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Development and validation of a chromatographic method for analysis of amines with low reactivity in biological matrices

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Kurzfassung

Diabetes 2 ist weltweit auf dem Vormarsch, deswegen ist es wichtig, dass individuelle Therapien entwickelt werden können. Die Identifikation von Einzelnukleotid-Polymorphismen (SNPs) auf Genen von Organischen Kationen Transportern (OCT), welche die Aufnahme von Medikamenten beeinflussen, ist von großem wissenschaftlichen Interesse.

Aus diesem Grund befasst sich eine aktuelle Studie in Zusammenarbeit mit der Medizinischen Universität Graz damit, wie die Behandlung von Diabetes mit Metformin verbessert werden kann. Mögliche SNPs werden klinisch, genetisch und chemisch identifiziert. Die aktuelle Arbeit ist auf die chemische Bestimmung von Metformin in Urin mittels HPLC fokussiert.

Für diese Methode wurde eine C18 Umkehrphasen-Säule (150 × 3 mm) verwendet. Folgende Eluenten werden für den Gradienten benötigt: Eluent 1 besteht aus Acetonitril, 0,5 M Tris(hydroxymethyl)aminomethan (TRIS) pH 8 und Wasser (5:21:74 v/v). Eluent 2 ist aus Acetonitril, Tetrahydrofuran und 0,5 M TRIS pH 8 und Wasser zusammengesetzt (65:10:10:15 v/v).

Die Flussrate war 0,5 ml/min und die Laufzeit 10 min. Die Retentionszeit von Metformin liegt bei 8,8 min. Die Detektion erfolgte mittels Fluoreszenz. Die Derivatisierung von Metformin mit Benzoin ist eine verlässliche Methode (Ohta et al., 1993). Im Vergleich zu Desylbromid war Benzoin empfindlicher. Als möglicher interner Standard wurde O-Tolylbiguanid getestet, aber er stellte sich als unpassend heraus. Derivatisierte Proben sollten aufgrund von Zersetzung des Fluorophores nicht über längeren Zeitraum gelagert werden.

Die aktuelle HPLC Methode wurde für folgende Parameter validiert: Selektivität, Linearität, Präzision, Genauigkeit und Robustheit. Die Wiederfindung beträgt 96 %. Die Nachweisgrenze beträgt 30 µg/ml, die Erfassungsgrenze liegt bei 60 µg/ml und die Bestimmungsgrenze bei 110 µg/ml. Die gemessenen Metformin-Konzentrationen liegen zwischen 0.1 mg/ml und 2.1 mg/ml, was mit anderen Studien vergleichbar ist.

Abstract

As type 2 diabetes cases still rise the development of individual therapies becomes more important. The identification of certain single nucleotide polymorphisms (SNPs) on genes of organic cation transporters (OCTs) that influence drug uptake is of great scientific interest.

For this reason, the current study in cooperation with the Medical University Graz is crucial for improvement of metformin medication. Possible SNPs are identified clinically, genetically and chemically. This thesis is focussed on the chemical metformin determination in urine via HPLC.

A C18 reversed phase column (150 × 3 mm) was used for this method. For the gradient elution, solvent 1 consists of acetonitrile, 0.5 M Tris(hydroxymethyl)aminomethane (TRIS) pH 8 and water (5:21:74 v/v), solvent 2 contains acetonitrile, tetrahydrofuran, 0.5 M TRIS pH 8 and water (65:10:10:15 v/v). The flow rate was 0.5 ml/min and the run time 10 min. The retention time of metformin was found to be 8.8 min.

The detection of the drug was carried out via fluorescence. The derivatization of metformin with benzoin is a reliable precolumn derivatization procedure (Ohta et al., 1993). In comparison to desyl bromide, benzoin was more sensitive. As a possible internal standard o-tolylbiguanide has been tested, but it was not suitable. Derivatized samples should not be stored, because of degradation.

The current HPLC method was validated for selectivity, linearity, precision, accuracy and robustness. The recovery was determined to be 96 %. The limit of detection is 30 µg/ml, the limit of decision is 60 µg/ml and the limit of quantification is 110 µg/ml. The found concentrations in urine samples range from 0.1 mg/ml to 2.1 mg/ml metformin, which is comparable to other studies.

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List of abbreviations

Acronym	Definition
AMPK	Adenosine monophosphate-activated protein kinase
BMI	Body mass index
FLD	Fluorescence detector
FSH	Follicle stimulating hormone
G6Pase	Glucose-6-phosphatase
HGP	Hepatic glucose production
HPLC	High performance liquid chromatography
IFG	Impairing fasting glucose
IGF	Insulin-like growth factor
IGT	Impaired glucose tolerance
LKB1	Liver kinase B1
OCT	Organic cation transporter
PCOS	Polycystic ovary syndrome
PPT	Protein precipitation
RSD	Relative standard deviation
RT	Retention time
SD	Standard deviation
SNP	Single nucleotide polymorphism
T1D	Type 1 diabetes
T2D	Type 2 diabetes
TMD	Transmembrane domains
TRIS	Tris(hydroxymethyl)aminomethane
λ_{EM}	Wavelength emission
λ_{EX}	Wavelength excitation

1 Introduction

1.1 The drug metformin

Metformin is a drug for treatment of diabetes type 2.

1.1.1 History

In the Middle Ages, the plant *Galega officinalis* (Goats rue) was used to treat the increased strangury that is a symptom of diabetes mellitus. The plant also increases lactation in cows and in times of the plague it was used as perspiration agent (Cusi & Defronzo, 1998). Only the leaves of the plant were used for medication (Bailey & Day., 1989).

The compound that is lowering the blood glucose level is called galegin. The structure is similar to metformin (Cusi & Defronzo, 1998). Galegin was used as antidiabetic drug in the 1920s (Muller & Rheinwein, 1927, Simonnet & Tanret, 1927).

The first synthetic diguanides (Synthalin A and B) were tested clinically in the 1920s. Soon they were declared to be toxic and less efficient, but also insulin became more popular. This led to the disappearing of Synthalin A in the 1930s and Synthalin B in the 1940s (Frank et al., 1926). Some of the guanides are too toxic for treatment of diabetes mellitus, but the group of biguanides has proven to be beneficial (Watanabe,1918).

In 1929 many biguanides were synthesized, also dimethylbiguanide, that was later called metformin (Hesse & Taubmann, 1929; Slotta & Tsesche, 1929).

Jean Sterne was a french scientist in the field of medicine and clinical pharmacology. He studied diabetology under Francis Rathery at the Hôspital la Pitie in Paris. Galegin and the blood glucose lowering effects were his special interest. In 1956 cooperation with Denise Duval he also discovered antihyperglycemic properties of other biguanides (Bailey & Day, 2004). Then he started a clinical trial with dimethyl biguanide (metformin) and gave it the commercial name “Glucophage” (Sterne, 1957).

In the 1950s there were found three active biguanides at the same time. Phenformin and buformin were the first ones and very common in the 1960s, but some of the adverse effects like cardiac mortality and lactic acidosis lead to taking them off the market. Metformin was used 20 years in Europe, and has proven to be safe, before it was released in US 1995 (Cusi & Defronzo, 1998).

Several years later some other beneficial effects of metformin were discovered:

- No weight gain or hyperglycaemia (Hermann, 1979; Campell & Howlett, 1995).
- Anti-insulin resistance (Bailey, 1992; Bailey & Turner, 1996).
- Reduced cardiac mortality (UK Prospective Diabetes Study (UKPDS) Group, 1998).
- Increased survival for obese diabetes type 2 patients (UK Prospective Diabetes Study (UKPDS) Group, 1998).

Metformin is now very common in the treatment of T2D, since it has so many additional positive effects, like anti-cancer activity. Also, women with PCOS and T2D can be treated with metformin.

1.1.2 Further applications of metformin

1.1.2.1 Metformin as anti-cancer drug

T2D is often correlated to a significantly higher chance of cancer, that often affects breast colon, prostate, kidney and pancreas (Giovanucci et al., 2010). The chronic increased plasma insulin levels act on cell growth (Jalving et al., 2010).

Other factors like, insulin resistance that goes along with hyperinsulinemia, can lead to carcinogenesis. That can occur “directly through the insulin receptor or indirectly by elevating levels of IGFs, steroid sex hormones, inflammatory processes and disrupting adipokines homeostasis” (Jalving et al., 2010). Also, the persistent increased plasma glucose levels might have an influence (Currie, 2009).

A pilot-case study with 12,000 patients has reported a relation between T2D and a higher chance of cancer. They also found a correlation between the development of cancer and metformin treatment (Evans et al., 2005). Other studies found that patients under metformin medication have lower risk of cancer mortality and abnormal growth of tissue (Bowker et al., 2010; Landman et al., 2010; Libby et al., 2009).

Metformin has beneficial effects on specific cancers, like prostate, pancreas and breast cancers (Bodmer et al., 2010; Li et al., 2009; Wright et al., 2009).

Not all mechanisms that support anti-cancer action are currently understood. Metformin has positive effects on blood glucose levels and insulin levels, which could explain the anti-cancer activity (Goodwin et al., 2008).

Metformin can kill cancer cells through activation of apoptotic pathways by caspase-dependent and independent mechanisms (Isakovic et al., 2007; Liu et al., 2009). Caspases are important proteins in the mechanism of apoptosis. Another theory

assumes that metformin has the ability to lower the energy status of the cell and changes the metabolism so that cancer cells cannot adapt to their new environment (Buzzai et al., 2007).

1.1.2.2 Polycystic Ovarial Syndrome

PCOS is a disease that affects 5 to 15 % of women in child-bearing age (Tang et al., 2012). The symptoms are “menstrual disturbance and/or hyperandrogenism and/or polycystic ovary on ultrasound” (Thessaloniki PCOS Consensus Workshop Group, 2008).

Insulin resistance is common in PCOS. For this reason, insulin sensitizers are often used for treatment in obese women with PCOS and insulin resistance. Some clinical trials have proven that metformin has positive effects on ovulation, menstrual cyclicity and leads to lower serum androgen levels (Tang et al., 2012).

The lower insulin levels through metformin treatment directly affect the ovary. Insulin affects some “steroidogenic enzymes in the ovary, like cytochrome P450, 3β-hydroxysteroid dehydrogenase and the steroidogenic acute regulatory protein” (Palomba et al., 2009).

The drug can diminish cytochrome P450 activity by improving insulin sensitivity. Repression of androstenedione production on ovarian theca cells and reduction of FSH are caused by metformin. The basal and FSH-stimulated progesterone and oestradiol levels are decreased (Palomba et al., 2009).

Women with PCOS are often more in danger to develop pregnancy complications. Metformin can lower the risk of abortion by activating other beneficial effects necessary for positive pregnancy progression. These are: IGF-binding protein 1, glycodelin levels, or uterine artery blood flow (Palomba et al., 2009).

The drug also suppresses some negative factors that affect a higher risk of abortion like plasminogen-activator inhibitor-1 levels and plasma endothelin-1. All these effects may be attributed to the improvement of insulin sensitivity (Viollet et al., 2012).

1.1.3 Chemical and physical properties

Metformin is strongly basic and belongs to the chemical group of biguanides.

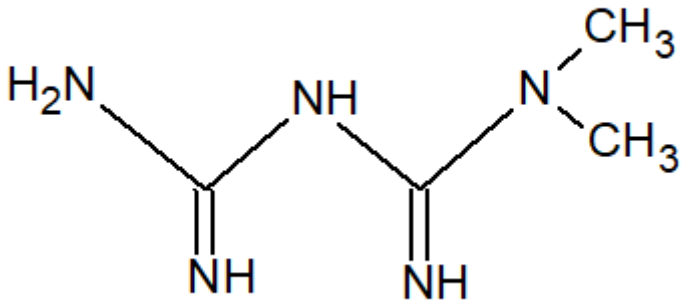

Figure 1: Structure of metformin.

It is soluble in water. The lipid solubility is low as given by the octanol-water partition coefficient (logP), that implies passive diffusion by cell membranes is very unlikely (Graham et al., 2011).

Table 1: Chemical and physical properties.
(from Graham et al., 2011; www.drugbank.ca, last access 5.7.2017)

Molecular weight	129.16 g/mol
pKa	12.4
logP	-1.8
Solubility in water	1.38 mg/ml
Melting point	223-226 °C

1.1.4 Pharmacology

Metformin takes acts primarily in the liver and influences many metabolic pathways like carbohydrate, lipid and protein (Zheng et al., 2015).

The OCTs play a very important role in uptake and clearance of metformin. In particular OCTs influence the intestinal absorption, hepatic uptake and renal excretion of metformin (Chen et al., 2013). There are three isoforms of OCTs, OCT1 and 2 are more common in the liver, whereas OCT3 is more distributed (Nies et al., 2011). OCT1 and 3 are responsible for the hepatic uptake, which are expressed on the basolateral

membrane of hepatocytes (Takane et al., 2008). The liver cells are enriched with OCT1 therefore metformin gets preferred accumulated in the liver (Graham et al., 2011).

The effect of improving hyperglycaemia can be explained by lowering the expression of hepatic gluconeogenic enzymes, phosphoenolpyruvate carboxykinase, and glucose-6-phosphatase in order to reduce HGP. Metformin uptake leads to activation of AMPK in the liver and turns on other signals that improve glucose and lipid metabolism (Zhou et al., 2001).

AMPK is a member of serine/threonine protein kinase family and its protein structure consists of α, β, γ subunits. "The activation of AMPK is due to an increase in the intercellular AMP:ATP ratio resulting from an imbalance between ATP production and consumption. AMP binds to regulatory sites on γ-subunits this causes conformational changes which activates the enzyme. Other kinases like LKB1 and Ca^{2+}/calmodulin-dependent protein kinase β are needed for the activation of AMPK" (Viollet et al., 2009).

There is new evidence that metformin can also act via AMPK-independent mechanisms. HGP is decreased through metformin by inhibiting gluconeogenesis this in turn leads to lower blood glucose levels (Rena et al., 2013).

AMPK has the ability to sense the cellular AMP:ATP ratio. If AMPK is activated, it switches the cell from an anabolic ta catabolic state, which restores energy by shutting down ATP-consuming pathways. This fact made AMPK a main target for metabolic disorders like T2D or liver diseases (Zheng et al., 2015).

"The consequence is an inhibition of glucose, lipid and protein synthesis and also cell growth, whereas fatty acid oxidation and glucose uptake are activated" (Viollet et al., 2012).

It was shown that inactivation of LKB1 results in elimination of metformin's ability to activate AMPK in vivo, which in turn leads to hyperglycaemia and increased expression of gluconeogenic enzymes (Shaw et al., 2005).

It is suggested that metformin does not directly act on LKB1 or AMPK. Foretz and coworkers (2010) have shown that mice that missing both AMPK catalytic subunits in the liver have blood glucose levels similar to wild-type mice. G6Pase repression in response to metformin is preserved in mouse primary hepatocytes in which AMPK and LKB1 has been depleted (Foretz et al., 2010).

Metformin inhibits hepatic gluconeogenesis by reducing the energy state (reduction of intracellular ATP content) without action of LKB1 and AMPK (Foretz et al., 2010; Hardie, 2006, Emami Riedmeier et al., 2013).

As metformin targets, also the respiratory chain complex 1 and the AMPK-activating effect of metformin might be a consequence of metformin actions on the mitochondria (Owen et al., 2000). Some studies have shown that in mitochondrial action of metformin intact cells are needed. (El Mir et al., 2000; Detaille et al., 2002; Guigas et al., 2004).

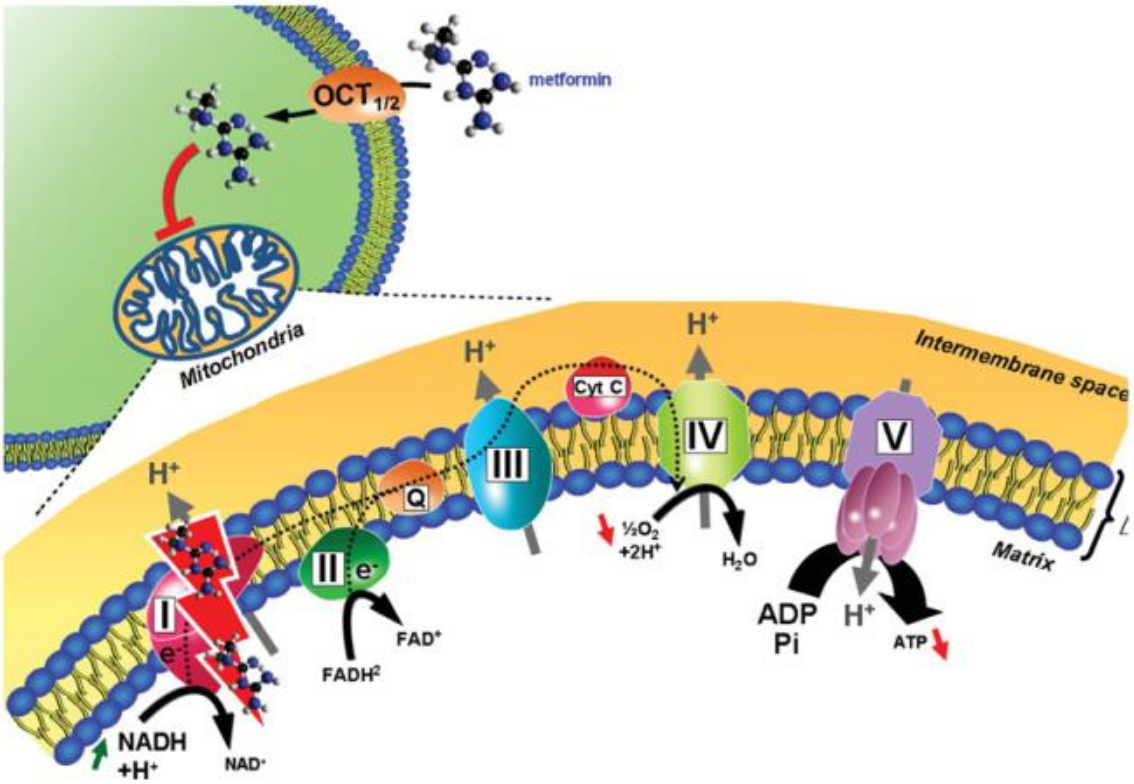

Figure 2: Metformin acts on the mitochondrial respiratory chain complex 1 (Viollet et al. 2012).

As metformin is positively charged the accumulation within the mitochondria is supported and is driven by the membrane potential (Owen et al., 2000). The nonpolar side chain of the drug can attribute to the binding to hydrophobic structures like phospholipids of mitochondrial membranes (Schafer et al., 1983).

Metformin affects the first step in the mitochondrial respiratory chain (figure 2). The drug can induce a “reduction in NADH oxidation, proton pumping across the inner mitochondrial membrane and oxygen consumption rate resulting in a reduction of the proton gradient” (Viollet et al., 2012). Finally, all of these steps lower the ATP synthesis (Viollet et al., 2012).

1.1.4.1 Pharmacological properties

Metformin is not metabolized in the body, clearance goes by tubular secretion (via kidneys) and into the urine (Bristol-Myers, 2008, Robert et al., 2003).

Table 2: Pharmacological properties

Pharmacological factor	Value	Author
Bioavailability	50-60 %	Bristol-Myers Squibb, 2008 Heller, 2007
Half-life in plasma	6.2 hours	Bristol-Myers Squibb, 2008

1.1.5 Genetic and biological aspects of OCTs

Under physiological conditions many organic compounds are positively charged. There are many molecules with tertiary or quaternary amine groups. The protonation state of tertiary amines depends on the pKa and the pH the solution. Quaternary amines are permanently charged. These molecules are mostly highly hydrophilic that makes them unable to cross a plasma membrane by simple diffusion (Lozano et al., 2013).

For this reason, special plasma membrane transport systems, like OCT1, for uptake are needed. OCTs have been classified in type 1 and type 2 based on of their structure (Meijer et al., 1990).

Biological transporters, which can move organic cations across the plasma membrane are called solute carrier family 22A (SLC22A) (Koepsell & Endou, 2004).

This family comprises of 13 plasma membrane proteins: “three OCTs, three Na+ zwitterion/cation cotransporters (OCTNs) and a group of transporters that is able of transporting organic cations or urate” (Koepsell, 2013).

Some family members are participating in the uptake of cationic and anionic compounds across the “sinusoidal membrane of hepatocytes” (Lozano et al., 2013).

The gene for OCT1 comprises of 11 exons and 10 introns (Gründemann & Schömig, 2000; Hayer et al.,1999) and the localization is on chromosome 6q26 (Koehler et al., 1997). The transporter proteins sequence is 554 amino acids long. Its tertiary structure has 12 α-helical TMDs and the N- and C-termini are found in the cell. These TMDs provide phosphorylation and glycosylation sites (Wright, 2005).

All OCTs have some highly conserved sequence motifs. A special sequence is found before TMD2 and is called a signature sequence of the OCT family (Wright, 2005).

Inter-individual drug response can be influenced by hepatic drug clearance and altered pharmacokinetic which is a result of genetic polymorphisms (Leabman, et al., 2003).

Many studies have shown that mutations in the SLC22A1 gene have influence on the pharmacologic effect. As many of the used drugs are cations this is important for clinical practice (Saito, et al., 2002; Itoda et al., 2004; Tarasova, et al., 2012).

Genetic variants of OCT1 are responsible for lowering the drug responses this may a consequence from reducing the hepatic uptake. Some examples are metformin (Shikata et al., 2007), sorafenib (Herraez et al., 2013), levodopa (Becker et al., 2011), and some platinum analogs (Li et al., 2011).

A SNP is defined as a variation of a single nucleotide on a certain position in the DNA among individuals. The variations are called SNP, if more than 1 % of a population doesn't have the same nucleotide at a specific position in the DNA sequence as defined by Nature Education, 2014. If the SNP is present within a gene, then the gene is said to have more than one allele. Some of them have no effect, whereas others may lead to certain diseases (Nature Education, 2014).

Chen and co-workers, 2009 have studied SNPs in OCT2. They found that he most frequent SNP was the base change in nucleotide 808 (G/T) that switches the amino acid from alanine to serine at position 270. This SNP had influence on the transporter activity and had a high allele frequency (10 % in different population groups) (Chen et al., 2009).

Interestingly 36 % of patients that are only on metformin therapy seemed to be bad responders (Hermann et al., 1994).

As an example, two Asian studies have shown that subjects carrying the 808G/T allele have a decreased metformin renal clearance (Song et al., 2008; Wang, et al., 2007).

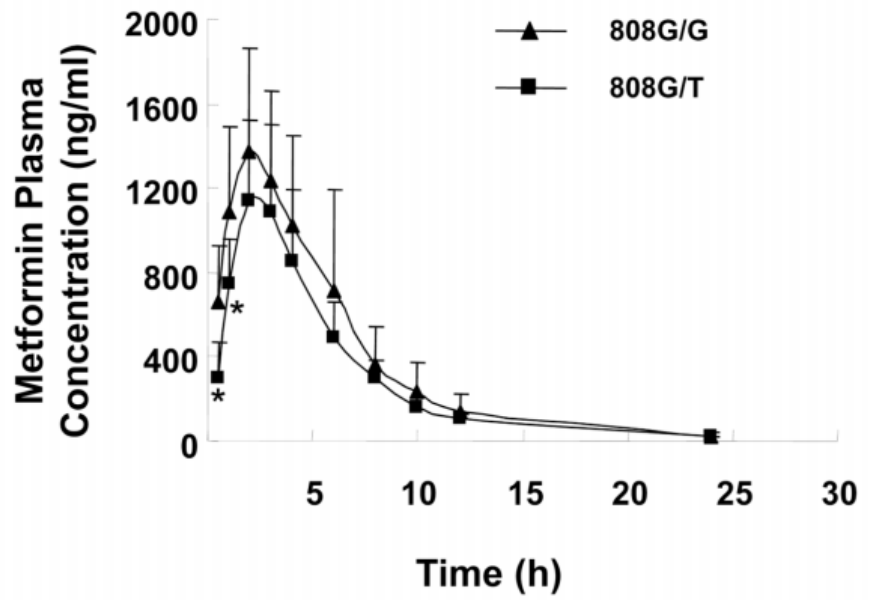

Figure 3: Metformin concentration in plasma after 850 mg administration. The stars mark the points of sample drawing (Chen et al., 2009).

For the study of Chen and co-workers (2009), 14 healthy persons who are homozygous for reference (808 G/G) were chosen. For the variant group 9 persons that are heterozygous for 808 G/T were selected.

They have determined higher metformin concentrations in subjects that are homozygous for the reference OCT2. This difference is visible at 0.5 to 1 h after drug intake (figure 3).

There was also a significant difference in renal clearance between variant and reference group. The renal clearance for the reference group was 441±108 ml/min and for the variant group 614±158 ml/min (Chen et al., 2009).

1.2 Type 2 diabetes

There are two types of diabetes:

- T1D is an autoimmune disease, which leads to destruction of ß cells. That cell type is responsible for insulin production. The patients need insulin injections to survive (Porte et al., 2003).
- T2D can develop, if the body fails to produce enough β cell insulin and the additional occurrence of insulin resistance. T2D is more common than T1D (Permutt et al., 2005).

Diabetes “results from an imbalance between the insulin-producing capacity of the islet β cell and the requirement for insulin action in insulin target tissues such as liver, adipose tissue and skeletal muscle” (Permutt et al., 2005) (figure 4).

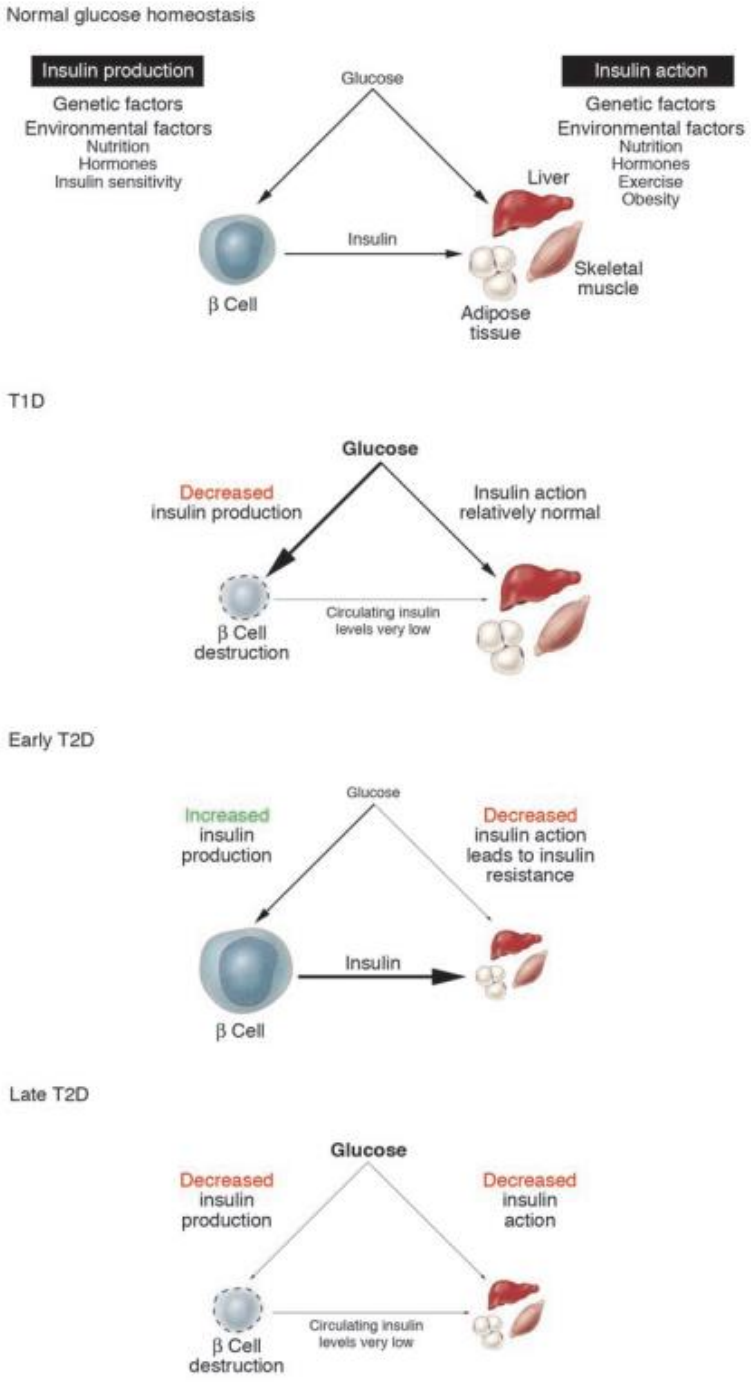

Figure 4: Mechanisms behind diabetes. (Permutt et al., 2005).

There are two conditions identified that lead to a rising risk of developing diabetes, which were determined by plasma glucose (Zimmet et al., 2003):

One is the IGT that is an intermediate between normal and diabetic levels following a glucose burden. The second is IFG, and is like IGT a risk factor for cardiovascular diseases and future diabetes (Misra & Vikrim, 2004; Meigs, et al. 2003).

Diabetes leads often to a higher rate of macrovascular and microvascular complications, which end up in large increases in morbidity and mortality (The Diabetes Control and Complications Trial Research Group, 1993).

Diabetics are the biggest part of patients, who receive dialysis (over 50 % of all cases) and kidney transplants (25 %). Also, a combination of peripheral vascular disease and neuropathy leads to a higher amount in lower extremity amputations (Engelgau et al., 2004). Adult diabetics have an age-adjusted mortality rate that is twice as high to that of nondiabetics mortality (The Diabetes Control and Complications Trial Research Group, 1993). Any risk factors for cardiovascular diseases, like systolic hypertension, increased cholesterol levels, and smoking, influence implications more in persons with diabetes (Stamler et al., 1993).

Modern medications and insulin therapy can improve the management, but according to WHO it is one of the biggest threats to human health in the 21st century (Hirsch, 2004, WHO, Global report on diabetes, 2016). Diabetes cases are rising around the world.

1.2.1 Diabetes trends around the world

The rise of diabetes in the last decades goes along with increased obesity of the population. Genetic factors have only a small influence. This was recently observed in Africa and this stands in context with ongoing urbanization (Hogan et al., 2003; Motala et al., 2003). Some other studies found evidence for the connection between obesity and diabetes that is affected by lifestyle and nutrition (Zimmet, 1999; Hu et al., 2001).

People with a BMI greater than 25 are called overweight and those with a BMI greater than 30 are specified as obese (Speakman et al., 2004).

“The risk for developing diabetes becomes higher, if people have a BMI greater than 23 for 16 years and for those, who have a BMI greater than 30, there was a 20-fold increase of the risk” (Motala et al., 2003).

A global study from 2016 (NCD Risk Factor Collaboration) shows the current situation of diabetes and forecasts the trend until 2025. The report summarizes results and numbers from all around the world. Data from 756 studies and 4.37 billion people were

included into this study. They used the Baysian hierarchical model to predict diabetes prevalence.

The data of 1980 was compared to 2014, the result was age-standardised prevalence of diabetes has increased or stayed the same (figure 5A). The consequence is a four times higher occurrence of diabetes that goes along with population growth and aging (figure 5B). The total number of diabetes cases has increased from 108 million in 1980 to 422 million in 2014 (WHO, Global report on diabetes, 2016).

Some summarized trends from around the world concerning the age-standardized diabetes prevalence (NCD Risk Factor Collaboration, 2016):

- In 2014, north- and southwestern Europe has the lowest (less than 5 %) and the highest was found in Polynesia and Micronesia with 25 %. This trend was followed by Melanesia, Middle East and North Africa with around 15 %.
- There was only a small alteration in adult women in Western Europe, although crude prevalence increased because of population.
- On the opposite in Polynesia and Micronesia there was a gain of 15 % among women and men.
- East Asia and South Asia have the largest total amount of diabetes cases in 2014, which is 106 million and 86 million. This is due to rise of the population growth and aging and rise in age-specific prevalence.
- Five countries count the half of people with diabetes in 2014, these are: China, India, the USA, Brazil and Indonesia. Together these countries hold the half of the world’s population.
- In China and India, the global share of diabetes cases has risen and in the USA, there was a decline.
- Some of the new top 10 countries with the most adults with diabetes in the low and middle-income range are Indonesia, Pakistan, Mexico and Egypt. These countries replaced European countries like Germany, UK, Ukraine and Italy.

There is an international aim of halting the rise in prevalence of diabetes by 2025 at the levels of 2010. Unfortunately, the chance of meeting this goal worldwide is lower than 1 % for men and is 1 % for women (figure 6). Only women and men in Western Europe have a 50 % higher probability reaching this objective. This means that the age-standardized prevalence will be 12.8 % in men and 10.4 % in women by 2025, if the post-2000 trends continue.

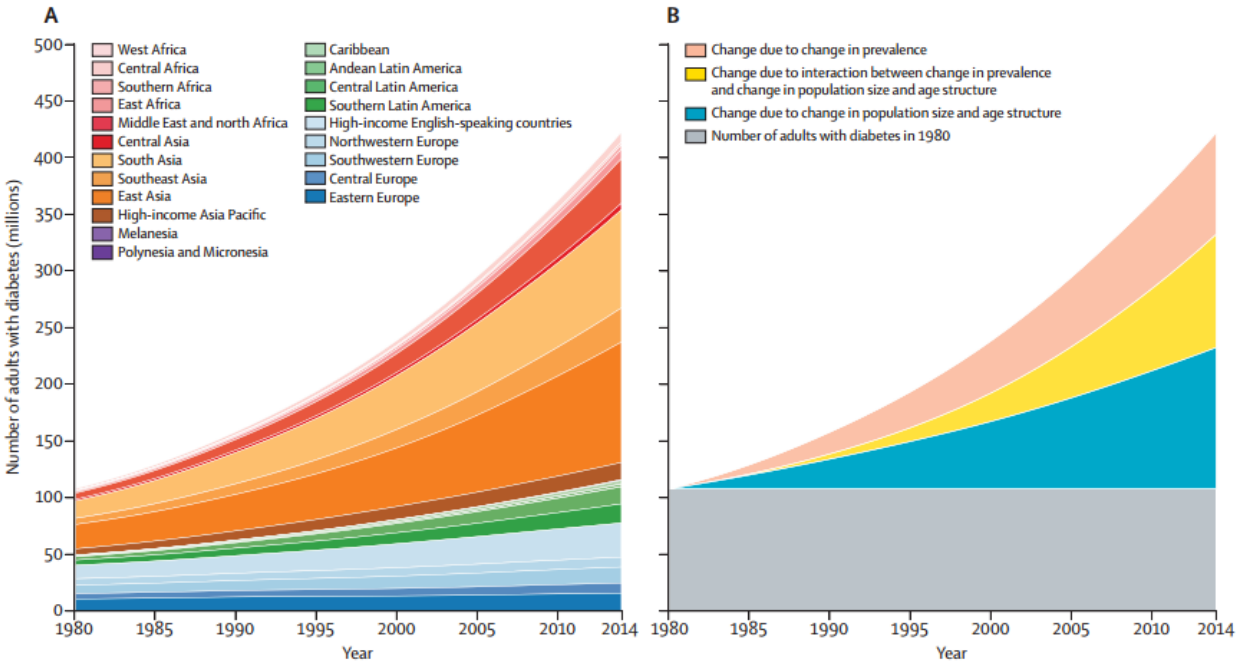

Figure 5: The trend of diabetes cases per number of million people and region (5A). The relation of population growth ageing, prevalence and interaction between the two concerning diabetes cases (5B). (NCD Risk Factor Collaboration, 2016).

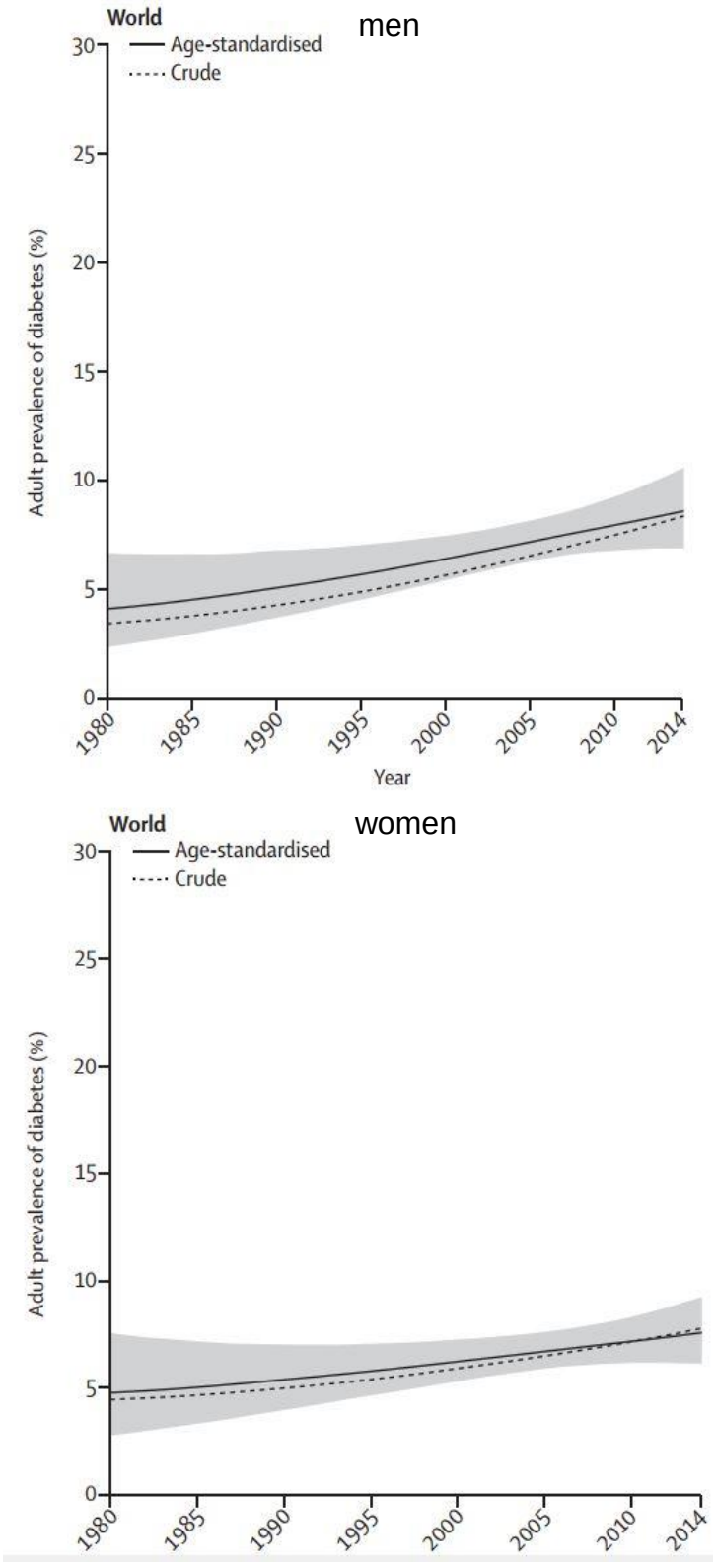

Figure 6: Diabetes frequency trend for men and women from 1980 to 2014. The lines (solid for age-standardised and dashed for crude) show the posterior mean estimates; the shaded area shows the 95% credible intervals for age-standardised prevalence (NCD Risk Factor Collaboration, 2016).

There are some theories that suggest a relation between nutrition habits, human evolution and development of diabetes.

1.2.2 The thrifty geno- and phenotype theory

“Thrifty genotype” hypothesis was made by the geneticist James V. Neel in 1962.

This hypothesis was suggested, that in times of reduced availability of food the storage of fat is accelerated. This goes back to the paleolithic history of humans. In developed countries, there is an oversupply of food and the physical activity is reduced, which is a risk factor for the population (Neel, 1999).

In population groups that kept traditional eating habits, there were not many cases of diabetes. As they started adapt to western diet, diabetes starts to spread rapidly (Baschetti, 1998).

The original hypothesis suggested that famine was common in paleolithic times and selection for the thrifty gene was intensified. In other studies, there was found evidence for a discrepancy in human evolution (Lee et al., 1968; Cohen, 1989; Baschetti et al., 1998; Speakman et al., 2007). Today it would be a disadvantage to store fat rapidly, because food is available in excess. This means the probability of existence of thrifty genes is not likely (Baschetti, 1998).

The incidence of low birth weight is associated with a higher diabetes risk later in life. This hypothesis is called “thrifty phenotype” (Hales et al., 1991; Hales & Barker, 2001).

The malnutrition of the foetus leads to impaired β cell development and insulin resistance. The progeny is also more vulnerable to diabetes and metabolic syndrome as long as enough food is available. The higher prevalence of T2D in progeny of diabetic mothers might be a consequence from environmental factors that act on a genetic level (NIH, Office of Extramural Research, 2003).

Not only environmental factors have an influence on the development of diabetes, also genetic factors.

1.2.3 Heritability of diabetes

In people with genetic predisposition environmental factors can accelerate the development of diabetes. “The regulation of food intake, energy expenditure, and variations in energy balance has to be understood on a genetical level” (Permutt et al., 2005). The majority of the obese population are resistant to insulin, but only 5-10 % are in danger to develop pancreatic β cell failure (Permutt et al., 2005).

If there are occurrences of diabetes type 1 and 2 in the family it’s more likely for the progeny to develop the disease. For T1D there is a 12 to 100-fold greater risk than for the general population (Redondo et al., 2001). It has turned out that monozygotic twins have a greater risk than dizygotic ones related to T2D (Beck-Nielsen et al., 2003).

Other factors like BMI, blood pressure, serum lipid and insulin sensitivity levels are heritable and contribute to the insulin resistance syndrome. Many studies found evidence for the heritability of these metabolic phenotypes (Hanson et al., 2001; Austin et al., 2004; Henkin et al., 2003; Pérusse et al., 2000).

1.3 Aim of the thesis

This project “Evaluation of SNPs associated with metformin resistance” is part of a medical study that goes over three years. There is a corporation between the Medical University of Graz and Graz University of Technology. The patients were chosen by the scientists of the Department of Internal Medicine, Division of Endocrinology and Metabolism.

The main aim is to assess the influence of SNPs, that are already known to modify the efficacy on renal clearance and hepatic uptake of metformin in healthy patients and in patients with PCOS and type 2 diabetes mellitus.

Furthermore, the impact of mostly intronic SNPs, which are not investigated yet, on metformin response, on hepatic uptake and renal clearance in patients with PCOS and T2D. Also, the relation of all selected SNPs on glucose monitoring parameters has to be determined.

Patients that are fully resistant to metformin, are called “non-responders” and those that are partly resistant are called “bad responders”. The identification of these two groups is done genetically, clinically and also chemically. Genetically “bad responders” are heterozygous and “non-responders” are homozygous. Clinically, for the “bad responders” the intake of an additional medication to metformin is necessary to reach the therapeutic goal. Some patients had a switch in their medication, because metformin had no effect on them, these might be “non-responders”. Chemically a difference between these two groups is recognizable according the blood levels and urinary concentration of metformin. In addition the correlation of the collected data (chemically, genetically, clinically) for non- and bad-responders will be examined. In the future the investigated SNPs in patients, will help to predict relevant response to metformin treatment.

The task was to establish a method for metformin detection in blood and urine including a validation. In this work only the urine samples were investigated. The plasma samples were measured by Laura Sonnleitner. The methods and results of this thesis are described in the following pages.

2 Methods

2.1 Metformin stock

As standard substance metformin hydrochloride from Hexal ® 850 mg was used. One tablet contains 663 mg of metformin. The tablet was crushed with a porcelain mortar and the resulting powder was dissolved in 85 ml of ethanol. Then the solution was put into an ultrasonic bath for 5 min. After that the insoluble compounds, like magnesium stearate, hypromellose, TiO_2, were removed by filtration through filter paper. Metformin is soluble in ethanol (Umapathi, Ayyappan & Darlin Quine, 2012).

The stock has a concentration of 7.8 mg/ml metformin. 1 ml aliquots were made and stored in the freezer at -20 °C.

$$g\ metformin\ per\ tablet = \frac{0.85\ g}{MW\ Metformin\ HCl\ \left(\frac{g}{mol}\right)}$$

$$= mol\ metformin\ HCl * MW\ metformin\ \left(\frac{g}{mol}\right)$$

Equation 1: Calculation of g metformin in an 850 mg metformin hydrochloride tablet.

Molecular weight (MW):

- Metformin HCl: 165.62 g/mol
- Metformin: 129.16 g/mol

The tablet consists of the following filling material[1]:

Coating of the tablet:

- Hypromellose: Hydroxypropylmethylcellulose, unsoluble in cold water
- Macrogol 4000: Polyethylenglycol, water soluble
- Titan dioxide: water insoluble

Tablet core:

- Magnesium stearate: water insoluble
- Povidon K90: Polyvinylpyrrolidon (PVP), amorphous white powder, hygroscopic, that binds the drug.

[1]http://www.apotheken-umschau.de/Medikamente/Beipackzettel/Metformin-HEXAL-850mg-Filmtabletten-2386506.html, last access 15.07.2017.

2.2 Sample preparation

2.2.1 Solid Phase Extraction (SPE)

The extraction was done according the user manual from Agilent technologies for Bond Elut Certify. The material is a mixed-mode strong cation exchanger (octyl- and benzene sulfonic acid) and a non-polar C8 sorbent. The cation exchanger is useful for basic compounds like metformin.

Sample preparation:

2 ml of urine and 2 ml of 100 mM phosphate buffer (pH 6.0) were mixed. The sample pH should be 6.0 ± 0.5. pH. The pH was adjusted with 1.0 M KOH, if necessary.

Column preparation/extraction:

1. Bond Elut Certify Cartridge Conditioning

a) 2 ml CH_3OH

b) 2 ml ultrapure H_2O

c) 1 ml100 mM phosphate buffer (pH 6.0)

A low vacuum (≤ 3 inches Hg) or gravity flow was used to prevent drying of sorbent.

2. Sample Loading

Load at 1 to 2 ml/minute

3. Column Wash

a) 2 ml ultrapure H_2O; draw through under vacuum

b) 2 ml 100 mM HCl; draw through under vacuum

c) 3 ml CH_3OH; draw through under vacuum

d) Dry column (5 minutes at ≥ 10 inches Hg)

4. Elution

2 ml CH_3OH /NH_4OH (98/2); collect eluate at 1 to 2 ml/minute

5. Dry Eluate

Evaporate to dryness at ≤ 40 °C under nitrogen gas stream.

6. Reconstitution of eluate in 150 µl ethanol and derivatization.

2.2.2 Protein precipitation with ethanol

To 200 µl urine sample the same amount of icecold ethanol was added. The sample tubes were centrifuged for 5 min at 14,000 g at 5 °C. The total volume is 400 µl. After centrifugation, the sample is mixed gently by inverting the tube, so that the pellet is not harmed. 150 µl of the sample are used for derivatization.

2.3 HPLC method

The HPLC 1100 Series is from Hewlett-Packard® and the fluorescence detector is obtained from Agilent Technologies®. As application software “ChemStation” from Agilent Technologies was used.

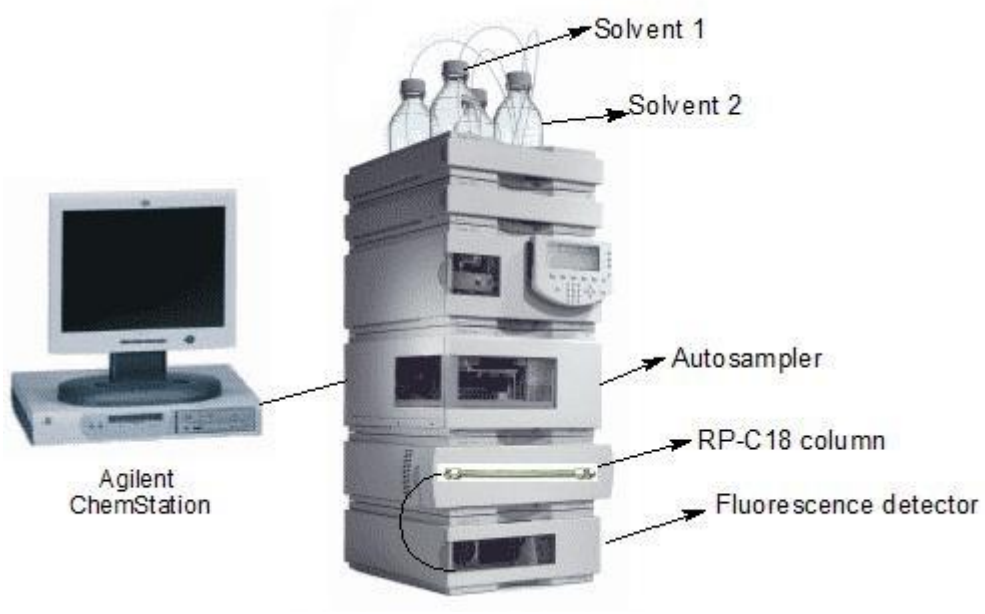

Figure 7: Setup of the HPLC system.

2.3.1 Column

For this LC-measurement a Kinetex® C18 reversed phase from Phenomenex® was used. The operating temperature was at 20 °C.

Table 3: Specifications of the column

Dimensions	150 × 3 mm
Particle size	5 µm
Pore size	100 Å

2.3.2 Gradients

Different gradients and solvent compositions were tested to find the optimal chromatographic separation for metformin from other components. The details are discussed in chapter 3.1. The gradient of method 5 was chosen for all measurements and was started with solvent 1 for 2 min and changes after 7 min to solvent 2.

See table 14 for composition of solvents.

2.3.3 Chromatographic conditions for metformin/benzoin

Table 4: Conditions for metformin and benzoin.

Flow	0.5 ml/min
Run time	10 min
Injection volume	10 µl
FLD setting	λ_{EX}: 280 nm
	λ_{EM}: 435 nm

2.3.4 Chromatographic conditions for metformin/desyl bromide

Table 5: Conditions for metformin and desyl bromide.

Flow	0.5 ml/min
Run time	10 min
Injection volume	10 µl
FLD setting	λ_{EX}: 281 nm
	λ_{EM}: 434 nm

2.4 Derivatization of metformin with benzoin and desyl bromide

2.4.1 Reaction of benzoin and desyl bromide with metformin

The reaction of metformin with benzoin/desyl bromide takes place at very basic conditions (pH 13-14) in presence of potassium hydroxide. Sodium thiosulfate and in the case of benzoin also β-mercaptoethanol are needed for reaction. Both reagents function as electron donor. The derivatization compounds form an imidazole ring with metformin. This reaction leads to the fluorophore, that makes the detection possible.

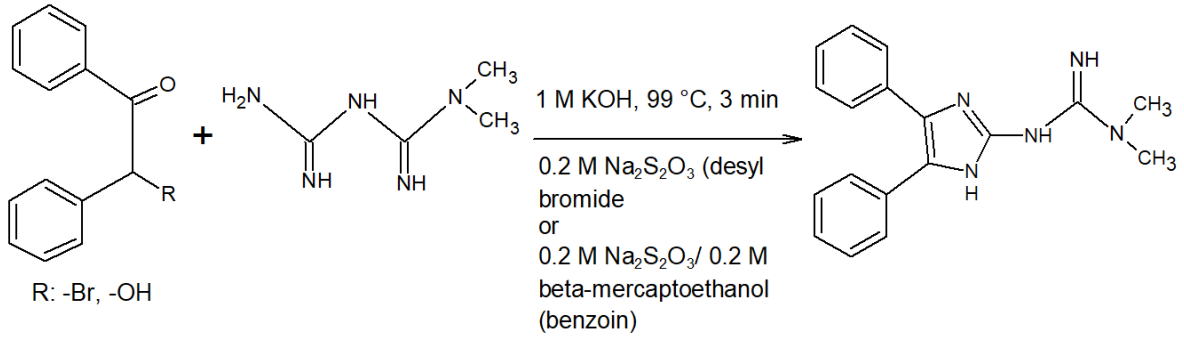

Figure 8: Reaction scheme of desyl bromide/benzoin with metformin forming a fluorophore.

The emission wavelength is 450 nm and the excitation wavelength is at 315 nm (Ohta et al., 1993).

2.4.2 Benzoin

The molecule benzoin is used for derivatization. The method was originally developed by Ohta and co-workers (1993) and adapted.

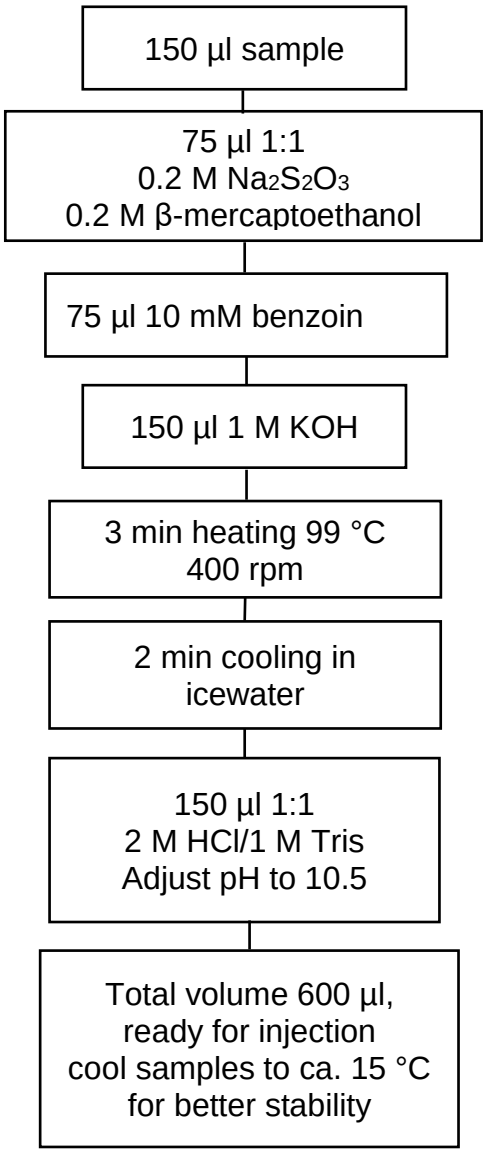

Figure 9: Flow chart for derivatization of metformin with benzoin

2.4.3 Desyl bromide

Instead of desyl chloride (Ohta et al., 1993), desyl bromide was used for derivatization.

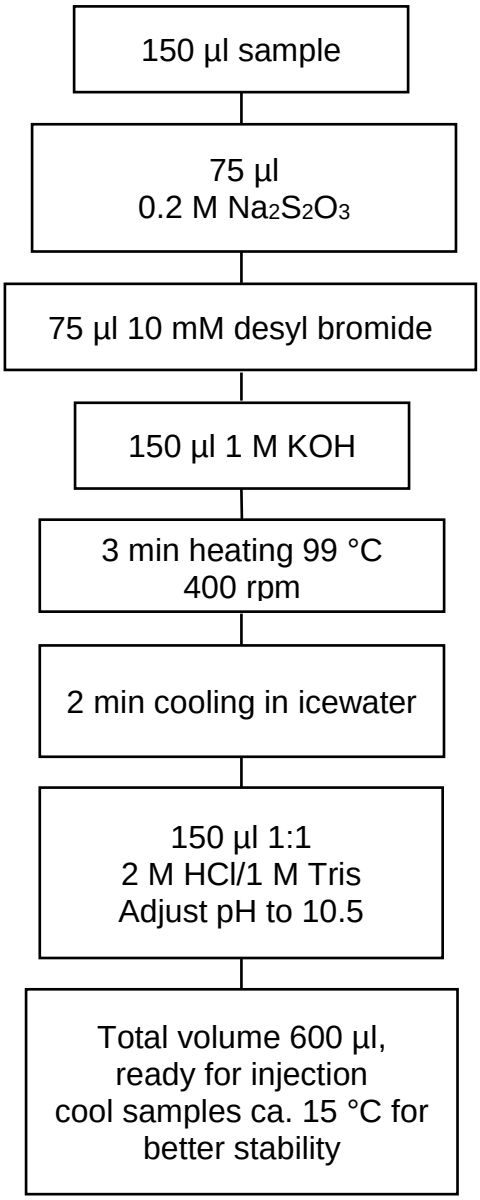

Figure 10: Flow chart for derivatization of metformin with desyl bromide.

2.5 Derivatization of o-tolylbiguanide with benzoin

O-tolylbiguanide has a similar structure to metformin, that is why it was chosen as a possible internal standard. The derivatization procedure is the same as described in 2.4.2.

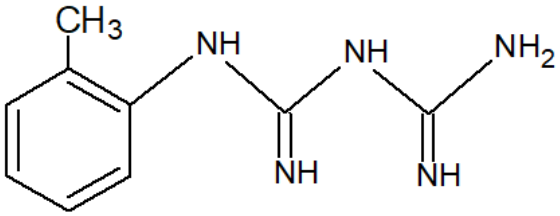

Figure 11: Structure of o-tolylbiguanide.

2.5.1 Changes in sample preparation

To 200 µl of sample additionally 50 µl of o-tolylbiguanide were added. Then 250 µl of icecold ethanol were pipetted into the reaction tube. The total volume is now 500 µl. The other steps are the same as described in chapter 2.2.2.

2.5.2 Experiments with o-toylbiguanide and metformin as analyte

The chromatographic conditions were the same as for metformin except for the run time and detector settings. See chapter 2.3.

Table 6: Chromatographic conditions of o-tolybiguanide

Flow	0.5 ml/min
Run time	12 min
Injection volume	10 µl
FLD settings	λ_{EX}: 295 nm
	λ_{EM}: 430 nm

In the first experiment, the volume of reagents was like described in chapter 2.4.2. The volume was increased by 25 µl for every reagent in the second experiment. The sample volume stayed the same.

Table 7: Composition of derivatization mixture.

Reagent	Volume (µl)
Sample	150
10 mM benzoin	100
200 mM β-mercaptoethanol/ 200 mM sodium thiosulfate	100
1 M KOH	175
1 M TRIS/2M HCl	175
Total volume	700

For the kinetic experiment, the volume of reagents was doubled and the sample volume stayed the same. The reaction temperature was 99 °C.

Table 8: Composition of derivatization mixture for kinetic experiment.

Sample in urine matrix	150
10 mM benzoin	150
200 mM β-mercaptoethanol/ 200 mM sodium thiosulfate	150
1 M KOH	300
1 M TRIS/2M HCl	300
Total volume	1050

2.6 ValiData for validation

The Excel-macro “ValiData” was programmed by Prof. Wegscheider and is used for validation of the method. The data of the calibration curve are needed. The lowest and highest concentration are measured in quadruplicate, for all other concentrations in between only duplicates are needed. This program enables the user to determine the linearity, variance homogeneity, limit of detection (LOD) and limit of quantification (LOQ).

Figure 12: ValiData the Excel-macro for validation.

3 Results and discussion

3.1 Gradients

Five different methods were tested for sufficient chromatographic separation of the metformin-benzoin fluorophore. In the beginning of the work the standard solvent composition (Ohta et al., 1993) was used. The emission wavelength is 450 nm and the excitation wavelength is at 315 nm. The flow rate was always 0.5 ml/min, the run time 10 min and the injection volume was 10 µl.

Table 9: Original composition of the solvents (method 1)

Compound	Solvent 1 (%)	Solvent 2 (%)
ACN	60	65
THF	-	10
0.5 M TRIS pH 8	10	10
H_2O	30	15

The linear gradient started with both solvents and switches after 7 min to 100 % solvent 2. This composition did not lead to a satisfying separation of metformin and other compounds. For further chromatographic measurements in the case of solvent 1 the amount of acetonitrile was decreased continuously until the separation was successful.

In method 2, the gradient started with solvent 1 for 2 min and after 7 min it switched to 100 % solvent 2.

Table 10: Method 2; adapted solvent 1 (ACN 30 %)

Compound	Solvent 1 (%)	Solvent 2 (%)
ACN	30	65
THF	-	10
0.5 M TRIS pH 8	5	10
H_2O	65	15

In the next step for method 3, solvent 1 was diluted to 10 % of ACN with water and this was done directly with a new gradient.

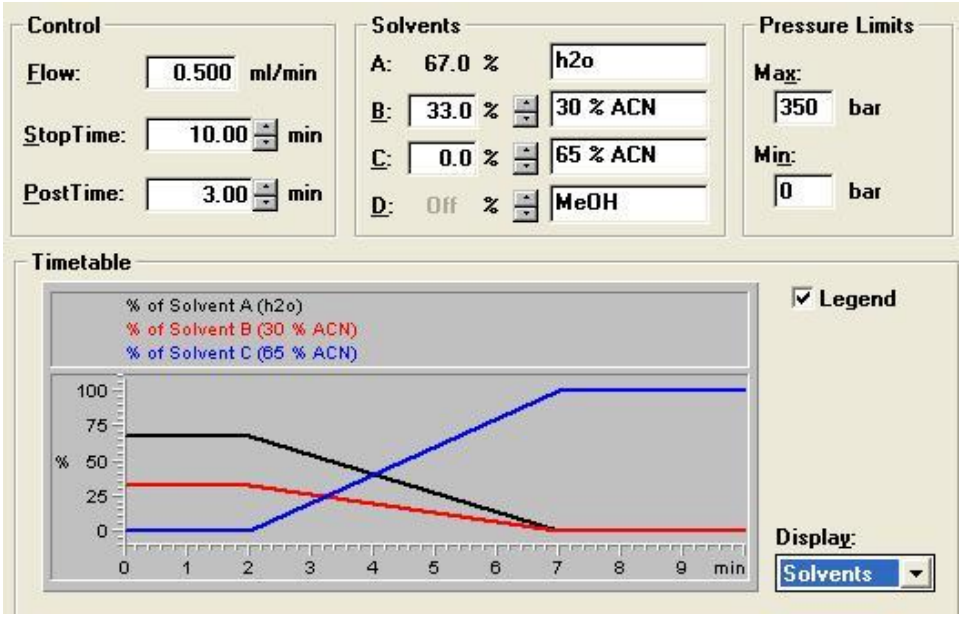

Figure 13: Gradient of method 3. 10 % ACN in solvent 1 was reached and solvent 2 stayed the same. This is a screenshot from Agilent's ChemStation.

Table 11: Composition of adapted solvent 1 for method 3.

Compound	Solvent 1 (%)	Solvent 2 (%)
ACN	10	65
THF	-	10
0.5 M TRIS pH 8	1.6	10
H_2O	88.4	15

The analyte eluted at 2 min in method 3.

Finally, the acetonitrile content of solvent 1 was decreased to only 5 %. The gradient started with solvent 1 for 3 min and after 8 min there was a change to solvent 2. Further dilution of solvent 1 for method 4 showed no improvement.

Table 12: Composition of adapted solvent 1 for method 4.

Compound	Solvent 1 (%)	Solvent 2 (%)
ACN	5	65
THF	-	10
0.5 M TRIS pH 8	0.8	10
H_2O	94.2	15

In method 5 the buffer content was increased to 21 %, because it was assumed that the solvent contained to much water related to the original solvent.

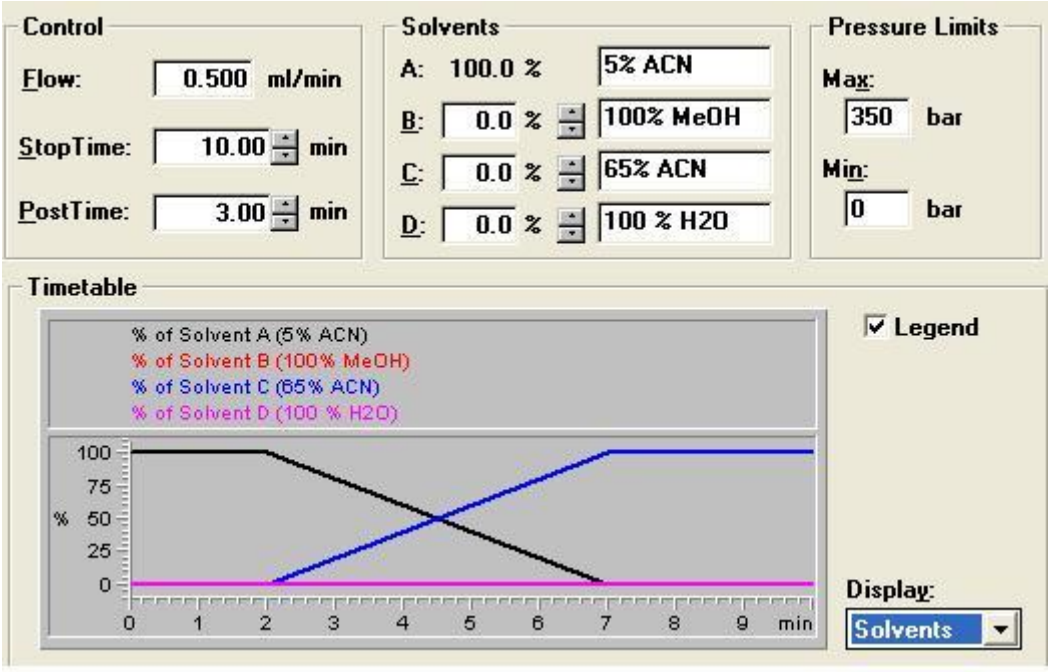

Figure 14: The gradient for method 5 was started with solvent 1 for 2 min and changes after 7 min to solvent 2. This is a screenshot from Agilent's ChemStation.

This method led to the successful separation of metformin from other compounds. Therefore, it was used for all further experiments.

Table 13: Final composition of the solvents from method 5.

Compound	Solvent 1 (%)	Solvent 2 (%)
ACN	5	65
THF	-	10
0.5 M TRIS pH 8	21	10
dd H_2O	74	15

Method 5 was chosen because it resulted in a good separation of the fluorophore from other compounds. The function of the TRIS buffer, pH 8 was to keep the fluorophore at a mostly uncharged state and make it nonpolar. The first solvent is more polar than the second.

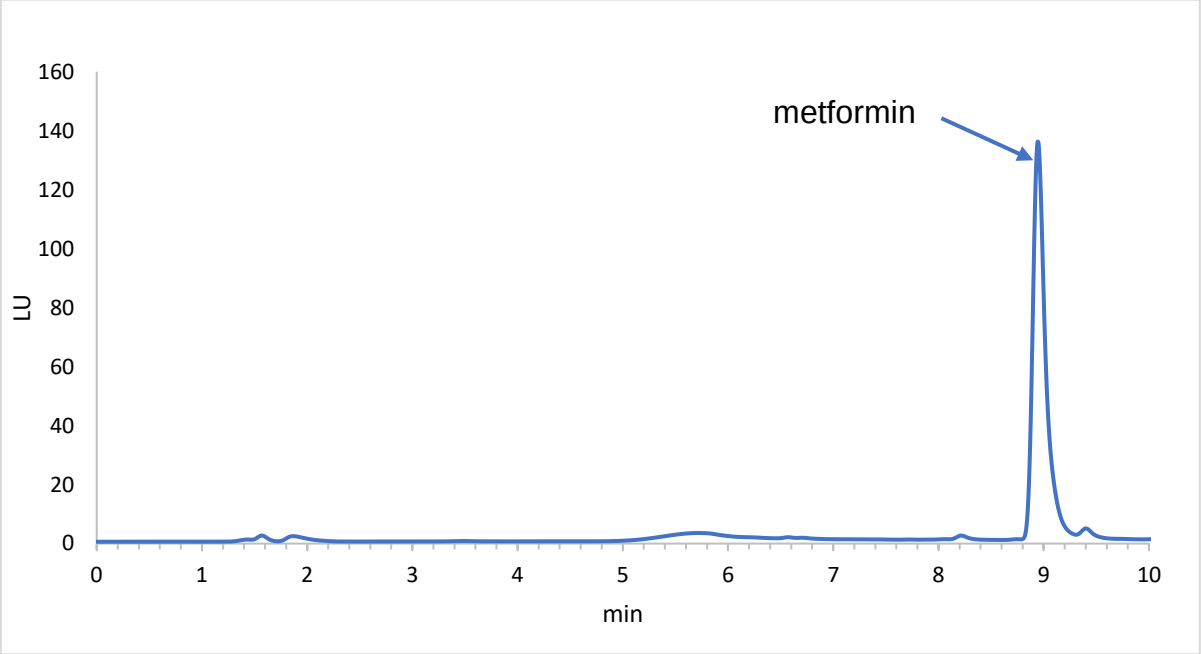

Figure 15: Method 5. 0.5 mg/ml metformin derivatized with desyl bromide in water.

As the gradient became increasingly nonpolar the fluorophore was migrating through the column and got eluted after 8.9 min.

In the original set up a TSK-gel® ODS-120T C18 (150 x 4.6 mm, 5 µm) was used (Ohta et al., 1993). The Phenomenex® Kinetex® C18 (150 x 3 mm, 5 µm) was the column of the provided LC system.

The column had a smaller inner diameter and the volume of the column can be reduced this way. This saved solvent and the peaks get smaller and higher in comparison to a bigger inner diameter. The original composition of the solvent leaded not to a separation. The organic content of solvent 1 was decreased from 60 % to only 5 %

ACN. The buffer content was increased from 10 % to 21 %. These changes made the chromatographic separation possible.

3.2 Fluorescence spectra of the reaction product with benzoin

A particular range of wavelengths was observed for determining the excitation and emission maxima. The HPLC method is the same as for all other measurements. For the excitation spectra, the wavelengths from 220 nm to 425 nm were measured. The wavelengths for the emission ranged from 340 nm to 550 nm.

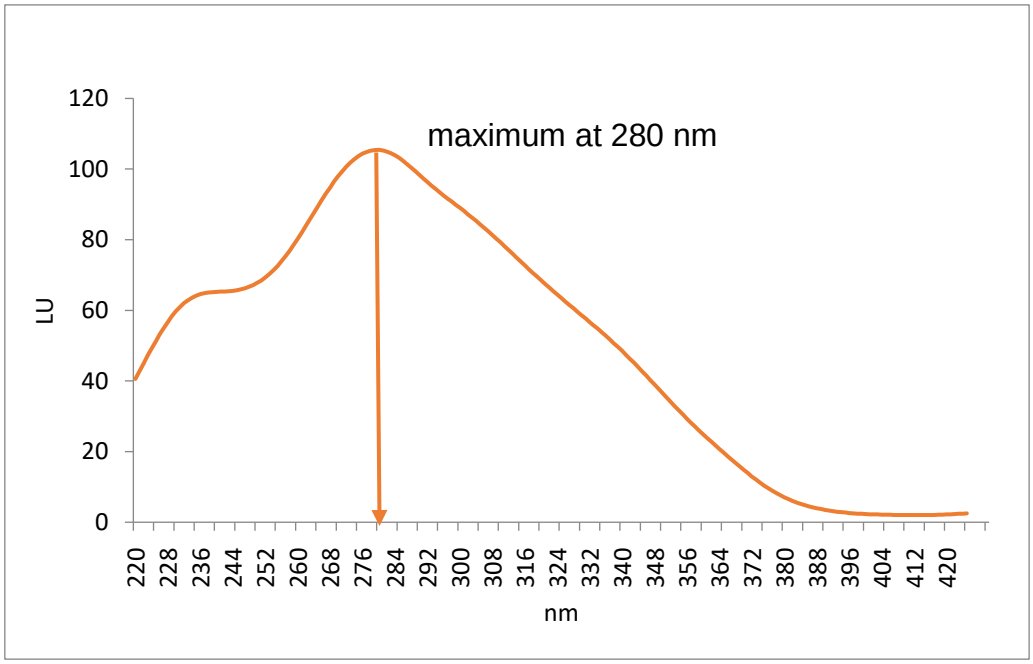

Figure 16: Excitation maximum of benzoin.

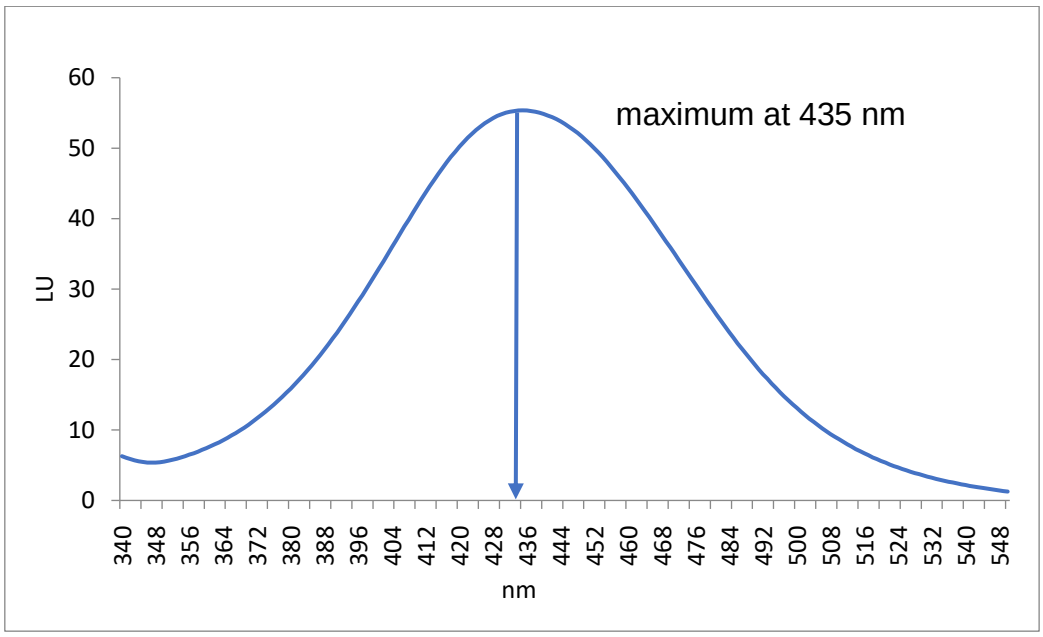

Figure 17: Emission spectrum of benzoin.

The determined maxima were used in all other measurements.

3.3 Fluorescence spectra of the reaction product with desyl bromide

The same procedure as for benzoin was applied to desyl bromide.

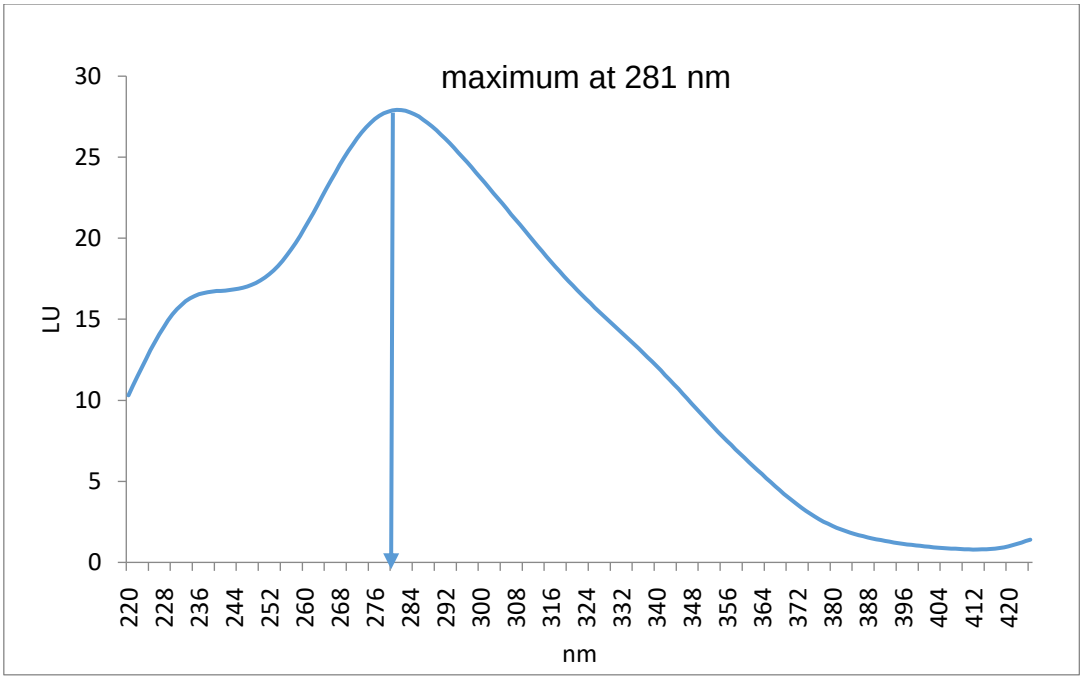

Figure 18: Excitation spectrum of desyl bromide.

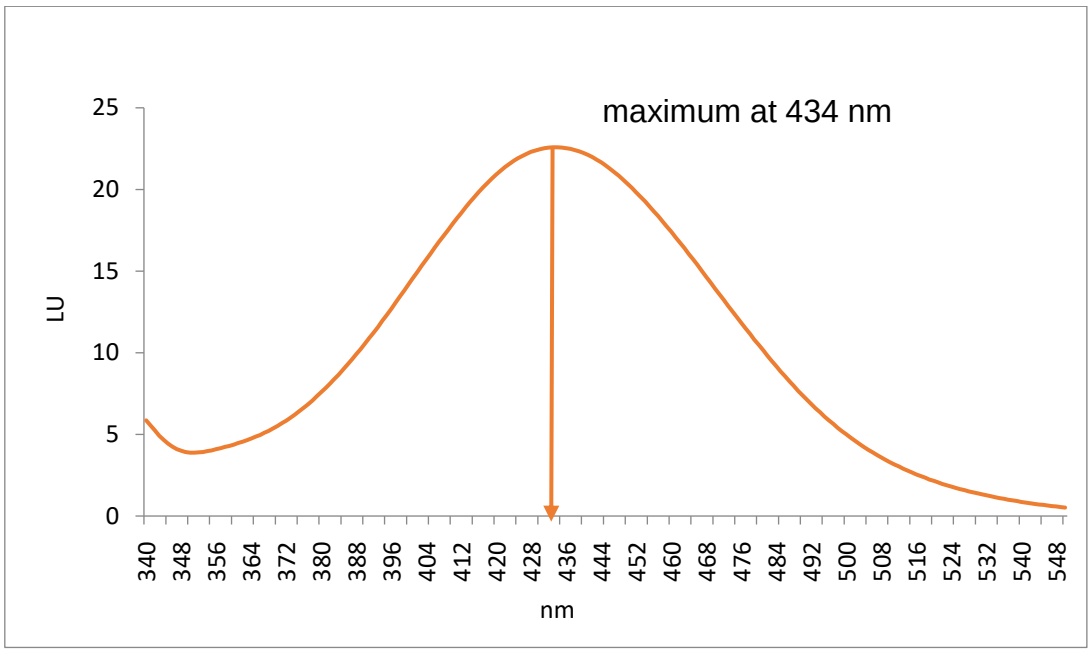

Figure 19: Emission spectrum for desyl bromide.

There is only 1 nm difference for the excitation and emission wavelength compared to benzoin. The found maxima were used for further measurements with desyl bromide.

3.4 Kinetics of benzoin

For determining the most suitable reaction time, the reaction time was continuously increased from 2 min to 6 min. The reactions were made in triplicates, the concentration was always 0.8 mg/ml metformin in urine matrix.

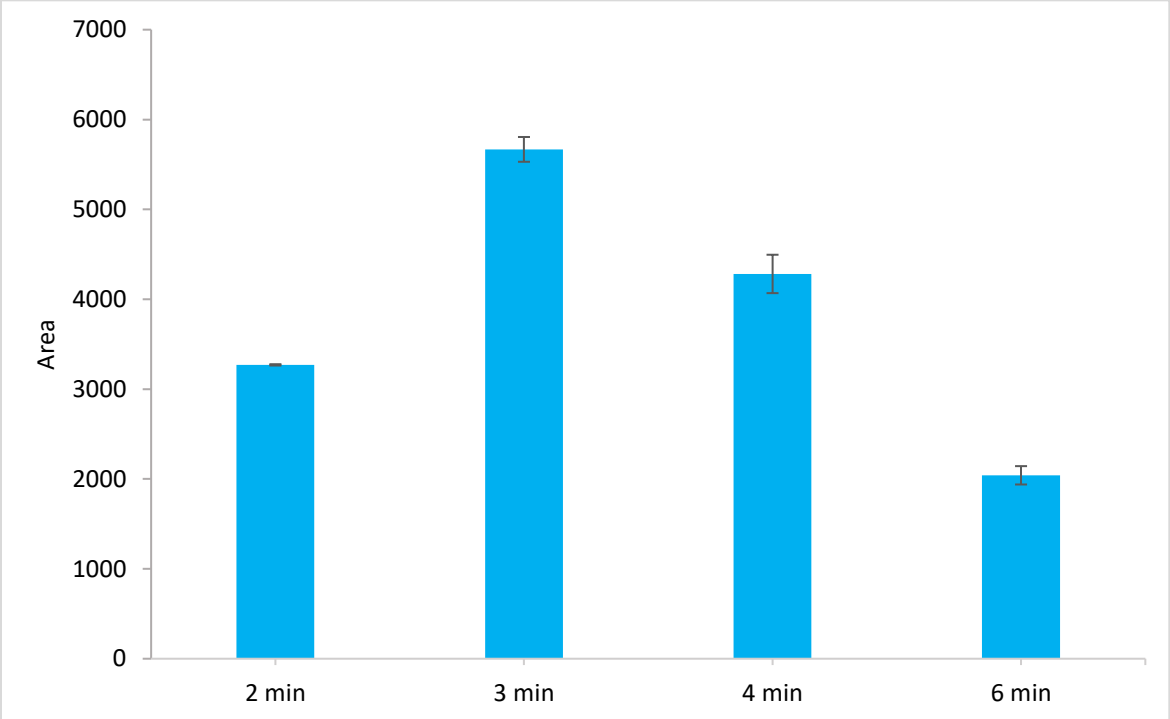

Figure 20: Results of the kinetic measurement.

Therefore, the standard reaction time was chosen to be 3 min at 99 °C. A similar kinetic behaviour was observed by Ohta et al., 1993. 3 min reaction time has proven to be optimal condition.

3.5 Derivatization comparison of benzoin and desyl bromide

Different concentrations of metformin were measured and compared to each other. The gained information was used to determine which reagent is more appropriate for further experiments.

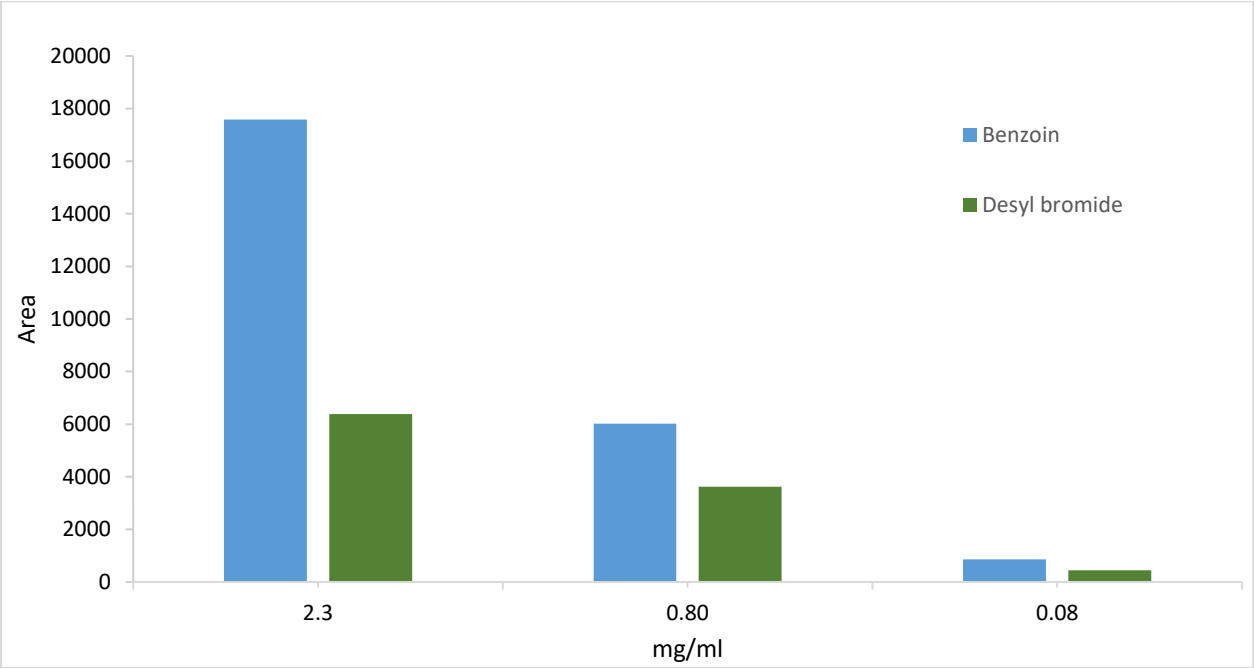

Figure 21: Mean of the areas for both reagents.

The same chromatographic method as for derivatization of benzoin with metformin was used for analysis of desyl bromide derivatized with metformin.

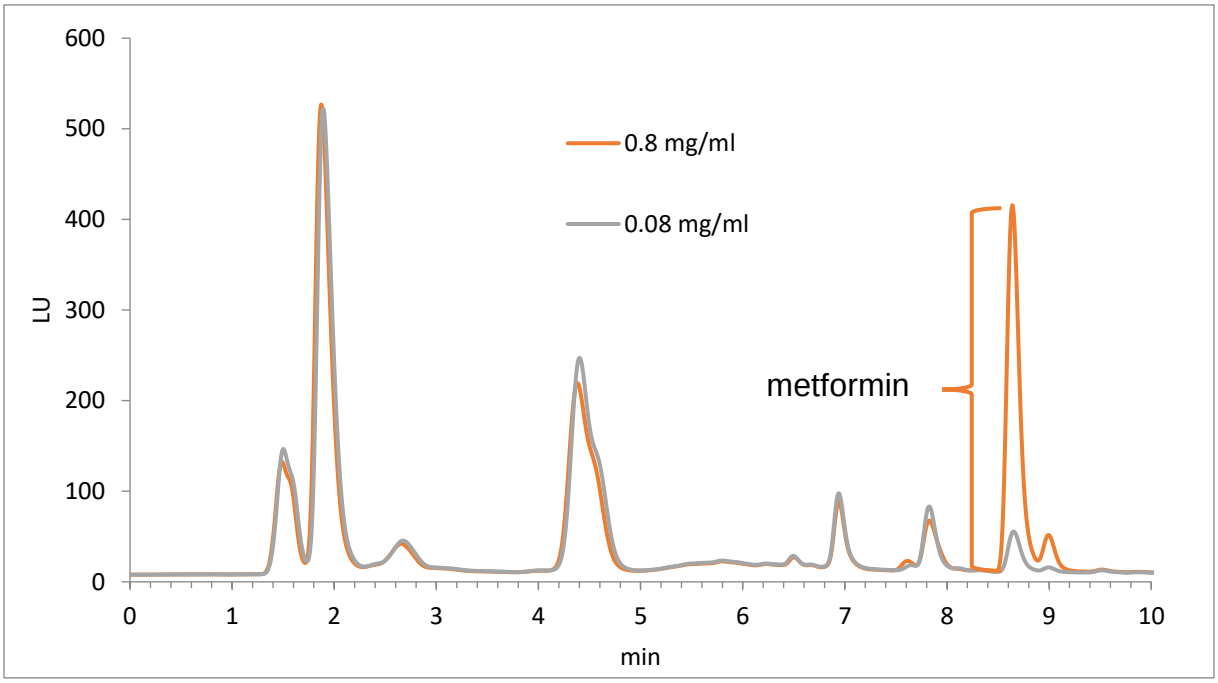

Figure 22: Chromatograms of metformin derivatized with desyl bromide.

The chromatograms looked like that for benzoin, but compared to benzoin the sensitivity is lower. The areas for desyl bromide were lower as for benzoin. The desyl bromide peak area for 2.3 mg/ml had to be higher.

For this reason, desyl bromide was not chosen for further analysis of samples. The retention time was almost the same as for benzoin.

As described by Ohta et al., 1993, desyl chloride had as similar sensitivity like benzoin. Desyl bromide, which has the same structure, but instead of a chloride a bromide is present. The reaction with metformin was not as good as that for desyl chloride.

3.6 Derivatization of o-tolylbiguanide with benzoin

3.6.1 Fluorescence spectra of o-tolylbiguanide

Also for o-tolylbiguanide the excitation and emission spectra were determined. For excitation wavelengths from 220 nm to 400 nm were measured. The measurement included a range of wavelengths from 340 nm to 550 nm for emission.

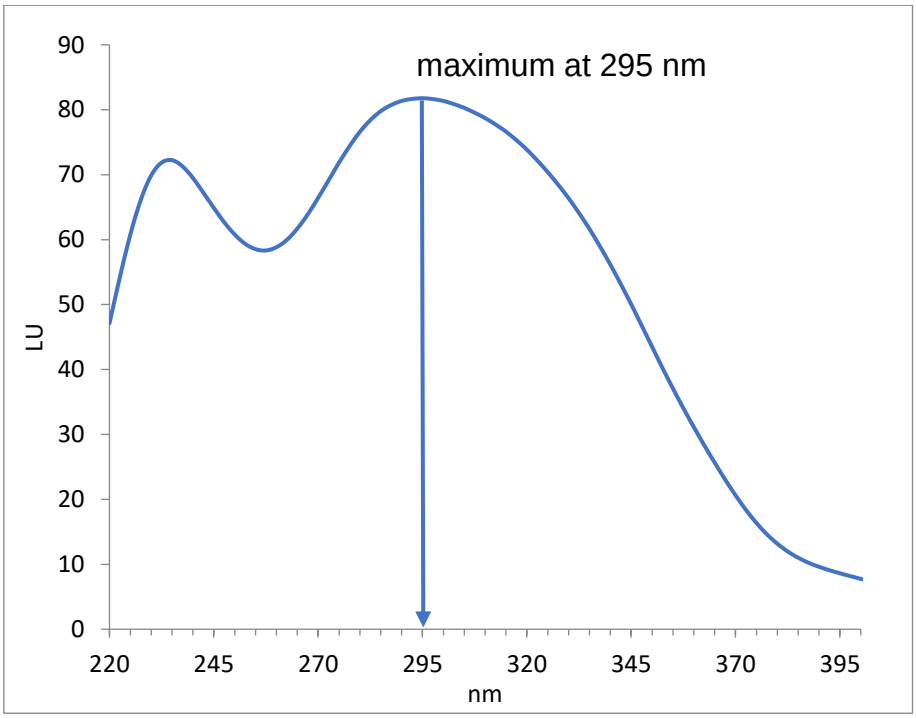

Figure 23: Excitation spectrum of o-tolylbiguanide.

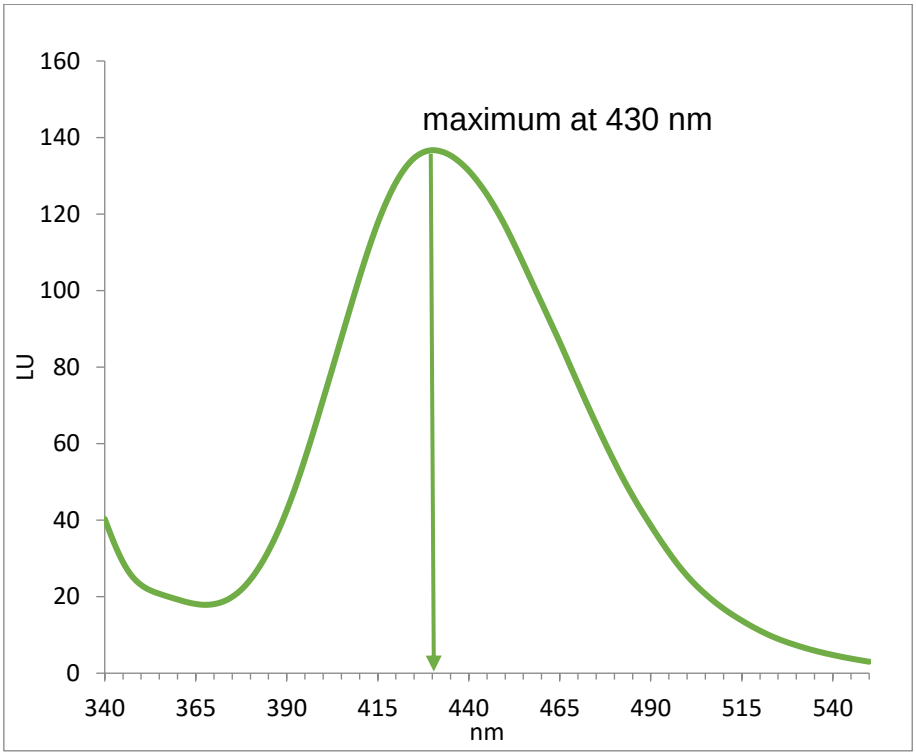

Figure 24: Emission spectrum of o-tolylbiguanide.

The identified maxima for excitation and emission were used in further measurements.

3.6.2 Different metformin concentrations spiked with o-tolylbiguanide

The first step was to identify the retention time for o-tolylbiguanide. Then different metformin concentrations were spiked with 1 mg/ml of o-tolylbiguanide. The aim was to find out if the compound was suitable as an internal standard.

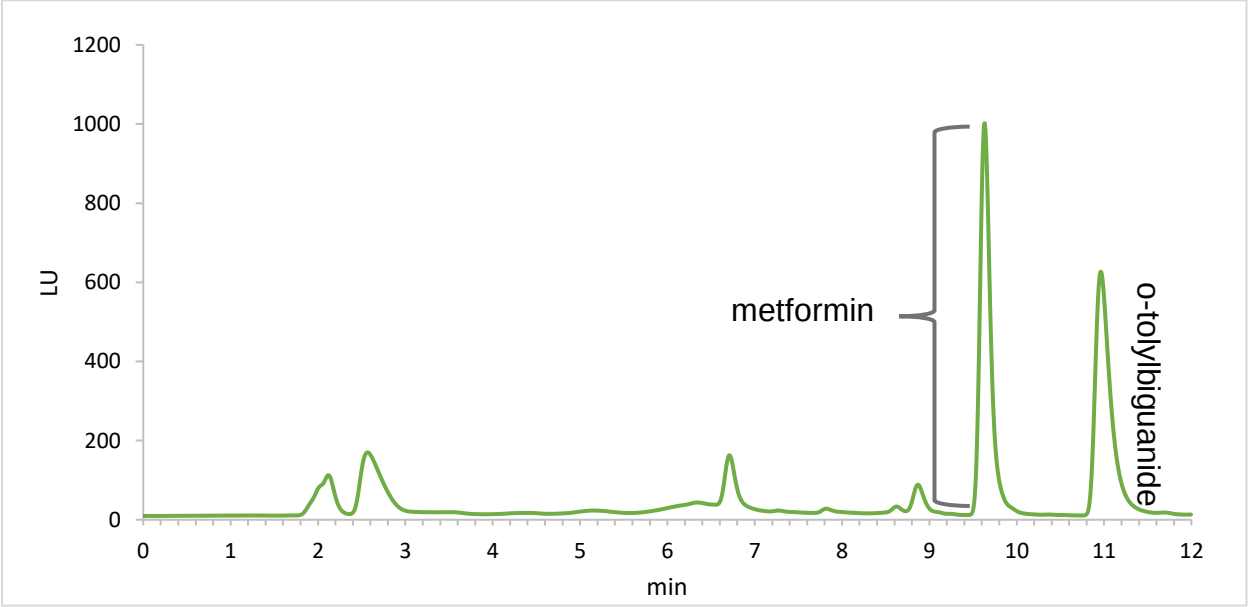

Figure 25: 2 mg/ml metformin and 1 mg/ml o-tolylbiguanide both were derivatized with benzoin.

The retention time of metformin was longer than normally observed. The difference was 50 sec. This could have been caused by an insufficient washing of the column.

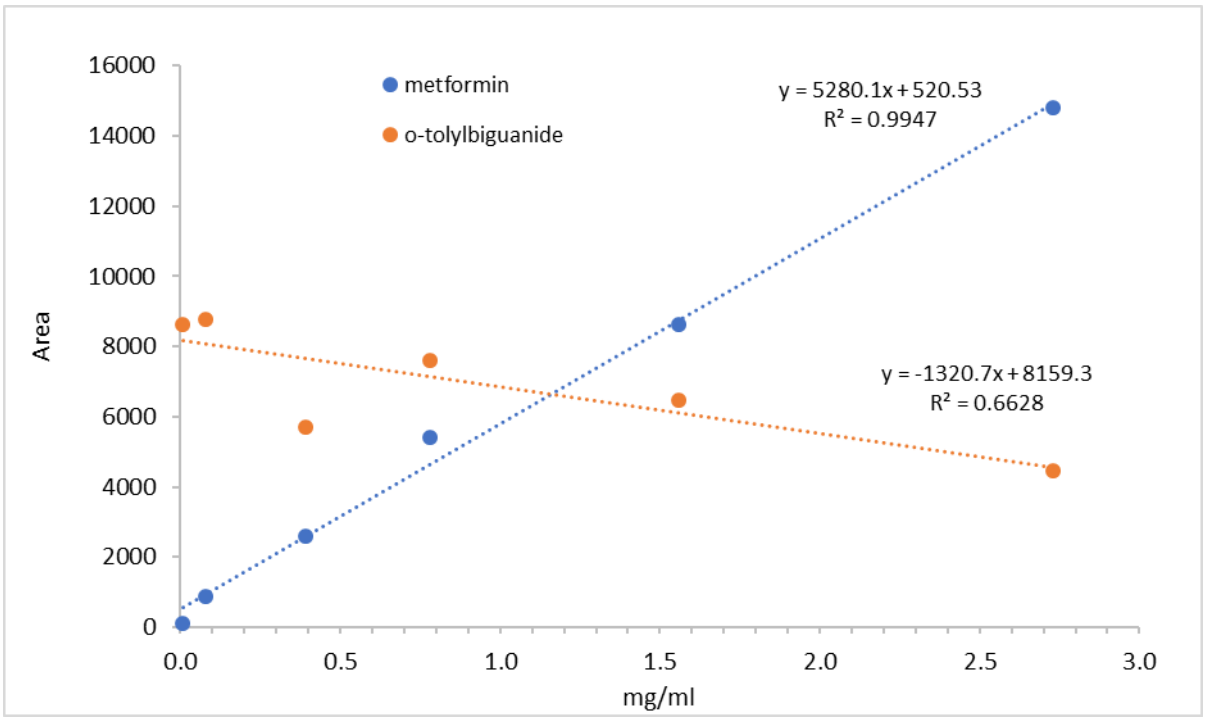

Figure 26: Response of the internal standard (o-tolylbiguanide) in relation to metformin at increasing metformin concentrations.

The higher the concentration of metformin was, the lower was the area of o-toylbiguanide and this relation was also inversely valid.

It was observed that both compounds compete with the reagents in the reaction tube. In further experiments the volume of benzoin, sodium thiosulfate with β-mercaptoethanol was increased. For composition see chapter 2.4.1.

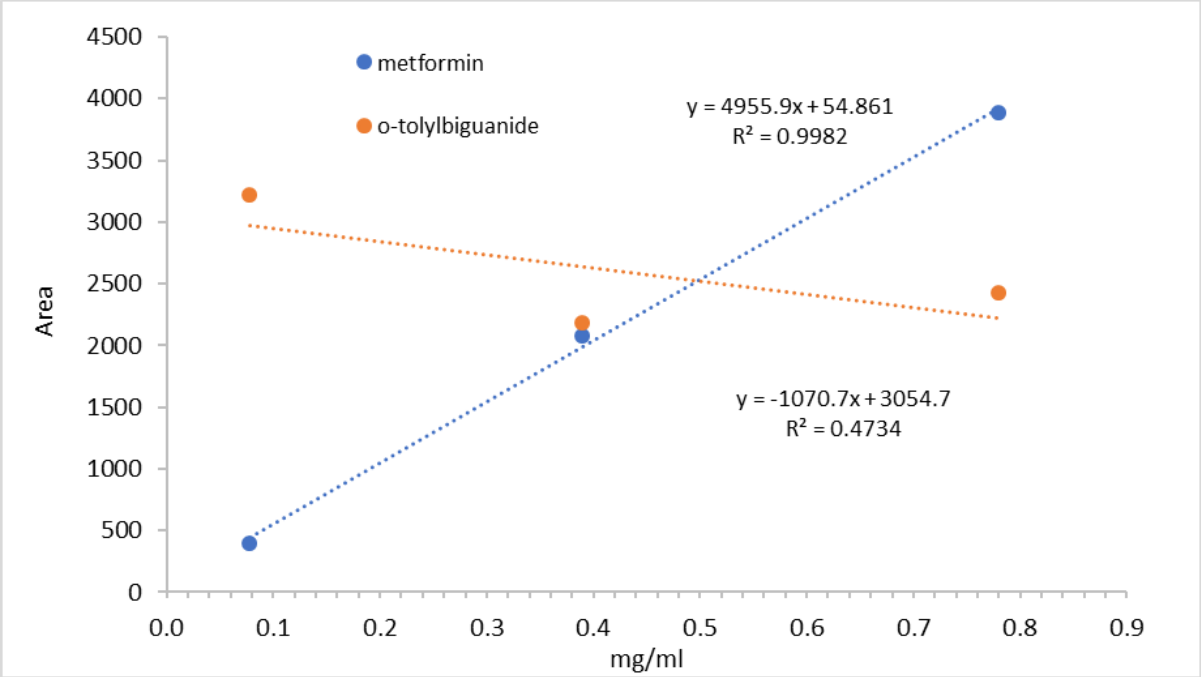

Figure 27: The effect of the competing reaction of metformin and o-tolylbiguanide.

Again at 0.4 mg/ml the area for o-tolylbiguanide was observed to be the lowest among these three concentrations. The standard deviation for the area has been reduced.

Table 14: Comparison of o-tolylbiguanide areas.

$C_{metformin}$ (mg/ml)	Area (standard conditions)	Area (more reagent volume)
0.8	7614	2423
0.4	5699	2179
0.08	8779	3226
SD	1555	548

3.6.3 Kinetics of o-tolylbiguanide

The reaction time was raised from 2 min until 8 min. The volume of reagents was also doubled. For composition see chapter 2.4.2.

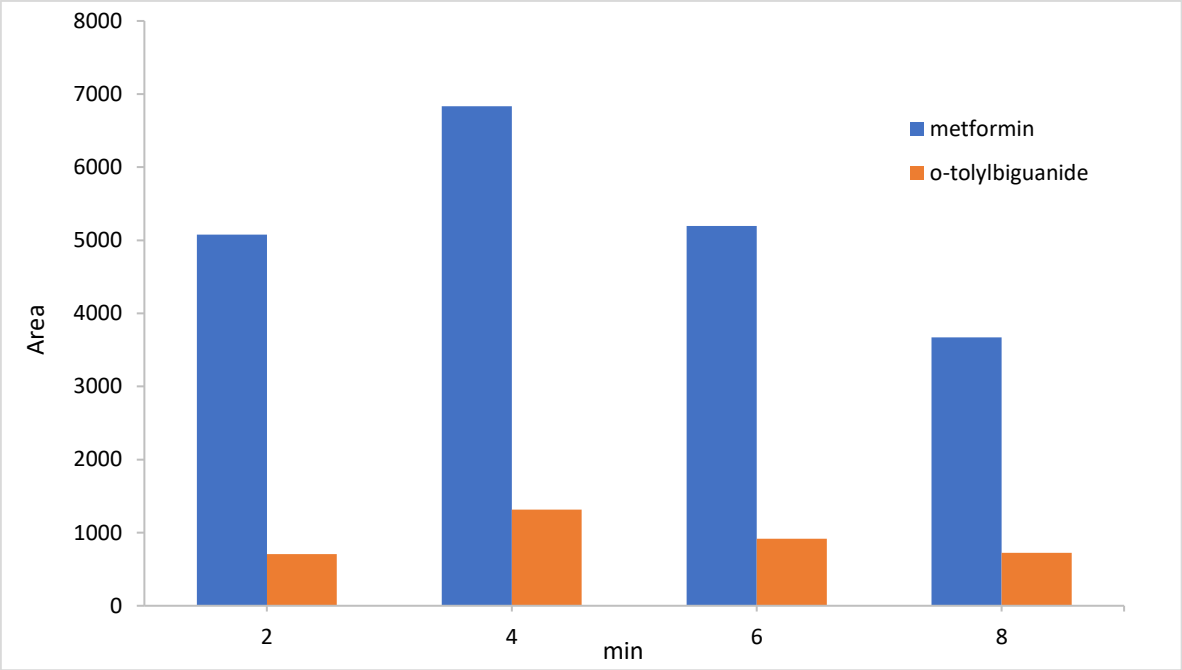

Figure 28: Kinetics of metformin and o-tolylbiguanide. The measurement was made in urine matrix.

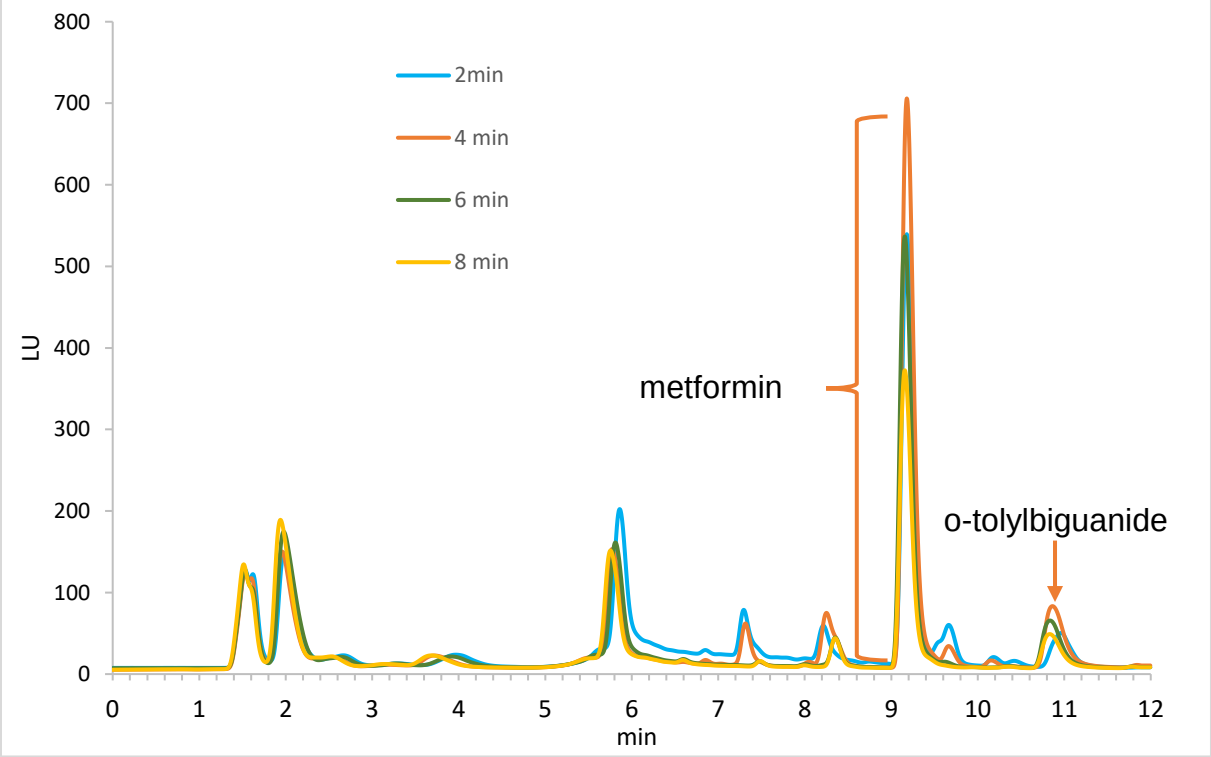

Figure 29: Chromatograms of kinetics of metformin and o-tolylbiguanide.

Although the derivatization of o-tolylbiguanide was successful and the kinetic behaviour was found to be the same, it could not be used as an internal standard for the method. The peak areas of o-tolylbiguanide varied too much so that a relation of the metformin area to the area o-tolylbiguanide is nonsense. Also, the addition of more reagent volume could not change this. The peak of the internal standard should always have the same area and peak height.

3.7 Sample preparation

3.7.1 Solid Phase Extraction (SPE)

For some samples SPE was used as a pre-treatment. All purifications were executed according the manual described in chapter 2.3.1.

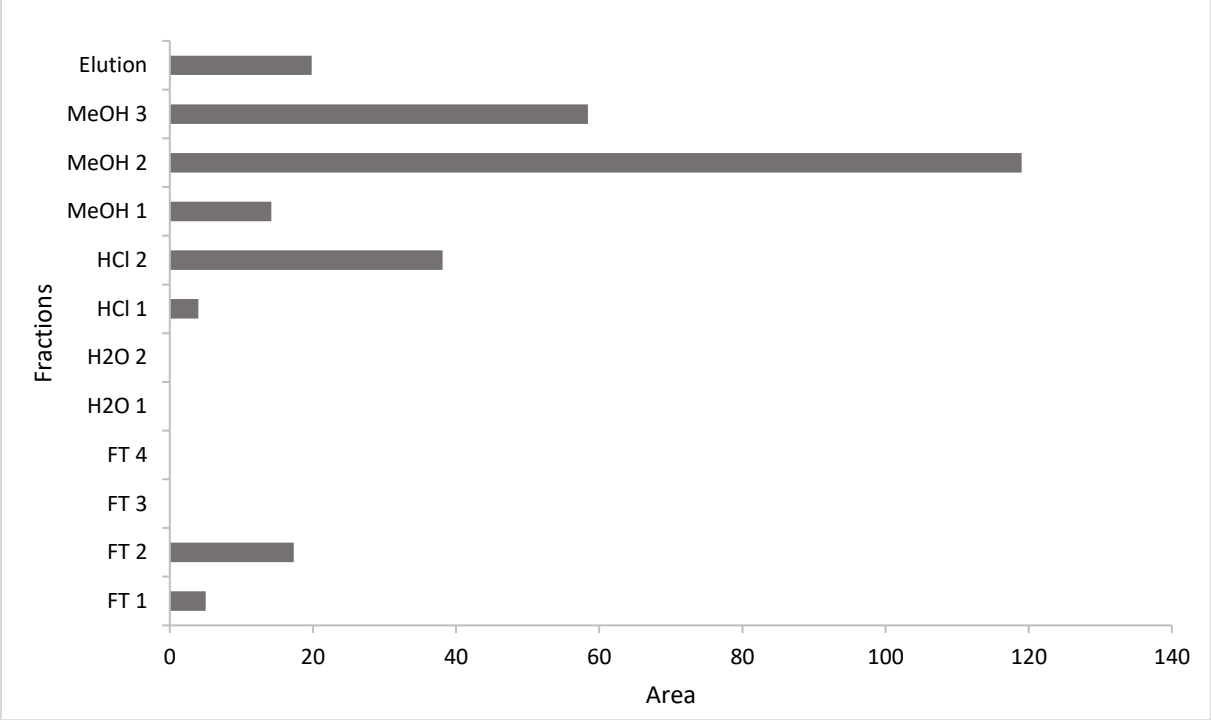

Figure 30: SPE 1. In this case 27.6 µl/ml metformin (in water) were loaded on the column and derivatized with desyl bromide.

The area of the elution fraction was 20. Without SPE the area of the same concentration was 364.

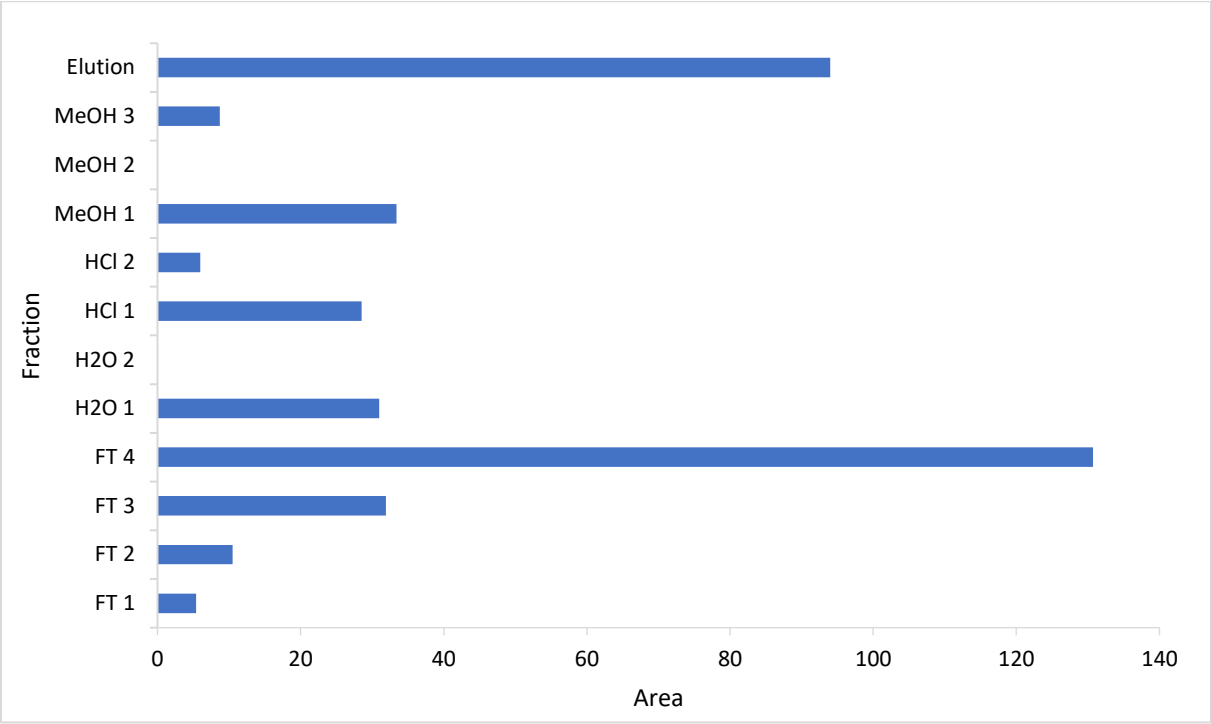

Figure 31: SPE 2. Again 27.6 µl/ml of metformin (in water) were loaded on the column and derivatized with desyl bromide.

Not even one third of the analyte was found in the elution fraction of SPE 2. The results are summarized in the following table:

Table 15: Results of SPE for metformin-desyl bromide.

	Without SPE	**SPE 1**	**SPE 2**
Area	364	20	94
Recovery (%)	100	5.5	25.8

The recoveries were very low this means that SPE in this case was not an option for sample preparation.

In the third SPE purification metformin in urine matrix were loaded on the column.

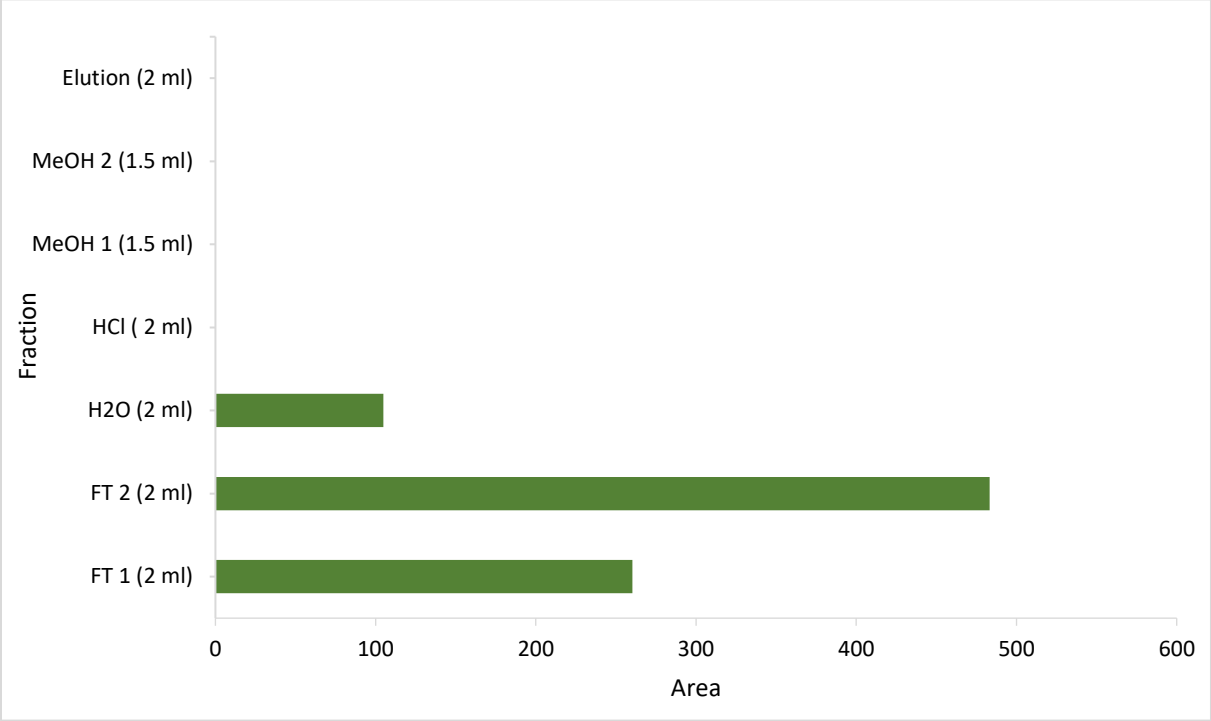

Figure 32: SPE 3. 78 µg/ml of metformin in urine matrix were loaded on the column. The derivatization was done with benzoin.

The last SPE was done with the same metformin concentration, but with water instead of urine.

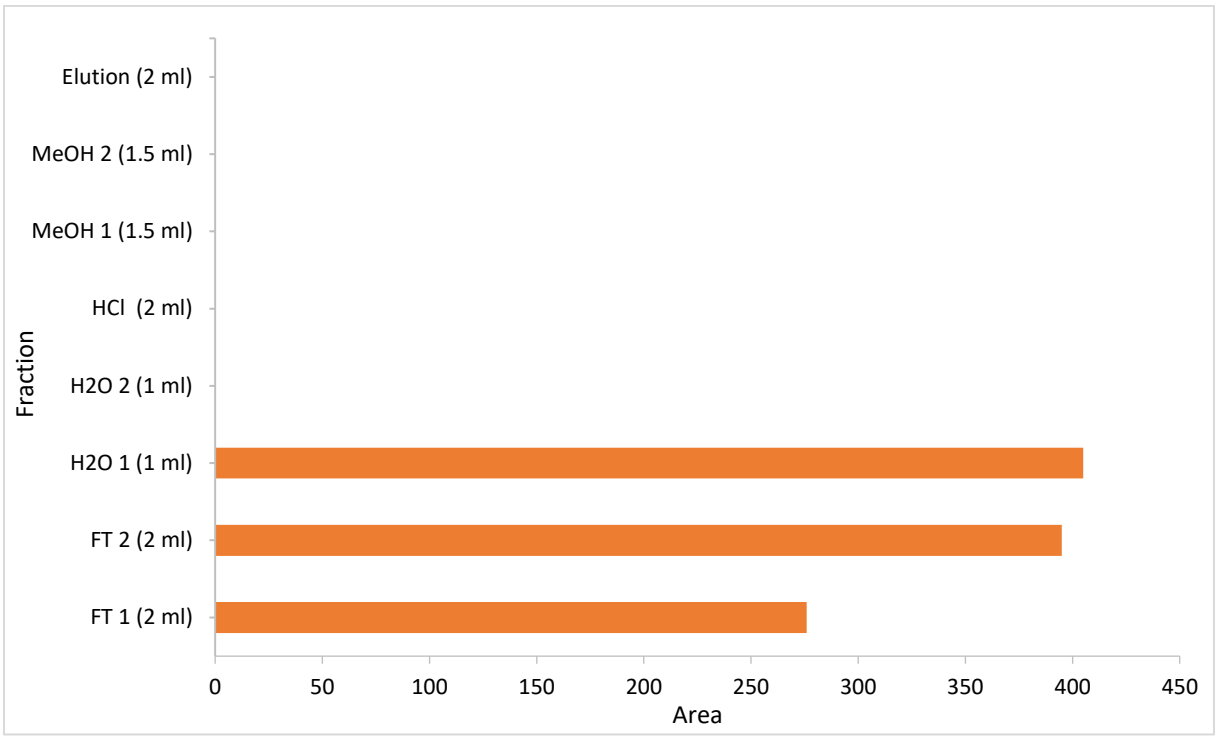

Figure 33: SPE 4. 78 µg/ml of metformin in water were loaded on the column. The derivatization was done with benzoin.

In SPE 3 and 4 nothing of the drug could be eluted, because everything got lost in the sample loading. A very small amount of metformin eluted in SPE 1 and 2. In this case no urine matrix was present.

For this reason, another sample preparation method must have been found. A simple protein precipitation with ethanol was chosen as new sample preparation method. There could be several reasons for failure. The column consisted of 130 mg of sorbent, which must be enough material to absorb the drug.

Before measuring urine samples, the pH was determined to make sure it was not above 6. At pH 6 metformin should be charged slightly positive and the SCX material negatively. Most of the drug got lost during sample loading for any reason, the interaction of the analyte with the sorbent might not have been strong enough.

For calibration, sample loading no pressure was applied on the column. In the washing steps, a pressure of 5 inches Hg was applied to achieve a flow rate of 1-2 ml/min. It was taken care, that the sorbent never runs out of fluid.

3.8 Validation of the HPLC-method

3.8.1 Determination of the recovery

For verification of the sample preparation method a standard addition experiment was done. The first step was to spike a sample (1_T0) with standard metformin solution with increasing concentration of analyte. The second step was a standard calibration.

After plotting these graphs, the given slopes of both are needed to determine the recovery.

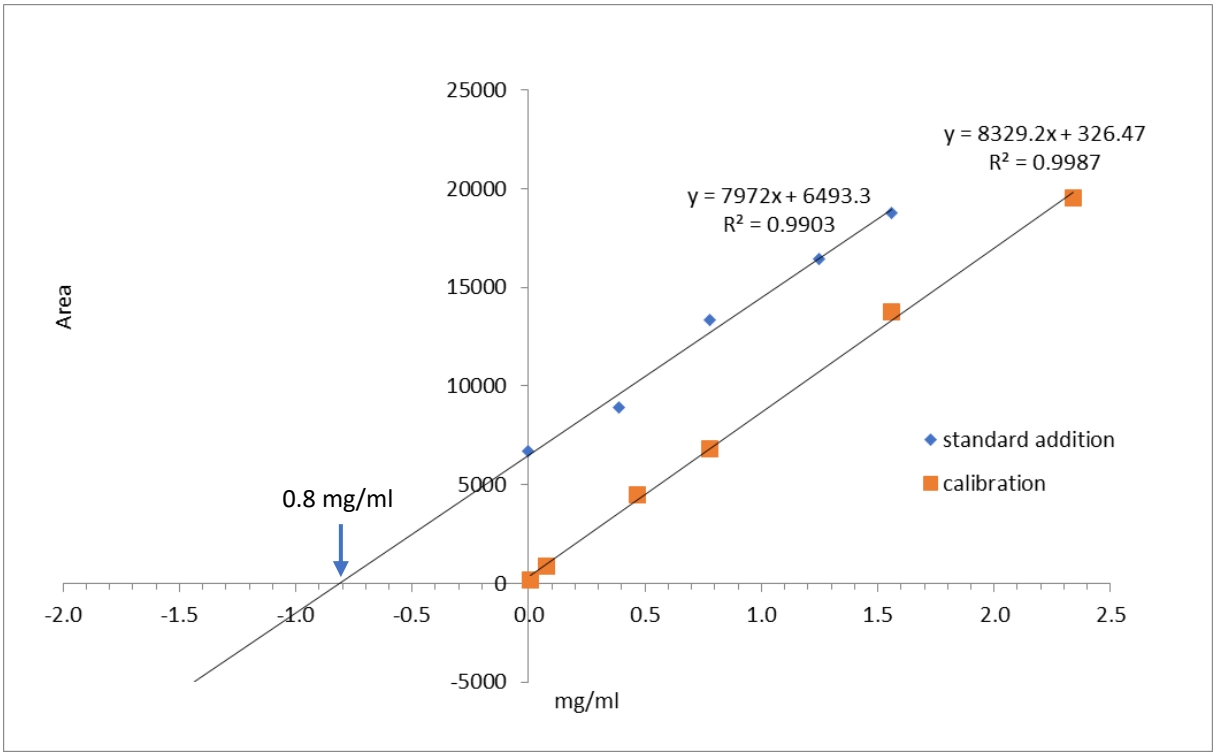

Figure 34: Calibration and standard addition.

As they were parallel to each other, the given slopes were used for calculation. An elongation of the standard addition graph gives the concentration of the sample.

$$Recovery\ (\%) = \frac{k_{STA}}{k_{CAL}} * 100$$

Equation 2: Calculation of the recovery in percent

k_{STA} ..... slope standard addition

k_{CAL} ..... slope calibration

The calculated recovery is 96 %. According to literature value between 80 % and 120 % are acceptable. Sample 1_T0 had a concentration of 0.8 mg/ml, this result was confirmed in further measurements.

3.8.2 Linearity

According to ValiData the linearity could be confirmed. The data can be found in the appendix.

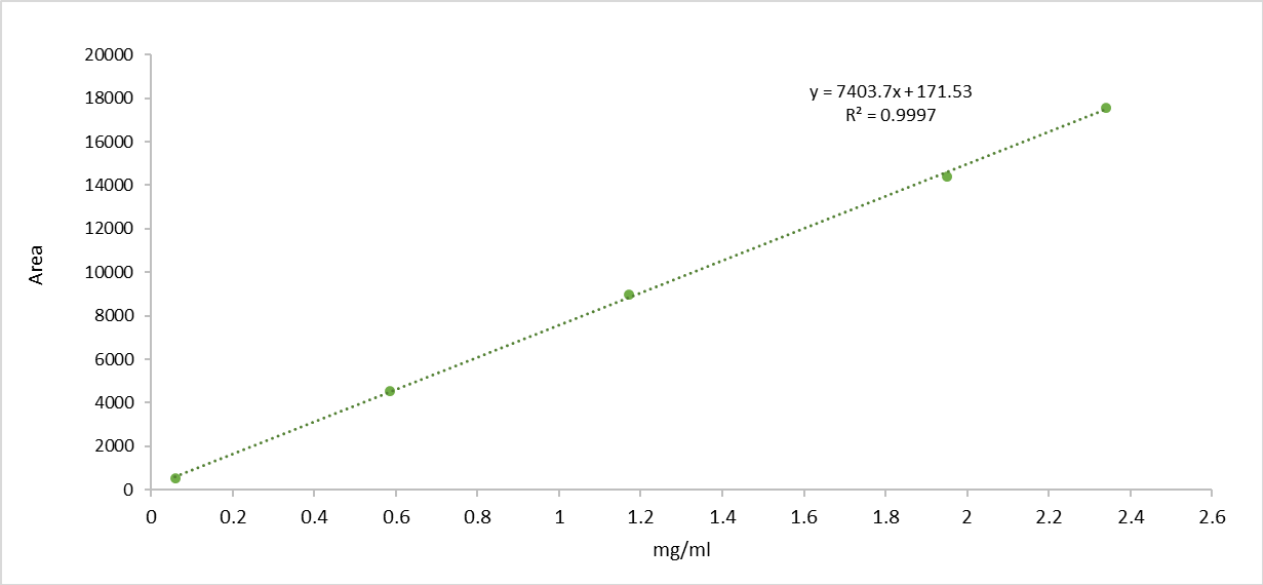

Figure 35: Calibration curve for metformin in urine samples.

The concentration of metformin was calculated with the following equation:

$$x = \frac{(y - d)}{k} * Recovery\ (\%)$$

Equation 3: Calculation of metformin concentration.

x .....concentration (mg/ml)

y .....area

d .....intercept

k .....slope

3.8.3 Precision

Before measuring of the real sample always a control sample in urine matrix has been injected. All controls were measured in triplicate and had a concentration of 0.4 mg/ml metformin.

The SD for the method is 0,018 mg/ml (from ValiData). The upper and lower limit for the controls were calculated with the linear equation from figure 35 and inserting the SD of the method.

$$Area\ (SD\ of\ method) = 7403{,}7 * \left(c_{SD\ of\ method}\right) + 171.53 = 302$$

Equation 4: Calculation of the area for SD of method

$$3\sigma = 3 * \left(c_{SD\ of\ method}\right) = 0{,}053\ mg/ml$$

Equation 5: three times the SD of method for defining the upper and lower limit.

The calculated concentration for three times the SD has been inserted again into the linear equation. This gave an area of 562. Insertion of 0.4 mg/ml into the linear equation leaded to an area of 3059. The upper limit was determined to be 3449 and the lower limit 2668.

Table 16: Calculated values for upper and lower limit according to SD of method

	mg/ml	**Area (calculated)**
SD of method (σ)	0.018	302
3 σ	0.053	562
Lower limit (- 3 σ)	0.337	2668
Upper limit (+ 3 σ)	0.443	3449

Table 17: Results of controls.

Date	Area (mean)	SD	RSD %
31.01.2017	2955	56.4	1.91
09.03.2017	3063	11.2	0.37
16.03.2017	3067	19.5	0.64
03.04.2017	2923	141.6	4.84
04.04.2017	3120	22.6	0.72
09.05.2017	3107	27.4	0.88
SD	**81.4**	All dates	
Mean area	**3039**		
RSD %	**0.03**		

The average area of the controls is found to be close at the calculated area for this concentration. The RSD was calculated to be under one percent for all dates.

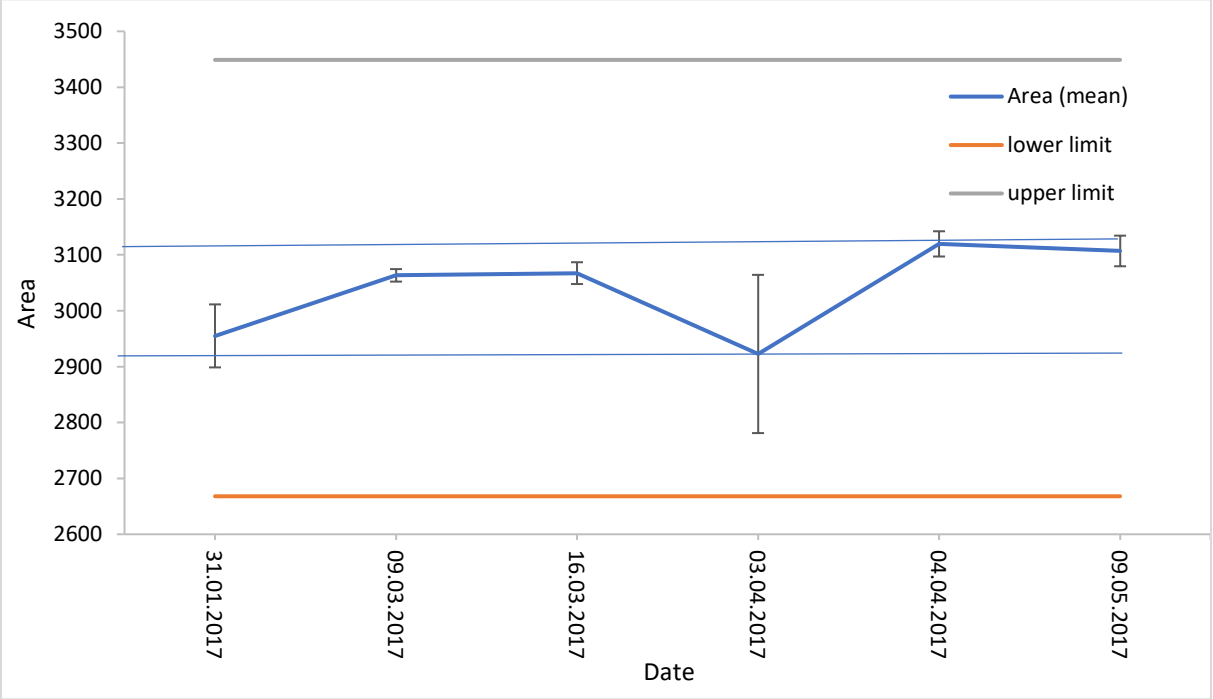

Figure 36: Control chart.

All controls are located in the middle of the range, that indicates for a good precision of the method. The data of all controls is listed in the appendix (6.7.2, table 45).

The lowest concentration of the samples was determined to be 0.1 mg/ml. The samples can be quantified, because the LOQ has been calculated to be 0.1 mg/ml and the limit of decision is 0.06 mg/ml. Results of validation are summarized in table 44.

The variance of the higher concentrations leads to an impairment of the limit of decision. In consequence, the limit of decision is found within the working range.

The used pipettes were calibrated to exclude pipetting errors as reason for the variance. As expected the pipettes were working very well, for results see chapter 6.7.2, table 46.

3.8.4 Robustness

Several parameters like flow rate, pH, temperature and amount of benzoin were changed to characterize the influence on the peak.

3.8.4.1 Change of the flow rate

The flow rate influences the retention time.

Table 18: Flow rate 0.4 ml/min. 0.7 mg/ml metformin in urine matrix.

	Area	**Height**	**RT (min)**
	5386	546	9.65
	5503	558	9.66
	5462	549	9.66
Mean	5450	550	9.66
SD	59.3	6.5	0.01
RSD in %	1.1	1.2	0.06

The retention time for metformin has elongated to 9.66 min.

Table 19: Flow rate 0.6 ml/min. 0.7 mg/ml metformin in urine matrix.

	Area	Height	RT (min)
	5433	711	8.26
	5502	726	8.25
	5541	729	8.24
Mean	5492	722	8.25
SD	55	9.6	0.01
RSD in %	1.0	1.3	0.15

The higher flow rate has shortened the retention time to 8.25 min. Compared to the flow rate of 0.4 ml/min the peak height became 200 units higher. The higher flow rate had an influence on the peak height.

3.8.4.2 Change of pH

The pH of the buffer was changed to pH 7 and pH 9. The sample was measured in water and urine matrix.

Table 20: 0.5 M TRIS pH 7. 0.8 mg/ml metformin in water.

	Area	Height	RT (min)
	4900	567	8.79
	4645	595	8.84
	4532	501	8.76
Mean	4692	554	8.80
SD	188.8	48.6	0.04
RSD in %	4.0	8.8	0.5

Table 21: 0.5 M TRIS pH 7. 0.8 mg/ml metformin in urine.

	Area	Height	RT (min)
	6020	628	8.70
	6011	626	8.69
	5426	547	8.50
Mean	5819	601	8.63
SD	340	46	0.12
RSD in %	5.8	7.7	1.4

Table 22: 0.5 M TRIS pH 9. 0.8 mg/ml metformin in urine.

	Area	Height	RT (min)
	4686	600	8.84
	4691	601	8.83
	4665	599	8.83
Mean	4681	600	8.83
SD	13.8	1.0	0.01
RSD in %	0.3	0.2	0.07

Table 23: 0.5 M TRIS pH 9. 0.8 mg/ml metformin in water.

	Area	Height	RT (min)
	5257	655	8.85
	5275	656	8.84
	5494	696	8.84
Mean	5342	669	8.84
SD	132	23	0.01
RSD in %	2.5	3.5	0.07

The areas were too low, but the retention time remained the same for both urine matrix and water. Also, the height and areas of both stayed mostly the same. At 0.5 M TRIS pH 7 in urine matrix the retention time was shorter than in other measurements. All experiments were carried out at the same day except that for 0.5 M TRIS pH 7 in urine matrix.

The pH of the buffer has only small influence on the retention time. The lower areas might be caused by pipetting error or the reagent was not freshly prepared (β-mercaptoethanol concentration might be decreased, because of oxidation).

3.8.4.3 Influence of the reaction temperature

Two different temperatures were examined for influence on the peak.

Table 24: Temperature 95 °C.

	Area	Height	RT (min)
	4940	549	8.88
	4874	550	8.81
	4817	544	8.81
Mean	4877	548	8.83
SD	61.5	3.2	0.04
RSD in %	1.3	0.6	0.5

Table 25: Temperature 98 ° C

	Area	Height	RT (min)
	5120	591	8.85
	5299	609	8.84
	5313	605	8.85
Mean	5244	602	8.85
SD	107.6	9.6	0.01
RSD in %	2.1	1.6	0.07

As expected the lower the temperature was set the lower are the peak areas and heights.

3.8.4.4 Changing the amount of benzoin and sodium thiosulfate

The amount of benzoin and sodium thiosulfate was reduced and once increased.

Table 26: 70 µl of benzoin and sodium thiosulfate/β-mercaptoethanol were added.

	Area	Height	RT (min)
	4648	540	8.82
	4655	543	8.81
	4526	525	8.81
Mean	4610	536	8.81
SD	72.5	9.6	0.01
RSD in %	1.6	1.8	0.07

Table 27: 80 µl of benzoin and sodium thiosulfate/β-mercaptoethanol were added.

	Area	Height	RT (min)
	5582	630	8.81
	5515	623	8.82
	5645	641	8.81
Mean	5581	631	8.81
SD	65.0	9.1	0.01
RSD in %	1.2	1.4	0.07

According the standard procedure 75 µl of each reagent were added to the reaction mixture. Reducing the volume by only 5 µl led to a smaller peak area, whereas increasing the volume by 5 µl had not caused this much difference in the peak area.

3.8.5 Stability of derivatization product

The samples were measured and then stored for 5 days in the fridge at 5 °C or in the freezer at -20 °C. The tables 29 and 30 show the resulting areas for the original and stored samples.

Table 28: Storage of sample 12.

12_T0		
Original	**Freezer**	**Fridge**
2539	2338	2296
Loss in %	7.9	9.6
12_T2		
9789	8475	7357
Loss in %	13.4	24.8

Table 29: Storage of sample 10.

10_T0		
Original	**Freezer**	**Fridge**
1882	1856	297
Loss in %	1.4	84.2
10_T2		
2558	1767	290
Loss in %	31	89

After five days, there was a degradation observed. The degradation in the fridge was higher, than in the freezer. The loss of sensitivity for sample 12 was found to be lower than for sample 10. Interestingly sample 10_T0 showed not much degradation in the

freezer. Storage in the fridge of both 10_T0 and 10_T2 led to a loss of over 80 % of the original sensitivity.

The differences between samples 10 and 12 could depend on the nutrition of the patient, other drugs might also be present or also redox reactions might have an influence on the stability of the fluorophore.

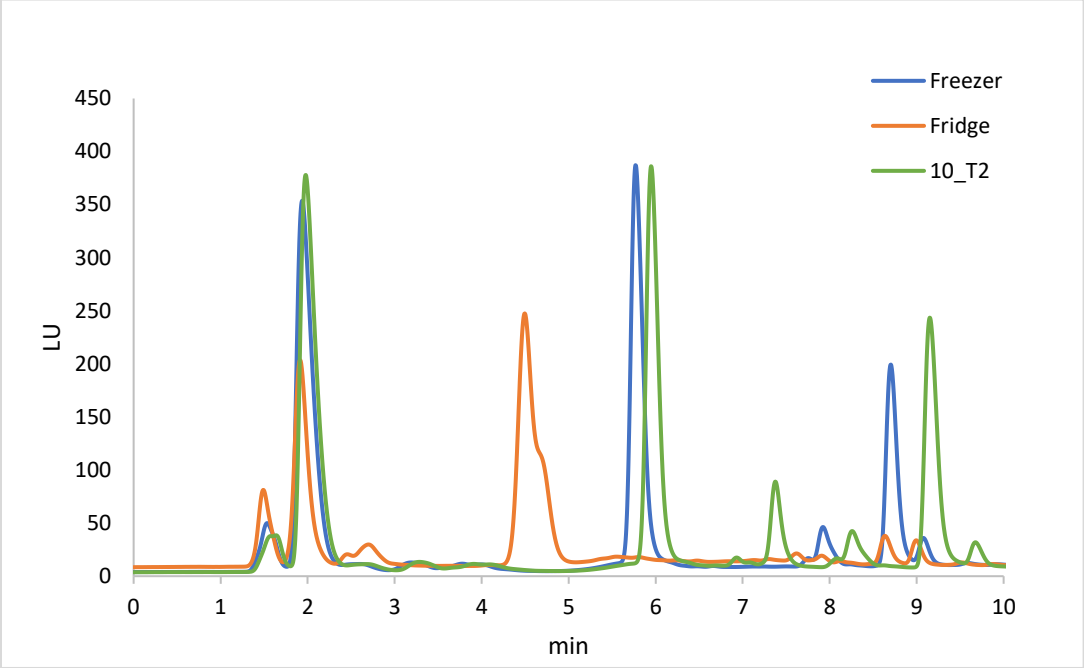

Figure 37: Chromatogram sample 10_T2 tested for stability.

The shift in the RT in original sample may be caused through insufficient equilibration of the column.

The peaks of the sample stored in the fridge differed clearly from the original. For the sample stored in the freezer the peaks showed not much variation compared to the original.

It is not recommended to store the derivatized samples for prolonged time, neither in the fridge nor in the freezer, since the loss of sensitivity is too high.

3.8.6 Comparison to other RP-HPLC methods

Some other published methods for detection of metformin in urine are listed:

Table 30: Comparison of HPLC-methods for metformin determination in urine.

Sample volume (µl)	Sample preparation	Analytical column	RT (min)	LOQ (µg/ml)	LOD (µg/ml)	Reference
200	Ethanol precipitation	Precolumn derivatization, RP-C18	8.8	110	30	Adapted from Ohta et al., 1993
10	Dilution with mobile phase	RP-C18	6.4	35	12	Troja et al., 2015
300	KOH, protein PPT with ACN and evaporation	RP-C18	8.8	-	0.05	Benzi et al., 1986
500	Dilution with water	Cation exchanger SCX	8.0	-	0.1	Bruce et al., 1981

The determined LOQ was 35 µg/ml and the retention time 6.4 min for the method of (Troja et al., 2015). In comparison to the current method the LOQ was three times lower.

The method described by Benzi et al. (1986) has determined a LOD of 0.05 µg/ml and a retention time of 8.8 min. Bruce and co-workers, 1981 have reported a LOD of 0.1 µg/ml and a retention time of metformin at 8 min. Instead of a RP-column they used a SCX cation exchanger column. All of the described methods used UV detection.

The linearity and the precision of the method were acceptable. As mentioned before the high variation has caused that the LOQ and LOD are not very low. If the variance could be improved the LOQ, LOD and limit of decision would also be better.

For further measurements, the sample could be more diluted after precipitation. As the concentration of metformin in urine was found to be high, a dilution should be possible without problems. A lower concentration of metformin and the same amount of benzoin and other reagents, might lead to a better ratio of metformin to benzoin that can react. Alamgir and co-workers, (2014) have studied the effect of benzoin and β-mercaptoethanol on the UV absorbance of the metformin derivatization complex. It was observed that increasing benzoin and β-mercaptoethanol concentrations led to higher UV absorbance. This is also a possible way to improve the derivatization reaction.

3.9 Metformin concentration in urine samples

The samples were provided by the Medical University of Graz and the patients were chosen by them. All samples were stored in the freezer at -20 °C and the volume was 10 ml. The sample preparation was done like described in chapter 2.2.2 and 2.4.2. The chromatographic conditions according chapter 2.3.2 and 2.3.3 were used for the measurements.

For every patient two time points were set for drawing the sample. The first is in the morning (fasting from metformin), the second 2 h after intake of the drug. The samples of time point two were collected after the maximum metformin concentration was present (Tucker et al., 1981; Al Hawari et al., 2007; Grün et al., 2013).

The following results were obtained from the received samples, the mean of the concentration and SD of every sample is given:

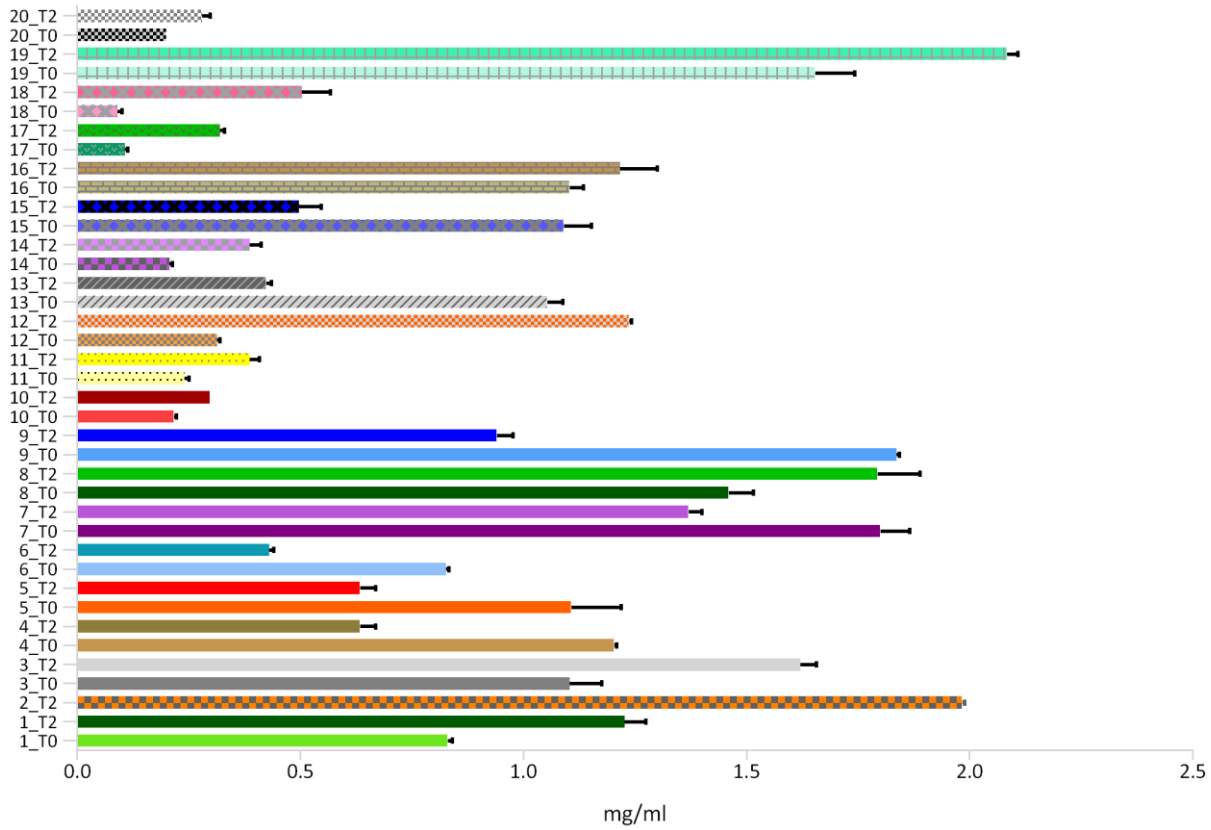

Figure 38: Metformin concentration in samples. T0: Fasting from metformin, T2: 2 h after intake, 1-20: Number of the patient.

*Table 31: Concentration of metformin in urine samples and corresponding daily metformin dosage. *mg metformin HCl in tablet converted into mg metformin. **2_T0 does not exist.*

Sample	c (mg/ml)	SD	Dose of metformin* (mg)
1_T0	0.8	0.01	780
1_T2	1.2	0.05	
2_T2**	2.0	0.01	780
3_T0	1.1	0.07	1560
3_T2	1.6	0.04	
4_T0	1.2	0.01	1560
4_T2	1.5	0.03	
5_T0	1.1	0.11	1560
5_T2	0.6	0.03	
6_T0	0.8	0.01	1326
6_T2	0.4	0.01	
7_T0	1.8	0.07	1560
7_T2	1.4	0.03	
8_T0	1.5	0.05	1560
8_T2	1.8	0.09	
9_T0	1.8	0.01	1443
9_T2	0.9	0.04	
10_T0	0.2	0.00	780
10_T2	0.3	0.01	
11_T0	0.2	0.01	780
11_T2	0.4	0.02	
12_T0	0.3	0.00	780
12_T2	1.2	0.01	
13_T0	1.1	0.03	1560
13_T2	0.4	0.01	
14_T0	0.2	0.00	1326
14_T2	0.4	0.02	
15_T0	1.1	0.06	1560
15_T2	0.5	0.05	
16_T0	1.1	0.03	1560
16_T2	1.2	0.08	

17_T0	0.1	0.01	780
17_T2	0.3	0.01	
18_T0	0.1	0.01	780
18_T2	0.5	0.06	
19_T0	1.7	0.09	1560
19_T2	2.1	0.02	
20_T0	0.2	0.00	1560
20_T2	0.3	0.02	

The lowest metformin concentrations were determined in patients 10, 14, 17, 18 and 20. Patients with the number 10, 17, and 18 have got a daily metformin dose of 780 mg. Patient 14 has received 1,326 mg and patient 20 1,560 mg of metformin.

In patients 2, 7, 8, 9 and 19 were the highest concentrations observed. 780 mg of metformin has been the daily intake of patient 2. For patients 7 and 8 it has been 1,560 mg and for patient 9 1,443 mg. 1,560 mg were taken by patient 19.

Interestingly the metformin concentrations in patient 20 were very low both times, although they daily intake was as high as for patient 19. Also, patient 2 got only 780 mg of metformin and a concentration of 2 mg/ml was determined in T2.

Shu and co-workers, (2008) have studied healthy subjects, which have confirmed OCT1 genotypes (variant group) got a daily metformin dose of 1,850 mg. They have reported higher plasma metformin concentrations in the variant group than in the reference group.

The renal clearance was found to be similar between the reference and variant group. It was observed that members of the variant group excrete more metformin into urine. The fraction excreted into urine ($f_{e,u}$) was 19 % (SD= 8.8) for the reference group and 28 % (SD= 16) for the variant group (Shu et al., 2008).

It can be expected, that patients with the highest metformin concentrations show relevant OCT polymorphisms.

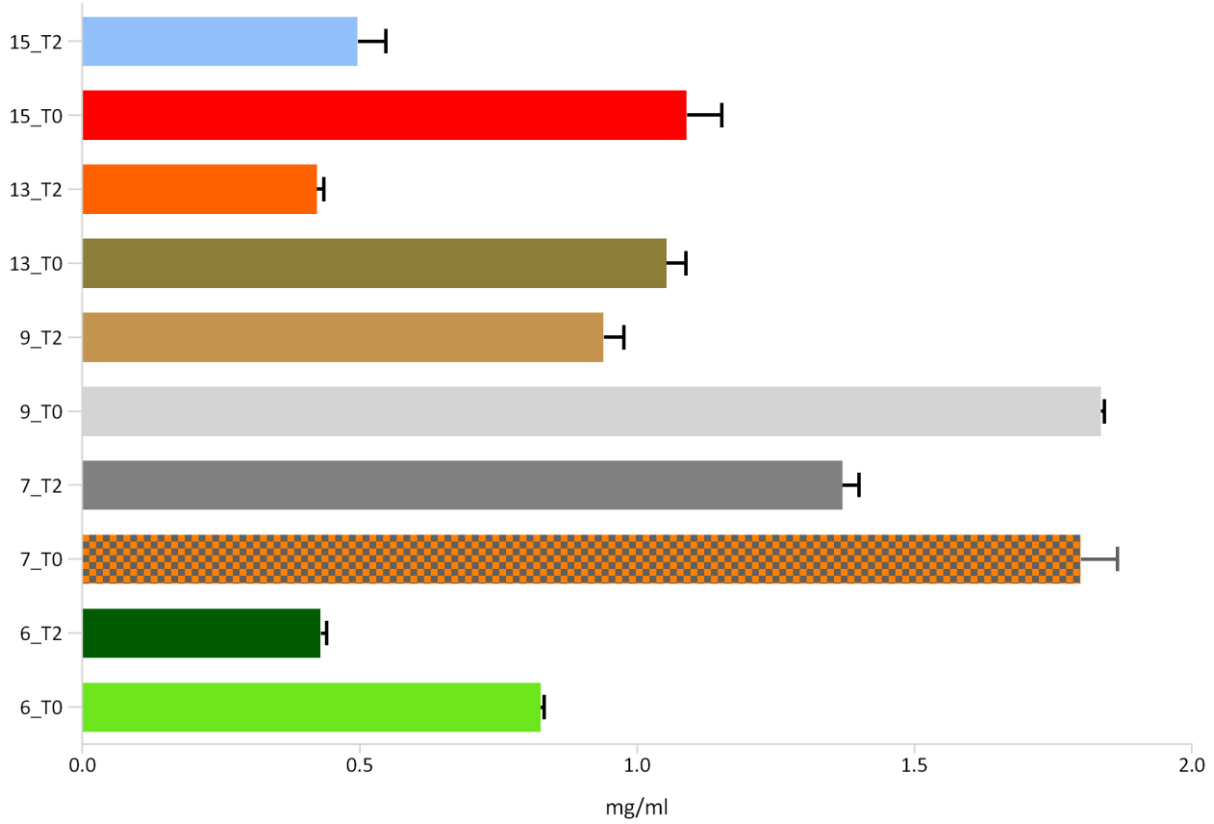

Figure 39: Five samples in which T0 is higher than T2 are shown.

As the second sample is drawn after the expected maximum of metformin concentration, the concentration of metformin should be higher than for the first sample. Probably these five patients could also show relevant OCT polymorphisms (figure 39). The rate of metformin adsorption is reduced if intake of food and drug occurs at the same time (Sambol et al., 1996). This could be a reason for lower concentrations found after 2 h.

The genotyping of all samples will be done at a later date. Unfortunately, there are no data at the moment that can confirm these results.

The current results of metformin concentration are comparable to previous work. Benzi and coworkers, 1986 found metformin concentrations in urine samples from 0.25 mg/ml to 1.5 mg/ml. The samples were drawn before 2 h after the administration of the last 500 mg tablet. Another work group (Bruce et al., 1981) has reported metformin concentrations in urine ranging from 0.04 to 1.2 mg/ml.

4 Conclusion

The derivatization of metformin with benzoin is a reliable pre-column derivatization procedure. In comparison to desyl bromide, benzoin was more sensitive.

As a possible internal standard o-tolylbiguanide has been tested. The experiments showed that metformin and o-tolylbiguanide compete for derivatization reagents. A higher volume of reagents at constant volume of sample has improved the behaviour, but the areas of o-toylbiguanide varied too much. Further optimization of the reaction conditions is necessary to make o-tolylbiguanide suitable as an internal standard.

Storage of the derivatized samples (in the fridge or freezer) is not recommended, because of their degradation.

The gradient is working very well with the RP-C18 column, but to save solvent and time the method should be adapted to a shorter column (100 × 3 mm). The RT of metformin is 8.8 min using the described conditions.

The current HPLC method has proven to be precise and accurate, although the variance of higher concentrations is high. This drawback must be improved to make the method more sensitive (lower LOD and LOQ). For future measurements, different sample dilution levels should be tested for a reduced variance. For improvement of the reaction of metformin with benzoin, the amount of β-mercaptoethanol should be increased. The effect on the stability of the fluorophore should be investigated. The pipettes should be calibrated once a year to assure precision and accuracy.

The robustness was also evaluated. Changes in the flow rate had an influence on the RT and on the peak height. Changes in the pH of the buffer, had only a small influence on the RT. As expected at 95 C° the peak areas and heights are lower. The amount of benzoin and sodium thiosulphate with β-mercaptoethanol also affected the peak areas and heights.

The LOD is 30 µg/ml, the limit of decision is 60 µg/ml and the LOQ is 110 µg/ml. The selectivity and linearity of the method are also acceptable. The found concentrations range from 0.1 mg/ml to 2.1 mg/ml, which is comparable to other studies. Genotyping data will be available at the end of 2017. Together with urine and plasma metformin concentrations it would be possible to identify some SNPs influencing metformin uptake.

5 Literature

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5.1 Links

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6 Appendix

List of tables

List of figures

6.1 Material

Table 32 List of material and devices

Product	Company
Balance	Mettler-Toledo
Bond Elut Certify 130 mg, 3 ml	Agilent technologies
Centrifuge 5804R	Eppendorf
Filter paper, MN 615 1/4 diam. 110mm	Macherey-Nagel
HPLC 1100 Series	HP
pH-meter pH3110	WTW
Pipettes pipetman, 1000, 200, 100 µl	Gilson
Reaction tubes, 1.5 ml, 2 ml	Eppendorf
Thermomixer, 2 ml	Eppendorf
Ultra-pure water system	Siemens
Vortexgenie 2	Scientific industries

Table 33: List of chemicals

Product	Company
Acetone	ChemLab
Acetonitrile, HPLC-grade	ChemLab
Ammonium (25 %)	Merck
Benzoin	Sigma-Aldrich
Desyl bromide	Sigma-Aldrich
Ethanol (100 %), HPLC-grade	ChemLab
Hydrochloric acid (38 %)	Merck
Methanol (100 %), HPLC-grade	ChemLab
o-Tolylbiguanide	Sigma-Aldrich
Potassium dihydrogen phosphate	Merck
Potassium hydroxide	Merck
Sodium hydroxide (25 %)	Merck
Sodium thiosulfate	Merck
Tetrahydrofuran, HPLC-grade	ChemLab
Tris(hydroxymethyl)aminomethane	Merck
β-Mercaptoethanol	Sigma-Aldrich

6.2 Gradients

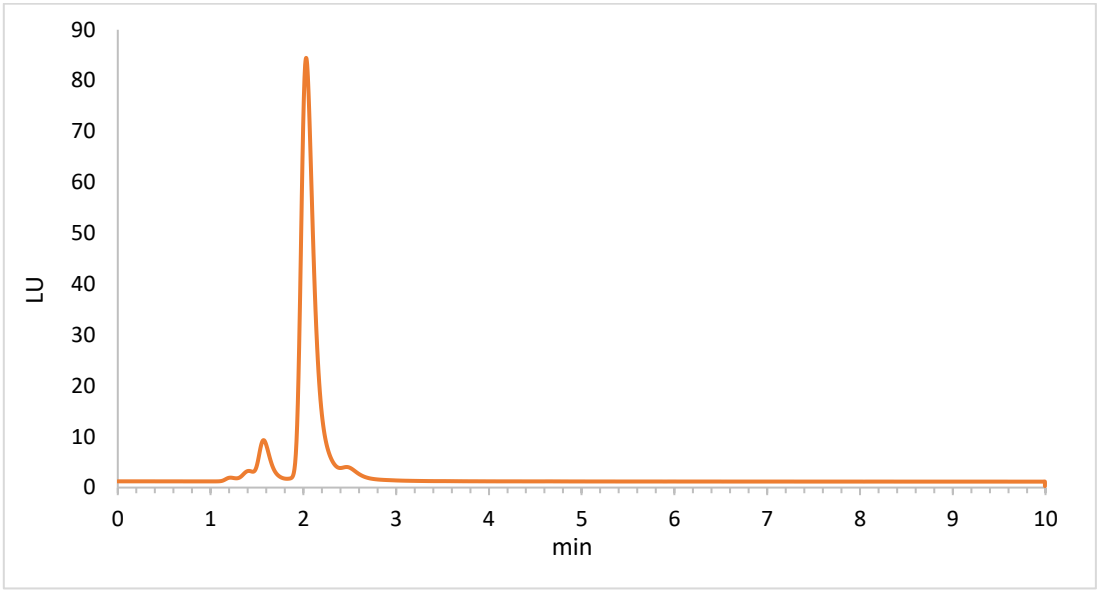

Figure 40: Method 1. Chromatogram of 53 µg/ml metformin derivatized with benzoin.

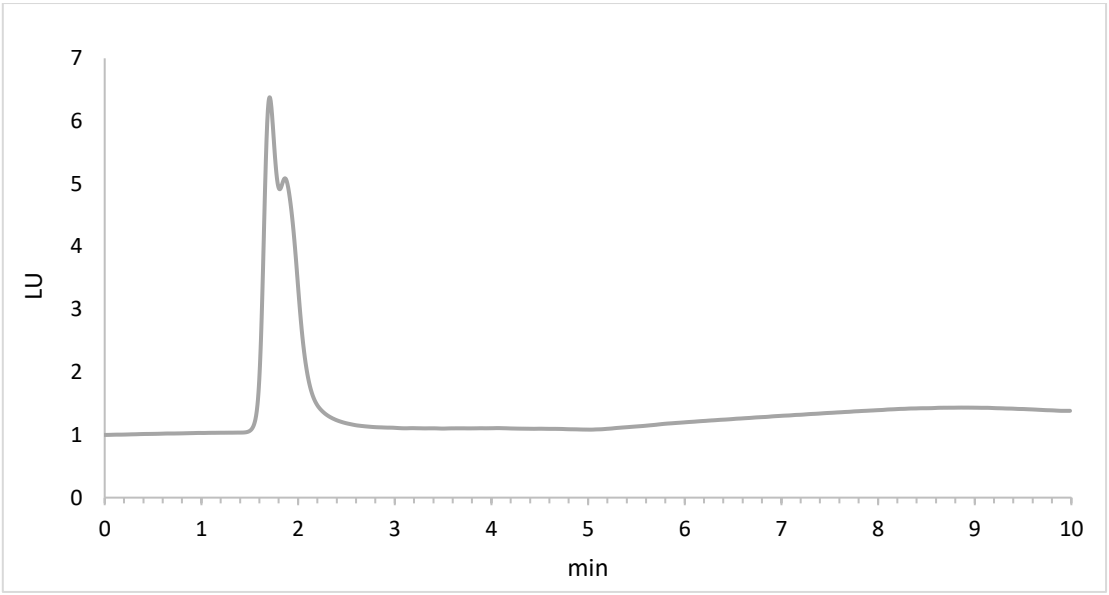

Figure 41: Method 2. 5 µg/ml metformin with benzoin.

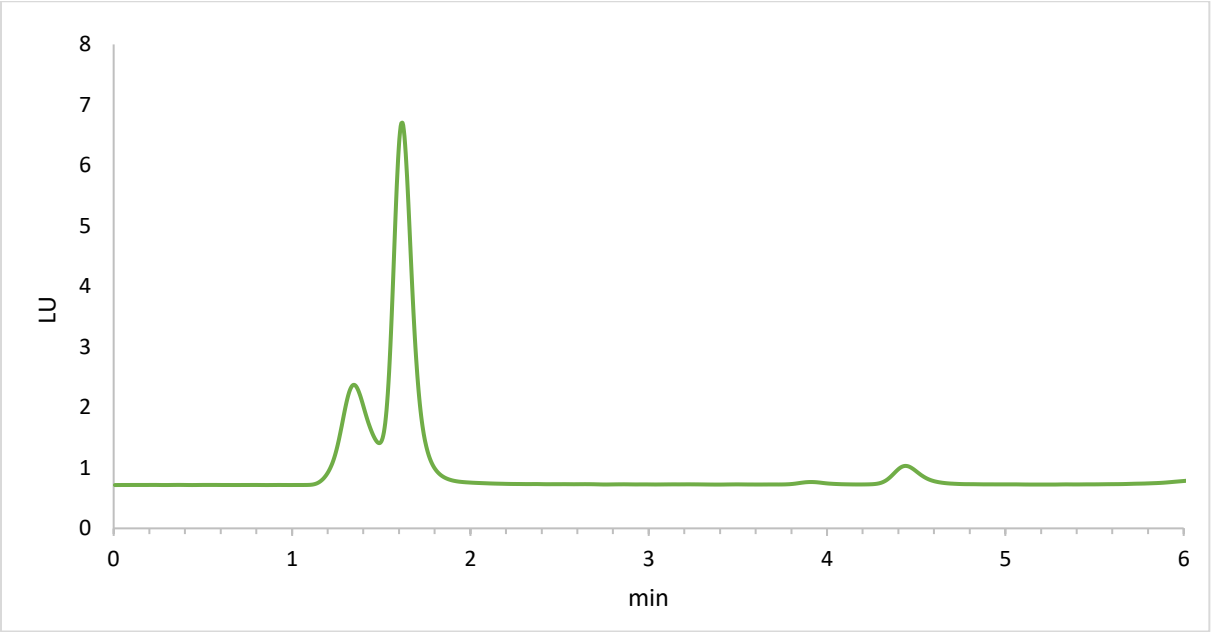

Figure 42: Method 4. 0.5 µg/ml metformin derivatized with desyl bromide.

6.3 Kinetics of benzoin

Table 34: Results of kinetic measurement.

	2 min		3 min		4 min		6 min	
	Area	**Height**	**Area**	**Height**	**Area**	**Height**	**Area**	**Height**
	3277	398	5827	674	4527	546	2154	241
	3271	396	5592	652	4136	475	2013	222
	3263	374	5585	663	4182	472	1956	216
mean	3270	389	5668	663	4282	497	2041	226
SD	7.4	13	134	11	214	42	102	13
RSD %	0.2	3.3	2.4	1.6	5.0	8.4	5.0	5.7

6.4 Comparison benzoin and desyl bromide

Table 35: Areas of three different concentrations for desyl bromide.

	Desyl bromide				
Concentration (mg/ml)	**Area**			**Mean**	**SD**
2.3	6395	6385	6378	6386	8.5
0.8	3612	3625	3617	3618	6.6
0.08	449	440	442	444	4.7

Table 36: Areas for benzoin for the given concentrations.

	Benzoin				
Concentration (mg/ml)	**Area**			**Mean**	**SD**
2.3	17579	17582	17583	17581	2.1
0.8	6020	6015	6011	6015	4.5
0.08	866	856	848	857	9.2

6.5 o-Tolylbiguanide

Table 37: Corresponding areas of metformin and o-tolylbiguanide (1 mg/ml).

	metformin			o-tolylbiguanide		
$c_{metformin}$ mg/ml	Area		mean	Area		mean
2.7	14750	14843	14797	5680	3230	4455
1.6	8620	8618	8619	7680	5276	6478
0.8	5781	5012	5397	8291	6937	7614
0.4	2582	2639	2611	5767	5631	5699
0.08	782	961	872	8307	9251	8779
0.008	112	111	112	8628	8586	8607

Table 38: Concentrations and related areas of both analytes with increased volume of reagents. o-tolylbiguanide conc. 1 mg/ml.

	metformin			o-tolylbiguanide		
$c_{metformin}$ mg/ml	Area		mean	Area		mean
0.8	3955	3810	3883	2380	2466	2423
0.4	1930	2216	2073	2297	2062	2179
0.08	384	404	394	3129	3322	3226

Table 39: Kinetic of o-tolylbiguanide. Areas related to time in minutes for both analytes. The metformin concentration was 1.56 mg/ml and that for o-tolylbiguanide 1 mg/ml.

	metformin			o-tolylbiguanide		
min	Area		Mean	Area		Mean
2	5101	5057	5079	720	693	707
4	6862	6805	6834	1338	1296	1317
6	5207	5188	5198	939	895	917
8	3696	3646	3671	742	705	724

6.6 Recovery

Table 40: Concentrations and areas of the original and spiked sample.

	c (mg/ml)	Area
Sample	-	6693
+ 50 µl standard	0.4	8901
	0.8	13374
	1.3	16420
	1.6	18792

Table 41: Concentrations used for calibration.

c (mg/ml)	Area
2.3	19502
1.6	13735
0.8	6808
0.5	4465
0.08	861
0.008	182

6.7 Validation of the HPLC-method

Table 42: Calibration data and calculated values of ValiData.

Data for linear regression									
Concentration (mg/ml)	Area	Estimated area	Residues	Confidence interval (-)	Confidence interval (+)	Prognosis-interval (-)	Prognosis-interval (+)	Calculated concentration	% Variance
0.0585	553	604.65	-51.65	0.04	0.08	0.02	0.09	0.05	-11.93
0.0585	548	604.65	-56.65	0.04	0.08	0.02	0.09	0.05	-13.08
0.585	4519	4502.70	16.30	0.57	0.60	0.55	0.62	0.59	0.38
0.585	4520	4502.70	17.30	0.57	0.60	0.55	0.62	0.59	0.40
1.17	8976	8833.86	142.14	1.16	1.18	1.14	1.20	1.19	1.64
1.17	8980	8833.86	146.14	1.16	1.18	1.14	1.20	1.19	1.69
1.95	14425	14608.75	-183.75	1.93	1.97	1.92	1.98	1.93	-1.27
1.95	14410	14608.75	-198.75	1.93	1.97	1.92	1.98	1.92	-1.38
2.34	17579	17496.19	83.11	2.32	2.36	2.30	2.38	2.35	0.48
2.34	17582	17496.19	85.81	2.32	2.36	2.30	2.38	2.35	0.50

Table 43: Summarized results of the validation from ValiData.

Result validation			
Date	11.07.2017		
Description	Metformin in urine matrix		
Operator	VB		
# Measurements	14	Unit conc.:	mg/ml
# Replica	2	Unit measurement:	Area
# Concentration level	5		

Model	Linear (conforming to standards)
y[Area] = 7403.7[Area/(mg/ml)] * x [mg/ml] + 171.53[Area]	

Variance	95% not ok! 99% not ok!		
Linearity	Ok		
lower variance	5.6		
upper variance	2327.3		
Slope	7290.9	7516.5	Area/(mg/ml)
Intercept	4.3	338.7	Area
Residual standard deviation	130.2		Area
SD for the method	0.018		mg/ml
RSD for the method	1.44		%

Limit of detection (LOD) and limit of quantification (LOQ)			
	Blank value meth.	Calibration meth.	
Confidence level	0.95	0.95	
LOD	N/A	0.03	mg/ml
Limit of decision	N/A	0.06	mg/ml
LOQ		0.11	mg/ml

6.7.1 Test for linearity

Table 44: Results of linearity test and calibration function 1st and 2nd grade. (ValiData)

Linearity test	
Test value	0.310
F_99	12.25
Ok, no significant difference (99% level)	

Calibration function 1st grade (y=a+b*x)			
slope	7403.70		Area/(mg/ml)
VB(slope)	7290.93	7516.47	Area/(mg/ml)
intercept	171.53		Area
VB(intercept)	4.32	338.74	Area
Mean (x)	1.22		mg/ml
Mean (y)	9209.23		Area
Residual standard deviation	130.16		Area
Standard deviation for the method	0.0176		mg/ml
Rel. standard deviation for method	1.44		%
t-value (95%)	2.31		
Qx	7.08		$(mg/ml)^2$

Calibration function 2nd grade (y=a+b*x+c*x^2)		
a	135.76	Area
b	7516.78	Area/mg/ml
c	-46.51	
Sensitivity	7403.22	Area/(mg/ml)
Mean (x)	1.22	mg/ml
Mean (y)	9209.23	Area
Residual standard deviation	136.16	Area
Standard deviation for the method	0.018	mg/ml
Rel. standard deviation for method	1.51	%
t-value (95%)	2.36	
Test value (solution)	80.80	mg/ml
Ok, no extreme value		

6.7.2 Precision

Table 45: Results of the measured controls.

Date	Area	Mean	SD
31.01.2017	3020	2955	56.35
31.01.2017	2925		
31.01.2017	2920		
09.03.2017	3073	3063	11.24
09.03.2017	3051		
09.03.2017	3066		
16.03.2017	3045	3067	19.50
16.03.2017	3076		
16.03.2017	3081		
03.04.2017	2760	2923	141.57
03.04.2017	2990		
03.04.2017	3018		
04.04.2017	3141	3120	22.59
04.04.2017	3096		
04.04.2017	3122		
09.05.2017	3076	3107	27.40
09.05.2017	3128		
09.05.2017	3117		

Table 46: Results of the pipette calibration. Date: 2017/21/03, Z-factor: 1.033, temperature: 22 °C.*

Pipette: Gilson® 100-1000 µl				**Pipette: Gilson® 20-200 µl**			
	1000 µl	**100 µl**	**500 µl**		**200 µl**	**20 µl**	**100µl**
	Weight (g)	Weight (g)	Weight (g)		Weight (g)	Weight (g)	Weight (g)
	0.998	0.0998	0.499		0.1980	0.0198	0.1000
	0.994	0.0994	0.498		0.1980	0.0200	0.0998
	0.998	0.0996	0.500		0.1990	0.0198	0.0997
	1.000	0.1000	0.501		0.1980	0.0197	0.0996
	0.998	0.0995	0.498		0.1980	0.0201	0.1000
	0.994	0.0995	0.497		0.1987	0.0199	0.0995
	0.997	0.1000	0.499		0.1994	0.0197	0.0998
	0.996	0.0995	0.498		0.1993	0.0199	0.0997
	0.995	0.0989	0.498		0.1989	0.0201	0.1001
	0.998	0.0997	0.497		0.1987	0.0197	0.0997
	0.997	0.0996	0.499	**Mean**	0.1987	0.0198	0.0998
Corr. Mean	1.000	0.099	0.500	**Corr. Mean**	0.199	0.019	0.100
SD	2.0×10^{-3}	3.2×10^{-4}	1.27×10^{-3}	**SD**	5.4×10^{-4}	1.6×10^{-4}	1.9×10^{-4}
RSD	0.20	0.32	0.25	**RSD**	0.27	0.78	0.19
% Inacc-uracy	0.022	-0.081	0.029	**% Inacc-uracy**	-0.322	-0.322	0.119
Result	PASS	PASS	PASS	**Result**	PASS	PASS	PASS

Acceptance criteria:

% Inaccuracy must be less than 2.0

No replicate may be greater than 2% from true volume.

RSD must be less than 1.0

*Excel-sheet created by the Wisconsin State Laboratory of Hygiene

6.7.3 Test of variances

Table 47: Results for ANOVA.

ANOVA for linear regression					
Source	FG	QS	QS/FG	F-Ratio	Probability
Model	1	388319380	388319380	22920.5	4.05×10^{-15}
Residues	8	135535.9	16941.9		
LOF	3	135398.7	45132.9	1645.5	6.63×10^{-8}
PE	5	137.15	27.43		

Table 48: Test of variances.

Test of variances		
	upper	lower
s(rel)	0.43	0.27
Degree of freedom	3	3
Variance	5.66	2327.29
Test value	410.70	
F_95Var	9.28	
F_99Var	29.46	
Attention: Significant difference on level 95%		
Attention: Significant difference on level 99%		

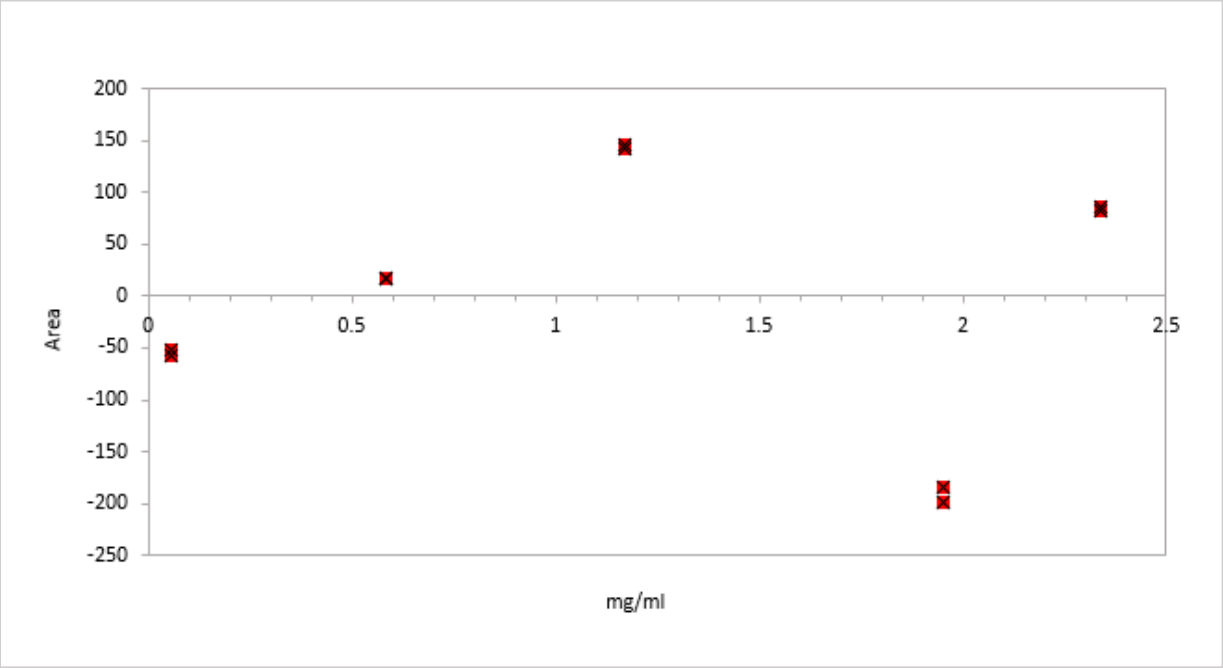

Figure 43: Residues of linear regression.

Table 49: Concentrations and areas of residues illustrated in figure 43.

Concentration (mg/ml)	Residues (area)
0.0585	-51.65
0.0585	-56.65
0.585	16.30
0.585	17.30
1.17	142.14
1.17	146.14
1.95	-183.75
1.95	-198.75
2.34	83.11
2.34	85.81

6.8 Metformin concentration in urine samples

Table 50: Areas and concentrations of all samples.

Sample	Area	mg/ml	Mean (mg/ml)	Result (mg/ml)	SD
1_T0	6693	0.84	0.83	**0.8**	0.01
	6534	0.82			
	6582	0.83			
1_T2	9398	1.19	1.23	**1.2**	0.05
	10104	1.28			
	9538	1.21			
2_T2	15604	2.00	1.99	**2.0**	0.01
	15570	1.99			
	15482	1.98			
3_T0	9045	1.15	1.08	**1.1**	0.07
	8049	1.02			
	9014	1.14			
3_T2	13034	1.66	1.62	**1.6**	0.04
	12481	1.59			
	12613	1.61			
4_T0	9538	1.21	1.20	**1.2**	0.01
	9415	1.20			
	9465	1.20			
4_T2	11565	1.47	1.50	**1.5**	0.03
	12000	1.53			
	11768	1.50			
5_T0	7989	1.01	1.11	**1.1**	0.11
	9685	1.23			
	8556	1.08			
5_T2	5321	0.67	0.63	**0.6**	0.03
	4833	0.60			
	5026	0.63			
6_T0	6596	0.83	0.83	**0.8**	0.01
	6595	0.83			
	6512	0.82			
6_T2	3546	0.44	0.44	**0.4**	0.01
	3534	0.43			
	3453	0.42			

7_T0	13654	1.74	1.81	**1.8**	0.07
	14658	1.87			
	14012	1.79			
7_T2	10567	1.34	1.37	**1.4**	0.03
	10974	1.40			
	10746	1.37			
8_T0	11003	1.40	1.46	**1.5**	0.05
	11563	1.47			
	11818	1.51			
8_T2	13326	1.70	1.79	**1.8**	0.09
	14027	1.79			
	14761	1.89			
9_T0	14324	1.83	1.84	**1.8**	0.01
	14414	1.84			
	14378	1.84			
9_T2	7106	0.90	0.94	**0.9**	0.04
	7703	0.97			
	7517	0.95			
10_T0	1836	0.22	0.22	**0.2**	0.00
	1856	0.22			
	1833	0.21			
10_T2	2559	0.31	0.29	**0.3**	0.01
	2339	0.28			
	2476	0.30			
11_T0	2142	0.25	0.24	**0.2**	0.01
	1956	0.23			
	2055	0.24			
11_T2	3109	0.38	0.39	**0.4**	0.02
	3316	0.41			
	3003	0.37			
12_T0	2539	0.31	0.31	**0.3**	0.00
	2571	0.31			
	2616	0.32			
12_T2	9795	1.24	1.24	**1.2**	0.01
	9789	1.24			
	9698	1.23			

13_T0	8058	1.02	1.05	**1.1**	0.03
	8586	1.09			
	8326	1.05			
13_T2	3327	0.41	0.42	**0.4**	0.01
	3525	0.43			
	3486	0.43			
14_T0	1752	0.20	0.21	**0.2**	0.00
	1802	0.21			
	1787	0.21			
14_T2	2994	0.36	0.39	**0.4**	0.02
	3357	0.41			
	3159	0.39			
15_T0	8088	1.02	1.08	**1.1**	0.06
	9010	1.14			
	8759	1.11			
15_T2	3935	0.49	0.52	**0.5**	0.05
	4389	0.55			
	3653	0.45			
16_T0	8931	1.13	1.10	**1.1**	0.03
	8465	1.07			
	8736	1.11			
16_T2	9070	1.15	1.17	**1.2**	0.08
	9386	1.19			
	10319	1.31			
17_T0	1021	0.11	0.11	**0.1**	0.01
	935	0.10			
	1029	0.11			
17_T2	2693	0.33	0.32	**0.3**	0.01
	2682	0.32			
	2605	0.31			
18_T0	809	0.08	0.09	**0.1**	0.01
	959	0.10			
	854	0.09			
18_T2	4344	0.54	0.50	**0.5**	0.06
	4334	0.54			
	3523	0.43			

19_T0	13341	1.70	1.71	**1.7**	0.09
	13382	1.71			
	12184	1.55			
19_T2	16251	2.08	2.07	**2.1**	0.02
	16100	2.06			
	16470	2.11			
20_T0	1694	0.20	0.20	**0.2**	0.00
	1723	0.20			
	1739	0.20			
20_T2	2443	0.29	0.28	**0.3**	0.02
	2211	0.26			
	2382	0.29			

6.8.1 Example Chromatograms

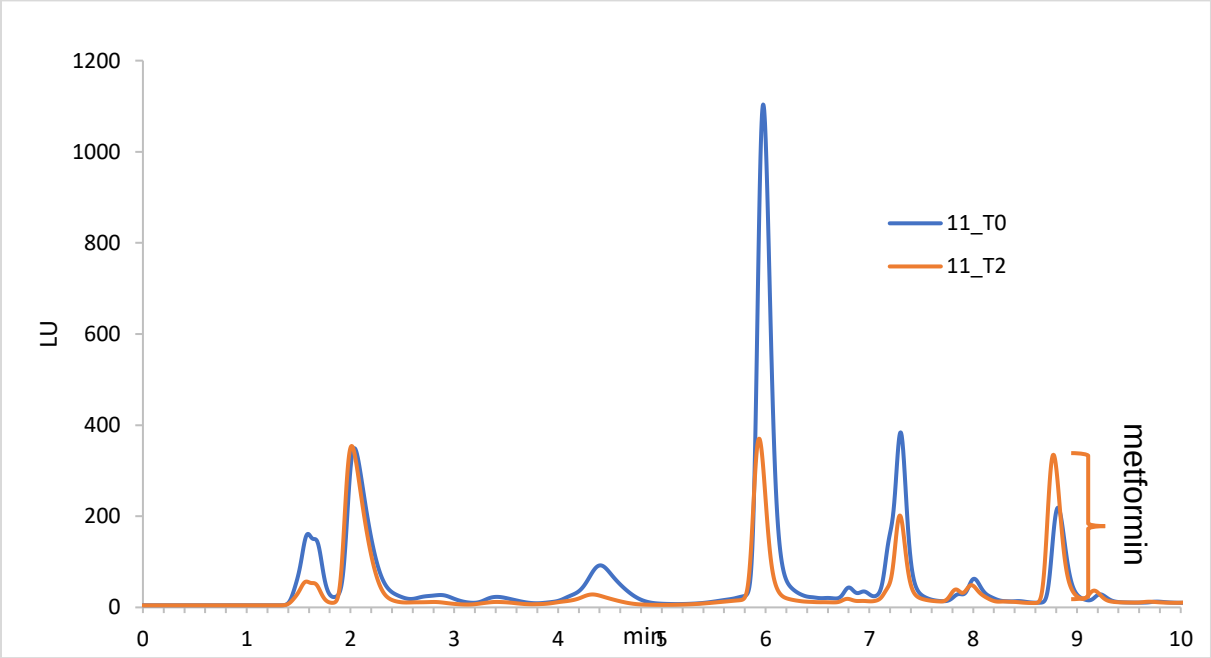

Figure 44: Chromatograms samples of patient 11.

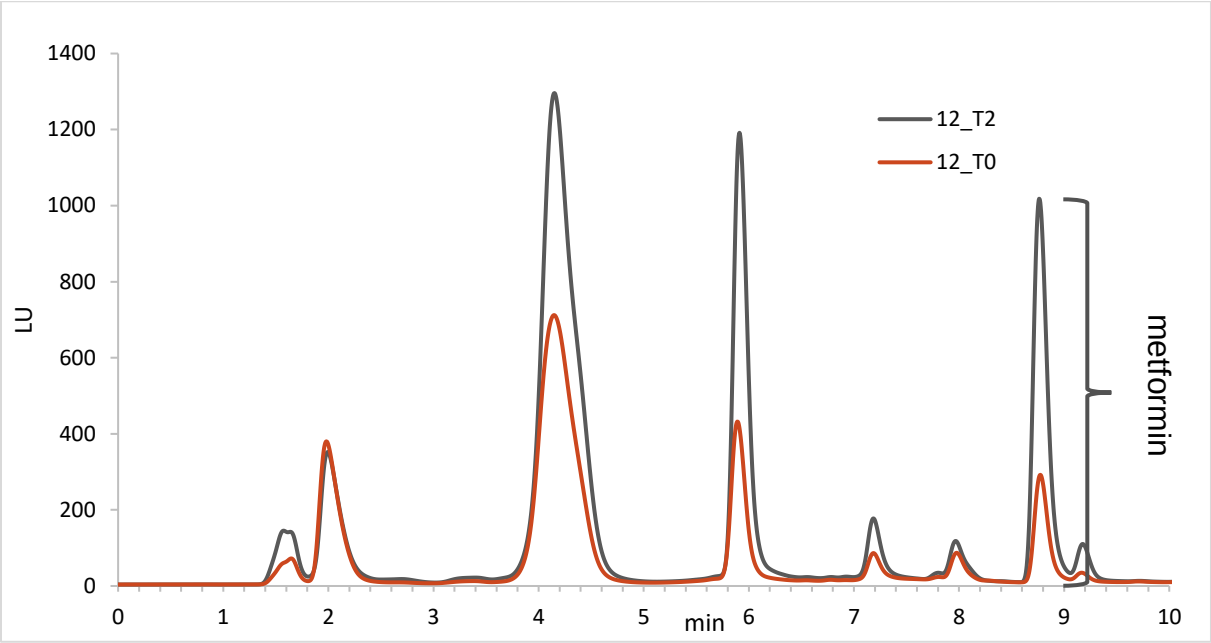

Figure 45: Chromatograms samples of patient 12.