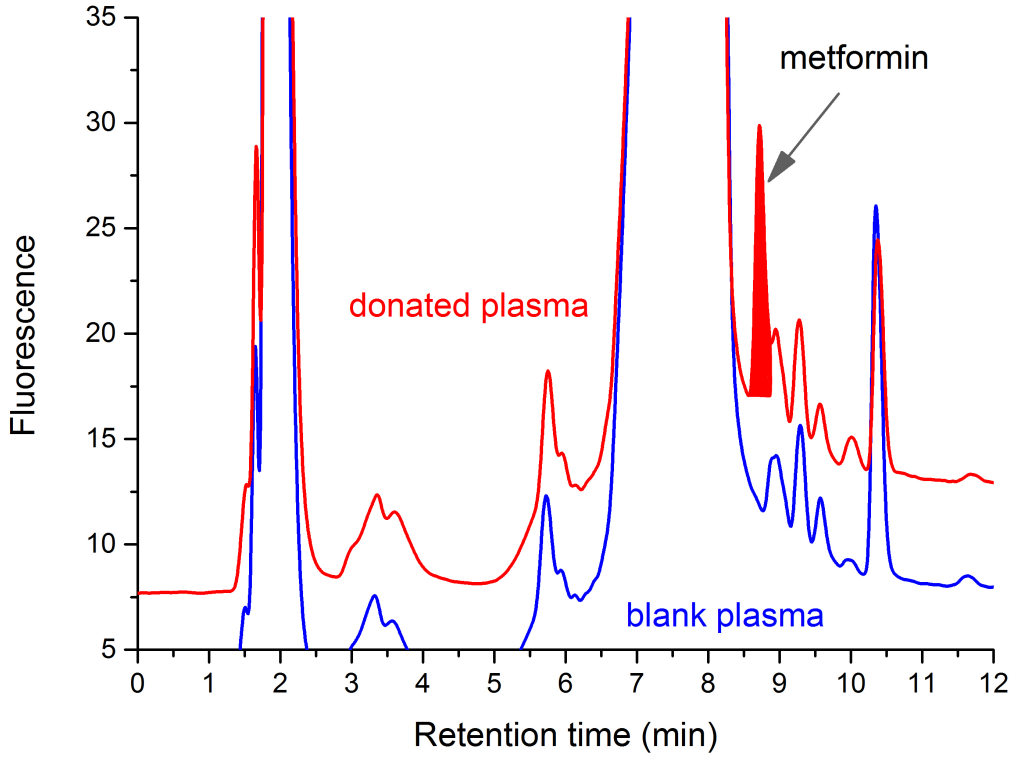


Figure 15. Chromatogram of metformin spiked plasma. The x-axis shows the retention time in minutes, the y-axis shows the fluorescent response signal. The blue line represents drug-free plasma, the red line shows 10 µg/ml metformin added. A retention time of 8.8 minutes for metformin was determined.

The most likely reason for the huge peak at 7.6 minutes are endogenous plasma proteins, which were not sufficiently precipitated with the chosen sample preparation method. Since metformin peak determination was not negatively affected, the sample preparation method with a 1:1 ACN to plasma ratio was applied further.

3.3 Derivatization of L-arginine and urea

For L-arginine and urea a qualitative HPLC determination was carried out. Bothe urea and L-arginine were successfully proven to contribute to the huge emerging peak from endogenous plasma substances at a retention time of 7.6 minutes.

3.3.1 L-arginine

L-arginine is present in human plasma in concentrations of 40-114 µmol/L. (Lüneburg et al., 2011) L-arginine was derivatized in the same manner as metformin and produced a signal at a

retention time of 7.7 minutes. **Figure 16** depicts the resulting chromatogram, the blue line represents the baseline signal from the derivatization reagents themselves, and the red line shows 1 mg/ml L-arginine added. The L-arginine peak at 7.7 min was clearly distinguishable from the baseline signal. All other peaks derive from derivatization reagents or chemicals in the mobile phases.

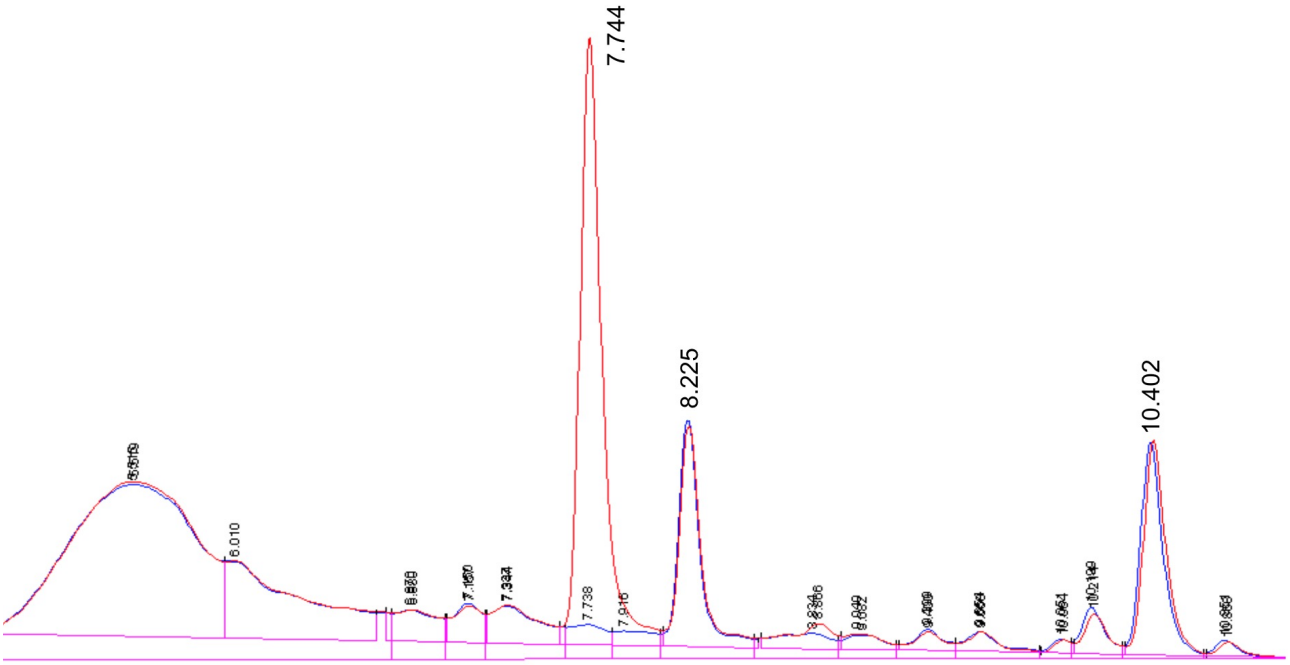


Figure 16. Chromatogram of L-arginine derivatized with benzoin. The blue line shows the baseline signal, the red line shows added L-arginine. The peak of 1 mg/ml L-arginine is visible at a retention time of 7.4 minutes.

3.3.2 Urea

Urea was derivatized with benzoin, the resulting chromatogram is depicted in **Figure 17**. The blue line shows the baseline signal, the red line shows mg/ml urea. At a retention time of 7.0 minutes a peak was detected. The peak of the baseline signal at 8.225 minutes shifted to a retention time of 8.031 minutes, although no changes in system parameters were made, which probably is due to matrix effects. The reference range of urea found in human plasma is approximately 2.5 - 7.8 mmol/l. (Higgins, 2016) Here 1 mg/ml (16.6 mmol/l) was tested. Urea is abundantly present in plasma samples, since it is a degradation product of several metabolic pathways.

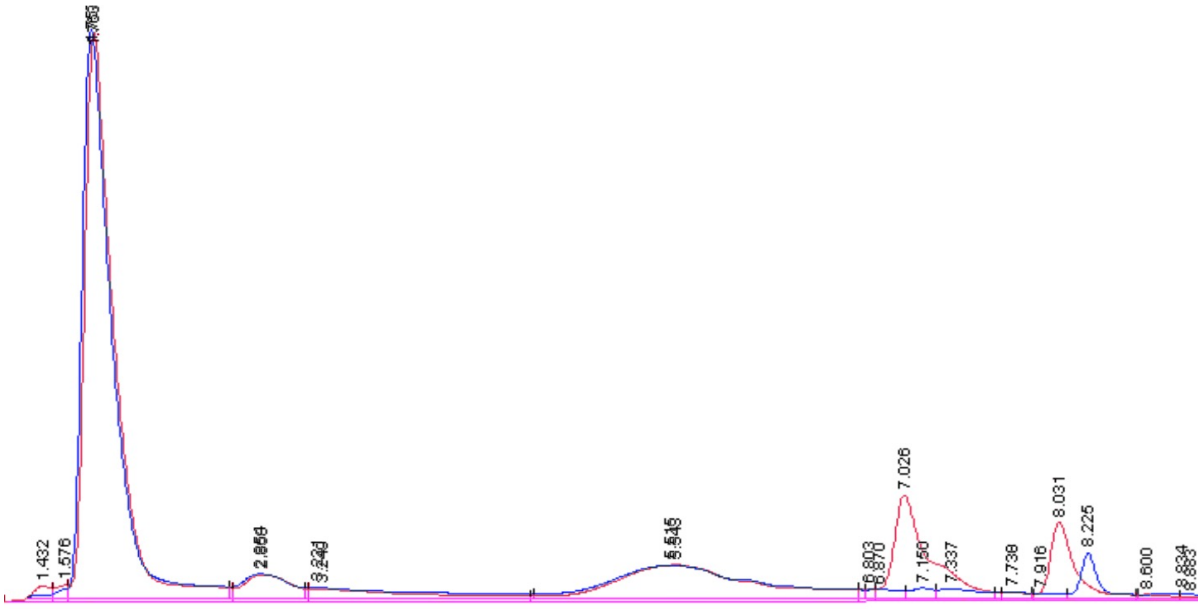


Figure 17. Chromatogram of urea derivatized with benzoin. The blue line shows the baseline signal, the red line shows added urea. The peak of 10 mg/ml urea is visible at a retention time of 7.0 minutes.

3.4 Stability of derivatized metformin signal

The stability of the formed metformin fluorophore (10 µg/ml) was tested over a period of 24 h. The first 19 measurements were taken continuously for 5 hours, whereas each measurement took 16 minutes. After 24 h passed, two additional measurements (m) 20 and 21 were taken (**Figure 18**). The same sample was tested more detailed in the five hour range, solely because this time period represents the maximum working time estimated for patient sample analysis, including derivatization reaction and HPLC analysis time. The emitted luminous intensity of metformin is consistent over 5 hours, with a detected initial amount of 316.9 LU (m-1) and an end concentration of 309.2 LU (m–19). The lowest detected amount was 303.7 (m–17), the highest was 317.5 (m–3). Variations in concentration seem to be a result of measuring inaccuracy, with a standard deviation of only 4.1 from the 310.9 peak area mean. After 24 hours, the detected amount increased slightly (326.2; 327.5), which may be a result of vaporized solvents (f. e. ethanol) and therefore concentration increase.

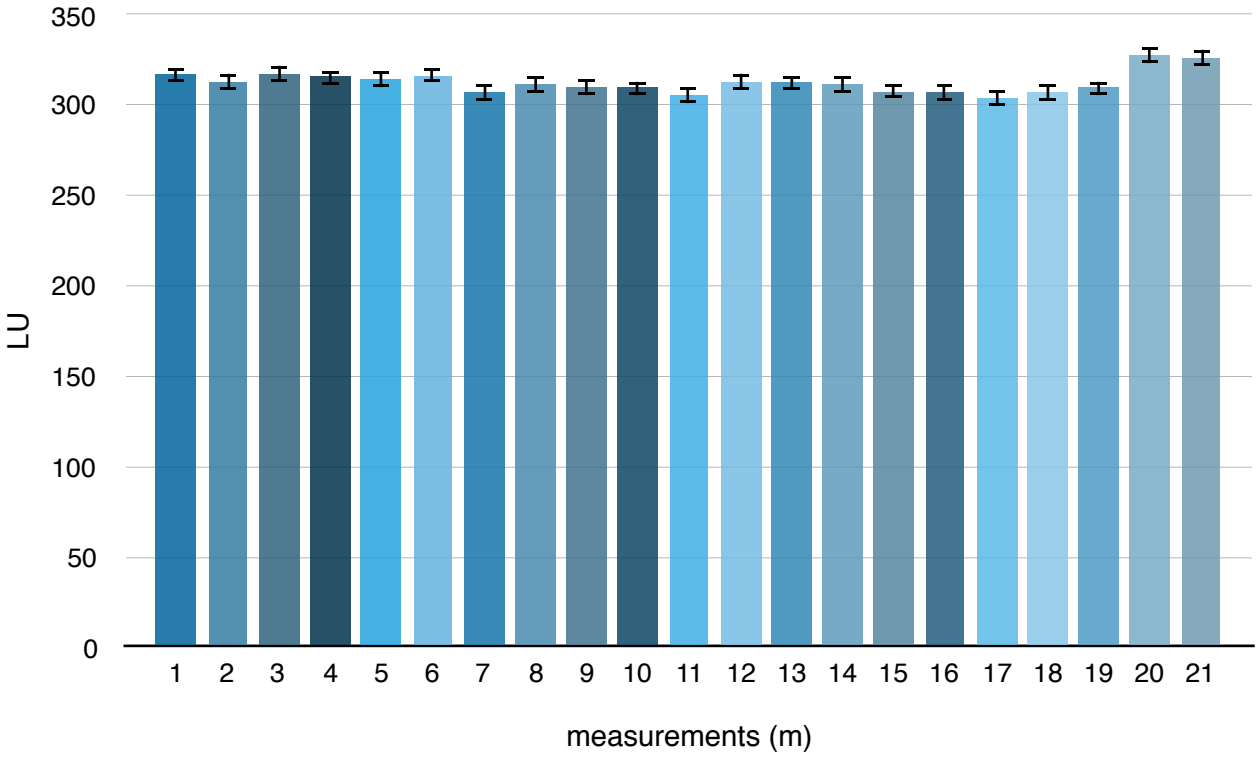


Figure 18. Stability of metformin fluorophore. Fluorescent signal strength of metformin fluorophore was determined over 24 h. The first 19 measurements were taken over a period of 5 h, measurements 20 and 21 were taken after 24 h.

3.5 Spectra determination

The excitation and emission spectra of metformin are visible in **Figure 19**. For excitation, the maximum wavelength was obtained with 279 nm. There is another fluorescence maximum at 230 nm which was not selected because a lot of compounds have their fluorescent maximum at 230 nm and the selection of the second maximum at 279 nm may aid in discrimination of different substances and increase specificity. For emission the detected fluorescence maximum was obtained at 440 nm.

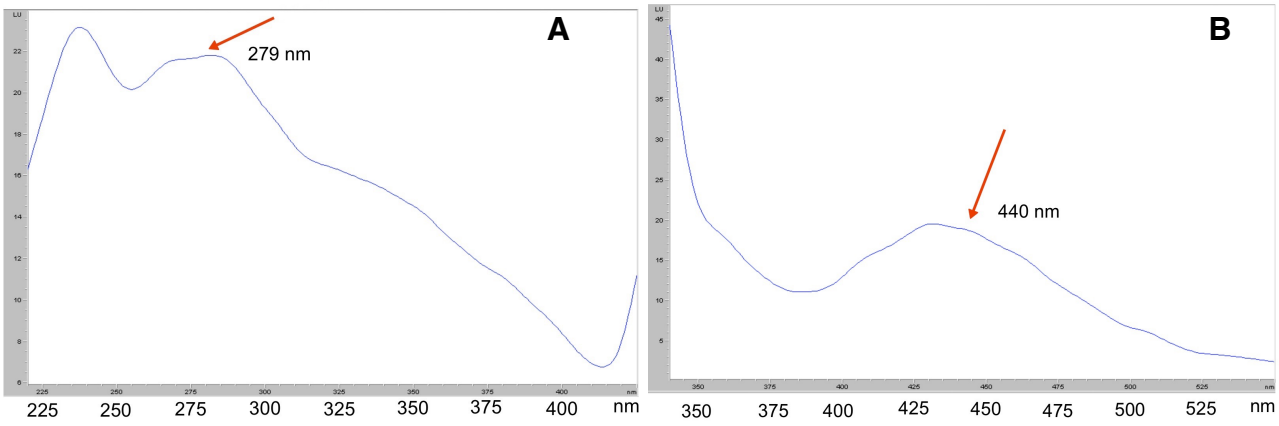


Figure 19. Fluorescence spectra of metformin. The x-axis shows the wavelength in nanometers, the y-axis shows the fluorescent response signal (LU). A - excitation spectrum of metformin; B - emission spectrum of metformin.

3.6 Linearity and precision

Metformin was extracted from human lithium heparin plasma with a simple and rapid protein precipitation method. Calibration curves were obtained from a 5 point calibration, where the concentration of metformin (µg/ml) was plotted on the x-axis and the response from the fluorescence detector (LU) was plotted on the y-axis.

Calibration of metformin standard was linear in the range of 1 µg/ml till 5 µg/ml. A correlation coefficient of 0.998 was obtained. For plasma which was spiked with metformin, the calibration curve was linear in the range of 0.95 µg/ml till 4.76 µg/ml, with a correlation coefficient of 0.997. Both curves show an excellent linearity (**Figure 20**).

The recovery was determined by comparing the response obtained from the standard metformin samples with the response obtained from metformin spiked plasma samples. The slopes of both reaction equations were used for recovery calculation, and was determined to be 109%. Recovery rates in the range of 80-110% are generally considered as acceptable when it comes to analytical testing. A recovery of 109% supports sensitive and accurate determination of metformin in human plasma. A high recovery rate gives confidence over plasma sample clean-up effectiveness. Apparently ACN protein precipitation extraction of the samples does not lead to any loss of metformin at all.

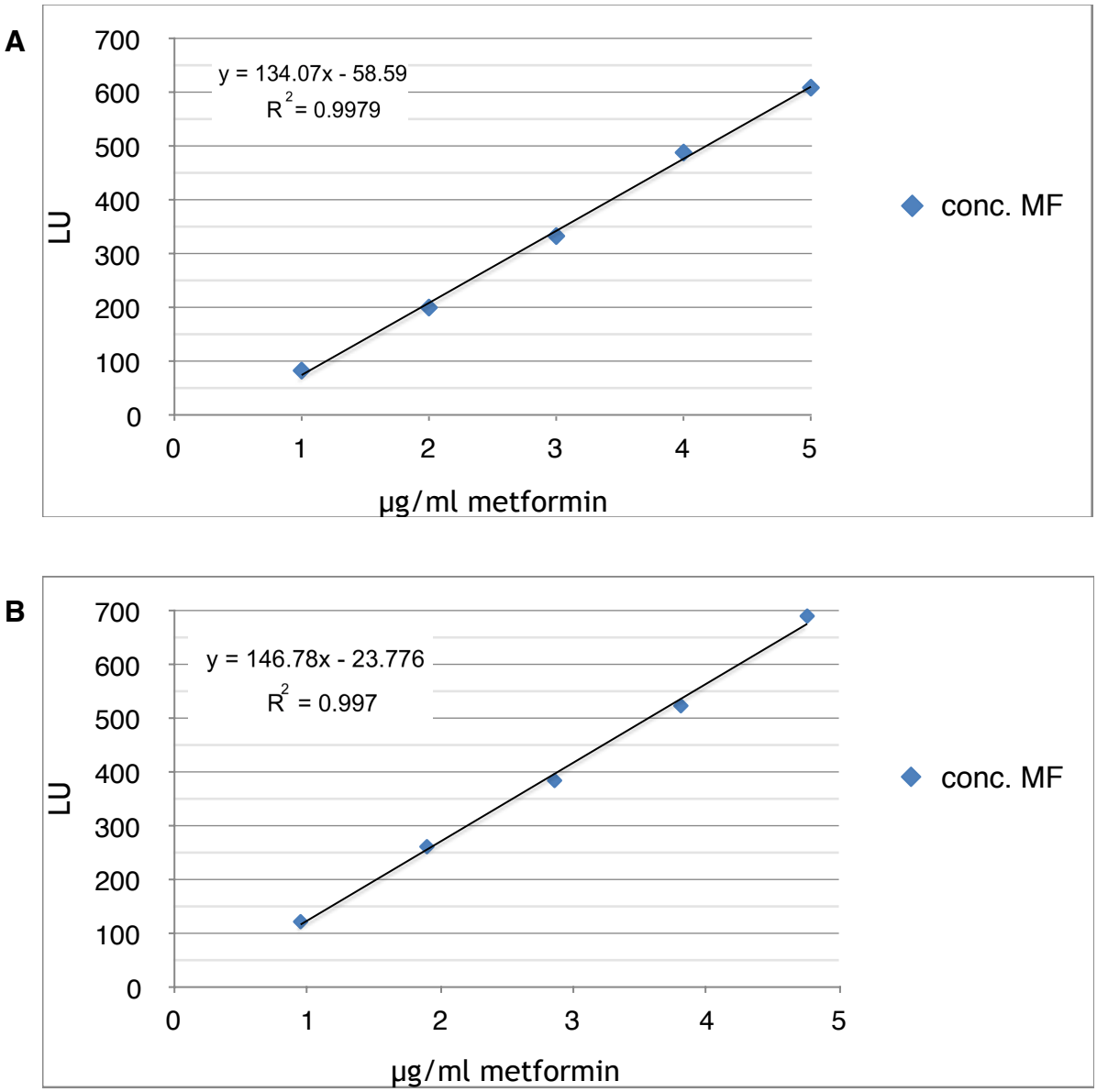


Figure 20. Calibration curves of MF standard and MF spiked plasma. A 5 point calibration was carried out for MF standard and MF spiked plasma. Excellent linearity was obtained in both cases. The x-axis shows µg/ml MF conc., the y-axis shows obtained fluorescence signal (LU).

The limit of detection (LOD) is the lowest quantity of an analyte that can be distinguished reliably from the blank value and was determined to be 190 ng/ml for metformin in plasma and 100 ng/ml for metformin standard solution. The LOD is defined as 3 times the standard deviation of the blank. The limit of quantification (LOQ) is the lowest amount of analyte in a sample which can be quantitatively determined reliably with suitable precision and accuracy and was determined to be 390 ng/ml for MF standard solution and 650 ng/ml for metformin

in plasma. The LOQ is defined as 10 times the standard deviation of the blank value. LOD and LOQ were obtained through the use of an Microsoft Excel macro called ValiData, a software program for the validation of analytical measurements. Both calibrations were sufficiently verified with this program.

3.7 Robustness

Column temperature was changed from 25 °C standard analysis temperature to 20 °C and 30 °C. Variations in peak height were minor, nevertheless the best result (162.1) was obtained with 25 °C (**Figure 21 - A**). Apparently column temperature plays hardly any or a minor role regarding metformin quantification. On the opposite, temperature reduction in the heating block during derivatization reaction led to a significant loss of detected metformin. Reduction of reaction temperature resulted in almost 18% loss of metformin (from 175.6 to 143.1) (**Figure 21 - B**). Ohta et al.,(1993), described, that the reaction of metformin and benzoin requires high temperatures. They observed, that the higher the temperature the more rapid fluorescence was formed. When the temperature was set to 99 °C, the fluorescence intensity reached a maximum. Here, the highest fluorescence response signal was also observable at 99 °C. It is recommended to conduct the derivatization reaction under high temperature conditions to obtain the highest response signal.

An effect of changes in pH concentration of metformin quantification was not detectable (**Figure 21 - C**). Minor concentration differences are more likely to be due to measuring inaccuracies or they maybe arose whilst sample preparation. Metformin is a very basic drug, with a pKa of 12.4. To ensure that all molecules are in the charged form, the pH should be at least two units lower. The used Phenomenex Kinetex C18 column has a rugged pH stability from 1-12, so even very high or very low pH concentrations can be applied.

It seems that besides temperature reduction during derivatization reaction, minor changes in the tested system parameters do not lead to any significant changes in metformin concentrations determination.

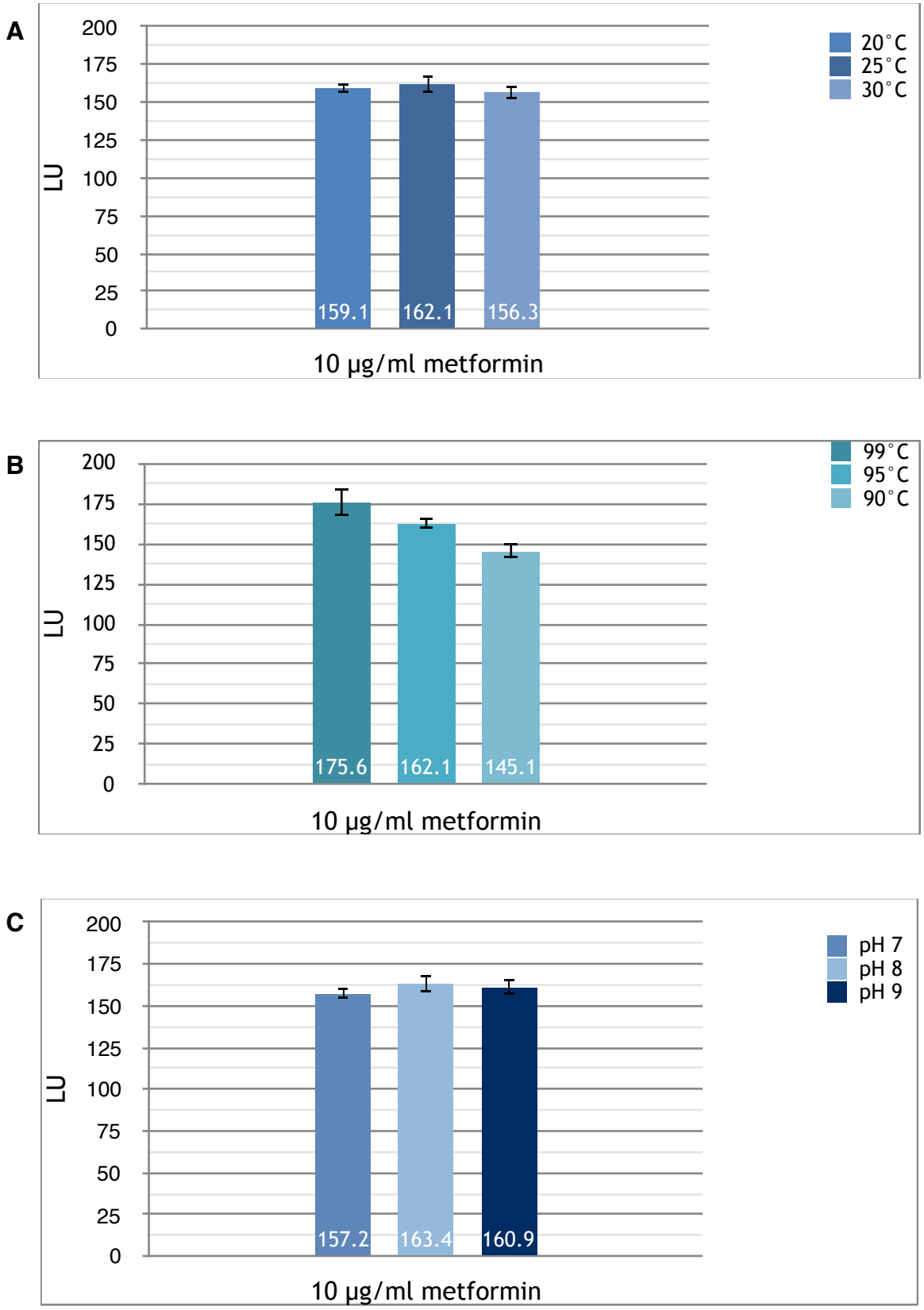


Figure 21. Robustness analysis. Alteration of system parameters. A - Variations in column temperature. B - Changes in temperature in the heating block during derivatization reaction. C - Change in pH of mobile phases. Every analysis was conducted as triplicates with a 10 µg/ml metformin sample.

3.8 Fluorescence formation over time

The fluorescence formation rate of metformin standard solution was observed over a period of ten minutes. According to Ohta et al., 1993, the maximum fluorescent response was obtained at 3 minutes exposure time in the heating block during derivatization reaction. In this experiment, the highest peak was detected at an exposure time of 8 minutes. Fluorescence increased with exposure time until 8 minutes, then a decrease in signal strength is visible. In **Figure 22**, the pink line shows the highest obtained signal after 8 minutes. Longer exposure than 8 minutes time led to a decrease in signal strength, visible through the green/ brownish line at an exposure time of 10 minutes. The same experiment was carried out with metformin spiked plasma. Here, the difference in peak heights between 4, 6 and 8 minutes was not as clear as in the metformin standard solution, however better overall peak characteristics, in particular shape, of the whole chromatogram were obtained with 8 minutes reaction time. A prolonged reaction time led to increased peak heights from derivatization and solvent reagents, which however did not interfere with the metformin peak. A derivatization reaction time of 7 minutes was chosen, one the one hand to ensure sufficient fluorescence signal formation, and on the other hand to be certain to prevent any possible signal losses when exposed too long at 99 °C

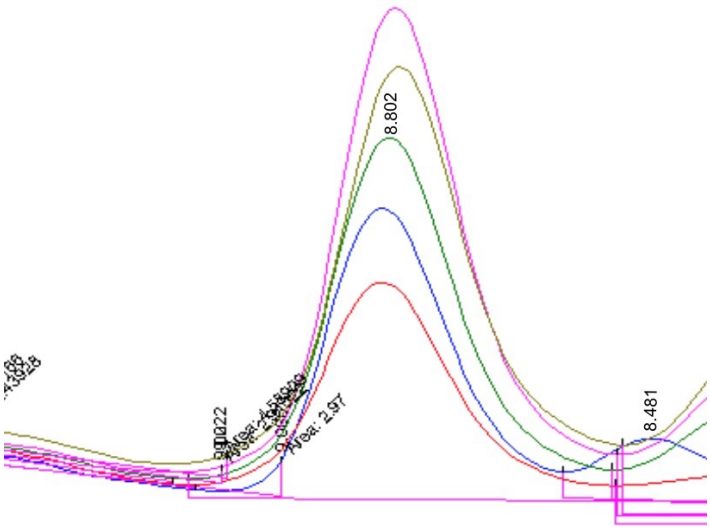


Figure 22. Observation of fluorescence formation over time. Metformin standard solution (conc. 5µg/ml) was derivatized and put on a heating block (99 °C) with different exposure times. 2 min.– red line; 4 min.– blue line; 6 min.– green line; 8 min.– pink line and 10 min.– light green.

3.9 Signal to noise ratio

A 2µg/ml metformin standard sample was analyzed. Fluorescence response signal strength is dependent on the PMT gain. PMT is short for photomultiplier, a special tube with ability to amplify weak light signals and transform them into electrical signals. Several different PMT gain values were tested, to see which value will give the best result. The best result was obtained when the PMT gain was set to 15, depicted through the pink line in **Figure 23**. Although the baseline signal was raised as well, the maximum peak area increased significantly with a higher detection sensitivity. Since metformin concentrations are expected rather low, a good sensitivity is crucial to ensure sufficient determination.

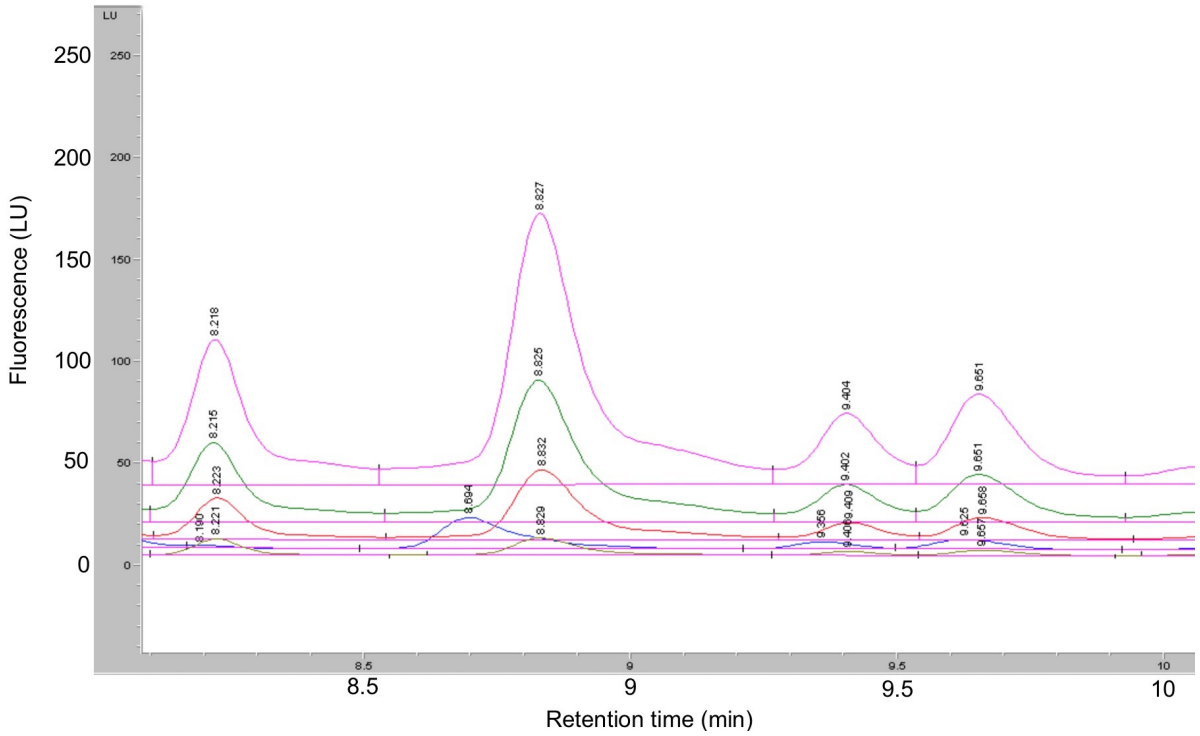


Figure 23. Signal to noise ratio of MF. A 2 µg/ml MF standard sample was tested with varying PMT gain values, increasing from 11 to 15. 15 - pink line; 14 - Green line; 13 - Red line; 12 - Blue line; 11 - Brown line.

3.10 Uniformity of metformin tablets

The metformin concentration of 10 different metformin Hexal tablets was determined. The solutions were diluted to a concentration of 8.5 µg/ml prior to HPLC analysis. The following **Table 2** shows the obtained results.

Table 2. Determination of uniformity of metformin tablets. Per tablet the measurements were conducted as triplicates. Shown are the distinct weight of the metformin tablets, the detected concentration in µg/ml and the standard deviation in µg/ml.

Tablet Nr.	1	2	3	4	5	6	7	8	9	10
weight (mg)	1085.0	1086.0	1087.8	1085.6	1077.5	1089.8	1083.1	1079.8	1086.8	1086.1
MF conc. (µg/ml)	8.51 ± 0.49	8.01 ± 0.31	8.55 ± 0.24	8.17 ± 0.41	8.47 ± 0.23	8.08 ± 0.44	8.52 ± 0.55	8.63 ± 0.46	8.35 ± 0.48	8.23 ± 0.26

According to the package insert of metformin Hexal tablets, each tablet should contain 850 mg metforminhydrochlorid. Tablets were diluted with ethanol and metformin concentrations of 8.5 µg/ml should have been determined. A great variability in the detected amount of MF is observable. The standard deviations are major and provide information about how representative the actual arithmetic mean actually is. The high differences between the single tablets combined with app. 0.5 µg/ml standard deviation are probably accounted to the manufacturing process of the metformin solutions. Although the dilutions were prepared carefully, an uneven distribution of metformin may be the most likely reason for the significant varying concentrations. For the preparation of metformin dilutions it is crucial to find a way which ensures an even distribution of the dissolved metformin in solution. The main purpose of this experiment was to determine if there are concentration differences in the metformin tablets themselves. With the obtained results, no significant information on the uniformity can be given, as the analytical method seems to be not accurate enough. It however shows that it is of utmost importance to prepare dilutions, f.e. for calibration extremely carefully to ensure adequate distribution of dissolved metformin.

3.11 Patient samples

Samples of 44 patients were analyzed. The retention time of metformin was determined to be at 8.8 min. Calculation of metformin concentration was carried out with the linear equation obtained from the 5 point calibration. Before substituting the obtained peak area in the equation, it was corrected with the blank value. In healthy humans, who receive the drug therapeutically, metformin plasma concentrations are usually in a range between 1-4 µg/ml.

(Baselt, 2008) Here most of the determined concentrations are in the expected range, with the mean metformin concentration in all 44 patients to be 3.18 ± 1.41 µg/ml for time point t2. The results are summarized in **Table 3**.

Table 3. Patient sample results. The metformin content in lithium heparin plasma of 44 people was analyzed. t0 - time point zero (at fasting). t2 - 2-2.5 h after metformin administration. All results are presented as µg/ml. A mean MF plasma concentration of 3.18 ± 1.41 µg/ml was determined. The orange marked values stand for a received dose of 500 mg MF per patient, the green marked values for 850 mg MF, the rest for 1000 mg MF. Circled in black is the lowest obtained value for time point t2, circled in red is the highest value for t2. Sample t2 of patient 25 was not obtained from the Med. University, t0 from patient 11&43 was not detectable.

patient nr.	1	2	3	4	5	6	7	8	9	10
t0	0.515	0.768	1.323	0.774	0.924	0.194	1.330	0.735	0.777	0.191
t2	1.369	3.646	3.300	2.141	2.930	0.957	4.324	3.547	1.262	1.644

patient nr.	11	12	13	14	15	16	17	18	19	20
t0	-	0.877	0.611	0.576	0.783	1.357	0.157	0.157	2.152	0.401
t2	1.772	3.671	3.136	2.445	2.097	3.534	2.601	1.489	5.112	2.284

patient nr.	21	22	23	24	25	26	27	28	29	30
t0	0.874	1.310	2.068	0.531	-	0.726	0.984	1.054	0.395	0.781
t2	2.170	4.493	4.167	3.413	4.126	2.858	3.912	1.640	7.075	1.520

patient nr.	31	32	33	34	35	36	37	38	39	40
t0	0.955	1.890	0.541	1.284	0.501	0.327	2.681	2.338	0.829	1.180
t2	2.518	5.304	1.927	1.898	1.463	2.552	3.769	5.013	2.149	3.489

patient nr.	41	42	43	44
t0	0.774	2.405	-	2.291
t2	2.321	5.893	5.716	4.14

Time point t2 represents the maximum metformin plasma concentration which is reached 2-3 hours post metformin intake. For time point to, which represents the basal plasma level of metformin concentration before tablet intake, a mean concentration of 1.01 ± 0.67 µg/ml was determined. Several different metformin tablet brands, among them Metformin Hexal and several generic medicaments, were used by the patients, in which metformin-hydrochlorid was either the single pharmacologically active ingredient or in combination with another antidiabetic drug, f.e. sitagliptin (Janumet; Velmetia) Sitagliptin belongs to the group of gliptines, antidiabetic drugs utilized for T2DM treatment. They promote insulin release and act as a blood glucose lowering agent.

Here the dosage information always refers to metforminhydrochlorid quantity. The obtained results were compared with administered tablet dosage. The majority of patients (n=29) received a metformin dosage of 1000 mg. 9 patients received a 500 mg tablet (marked orange) and 6 patients received a 850 mg tablet (marked green).

For patient 25, time point to, no sample was obtained from the Medical University, so no determination was possible. For patient 11 and 43, no peak at time point t0 could have been detected.

As expected in all samples, t2 concentrations were always higher than to concentrations. The lowest detected amount was 1.0 µg/ml whereas the highest detected amount was 7.1 µg/ml. Several values exceed the expected concentration of 1-4 µg/ml by far, namely patient 19, 29, 32, 38, 42 and 43. The concentration were 5 µg/ml or higher.

According to Cheng and Chou (2001), the amount of metformin after an administration of 500 mg should be approximately 1.3~1.4 µg/ml. When comparing the results to the findings of Cheng and Chou, most of the obtained results are way above the proposed range. Results of the 500 mg users are just slightly higher, but three patients exceed the supposed concentration by double or more. Considering the results of Cheng and Chou, an 850 mg dosage should result in app. 2.1 µg/ml. Here the results are significantly higher than expected. With 1000 mg, concentrations of app. 2.8 µg/ml are anticipated. When looking at the results, a great variability in determined metformin concentrations is visible.

A definite conclusion over the different metformin concentrations can only be made when the genotyping analysis is done, which unfortunately is not yet available. No further information regarding the possible effects of SNPs can be presented currently. However when looking at findings of researches dealing with possible SNP effects on metformin performance, it seems that SNPs do influence metformin pharmacokinetics.

Shu et al. (2008), described the effect of genetic variations (SNPs) in the organic cation transporter 1 (OCT1,) which is responsible for the uptake of metformin into hepatocytes. They analyzed 20 healthy patients, with known OCT1 genotype, of those 8 who only carry OCT1 reference alleles and 12 who carry an OCT1 variant allele. They discovered, that individuals with a reduced function OCT1 allele had higher maximal plasma concentrations, with a detected mean metformin plasma concentration of 1.5 µg/ml compared to the findings of mean plasma concentrations of OCT1 reference alleles, 1.3 µg/ml. Apparently genetic variations play a significant role in metformin uptake.

To assess the concentration correctly several parameters have to be considered. Besides possible genetic variations, the prescribed cumulative dosage has to be taken into account as well. For patient 29, where 7.1 µg/ml were determined, a measuring inaccuracy can be ruled out, since all measurements were carried out as duplicates, and the peak appeared unchanged in both sample reaction mixtures. A possible reason might be, that somehow the patient confused the prescribed dosage and took more pills than supposed to. Given that the initial concentration of MF at t0 is also pretty low, this might be the most likeliest reason.

Some of the obtained results were outside of the linear range, so that their values are not completely reliable. They are presented here for the sake of completeness.

Figure 24 provides a quick overview for the detected metformin concentrations.

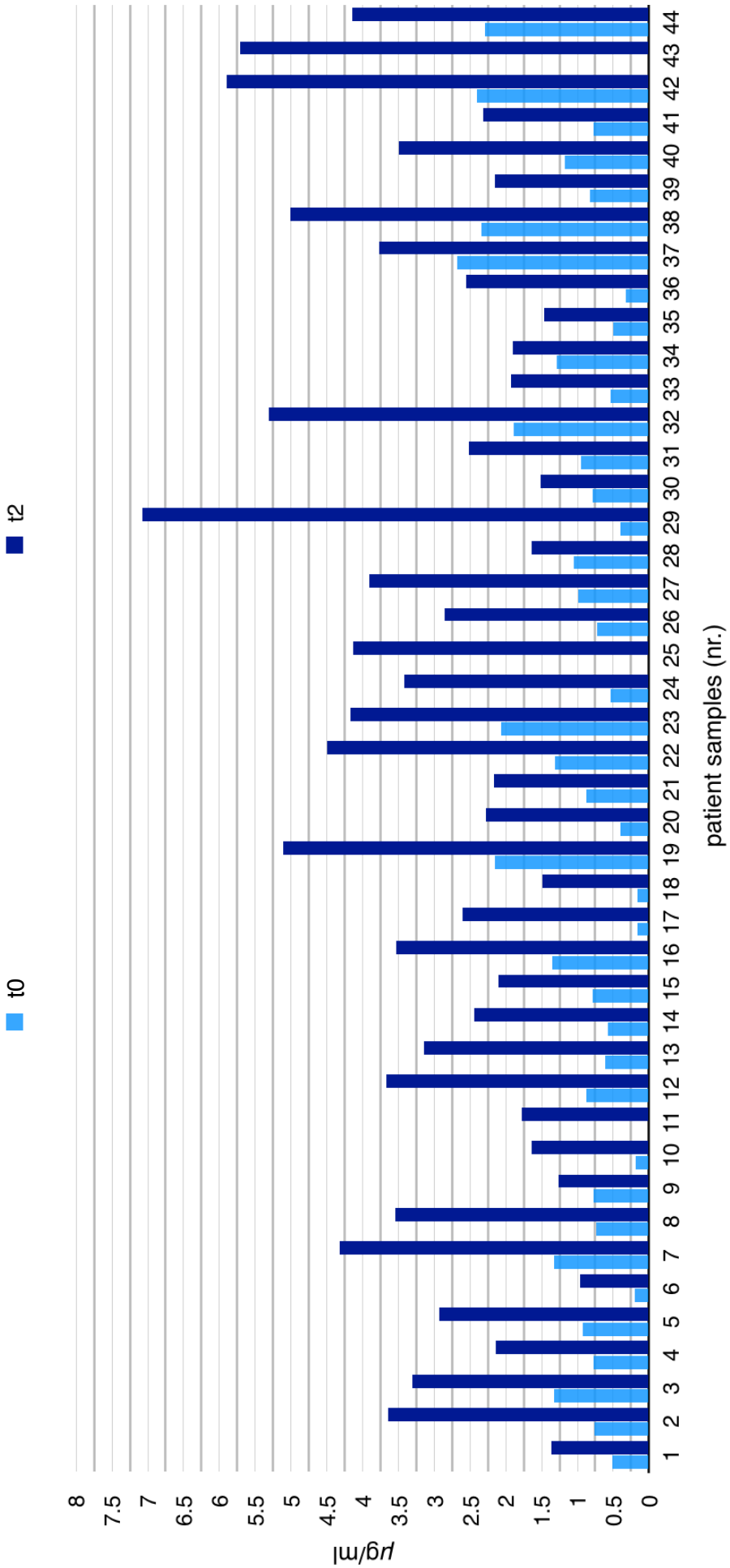


Figure 24. Overview of MF concentration in 44 patients. Determination of MF content was carried out at 2 different time points, t0 (at fasting) and t2 (after MF administration). The x-axis shows patient identification in numbers, the y-axis shows the determined MF concentration in µg/ml. t0 is depicted through light blue bars, t2 is depicted trough dark blue bars. For patient 25, no t0 sample was obtained from the Medical University of Graz. No peaks were detecable for patient 11, t0 and patient 43, t0.

4. Conclusion

Determination of metformin in human lithium heparin plasma samples with the adapted method from Ohta et al, (1993), has proven to be simple, precise and specific. Selectivity in metformin detection is verified given that there were no endogenous components found in plasma which interfered with metformin measurements. Protein precipitation was carried out with acetonitrile, which has proven to be a suitable plasma protein precipitant. At a retention time of 7.6 minutes, a major peak was detected which resulted from endogenous plasma components. This could possibly be overcome by a different precipitant/sample ratio, in general a 2:1 ratio of ACN to sample is recommended. However, since this peak did not interfere with drug detection and the to determine concentrations were relatively low, the ratio of 1:1 was kept. Although fluorescence detection usually provides an enhanced sensitivity compared to the more common UV-Vis detection, a relatively low LOQ of 650 ng/ml in plasma samples was obtained. The 5 point calibration was conducted in the range of 0.95 - 4.76 µg/ml. A solution for an even more specific and sensitive determination would be to couple an MS device to the HPLC device, since MS detection offers lower detection limits compared to HPLC analysis. However MS is more expensive and is often not widely available in laboratories.

As no information is currently available regarding the genetic variations possibly found in the analyzed patients, it is quite difficult to interpret the results. This part covers the results of the first 44 patients, the metformin plasma concentrations of 256 patients still needs to be determined. Comparison with those results and the following genotyping will provide more insight in the detected values.

5. Standard Operating Procedure

Determination of metformin in human lithium heparin plasma

Laura Sonnleitner, October 2017

Introduction

Metformin is an antidiabetic drug and belongs to the chemical group of biguanides. It is nowadays the preferred medication for the treatment of type 2 diabetes mellitus. The determination of metformin concentrations in plasma is important for pharmacokinetics and selection of the right treatment dosage. Metformin concentration is determined with RP-HPLC analysis, with pre-column fluorescence derivatization. For sample clean-up, plasma samples are deproteinized with ice cold acetonitrile. Since metformin is not fluorescent by itself, a derivatization step prior to HPLC analysis is employed. Benzoin was chosen to be the fluorogenic reagent. A linear gradient was run, with increasing concentrations of acetonitrile from 5-65%. Fluorescence detection occurred with 279 nm for excitation and 440 nm for emission.

List of devices

Pipettes - Pipetman neo P200 N, 20 -200 μl 100 - 1000 μl.
Siemens Ultra Clear Water Purification System
Mettler Toledo Analytical Scale AG245.
Elma Transsonic T460
pH meter WTW pH 3110
Eppendorf 5804 R centrifuge
hp Hewlett-Packard series 1000 HPLC
Phenomenex Kinetex 5 μm EVO C18 column (150 x 30 mm; 100 Å)
Chemstation for LC 3D, Agilent Technologies software

List of chemicals

Mobile phase A:	5% ACN
	74% H_2O
	21% tris buffer pH8 (0.5 M)
Mobile phase B:	65% ACN
	15% H_2O
	10% THF
	10% tris buffer pH8 (0.5 M)
Protein precipitation:	100% ACN (cooled down at -70 °C)

Derivatization reaction: 10 mM benzoin
0.2 M sodium thiosulfate / 0.2 M 2-mercaptoethanol (v:v)
1 M potassium hydroxide
1 M tris / 2 M HCl (v:v)
MF standard solution: EtOH, 100% abs.

Workflow

Sample preparation

Plasma samples are thawed at room temperature. Once thawed and flipped for several times, 1 ml patient sample is mixed with 1 ml ice cold acetonitrile (1:1) in a 2 ml Eppendorf tube. The sample is then vortexed and incubated for 5 minutes, then centrifuged at max. speed (14000 rpm) for 5 minutes.

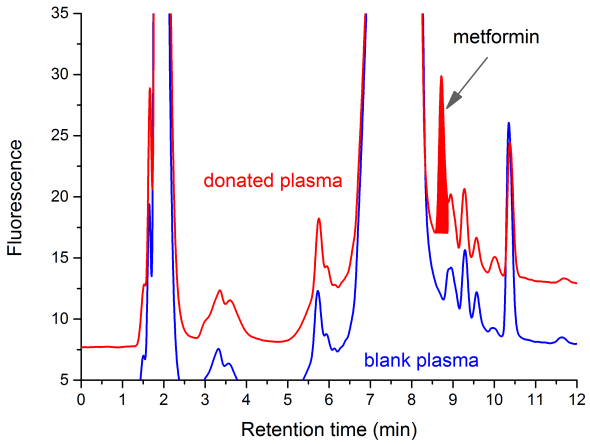
Derivatization

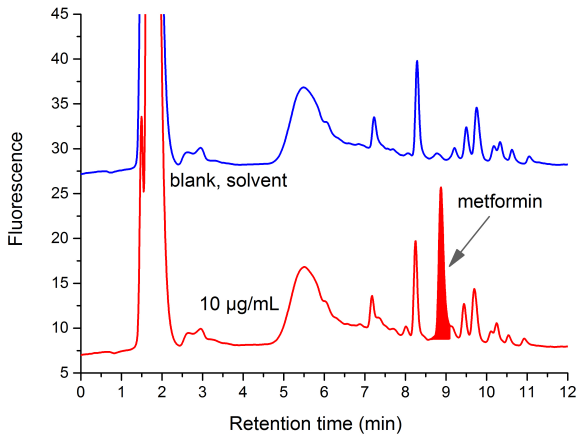
300 µl clear supernatant is then mixed with 150 µl benzoin, 150 µl sodium thiosulfate / 2-mercaptoethanol and 300 µl KOH. The sample is put on a heating block for 7 minutes at 99 °C. The use of a safety lock cap for the Eppendorf tube is advised. Afterwards the solution is cooled on ice for app. 5 minutes. Then 300 µl tris / HCl is added. The whole reaction mixture is then transferred to an HPLC vial.

RP-HPLC analysis

20 µl of the reaction solution are injected into the HPLC system. A gradient elution is run, with a starting point of 100% mobile phase **A** until 2 minutes, and mobile phase **B** at 100% at 7 minutes. Reaction end time was set to 12 minutes including a post time of 3 minutes. Wavelengths were set at 279 nm for excitation and 440 nm for emission. Column temperature was 25°C, flow rate was maintained at 0.6 ml. PMT gain value was set at 15.

Chromatograms





Metformin has a retention time of 8.8 minutes in plasma and in metformin standard solution. The chromatogram at the left side shows the peak patterns of metformin in plasma, the chromatogram on the right shows it in metformin standard solution.

Results

Metformin concentration is calculated with the equation obtained from a 5 point calibration curve in the range of 0.95, 1.9, 2.86, 3.81, 4.76 μg/ml in MF spiked plasma. The resulting equation is in the form of $y = kx \pm d$; k is the slope of the reaction, whereas x represents the searched for MF concentration and y represents the obtained peak area value from the sample. All results are presented in μg/ml.

LOD and LOQ can be calculated with the software ValiData 3.02, a Microsoft Excel macro. For that the highest and lowest concentration have to be analyzed 3 times, the concentrations in between only once.

Recovery can be determined with the aid of the 5 point calibration of MF spiked plasma (in the range of 1, 2, 3, 4 and 5 μg/ml), and the 5 point calibration of MF spiked plasma. The slope of the reaction equations from MF spiked plasma is divided through the slope of MF standard solution and then multiplied with 100. This gives the recovery in percent (%).

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