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Quantitative determination of metformin and melatonin using high performance liquid chromatography

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Abbreviations

Å	Angstrom
ACN	Acetonitrile
H_2O	Water
HCl	Hydrochloric acid
HPLC	High-performance liquid chromatography
LC	Liquid chromatography
n	Number of samples
n.d.	None detected
N_2	Nitrogen
rpm	Revolutions per minute
t_0, t_{2h}	Time point 0 and 2 hours after intake
THF	Tetrahydrofuran
tris	Tris(hydroxymethyl)-amino methane
UV-light	Ultra violet light

Abstract

The first part of this thesis deals with the quantitative analysis of metformin, which is part of a bigger project in cooperation with the Medical University of Graz. Metformin is administered to patients affected by type 2 diabetes mellitus either alone or in combination with other medications. Patient's urine (n = 102) and plasma (n = 78) samples taken 0 and 2 hours after administration of metformin medication are analyzed for their metformin content. The data is correlated with genetic data from the patients by the Medical University.

Samples are subjected to precolumn fluorescence derivatization using benzoin and analyzed using a linear gradient elution with a C18-column on the HPLC. A flow rate of 0.5 ml/min for urine and 0.6 ml/min for plasma samples was used. Solvent 1 (5% ACN, 21% 0.5 M HCl/tris buffer pH 8, 74% ultrapure H_2O) was decreased from 100% to 0% over the timeframe of 5 minutes, while solvent 2 (65% ACN, 10% 0.5 M HCl/tris buffer pH 8, 10% THF, 15% ultrapure H_2O) was increased accordingly for a total run time of 10 and 12 minutes, respectively. The retention time for the metformin derivative was 8.6 minutes for urine samples and 8.1 minutes for plasma samples. The fluorescence maxima were detected at 280 nm for excitation and 440 nm for emission.

The second part of the thesis is concerned with the quantitative analysis of melatonin. Cranberry and white mustard seeds are analyzed both involving precolumn derivatization and direct measurement with the HPLC using a C18 column. In this thesis, the procedure involving precolumn derivatization did not give any results. Therefore, melatonin was analyzed directly using its own fluorescence at 280 nm (excitation) and 350 nm (emission). With a run time of 15 minutes, the melatonin peak is found at 6.7 minutes. Starting at 20% ACN and 80% ultrapure H_2O, the gradient increases to 35% ACN and 65% ultrapure H_2O over 6.5 minutes and then to 100% ACN over 1 minute. 100% ACN is maintained for the rest of the running time.

The HPLC methods were validated for linearity, precision, accuracy, and robustness.

Zusammenfassung

Der erste Teil dieser Arbeit beschäftigt sich mit der quantitativen Analyse von Metformin, welche Teil eines größeren Projekts ist, das in Kooperation mit der Medizinischen Universität Graz erfolgt. Dabei wird Patienten, die an Typ 2 Diabetes Mellitus leiden, Metformin verabreicht. Die Therapie kann ausschließlich mit Metformin oder als Teil einer Kombitherapie mit anderen Medikamenten erfolgen. Von diesen Patienten wurden 0 und 2 Stunden nach Einnahme von Metformin Plasma- (n = 102) und Urin-Proben (n = 78) bezogen und auf ihren Metformin-Gehalt untersucht. Die Ergebnisse werden von der Medizinischen Universität mit genetischen Daten der Patienten korreliert.

Vor der Analyse wurden die Proben mithilfe von Benzoin derivatisiert und anschließend mittels linearer Gradientenelution am HPLC mit C18-Säule gemessen. Die Flussrate betrug 0,5 ml/min für Urin- und 0,6 ml/min für Plasma-Proben. Laufmittel 1 (5% ACN, 21% 0,5 M HCl/tris Puffer pH 8, 74% hochreines H_2O) wurde von 100% auf 0% über 5 Minuten reduziert während Laufmittel 2 (65% ACN, 10% 0,5 M HCl/tris Puffer pH 8, 10% THF, 15% hochreines H_2O) in selber Weise von 0% auf 100% erhöht wurde. Die Gesamt-Laufzeit der Urin-Proben betrug 10 Minuten und die der Plasma-Proben 12 Minuten. Die Retentionszeit des Metformin-Derivates betrug 8,6 bzw. 8,1 Minuten. Das Fluoreszenz-Maximum der Anregung wurde bei 280 nm detektiert und das der Emission bei 440 nm.

Der zweite Teil der Arbeit beschäftigt sich mit der quantitativen Analyse von Melatonin. Untersucht wurden Cranberries und gelbe Senfsamen, die sowohl vor der Fluoreszenzmessung derivatisiert als auch direkt per HPLC mit einer C18-Säule analysiert wurden. Die Messung der derivatisierten Proben lieferte im Rahmen dieser Arbeit keine Ergebnisse. Infolgedessen wurde Melatonin mithilfe der eigenen Fluoreszenz detektiert. Das Anregungs-Maximum dieser Fluoreszenz liegt bei 280 nm und das Emissions-Maximum liegt bei 350 nm. Mit einer Laufzeit von 15 Minuten elutierte der Melatonin-Peak bei 6,7 Minuten. Der Gradient wurde von 20% ACN und 80% hochreines H_2O auf 35% ACN und 65% hochreines H_2O über 6,5 Minuten linear erhöht und danach auf 100% ACN innerhalb einer Minute. Die restliche Zeit wurde mit 100% ACN eluiert.

Die Linearität, Präzision, Exaktheit und Robustheit der angewandten HPLC-Methoden wurden verifiziert.

1 Introduction

The dimethylated biguanide metformin is used as an antidiabetic medication for type 2 diabetes mellitus [1]. Type 2 diabetes is also called non-insulin-dependent diabetes mellitus (NIDDM), where an insulin resistance leads to increased blood glucose levels. Metformin is administered orally to improve insulin sensitivity of peripheral tissue and the liver which counteracts the resistance both individually or in addition to existing antidiabetic therapy [2], [3]. The structure of metformin is depicted in Figure 1 [4].

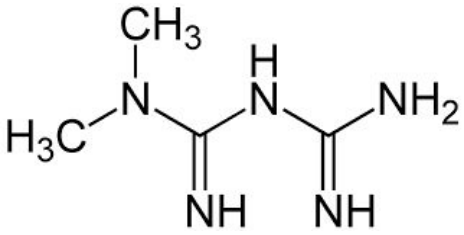

Figure 1: Structure of metformin

Metformin can be analyzed by high-performance liquid chromatography (HPLC) with UV- [5]–[7] or fluorescence detection with precolumn derivatization [8]–[10] as well as by gas chromatography (GC) [11]. The method used for this master thesis is based on the work of Laura Sonnleitner and Verena Buchgraber in their respective master theses [12], [13]. In this work, precolumn derivatization to enable the UV-detection of metformin on the HPLC is conducted to determine metformin concentrations in plasma and urine samples received from the Medical University of Graz.

Melatonin is a molecule widely produced in nature, in vertebrates it is primarily synthesized in the pineal gland [14], [15]. It was first discovered as a hormone of the pineal gland and characterized as N-acteyl-5-methoxytryptamine [15]–[17]; the structure is shown in Figure 2 [18].

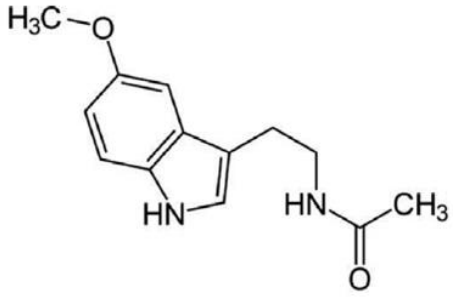

Figure 2: Structure of melatonin

The synthesis of melatonin is regulated by daylight, which in turn regulates the cyclical body activities like sleep/wake or seasonal cycles [14], [17]. Other functions include antioxidative protection, immunostimulation and cytoprotective properties [14], [17]. Evidence has been found that melatonin may also influence mood, tumor growth, aging and neurodegenerative diseases [14], [17], [19].

Melatonin is also produced by plants in high concentrations, especially in seeds and leaves [14], [17], [20]. It is part of the antioxidant defense system as a free radical scavenger protecting the plant from drought, extremes in temperature, chemical pollutants and UV-light [21]–[23] and plays a role in the regulation of rhythmic phenomena e.g. promoting photosynthesis [23]–[25].

Melatonin is most commonly analyzed with radioimmunoassays or HPLC with electrochemical or fluorescence detection or coupled with mass-spectrometry [20], [26], [27].The first method for the analysis of melatonin is based on the work of Iinuma et. al [27]. The second method is based on the thesis of Patricia Mürzl [28].

Metformin contains three primary amine groups and one secondary amine group while melatonin contains only two secondary amine groups [29]. The primary amine groups present in metformin enable the derivatization reaction depicted in Figure 3 [5], [9].

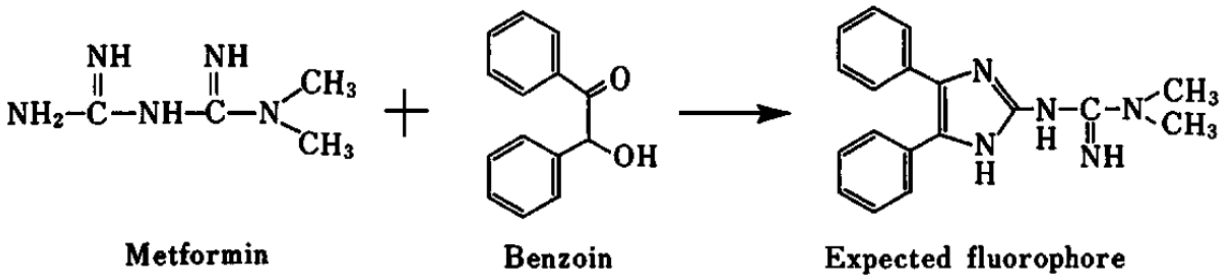

Figure 3: Derivatization reaction of metformin with benzoin yielding the fluorophore

Melatonin lacks those primary amine groups and therefore the derivatization method is based on a different mechanism. Melatonin is an indole compound which are known to show strong fluorescence signals under alkaline conditions in presence of hydrogen peroxide [27]. The mechanism for this fluorescence is shown to be dynamic without any ground-state complex formation. It is thought to result from an electron transfer from the excited indole ring to the hydrogen peroxide [30].

2 Method

2.1 Part 1 – Metformin

2.1.1 Metformin stock

Commercial metformin 850 mg tablets (Hexal, Sandoz, Holzkirchen, Germany) received from the Medical University of Graz were used to prepare a stock solution of metformin hydrochloride prior to receiving the certified reference material.

As the tablets contain metformin hydrochloride, which has a molecular weight of 165.625 g/mol, the concentration of metformin, which has a molecular weight of 129.16 g/mol, was determined as follows:

$$\frac{850\ mg}{165.625\ mg/mmol} * 129.16\ mg/mmol = 663\ mg$$

One tablet contains 663 mg of metformin which was crushed using a porcelain mortar and pistil. The powder was dissolved in 85 ml of ethanol and put into an ultrasonic bath for 5 min. To remove insoluble components used as the matrix in the tablet, the solution was filtrated. The resulting clear liquid was frozen at -20 °C in 1 ml aliquots for further use.

The concentration of those aliquots was calculated as follows:

$$\frac{663\ mg}{85\ ml} = 7.8\ mg/ml$$

2.1.2 Metformin standard

Calibrations were done by dissolving metformin hydrochloride (Pharmaceutical Secondary Standard; Certified Reference Material obtained from Sigma Aldrich) in ethanol.

2.1.3 Sample preparation

All samples were received from the Medical University of Graz in deep frozen state and stored at -20 °C prior to analysis.

<u>Urine</u>

The urine samples were thawed at room temperature. For protein precipitation, 200 µl of ice cold ethanol were added to 200 µl of urine sample in a 1.5 ml reaction tube. The solution was

centrifuged for 5 min at 14,000 rpm. The tube was then gently mixed by inversion to keep the protein pellet intact. 150 µl of the supernatant was used for derivatization.

Plasma

For the sample preparation, the plasma samples were thawed at room temperature and mixed by gentle flipping by hand. 1 ml of the supernatant was transferred into a 2 ml reaction tube where 1 ml of ice cold acetonitrile was added. The solution was vortexed and incubated at room temperature for 5 min. After centrifugation for 5 min at 14,000 rpm, 300 µl of the supernatant was used for derivatization.

2.1.4 Derivatization

Urine

For the derivatization of the urine samples, 150 µl of the previously produced supernatant was transferred to a 1.5 ml reaction tube. Then, 75 µl of a 10 mM benzoin solution, 75 µl of a mixture of sodium thiosulfate solution (0.2 M) and a 2-mercaptoethanol solution (0.2 M) (sodium thiosulfate/2-mercaptoethanol) and 150 µl of a 1 M potassium hydroxide solution were added to the supernatant. After mixing, the tube was heated on a thermomixer for 3 min at 99 °C and 400 rpm. The tube was then cooled in ice water for 2 min. Afterwards, 150 µl of a mixture of tris solution (1 M in water) and HCl (2 M) was added (tris/HCl). If necessary, the pH was adjusted to pH 10.5 before the solution was transferred into a HPLC vial for analysis.

Plasma

The derivatization of the plasma samples was conducted by transferring 300 µl of clear supernatant into a 1.5 ml reaction tube. Then, 150 µl of 10 mM benzoin solution, 150 µl of sodium thiosulfate/2-mercaptoethanol and 300 µl of a 1 M potassium hydroxide solution were added to the supernatant. After mixing, the tube was heated on a thermomixer for 7 min at 99 °C. The tube was then cooled on ice for approximately 5 min. Afterwards, 300 µl of tris/HCl was added. If necessary, the pH was adjusted to pH 9-10.5 before the solution was transferred into a HPLC vial for analysis.

2.1.5 HPLC method

For the analysis of the metformin derivative, a linear gradient elution using a reversed phase C18 column was applied with the parameters listed in Table 1.

Table 1: HPLC method for the analysis of derivatized plasma and urine samples from diabetes patients who had received metformin medication

	Urine	Plasma
Injection volume	10 µl	20 µl
Flow rate	0.5 ml/min	0.6 ml/min
Run time	10 min	12 min
Post time	3 min	3 min
Detection	Fluorescence	
Excitation	280 nm	279 nm
Emission	435 nm	440 nm
Column	Kinetex® 5 µm EVO C18 100 Å (LC column 150 × 3 mm, Phenomenex, Aschaffenburg, Germany)	
Temperature	20 °C	25 °C
Solvent 1	5% ACN, 21% 0.5 M HCl/tris buffer pH 8, 74% ultrapure H_2O	
Solvent 2	65% ACN, 10% 0.5 M HCl/tris buffer pH 8, 10% THF, 15% ultrapure H_2O	
Gradient	0-2 min: 100% solvent 1 7 min: 100% solvent 2	

The HPLC consisted of Hewlett Packard Series 1100 modules, while the fluorescence detector was from Agilent Technologies 1200 Series. The software used was ChemStation for LC 3D (Rev. A. 10.02 [1757], Agilent Technologies 1990-2003). The mobile phases were degassed in an ultrasonic bath prior to use. The gradient for analysis of the urine samples is depicted in Figure 4, while Figure 5 shows the gradient for the plasma sample analysis.

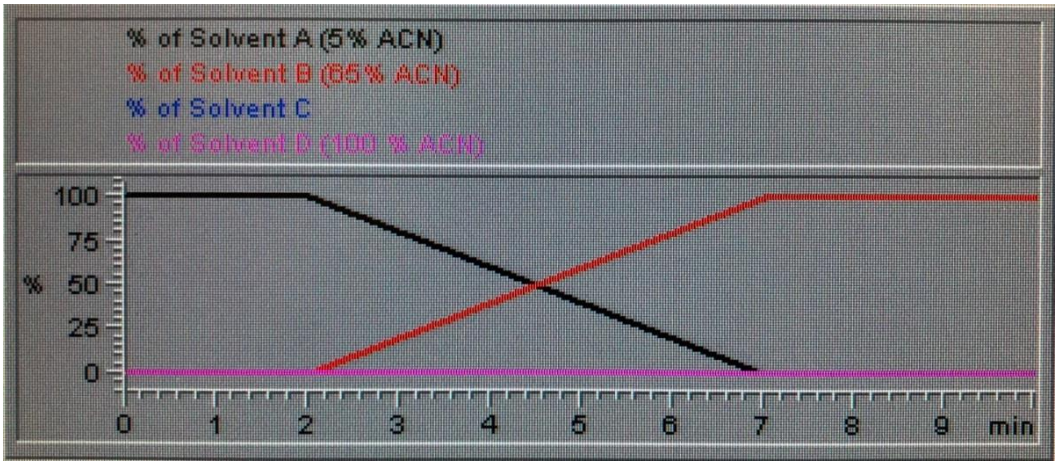

Figure 4: Gradient for the analysis of urine samples containing derivatized metformin

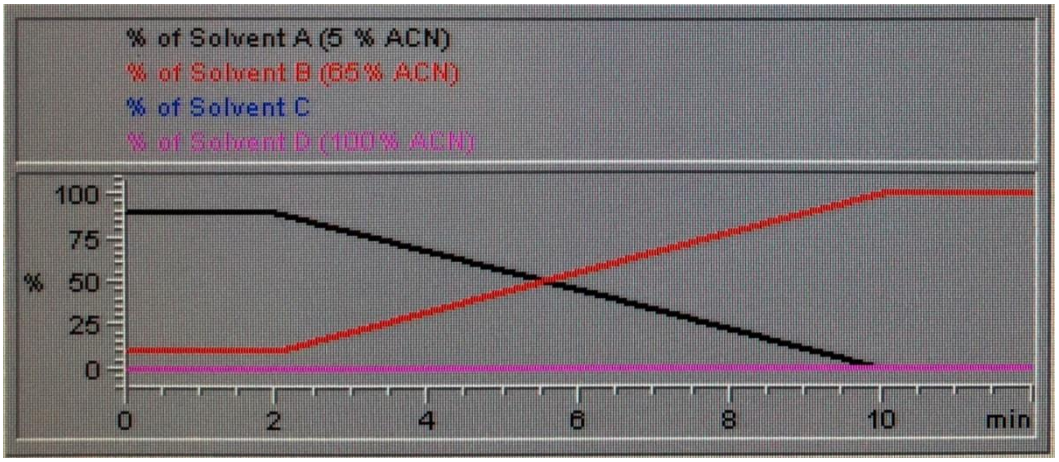

Figure 5: Gradient for the analysis of plasma samples containing derivatized metformin

2.2 Part 2 – Melatonin

2.2.1 Melatonin standard

Calibrations were done by dissolving melatonin (purchased from Carl Roth) in ultrapure water.

2.2.2 Sample preparation

The samples were deep frozen at -85 °C prior to milling to receive a fine powder.

Cranberries

0.1 g of milled cranberries were used for extraction with 500 µl of methanol. The solution was put on a shaker for 5 min and then centrifuged for 5 min at 14,000 rpm. The liquid layer was then transferred to another reaction tube and dried under N_2. The residue was then dissolved in 500 µl H_2O.

White mustard seeds

1 g of milled mustard seeds was used for extraction with 3 ml of methanol. To remove lipids present in the seeds, 3 ml of hexane were added. To prevent the melatonin from moving into the hexane phase, a drop of HCl was used to ensure protonation. The solution was put on a shaker for 5 min, then the hexane phase was removed and the remaining solution put into the centrifuge for 5 min at 14,000 rpm. The liquid layer was then used for analysis.

2.2.3 Derivatization

First method

Derivatization was done by pipetting 200 µl of the extract into an HPLC vial, adding 20 µl of sodium carbonate in H_2O (300 mM) and 20 µl of hydrogen peroxide in H_2O (10 mM). The vial was tightly capped and heated at 90 °C for 30 min. It was then put into the HPLC for analysis.

Second method

The melatonin was analyzed without derivatization.

2.2.4 HPLC method

First method

For the analysis of the melatonin derivative, an isocratic elution using a reversed phase C18 column was applied with the parameters listed in Table 2.

Table 2: HPLC method for the analysis of derivatized melatonin samples

Injection volume	10 µl
Flow rate	0.6 ml/min
Run time	7 min
Post time	-
Detection	Fluorescence
Excitation	244 nm
Emission	378 nm
Column	Kinetex® 5 µm EVO C18 100 Å (LC column 150 × 3 mm, Phenomenex, Aschaffenburg, Germany)
Temperature	40 °C
Solvent	100 mM sodium phosphate buffer pH 7, 12% ACN

Second method

For the analysis of the underivatized samples, a gradient elution with the parameters listed in Table 3 was applied.

Table 3: HPLC method for the analysis of underivatized melatonin samples

Injection volume	10 µl
Flow rate	0.5 ml/min
Run time	15 min
Post time	5 min
Detection	Fluorescence
Excitation	280 nm
Emission	350 nm
Column	Kinetex® 5 µm EVO C18 100 Å (LC column 150 × 3 mm, Phenomenex, Aschaffenburg, Germany)
Temperature	Not controlled
Solvent 1	100% ACN
Solvent 2	100% ultrapure H_2O
Gradient	0-6.5 min: 20% ACN/80% H_2O increase to 35% ACN/65% H_2O 6.5-7.5 min: increase to 100% ACN 7.5-15 min: 100% ACN

The gradient for the analysis of the underivatized samples is depicted in Figure 6.

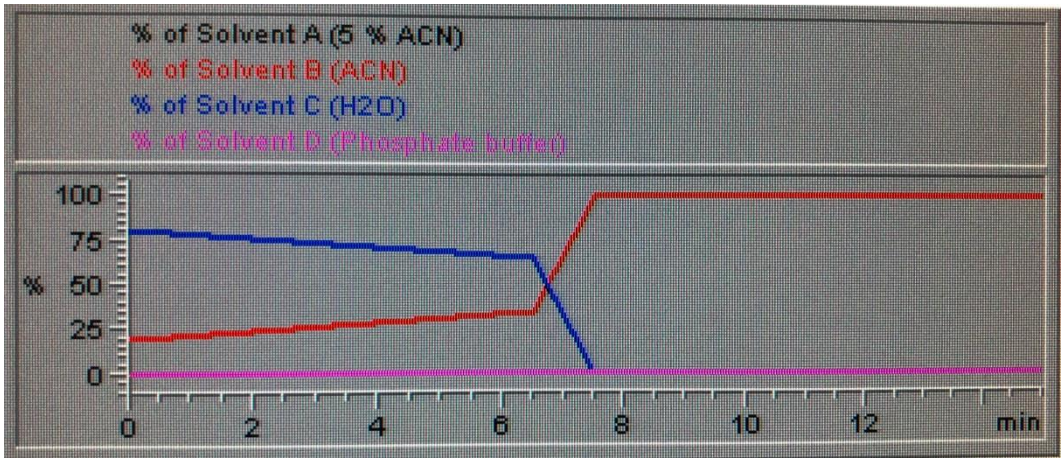

Figure 6: Gradient for the analysis of underivatized melatonin samples

3 Results

3.1 Part 1 – Metformin

3.1.1 Calibration

The initial calibration for tests of the urine procedure were done with the stock prepared from the provided metformin tablets shown in Figure 7.

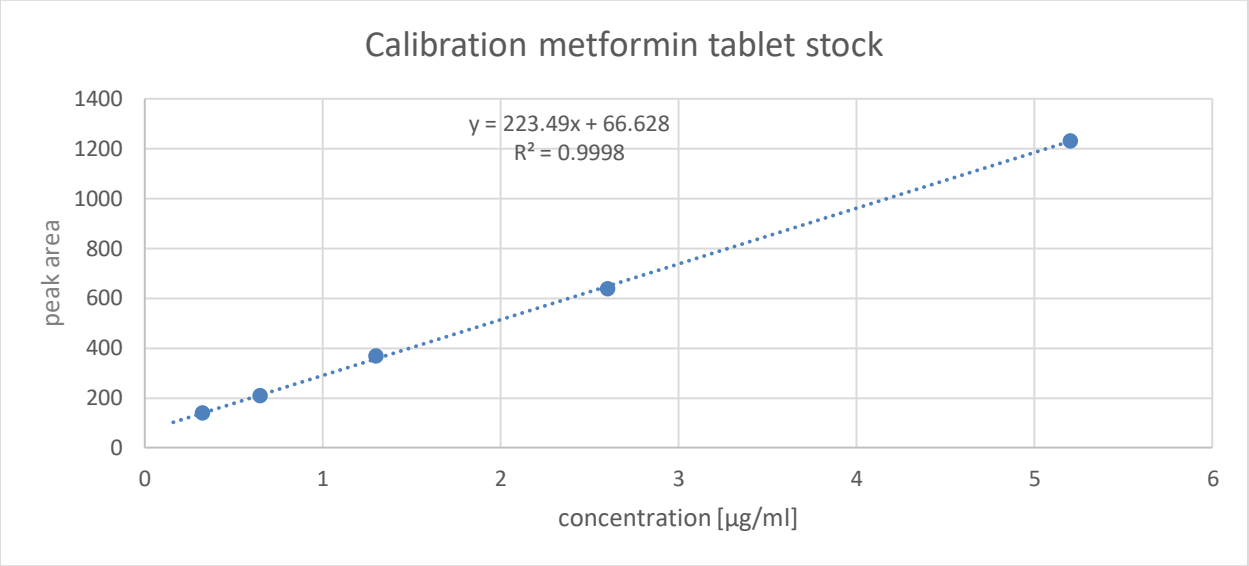

Figure 7: Calibration of metformin using the metformin Hexal tablets

All calculations to determine sample concentrations were done based on the calibrations using the metformin standard depicted in Figure 8 and Figure 9.

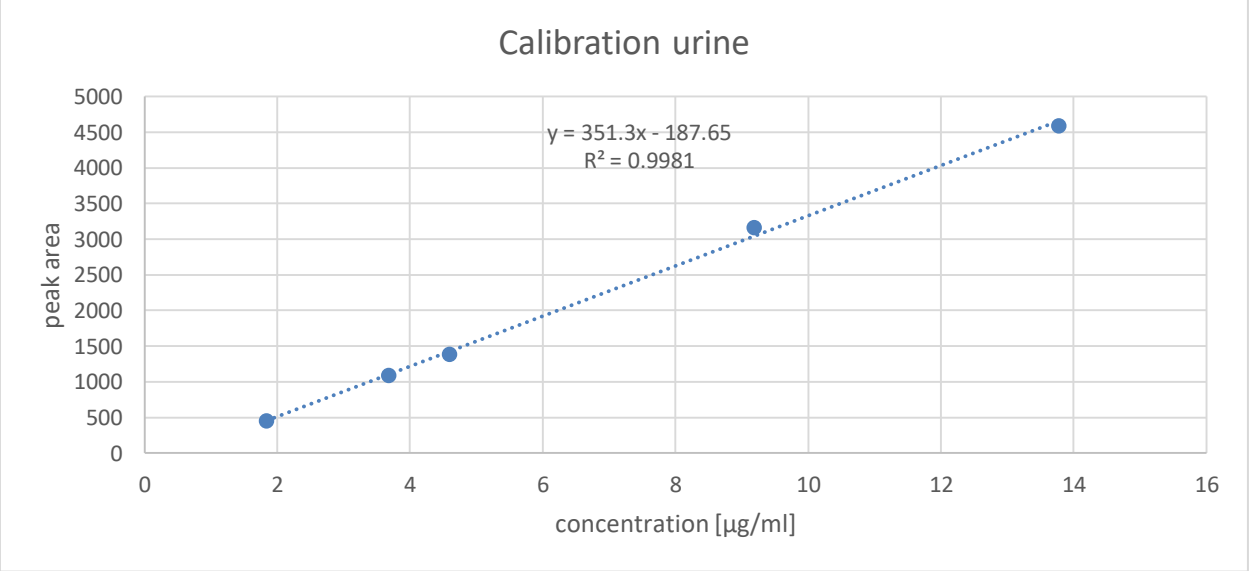

Figure 8: Calibration of metformin in urine using the metformin standard purchased from Sigma Aldrich

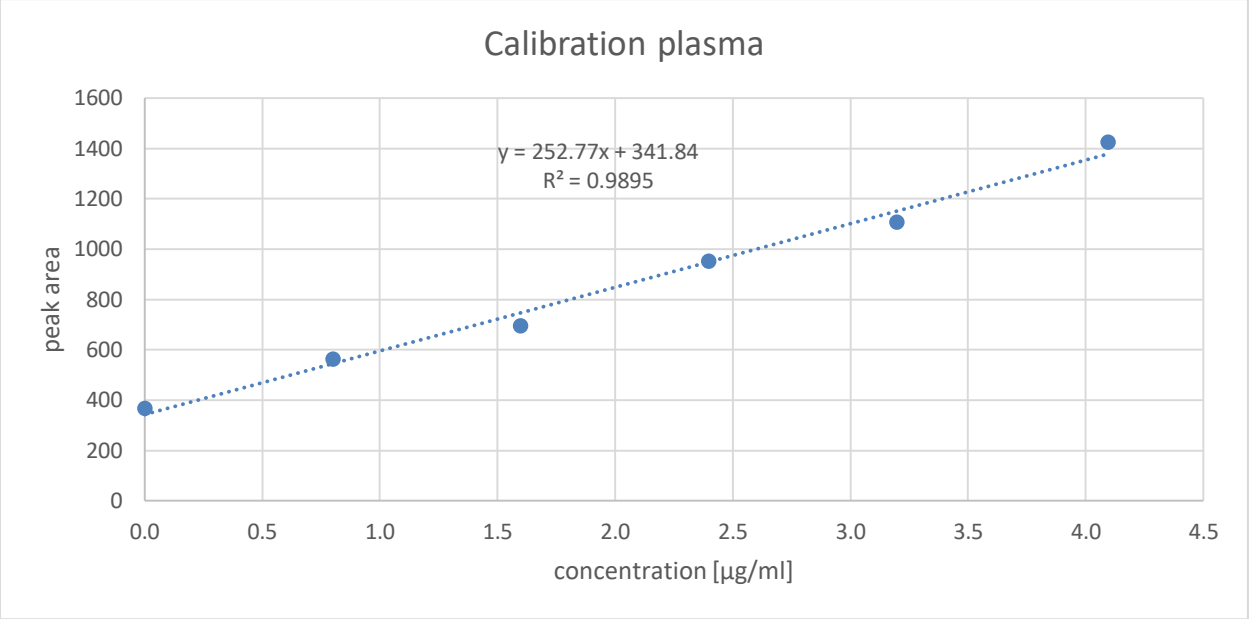

Figure 9: Calibration of metformin in blank plasma using the metformin standard purchased from Sigma Aldrich

3.1.2 Standard addition

For the standard addition, 800 µl of a sample was spiked with 200 µl of different concentrations of melatonin as shown in Figure 10 and Figure 11.

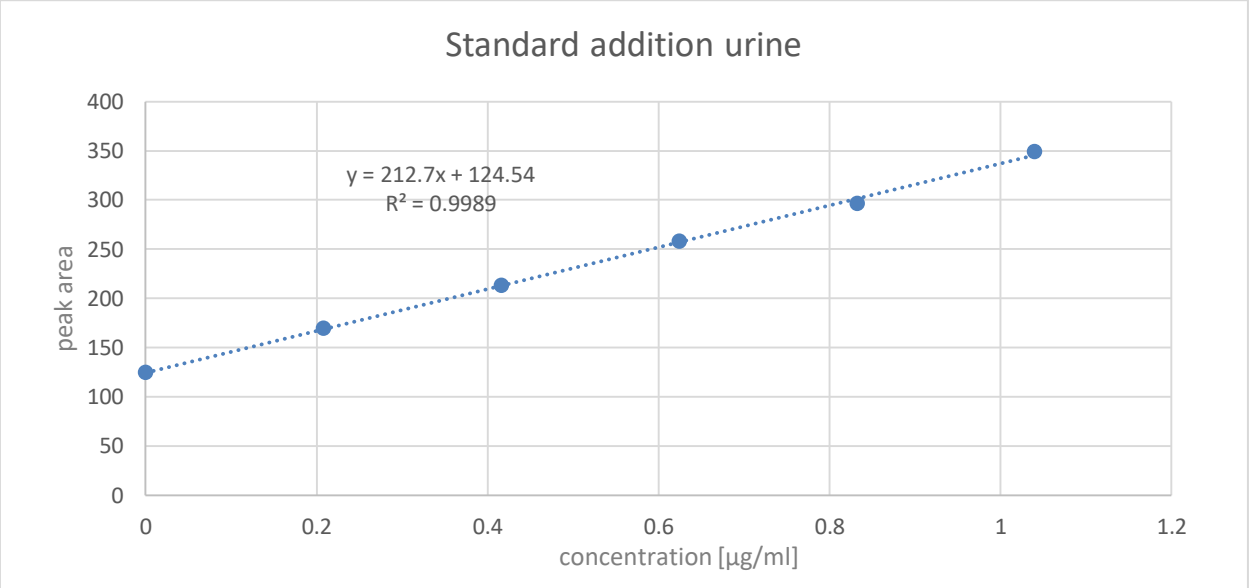

Figure 10: Standard addition of metformin standard to the urine sample 108 t_{2h}

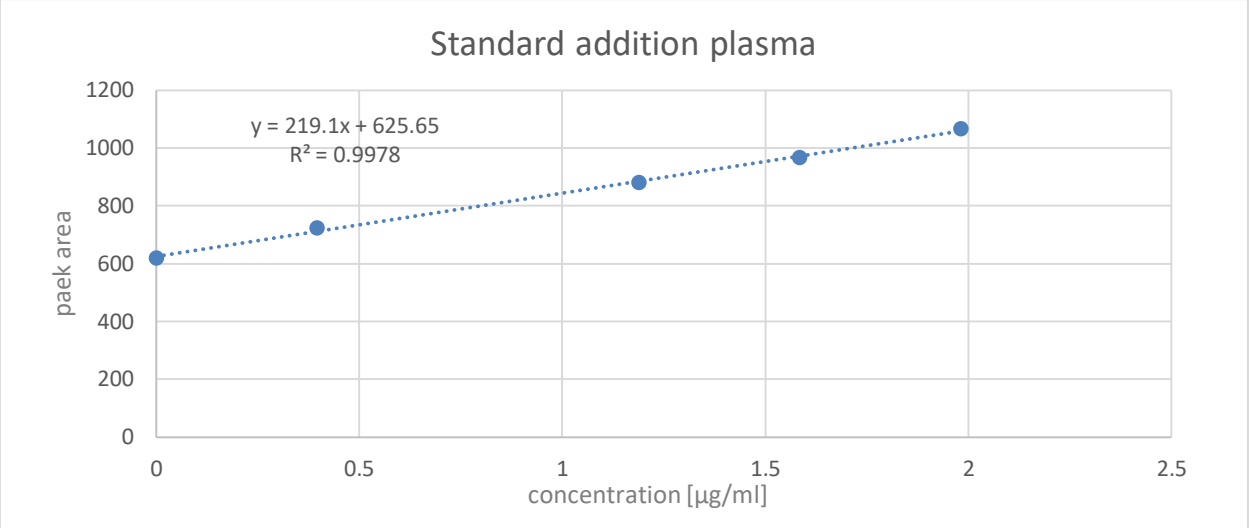

Figure 11: Standard addition of metformin standard to the plasma sample 45 t_{2h}

The data from the standard addition can be used to calculate the recovery by dividing the slope of the standard addition with the slope of the calibration. As the standard addition for urine was still done with the stock produced from the tablets, the slope of the calibration with the tablets was used.

Recovery for urine:

$$\frac{212.7}{223.5} * 100 = 95\%$$

Recovery for plasma:

$$\frac{219.1}{252.8} * 100 = 87\%$$

3.1.3 Samples

The urine samples were diluted 1:100 or 1:50 to achieve good results while the plasma samples were analyzed undiluted. The concentration was calculated as follows:

$$\frac{(peak\ area * dilution) - intercept}{slope} = concentration$$

The results are listed in the appendix in Table 7 along with the doses and other pharmaceuticals administered to the patient by the Medical University of Graz.

Selected probes and standards were analyzed several times to monitor HPLC performance over time.

3.2 Part 2 – Melatonin

3.2.1 Calibration

All calculations of melatonin concentrations were done based on the calibration using the melatonin standard. Two calibrations were done, one for the derivatized samples depicted in Figure 12 and one for the underivatized samples shown in Figure 13.

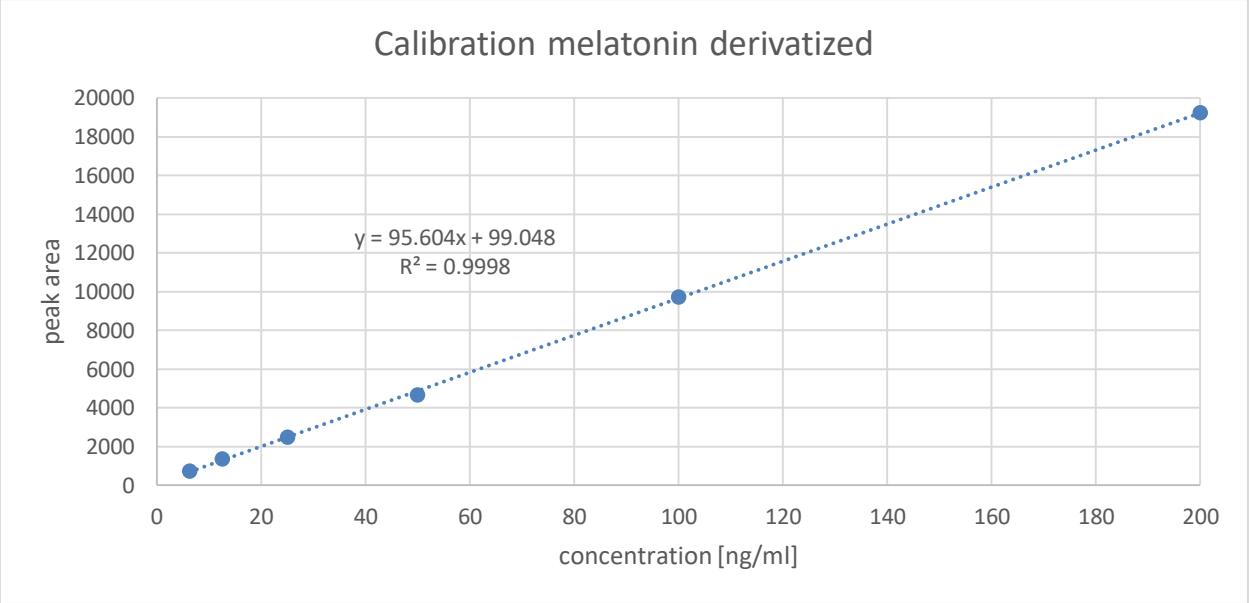

Figure 12: Calibration of melatonin standard using the derivatization method

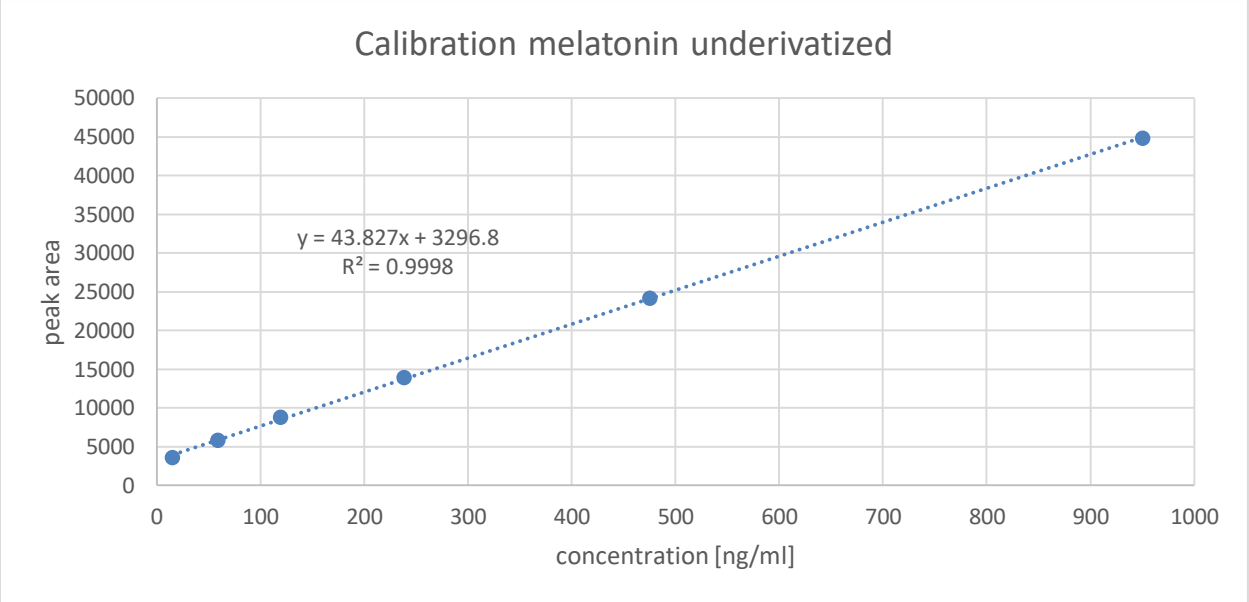

Figure 13: Calibration of melatonin standard using the method without derivatization

3.2.2 Standard addition

As the analysis with the derivatized samples was without success, standard addition was only conducted with underivatized samples. 200 µl of sample was spiked with 200 µl of melatonin in different concentrations as shown in Figure 14.

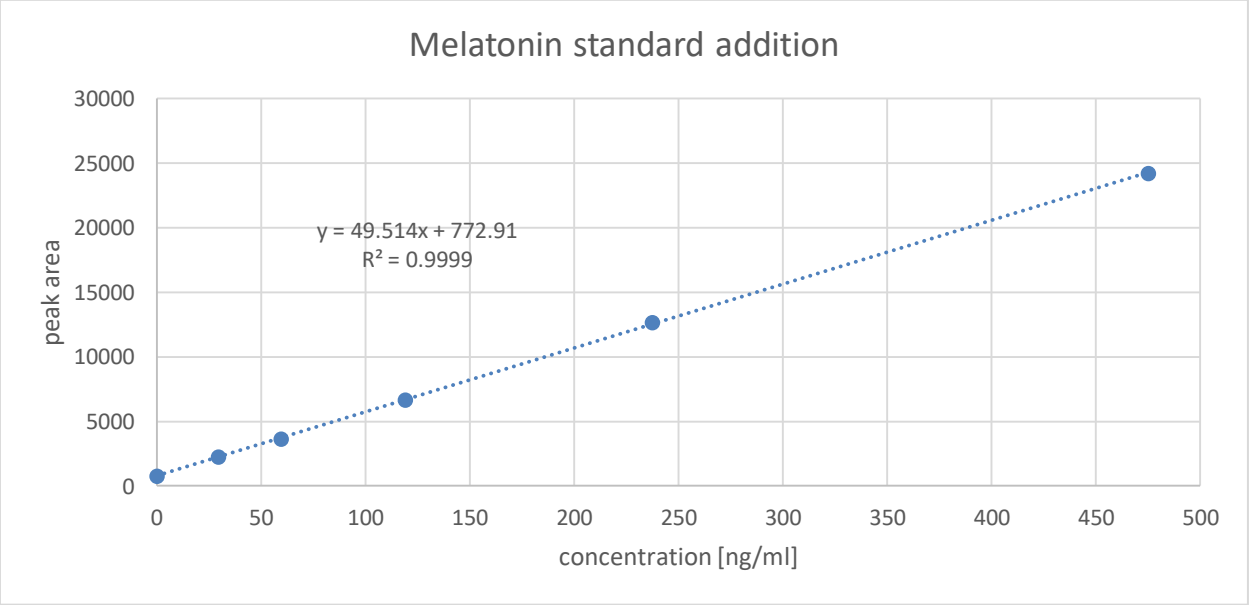

Figure 14: Calibration of melatonin standard using the method without derivatization

The recovery was therefore also only calculated for the underivatized samples:

$$\frac{49.5}{43.8} * 100 = 113\%$$

3.2.3 Samples

The samples were measured undiluted. The concentration was calculated as follows:

$$\frac{(peak\ area * dilution) - intercept}{slope} = concentration$$

The results are listed in Table 4.

Table 4: Results of the analysis of melatonin in cranberries and white mustard seeds

Sample	Underivatized	Derivatized
Cranberries	n.d.	n.d.
White mustard seeds	145 ng/g	

4 Discussion

4.1 Part 1 – Metformin

The derivatization method enabled sensitive detection and appropriate chromatographic separation of the metformin fluorophore from other compounds present due to the sample matrix. The emission spectrum of the metformin derivative is depicted in Figure 15.

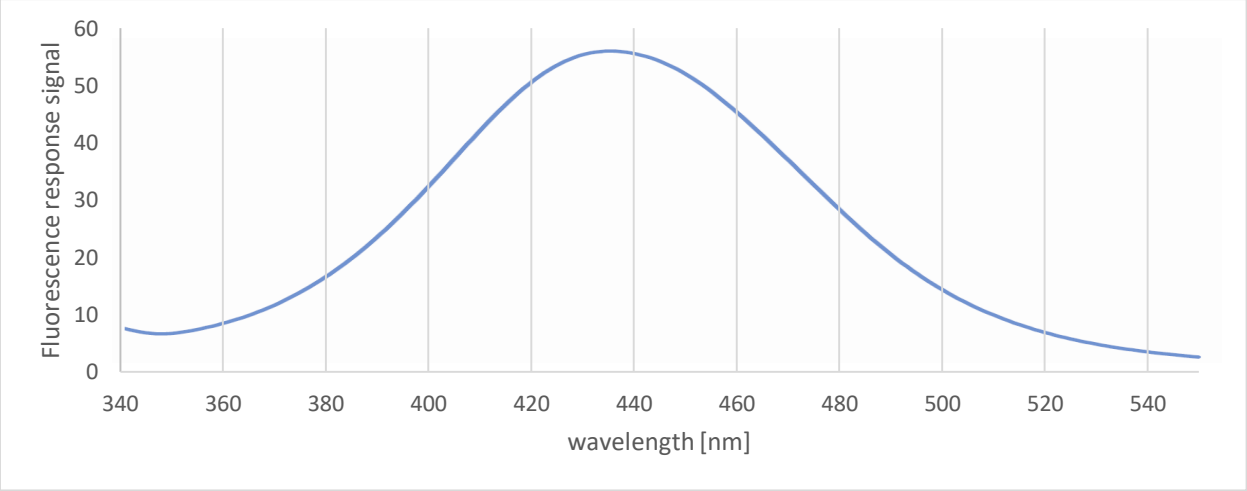

Figure 15: Emission spectrum for the metformin fluorophore, excitation at 280 nm

The increase of polarity in the mobile phase over time eluted the fluorophore at 8.6 minutes for the urine samples and at 8.1 minutes for the plasma samples. Example chromatograms of both sample types are depicted in Figure 16 and Figure 17.

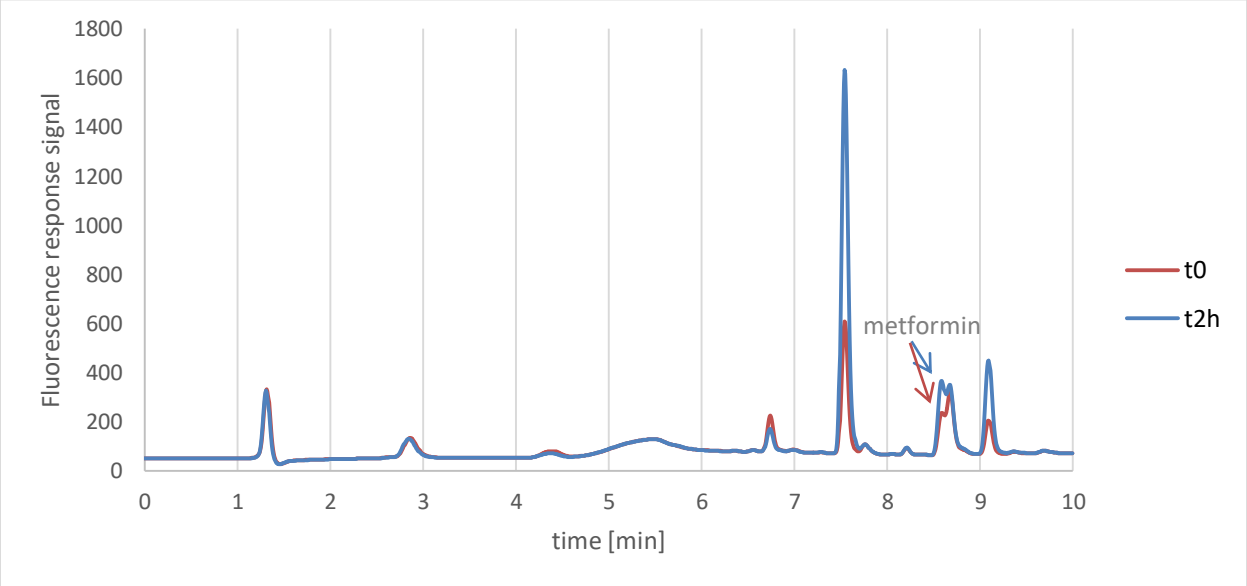

Figure 16: Example chromatogram of urine sample 112 at time point 0 and 2 hours after administration

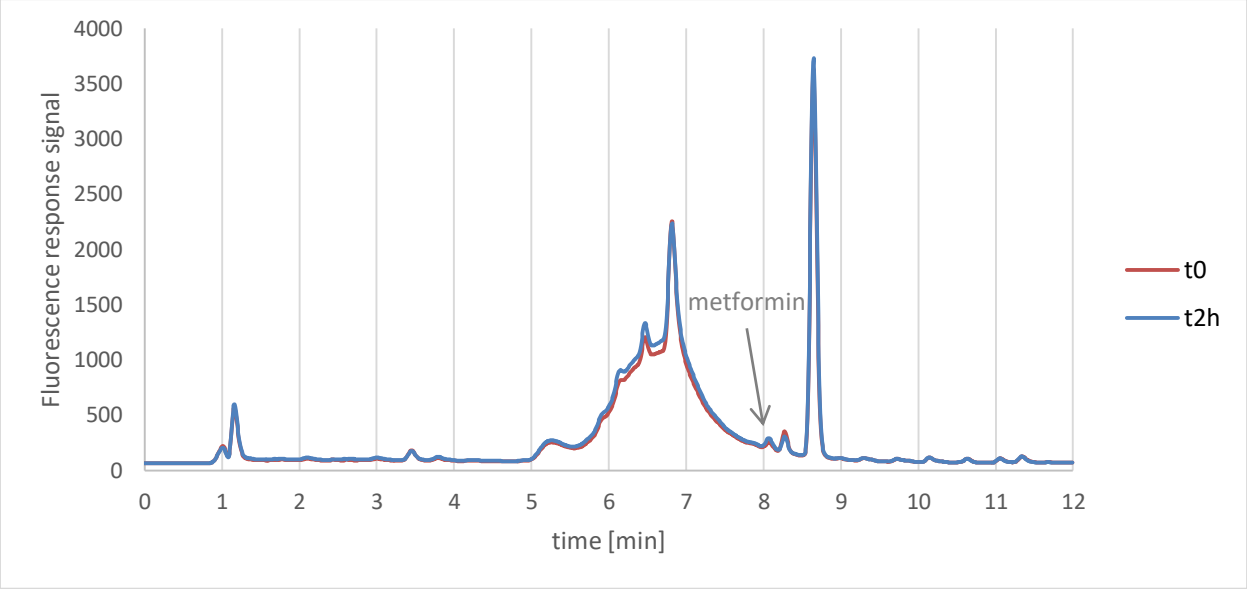

Figure 17: Example chromatogram of plasma sample 100 at time point 0 and 2 hours after administration

For the validation of the HPLC-method, standard addition experiments were conducted and the recovery was calculated. A recovery of 95% for the urine and 87% for the plasma samples was achieved. In general, recovery values between 80 and 120% are acceptable. Linearity was also confirmed by calibrations as can be seen in Figure 8 and Figure 9 which were then used to determine the concentrations of metformin in the samples. The limit of detection was found to be about 40 µg/ml in urine samples and 200 ng/ml in plasma samples, while the limit of quantification was approximately three times higher at about 120 µg/ml for urine samples and 650 ng/ml in plasma samples.

The results obtained from the samples are listed in Table 7 in the appendix. In total, 120 patients were part of this project with samples collected 0 and 2 hours after administration of metformin medication for both urine and plasma. Time point 0 hours represents the base metformin level in the individual while around the time point 2 hours the maximum concentration of metformin in the plasma is reached [31]. A short excerpt of results for both urine and plasma is shown in Figure 18 and Figure 19.

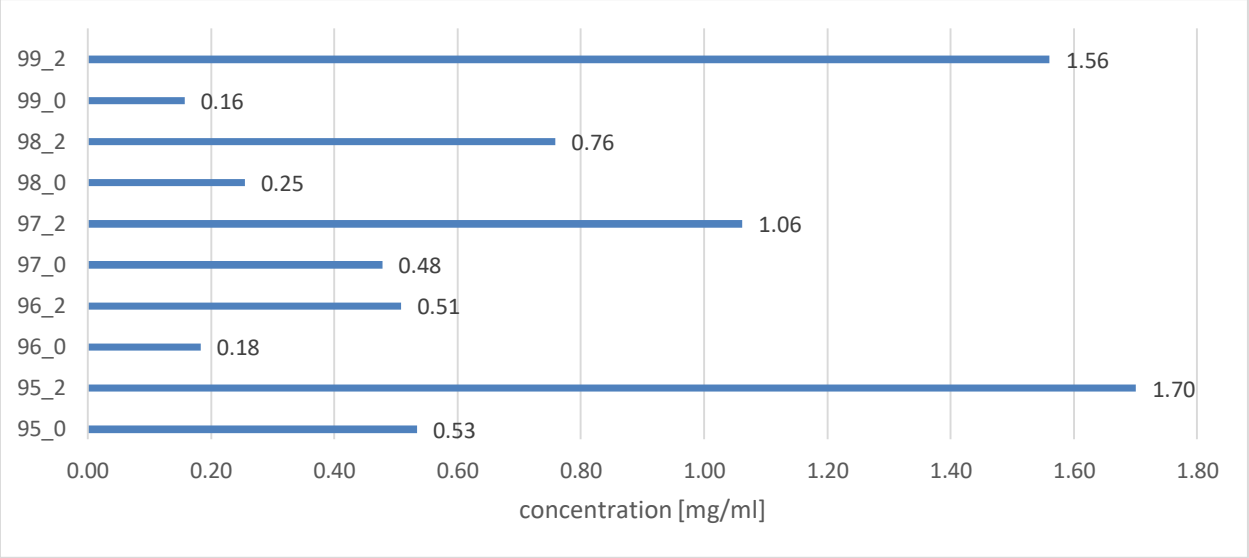

Figure 18: Excerpt of results for the urine samples of patients 95-99 at both time points labeled as "patient_timepoint"

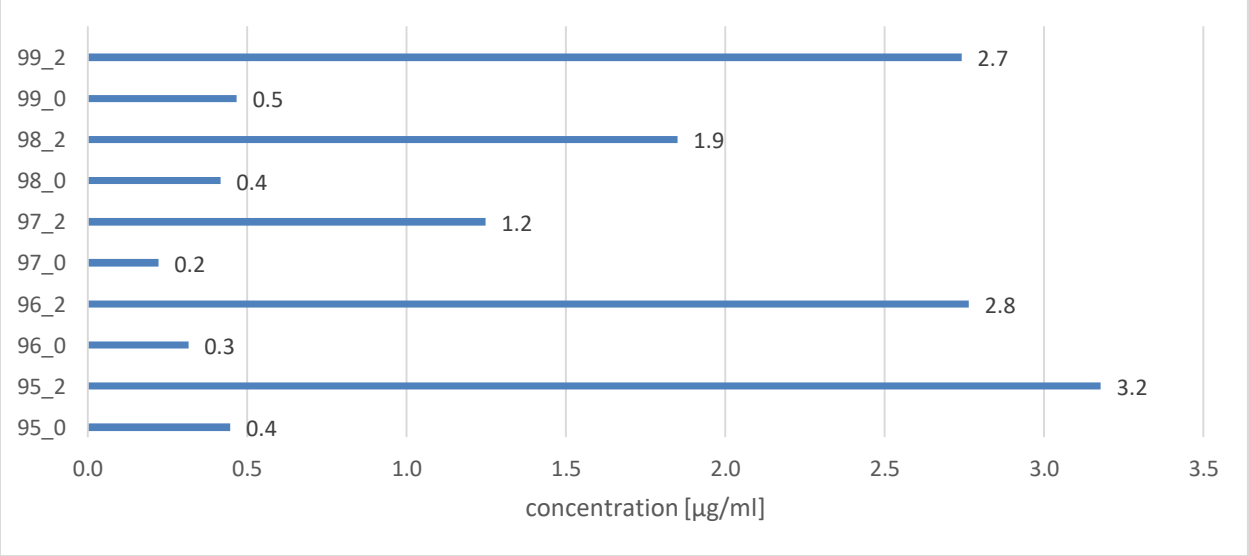

Figure 19: Excerpt of results for the plasma samples of patients 95-99 at both time points labeled as "patient_timepoint"

These samples show a low concentration for the time point at 0 hours and an appropriate increase for the time point at 2 hours. Patient 97 received a dose of 850 mg metformin, the dose for the others shown in this excerpt was 1000 mg metformin. This is also reflected by a slightly lower concentration in the plasma of patient 97.

According to literature, a dose of 500 mg of metformin medication leads to 1-1.2 µg/ml metformin in the plasma after 2 hours [31]. On the assumption of a linear relationship, doses of 850 mg and 1000 mg would then lead to a concentration of about 1.7-2 and 2-2.4 µg/ml,

respectively. Subtracting the concentration at t_0, the plasma t_{2h} samples shown in Figure 19 would only partly support that statement. Patient 97 and 98 show lower concentrations than expected, while patient 95 shows slightly higher concentrations. These variations might be better understood when the data is paired with a genotyping analysis done by the Medical University of Graz.

The concentration of metformin in urine varies greatly depending on dose, additional medication and individual from below detection limit to 2.53 mg/ml at t_0 and 4.16 mg/ml at t_{2h}. The average concentration is 0.49 mg/ml for t_0 and 0.87 mg/ml for t_{2h}. The same is true for the plasma samples; here the concentration varies from below detection limit to 6.2 µg/ml at t_0 and 6.8 µg/ml at t_{2h}. The average concentration is 1.1 µg/ml for t_0 and 2.7 µg/ml for t_{2h}.

4.2 Part 2 – Melatonin

The fluorescence signal emitted by melatonin itself enabled quantification of melatonin in samples using HPLC without precolumn derivatization. The emission spectrum at 350 nm is depicted in Figure 20.

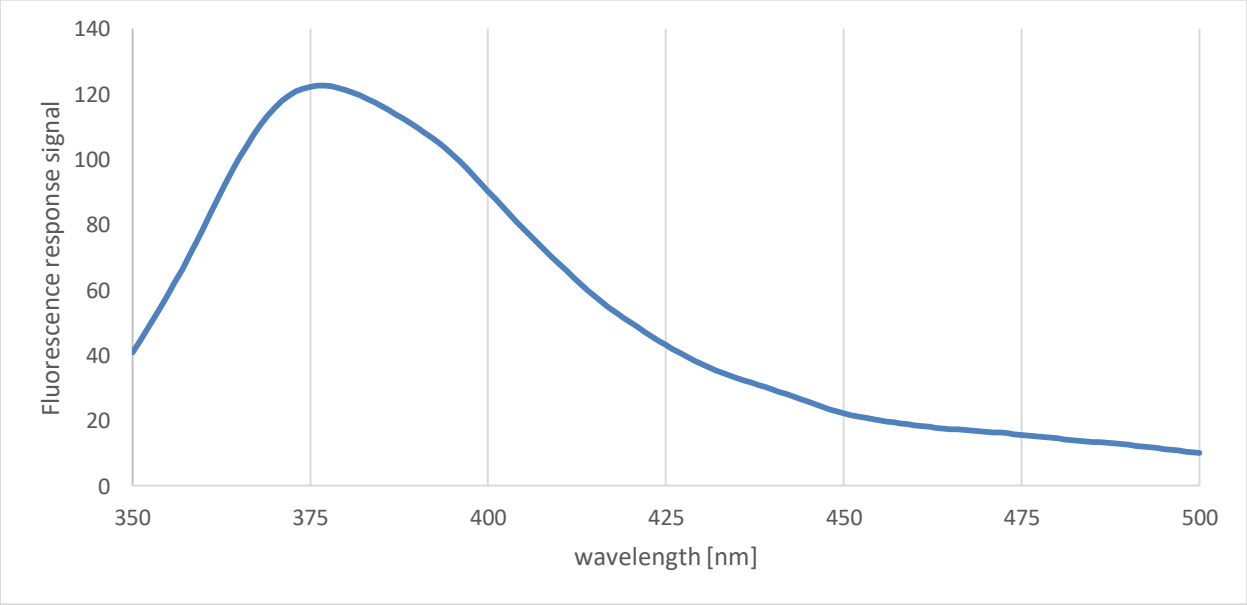

Figure 20: Emission spectrum for melatonin, excitation at 280 nm

An increase in the ACN concentration in the mobile phase over time eluted melatonin at 6.7 minutes. The chromatogram of the melatonin standard at a concentration of 950 ng/ml is depicted in Figure 21.

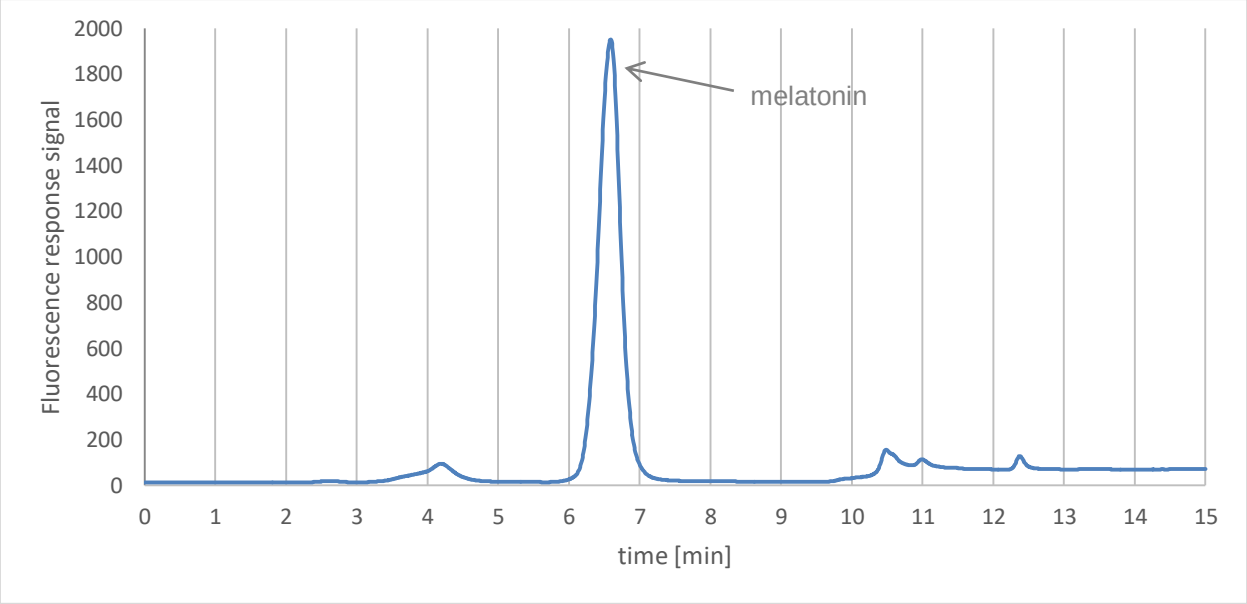

Figure 21: Chromatogram of melatonin standard, 950 ng/ml

Again, for the validation of the HPLC-method, standard addition experiments were conducted and the recovery was calculated. A recovery of 113% for the white mustard seeds was calculated. Linearity was confirmed by calibrations as can be seen in Figure 14. The calibration was then used to determine the concentrations of melatonin in the samples. The limit of detection was found to be about 50 pg/ml, while the limit of quantification was approximately four times higher at about 220 pg/ml.

Using the cranberry sample “Arándanos deshidratados” received from a contact in Chile, neither of the methods delivered any results.

Whole white mustard seeds purchased from Kotányi (Wien, Austria, lot number L 343543 061849) were then only measured without derivatization and showed concentrations of 145 ng/g on average. According to literature, white mustard seeds have an average melatonin concentration of 129 ng/g [21], [22].

5 References

[1] B. J. Goldstein and D. Müller-Wieland, Type 2 diabetes: principles and practice, *Informa Healthcare*, p. 608, 2016.

[2] C. J. Bailey and R. C. Turner, Metformin, *N. Engl. J. Med.*, **334**, 574–579, 1996.

[3] C. J. Dunn and D. H. Peters, Metformin, *Drugs*, **49**, 721–749, 1995.

[4] S. M. D. Danish *et al.*, Role of anti-diabetic drugs as therapeutic agents in Alzheimer's disease, *EXCLI J.*, **14**, 684–696, 2015.

[5] M. Ohta *et al.*, Determination of a biguanide, metformin, by high-performance liquid chromatography with precolumn fluorescence derivatization, *Anal. Sci.*, **9**, 217–220, 1993.

[6] A. Zarghi *et al.*, Rapid determination of metformin in human plasma using ion-pair HPLC, *J. Pharm. Biomed. Anal.*, **31**, 197–200, 2003.

[7] K. H. Yuen and K. K. Peh, Simple high-performance liquid chromatographic method for the determination of metformin in human plasma, *J. Chromatogr. B Biomed. Sci. Appl.*, **710**, 243–246, 1998.

[8] D. Ruggiero-Lopez *et al.*, Reaction of metformin with dicarbonyl compounds. Possible implication in the inhibition of advanced glycation end product formation., *Biochem. Pharmacol.*, **58**, 1765–1773, 1999.

[9] S. Tanabe, T. Kobayashi, and K. Kawanabe, Determination of oral hypoglycemic biguanides by high performance liquid chromatography with fluorescence detection, *Anal. Sci.*, **3**, 69–73, 1987.

[10] G. Mubeen and K. Noor, Spectrophotometric method for analysis of metformin hydrochloride, *Indian J. Pharm. Sci.*, **71**, 100–102, 2009.

[11] S. A. Majidano and M. Y. Khuhawar, GC determination of famotidine, ranitidine, cimetidine, and metformin in pharmaceutical preparations and serum using methylglyoxal as derivatizing reagent, *Chromatographia*, **75**, 1311–1317, 2012.

[12] L. Sonnleitner, Antidiabetic drug metformin in human lithium heparin plasma with RP-HPLC analysis, Master Thesis, Technical University of Graz, 2017.

[13] V. Buchgraber, Development and validation of a chromatographic method for analysis of amines with low reactivity in biological matrices, Master Thesis, Technical University of Graz, 2017.

[14] S. R. Pandi-Perumal *et al.*, Melatonin: Nature's most versatile biological signal?, *FEBS J.*, **273**, 2813–2838, 2006.

[15] H.-S. Yu and R. J. Reiter, Melatonin: Biosynthesis, physiological effects, and clinical applications, *CRC Press*, 1992.

[16] J. Arendt, Melatonin, *Clin. Endocrinol. (Oxf).*, **29**, 205–229, 1988.

[17] R. Hardeland, S. R. Pandi-Perumal, and D. P. Cardinali, Melatonin, *Int. J. Biochem. Cell Biol.*, **38**, 313–316, 2006.

[18] A. Chabra *et al.*, Melatonin ameliorates oxidative stress and reproductive toxicity induced by cyclophosphamide in male mice, *Hum. Exp. Toxicol.*, **33**, 185–195, 2014.

[19] A. Brzezinski, Melatonin in humans, *N. Engl. J. Med.*, **336**, 186–195, 1997.

[20] M. B. Arnao and J. Hernández-Ruiz, The physiological function of melatonin in plants, *Plant Signal. Behav.*, **1**, 89–95, 2006.

[21] L. C. Manchester *et al.*, High levels of melatonin in the seeds of edible plants: Possible function in germ tissue protection, *Life Sci.*, **67**, 3023–3029, 2000.

[22] M. M. Posmyk and K. M. Janas, Melatonin in plants, *Acta Physiol. Plant.*, **31**, 1–11, 2009.

[23] H. Shi, J. Love, and W. Hu, Editorial: Melatonin in Plants, *Front. Plant Sci.*, **8**, 1666, 2017.

[24] R. Caniato *et al.*, Melatonin in plants, *Adv. Exp. Med. Biol.*, **527**, 593–597, 2003.

[25] D.-X. Tan *et al.*, Functional roles of melatonin in plants, and perspectives in nutritional and agricultural science, *J. Exp. Bot.*, **63**, 577–597, 2012.

[26] A. Hattori *et al.*, Identification of melatonin in plants and its effects on plasma melatonin levels and binding to melatonin receptors in vertebrates, *Biochem. Mol. Biol. Int.*, **35**, 627–34, 1995.

[27] F. Iinuma *et al.*, Sensitive determination of melatonin by precolumn derivatization and reversed-phase high-performance liquid chromatography, *J. Chromatogr. A*, **835**, 67–72, 1999.

[28] P. Mürzl, Melatonin, eine physiologisch wirksame Substanz in Lebensmitteln, Master Thesis, Technical University of Graz, 2012.

[29] H. S. Stoker, General, organic, and biological chemistry, *Cengage Learning US*, p. 1232, 2015.

[30] P. Cavatorta, R. Favilla, and A. Mazzini, Fluorescence quenching of tryptophan and related compounds by hydrogen peroxide, *Biochim. Biophys. Acta - Protein Struct.*, **578**, 541–546, 1979.

[31] C.-L. Cheng and C.-H. Chou, Determination of metformin in human plasma by high-performance liquid chromatography with spectrophotometric detection, *J. Chromatogr. B Biomed. Sci. Appl.*, **762**, 51–58, 2001.

6 Appendix

Table 5: List of chemicals

Substance	Specifications	Producer
2-Mercapto-ethanol	≥99.0%	Sigma Aldrich
Acetonitrile	HPLC grade	Chem-Lab
Benzoin	≥99.5%	Sigma Aldrich
di-Sodium hydrogen phosphate dihydrate	≥99.5%	Roth
Ethanol	96%	Chem-Lab
n-Hexane	95%	J.T.Baker
Hydrochloric acid (HCl)	Fuming 37%	Roth
Hydrogen peroxide	30%	Fluka
Melatonin	≥97%	Roth
Metformin hydrochloride		Sigma Aldrich
Methanol	HPLC grade	Chem-Lab
Potassium hydroxide (KOH)		J.T.Baker
Sodium carbonate monohydrate		Merck
Sodium dihydrogen phosphate monohydrate		Merck
Sodium thiosulfate pentahydrate		Merck
Tetrahydrofuran (THF)	For liquid chromatography	Merck
Tris(hydroxymethyl)amino methane (TRIS)	≥99.9%	Roth

Table 6: List of equipment

Equipment	Specifications	Producer
Pipettes	Pipetman 100-1000 µl, 20-200 µl	Gilson
	Transferpettor 1000 µl	Brand
Pipette tips	Pipettor tips standard universal, 100-1000 µl	Roth
	Plastibrand 200 µl	Brand
Micropipettes	10 µl, 20 µl	Brand
Water purification	Ultra Clear System	Siemens
Scales	Entris2201i-1S	Sartorius
	AG245	Mettler Toledo
Thermomixer	Thermomixer comfort	Eppendorf
Shaker	Vibrax VXR basic	IKA
Centrifuge	5804 R	Eppendorf
	Z 323	Hermle
Ultrasonic bath	Transsonic T460	Elma
Reaction tubes	Microcentrifuge tubes with lid, PP, 2.0 ml	Brand
	Microcentrifuge tubes with lid, 1.5 ml	Brand
pH meter	pH 3110	WTW
pH paper		Macherey-Nagel
Lid locks	Multi-lid locks for microcentrifuge tubes 1.5/1.7 ml	Roth
HPLC vials	1.5 ml Crimp Neck Vial, 32 x 11.6 mm, clear glass	LLG labware
	11 mm Combination Seal: Aluminium cap	LLG
Ice machine	AF 200	Scotsman
Filter paper	MN 615 ¼, ø150 mm	Macherey-Nagel
Mill	A11 basic	IKA
HPLC	1100 Series	Hewlett Packard

Table 7: Samples analyzed with patient number, time point of sampling, dose of metformin and additional pharmaceuticals administered as well as the detected mean values of metformin in urine and plasma

patient	time point [h]	dose [mg]	mean urine [mg/ml]	mean plasma [µg/ml]	additional pharmaceuticals: dose morning-noon-evening
13	0	1000	0.62		
	2		2.37		
19	0	1000	1.91		Forxiga (10.01.2017-06.02.2017)
	2		3.45		
20	0	1000	0.02		Insulin
	2		0.20		
21	0	1000	0.57		
	2		0.90		
22	0	850	0.69		
	2		1.30		
23	0	1000	1.17		
	2		0.62		
24	0	850	0.15		
	2		0.52		
25	0	850	0.33		
	2		0.25		
26	0	850	0.16		
	2		0.64		
27	0	1000	0.22		
	2		1.89		
28	0	1000	0.82		
	2		0.41		
29	0	1000	0.09		
	2		1.03		
30	0	500	0.08		
	2		0.24		
31	0	1000	0.66		
	2		1.12		
32	0	1000	1.46	1.2	Diamicron 30 mg: 1-0-0
	2		3.75	4.7	
33	0	500	0.17		
	2		0.49		
34	0	1000	0.31		Diamicron 30 mg: 1-0-0
	2		0.40		
35	0	1000	0.16		Amaryl 2 mg: 1-2 per month
	2		0.50		
36	0	1000	0.001		
	2		0.001		

37	0	1000	1.71		
	2		0.68		
38	0	1000	0.54		Forxiga 10 mg: 1-0-0
	2		0.65		
39	0	1000	0.55		
	2		0.46		
40	0	1000	0.17		Forxiga 10 mg: 1-0-0
	2		0.17		
41	0	1000	0.02		Forxiga 10 mg: 1-0-0
	2		0.02		
42	0	1000	0.39	2.5	Trajenta 10 mg: 1-0-0
	2		0.32	4.5	
43	0	1000	0.61		Diamicron 30 mg: 1-0-0
	2		0.72		
44	0	1000	1.56	2.0	
	2		1.06	2.6	
45	0	1000	0.35	0.9	Glizlazid 30 mg: 1-0-0
	2		1.93	4.0	
46	0	1000	0.55	2.7	Glicada 60 mg: 1-0-0
	2		0.49	2.8	
47	0	1000	0.21	0.8	Jardiance 10 mg: 1-0-0
	2		0.40	1.6	
48	0	850	0.11	0.7	
	2		0.37	2.1	
49	0	1000	0.25	0.9	Insulin Lantus: 20 mg in the evening Novo Rapid: to meals
	2		0.52	1.5	
50	0	1000	0.11	1.2	Insulin, Novo? Patient does not know
	2		2.32	6.1	
51	0	1000	0.05	0.9	
	2		1.58	2.3	
52	0	1000	0.09	0.7	NovoMix 70: 12 units in the morning NovoMix 50: 12 units in the evening
	2		0.21	1.7	
53	0	850	0.97	1.0	Jardiance 10 mg
	2		0.93	2.4	
54	0	1000	1.03	1.1	Lantus: 18-0-32 units Aprida: 24-20-16 units
	2		3.13	3.3	
55	0	1000	2.24	1.7	Repaglinid 0.5 mg: 1-1-1 Levemir Penfill 100 units/ml 3 ml: 0-0-32 units
	2		0.43	3.0	Victoza Pen 6 mg/ml: 1.8-0-0 units, before breakfast
56	0	1000	0.63	1.9	Bydureon 2 mg: once per week
	2		1.58	3.3	

57	0	1000	1.04	1.9	
	2		0.98	3.2	
58	0	1000	0.07	0.7	Novorapid: 3 × 8 units Insulatad: 3 × 22 units Victoza Pen 6 mg/ml: 1 × 1.8
	2		0.67	1.7	
59	0	1000	0.07	1.1	Humalog 100 units/ml: 0-0-34 units Humaninsulin 100 units/ml: depending in the food
	2		0.09	3.1	
60	0	850	0.01	0.6	
	2		0.09	0.7	
61	0	1000	0.27	1.2	Jardiance 10 mg: 1-0-0
	2		0.51	2.4	
62	0	850	0.05	0.3	Diamicron 30 mg: 2-0-0 Insulin: long-term 24 units/day in the evening
	2		1.11	2.1	
63	0	1000	0.55	0.7	
	2		0.17	2.9	
64	0	850	0.17	1.3	
	2		0.07	2.0	
65	0	1000	1.94	1.4	Glizlazid 30 mg: 1-0-0
	2		2.32	2.8	
66	0	1000	0.05	0.3	
	2		0.45	3.0	
67	0	1000	0.06	n.d.	Humalog 100 units/ml: depending on the food Humaninsulin Lilly 100 units/ml: basal between 4 and 10 units
	2		0.25	1.6	
68	0	500	0.01	0.2	
	2		0.08	0.3	
69	0	1000	0.44	0.1	
	2		1.89	1.7	
70	0	1000	0.80	0.2	
	2		0.20	1.5	
71	0	850	1.28	0.7	Humalog 50 30 units: 1-0-1
	2		2.35	1.5	
72	0	1000	0.04	0.1	Damicron 30 mg: 0-1-0 Jardiance: 1-0-0
	2		0.43	3.0	
73	0	1000	0.45	0.6	Lanuts: 0-0-23 units
	2		2.18	1.7	
74	0	850	1.69	0.5	Glimeperid 2 mg: 1-1-0
	2		0.88	1.8	
75	0	1000	0.43	0.4	Forxiga 10 mg: 1-0-0
	2		0.05	1.8	
76	0	1000	0.54	0.5	Repaglinid 1 mg Diabeticur pro
	2		0.13	1.8	

77	0	1000	n.d.	n.d.	
	2		1.68	3.9	
78	0	1000	0.92	1.5	Forxiga 10 mg: 1-0-0
	2		1.87	2.6	
79	0	1000	0.19	0.5	Forxiga 10 mg: 1-0-0
	2		0.27	2.5	
80	0	1000	0.03	0.2	
	2		0.39	2.0	
81	0	1000	0.85	1.3	Bydureon 2 mg: once per week
	2		0.37	2.6	
82	0	1000	0.38	0.9	
	2		0.23	1.9	
83	0	1000	0.23	n.d.	Jardiance 10 mg: 1-0-0
	2		0.04	3.2	
84	0	1000	0.55	0.6	
	2		0.25	1.7	
85	0	1000	0.30	1.5	Jardiance 10 mg: 1-0-0
	2		0.47	2.9	
86	0	850	0.12	0.4	
	2		0.74	1.6	
87	0	1000	0.04	0.5	
	2		0.22	1.6	
88	0	1000	2.53	0.9	
	2		4.16	2.7	
89	0	1000	0.05	0.1	Jardiance 10 mg: 1-0-0
	2		0.28	1.7	
90	0	1000	2.02	1.3	
	2		0.34	2.6	
91	0	1000	0.03	n.d.	
	2		1.21	0.6	
92	0	1000	1.49	0.9	Jardiance 10 mg: 1-0-0
	2		3.19	1.4	
93	0	1000	0.16	n.d.	
	2		0.34	1.6	
94	0	1000	0.04	0.0	
	2		0.37	1.4	
95	0	1000	0.53	0.4	
	2		1.70	3.2	
96	0	1000	0.18	0.3	
	2		0.51	2.8	
97	0	850	0.48	0.2	Xultophy: as needed
	2		1.06	1.2	

98	0	1000	0.25	0.4	Jardiance 10 mg: 1-0-0
	2		0.76	1.9	
99	0	1000	0.16	0.5	Diamicron: 2-0-1 Victoza: 1-0-0
	2		1.56	2.7	
100	0	1000	0.48	1.2	
	2		0.83	2.7	
101	0	1000	0.84	3.1	Apidra Solastar pre-filled pen: 5-5-4 Lantus Solastar pre-filled pen: 0-0-18
	2		1.61	6.2	
102	0	1000	0.27	0.9	
	2		0.88	3.6	
103	0	1000	0.16	0.7	Huminsulin Lilly: basal 0-0-20
	2		0.87	3.3	
104	0	1000	0.35	0.9	
	2		0.16	2.3	
105	0	500	n.d.	0.9	
	2		0.57	2.1	
106	0	850	0.87	1.1	Januvia 100 mg: 1-0-0
	2		0.24	2.2	
107	0	1000	0.29	1.2	
	2		2.10	3.2	
108	0	850	0.26	1.6	Trulicity 1.5 mg: once per week
	2		0.12	2.4	
109	0	1000	0.36	2.2	Victoza
	2		0.26	3.3	
110	0	1000	0.33	2.0	
	2		0.81	4.0	
111	0	500	0.20	1.2	
	2		0.69	3.1	
112	0	500	0.46	1.4	
	2		0.93	2.7	
113	0	1000	0.19	1.0	Dydureon pre-filled pen 2 mg Glime Pirid 6 mg: 0-0-1
	2		0.55	3.5	
114	0	500	0.01	0.1	
	2		0.004	0.8	
115	0	1000	1.09	0.4	Repaglinid 2 mg: 0-0-1
	2		1.18	1.0	
116	0	500	0.11	1.8	
	2		0.38	1.6	
117	0	500	0.70	5.2	
	2		0.38	6.7	
118	0	1000	0.17	6.2	
	2		0.44	6.3	

119	0	500	0.15	3.9	
	2		0.47	5.8	
120	0	1000	0.14	3.8	
	2		1.29	6.8	

Table 8: Pipette calibration [g] based on the Excel sheet from Wisconsin State Laboratory of Hygiene; Z-factor 1.0033 at 22 °C

	Gilson Pipetman 100-1000 µl				Gilson Pipetman 20-200 µl		
	1000 µl	500 µl	100 µl		200 µl	100 µl	20 µl
	1.0023	0.4943	0.1000		0.1972	0.0990	0.0197
	0.9956	0.4947	0.0999		0.1967	0.0983	0.0197
	0.9971	0.4945	0.0997		0.1964	0.0992	0.0198
	1.0053	0.4943	0.1013		0.1978	0.0997	0.0198
	0.9986	0.4932	0.1007		0.1973	0.0990	0.0197
	0.9957	0.4930	0.0992		0.1987	0.0989	0.0201
	0.9961	0.4937	0.1006		0.1980	0.0993	0.0198
	0.9927	0.4927	0.0995		0.1978	0.0992	0.0200
	0.9923	0.4919	0.1004		0.1972	0.0993	0.0197
	0.9871	0.4951	0.1000		0.1986	0.0988	0.0202
Mean	0.9963	0.4937	0.1001		0.1976	0.0991	0.0199
Corr. mean	0.9996	0.4954	0.1005		0.1982	0.0994	0.0199
STD dev.	0.0051	0.0010	0.0006		0.0008	0.0004	0.0002
RSD	0.5138	0.2050	0.6226		0.3798	0.3736	0.9244
% inacc.	0.0432	0.9261	0.4604		0.8890	0.6031	0.4225
	PASS	PASS	PASS		PASS	PASS	PASS