The effect of experimental diabetes on the intestinal elimination of paracetamol

PhD Thesis

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INTRODUCTION

The role of the small intestine in the elimination of xenobiotics

The small intestine is functionally suitable for the absorption, metabolism and excretion of nutrients, water and various xenobiotics. In case of oral drug intake, drugs first enter the gastrointestinal tract, via the portal vein reach the liver, and then via the hepatic vein get into the site of action or may be secreted into the bile or intestine, from where they may be reabsorbed or eliminated. This process is called enterohepatic circulation. During this process, the given xenobiotic may be transformed, thus reducing the oral bioavailability of a given drug. Although the liver is considered the main metabolizing organ, several phase I and phase II enzymes and transporters are also expressed in the enterocytes that form the surface of the small intestine. The most important of the expressed enzyme families are: cytochrome P450, UDP-glucuronyl transferase, sulfotransferase, glutathione-S-transferase, acetyltransferase, and various peptidases are also expressed. In terms of transporters, the P-gp, BCPR, and MRP2 transporters expressed on the apical (intestinal lumen side) side of enterocytes are involved in the excretion of metabolites produced in enterocytes or delivered via the bloodstream into the small intestine.

Paracetamol

Paracetamol is a moderately water soluble, weak acid (pK_a= 9.3–9.7). The absorption of paracetamol mostly occurs by passive diffusion. Paracetamol is absorbed in the proximal part of the small intestine, mostly in the duodenum and jejunum.

Paracetamol can be metabolized both hepatically and extrahepatically. The largest part is conjugated with UDP-glucuronic acid to form paracetamol β-D-glucuronide (50-70%). A smaller part (25-35%) is sulfated by 3'-phosphoadenosine-5'-phosphosulfate (PAPS), a reaction catalyzed by the sulfotransferase enzyme family. Another possible route of paracetamol metabolism (5-15%) is oxidative transformation catalyzed by CYP 450 enzymes, as a result of which reactive N-acetyl-para-benzoquinone imine (NAPQI) is formed. It has been shown that in addition to CYP enzymes, peroxidases, such as COX enzyme, also participate in the oxidation of paracetamol. If sufficient amounts of glutathione are present in the body, NAPQI is detoxified by conjugation with glutathione. The glutathione conjugate enters the kidney and is degraded to the cysteine conjugate by the enzymes γ-glutamyl transferase (γGT) and cysteinylglycine dipeptidase. It is then acetylated by the enzyme N-acetyl transferase (NAT) and excreted into the urine.

The paracetamol-cysteine conjugate which formed in the kidney can be reabsorbed from the renal tubular lumen into the circulation, reach the liver, where some of it is acetylated by N-acetyl transferase to form paracetamol mercapturate The two metabolites formed are excreted in the bile and reach the intestine, but also can be reabsorbed into the circulation, enter the kidney, and can be excreted into the urine. γGT and dipeptidases (DP) are also found in the bile duct and the intestinal lumen, so the cysteine conjugate can also be formed in these places.

Experimental diabetes and the oxidative stress

Many factors can influence the metabolism of xenobiotics, such as the physicochemical properties of the compound or the pathological state of the organism (hormonal factors, diabetes mellitus). Diabetes mellitus is an endocrine metabolic disorder that affects a significant proportion of the world's population and is currently showing an increasing tendency. The main characteristics of diabetes are hyperglycemia and glucosuria, which are caused by the lack or impairment of the secretion of insulin and/or reduced sensitivity of cells. In addition, other physiological and metabolic functions are also altered, in which carbohydrate, protein and fat metabolism are also affected, and the pharmacokinetics and pharmacodynamics of xenobiotics are also changed.

Despite the numerous in vitro methods, the use of rodent models is a common experimental method in diabetes research. During my doctoral work, experimental diabetes was induced using streptozotocin (STZ). Streptozotocin is a glucosamine nitrosourea antibiotic that selectively causes the destruction of pancreatic β-cells, a property that makes it suitable for inducing various forms of experimental diabetes using different treatment protocols.

Pathological conditions such as prolonged hyperglycemia and the development of diabetes mellitus can affect the metabolism of xenobiotics and can cause an increase in the level of reactive oxygen species (ROS) and thus free radicals, which leads to oxidative stress. These reactive oxygen species, free radicals and endogenous compounds are also eliminated by conjugation with glutathione, which can occur spontaneously and enzymatically by the enzyme glutathione-S-transferase, contributing to the decrease in glutathione and cysteine levels in cells, further worsening the body's antioxidant defense system. Oxidative stress plays a significant role in the development of both types of diabetes mellitus and the associated micro- and macrovascular complications such as retinopathy, nephropathy, neuropathy and endothelial dysfunction.

AIMS OF THE STUDY

The aim of my doctoral studies was to investigate the metabolism and excretion of paracetamol in the small intestine in untreated (control) and diabetic animals. The intestinal tract is an important and less often investigated site of drug metabolism especially in pathological conditions such as in diabetes. Therefore, we consider it is important to perform studies those are related to the altered biotransformation of xenobiotics due to disease.

In summary, the following objectives were formulated:

1. Development of a reversed-phase HPLC UV-Vis analytical method for the identification and quantification of paracetamol and its metabolites in rat small intestinal perfusate.
2. Investigation of the role of the small intestine in the first-pass effect and the absorption capacity through the small intestinal wall by *in vivo* animal experiments during intestinal perfusion of 250 and 500 μM paracetamol.
3. Investigation of the effect of experimental diabetes on the absorption and elimination activity of the small intestine by *in vivo* animal experiments.
4. Determination of the activity of enzymes involved in the metabolism of paracetamol (CYP3A4, CYP2E1, COX) in small intestinal homogenates of control and experimental diabetic rats.
5. Determination of the amount of oxidized and reduced glutathione and cysteine in small intestinal homogenates of control and experimental diabetic rats by reversed-phase HPLC UV-Vis method.

METHODS

Experimental procedure

Our animal experiments were performed on male Wistar rats (Toxi-Coop Zrt, Budapest) weighing 240-300 g. The animals were anesthetized with urethane (dissolved in isotonic saline) (1.2 g/kg, i.p.) before the start of the experiment. The abdomen was opened by a mid-line incision, and a jejunal loop (length about 9–12 cm) was isolated and cannulated. In order to examine the role of the small intestine in metabolism, the bile duct was also cannulated.

The lumen of the jejunal loop was flushed with a warmed isotonic solution (30–40 mL) to remove digesta and food residues and then, blown empty with 4–5 mL air. Perfusion through the lumen of the jejunal loop with a 250 μM and 500 μM solution of paracetamol was circulated through the intestinal lumen.

The flow rate of the solution was 12 ml/min. The initial perfusion volume was 16,5 mL. During the 90-min-long experiment, 250 µL samples were collected. During the experiment, the conditions for normal physiological function were ensured, and the temperature of the animals and the perfusion medium was maintained at 37 °C. Each group had 5 rats.

The experimental diabetes test was induced by the i.v. administration of 65 mg/kg STZ freshly dissolved in 0.1 M citrate buffer (pH 4.0). The experiments were performed on the 7th day after administration of STZ after 12-hour fasting. Blood glucose levels and the development of hyperglycemia (>20 mmol/l in rats) were monitored with an Accu-Chek® (Roche) digital blood glucose meter.

At the end of the experiment, the animals were anesthetized with urethane and certain organs were removed for further examination (liver, kidney, small intestine). The samples were then stored in -70 °C.

The study was designed and conducted according to European legislation (Directive 2010/63/E.U.) and Hungarian Government regulation (40/2013., II.14.) on the protection of animals that are used for scientific purposes. The project was approved by the Animal Welfare Committee of the University of Pécs and by the Government Office of Baranya County (license No. BAI35/51-61/2016 and license supplement No. BAI35/90-5/2019).

Method development for HPLC UV-Vis analysis of small intestinal perfusate containing paracetamol

The chromatographic separation and quantification of paracetamol and its formed metabolites was performed using an Agilent 1100 Series HPLC. A PerfectSIL 120 ODS C18 (4.6 mm × 100 mm, 5 µm) chromatography column was used for the analytical study. The separation was performed using an isocratic elution method, the mobile phase consisted of a mixture of water:acetonitrile:triethylamine (92.95:7.00:0.05 v/v%) (pH 2.25, pH value was adjusted by adding HCOOH). The eluent flow rate was 1 ml/min. The injected sample volume was 20 µl, and the measurements were performed at room temperature (21 ± 1 °C). The detection wavelength was 245 nm.

The structure identification of paracetamol and the metabolites was performed using a Thermo Dionex UltiMate 3000 liquid chromatography system connected to a Thermo Q Exactive Focus quadrupole-Orbitrap hybrid mass spectrometer.

Determination of enzyme activities in small intestinal homogenate

Five cytochrome P450 isoenzymes play a role in the phase I metabolism of paracetamol: CYP 1A2, 2E1, 2A6, 2D6 and 3A4 and the cyclooxygenase enzyme (COX). Among the five CYP enzymes, we aimed to determine the activity of CYP3A4 and CYP2E1, as well as COX, in control and STZ-treated animals. For this purpose, microsomes were prepared from small intestinal homogenate, the protein content was determined by UV-Vis spectroscopy using biuret reagent.

1. Spectrophotometric method to determine CYP3A4 activity in the small intestine

The prepared microsomes were incubated with erythromycin estolate solution, phosphate buffer, and NADPH solution. After addition of Nash reagent, absorbance was measured at 412 nm with a UV-Vis spectrophotometer. For quantitative determination, a calibration curve was prepared (100-1000 μM formaldehyde, dissolved in buffer). The CYP3A4 enzyme activity was expressed in μmol formaldehyde per minute, per 1 mg protein. The experiment was also performed with the addition of the CYP3A4 enzyme inhibitor ketoconazole (10 μM).

2. HPLC measurement to determine CYP2E1 activity in the small intestine

The prepared microsomes were incubated with 4-nitrophenol and NADPH solution. The reaction was stopped by the addition of acetone and 1 M hydrochloric acid. Subsequently, salicylamide solution (10 μl, dissolved in Krebs-Tris buffer) (internal standard) was added to 0.1 ml of the sample, later on it was acidified with hydrochloric acid and separated with diethyl ether. The ether phase was evaporated with nitrogen gas, then the evaporated residue was dissolved in the eluent used for HPLC UV-Vis determination.

A reversed-phase Knauer C18 (4.6 mm x 250 mm, 5 μm) chromatography column was used for analytical separation. The separation was performed using an isocratic elution method, the mobile phase was a mixture of acetonitrile:water:glacial acetic acid (22:77:1 v/v%) to which 3.035 g/l triethylamine was added (pH 3.0, adjusted with H_3PO_4). The flow rate was 1 ml/min. The measurements were performed at 25 °C, the detection wavelength was 250 nm.

The activity of the CYP2E1 enzyme was expressed as the amount of 4-nitrocatechol converted (nmol) per minute, relative to 1 mg of protein.

3. Spectrophotometric method to determine COX activity in the small intestine

The prepared microsomes were incubated with Tris-hydrogen chloride buffer and N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD). Then, the absorbance change was recorded with a UV-Vis spectrophotometer after the addition of arachidonic acid.

A calibration curve was prepared for the quantitative determination (100-1000 μM TMPD, dissolved in buffer). The COX enzyme activity was expressed in μmol TMPD per 1 mg protein per 30 seconds.

HPLC measurement to determine the amount of cysteine and glutathion

Considering the paracetamol metabolites measured in the small intestine, we used an HPLC UV-Vis method for the determination of cysteine and glutathione. The study was performed on rat small intestine samples belonging to 4 different groups: paracetamol-free control, paracetamol-free STZ-treated, 250 μM paracetamol-perfused control, 250 μM paracetamol-perfused STZ-treated samples. The basis for the determination was the reaction of cysteine and glutathione with 5,5'-dithio-bis-(2-nitrobenzoic acid), which was added to the small intestine samples during sample preparation.

The prepared samples were separated using a reverse-phase Teknokroma NUCLEOSIL 100 C18 (4.6 mm × 250 mm, 5 μm) column with gradient elution. The eluent "A" was 10 mM KH2PO4 solution (pH 6.0 KOH), the eluent "B" was 40% "A" and 60% acetonitrile. The flow rate was 1 ml/min.

The analysis was performed at 25 °C, the detection wavelength was 330 nm. The amounts of cysteine and glutathione were calculated using a calibration curve from the standards prepared in the homogenization medium (10-100 μM cysteine and 50-500 μM glutathione).

HPLC measurement to determine the amount of oxidized and reduced glutathion

The amount of oxidized and reduced glutathione was determined from rat small intestine samples belonging to 4 different groups: paracetamol-free control, paracetamol-free STZ-treated, 500 μM paracetamol-perfused control, 500 μM paracetamol-perfused STZ-treated samples.

The small intestine samples were mixed with a homogenizing solution (sucrose, DL-dithiothreitol, Hepes-Tris), treated with acid and then alkali and centrifuged before being injected.

A reversed-phase Kinetex Hypersil C18 (4.6 mm × 150 mm, 5 μm) chromatography column was used for analytical separation. The eluent during the isocratic separation was 0.1% TFA solution (pH 2.0, NaOH). The eluent flow rate was 1 ml/min. The sample volume was 10 μl, and the measurements were performed at 25 °C. Detection was performed at 215 nm.

CALCULATIONS, STATISTICAL ANALYSIS

The concentration of paracetamol and its metabolites was determined by reversed-phase HPLC UV-Vis method after method validation.

At the beginning of the experiment, the initial volume of the perfusion medium containing 250 μM and 500 μM paracetamol was 16.5 ml, which changed during the 90-minute perfusion period during sampling and the absorption and excretion of fluid by the small intestine. The volume of the perfusion medium was determined with decimal accuracy at the end of the experiment, thus the current perfusion volume was calculated taking into account the two factors for a given time point. The amount of the given metabolite was calculated by multiplying the corrected volume and the determined concentration. The cumulative luminal appearance represents the aggregated metabolite quantitative data for the entire 90-minute experimental period.

The method for determining enzyme activities was described in the chapter entitled “Methods”. The protein content of the homogenates was determined by measuring the absorbance at 545 nm using a UV-Vis spectrophotometer using biuret reagent. For this, a calibration curve was prepared in advance using BSA solutions of known concentrations. Thus, the enzyme activities were expressed as the amount of converted substrates per 1 mg of protein and per 1 minute for CYP2E1 and CYP3A4 enzymes, and per half a minute for COX.

Significance was calculated using Student's one-sample t-test, while for the determination of the level of reduced glutathione, an ANOVA test was performed in control and STZ-pretreated animals, as well as in the case of different concentrations of paracetamol perfusion, to determine significant differences between the different experimental groups.

RESULTS

Method development for HPLC UV-Vis analysis of small intestinal perfusate containing paracetamol

A reverse-phase HPLC UV-Vis chromatography method was developed for the analysis of samples collected during small intestinal perfusion, which was suitable for the identification and quantification of paracetamol and its conjugated metabolites. The following parameters were investigated for the validation of the chromatographic method: linearity, selectivity, precision, limit of detection (LOD) and limit of quantification (LOQ) values.

For the linearity test, a series of solutions of paracetamol and metabolites with known concentrations were prepared, where the compounds were dissolved in the control intestinal perfusate in the concentration range of 10-100 μM for paracetamol, 2-100 μM for paracetamol β-D-glucuronide, and 2-10 μM for the other metabolites.

During the selectivity test, no signal interfering with the chromatographic determination – the baseline – was visible in the retention region of the expected components. The retention times were as follows: peak 1 was paracetamol β-D-glucuronide (t_R=2.305 min), peak 2 was paracetamol cysteine (t_R=2.776 min), peak 3 was paracetamol sulfate (t_R=3.904 min), peak 4 was paracetamol (t_R=4.354 min), peak 5 was theophylline (internal standard) (t_R=5.701 min), and peak 6 was paracetamol mercapturate (t_R=10.271 min).

In order to examine the precision of the method, the intra-day and inter-day repeatability, the relative standard deviation (RSD %) of the areas under the curve and the retention times (t_R) were determined. The limit of detection LOD= 3 × RMSE/m, and the limit of quantification LOQ= 10 × RMSE/m (where m is the slope of the calibration curve) were determined using formulas. Based on the calculation of the root mean square error (RMSE) of the concentration, the limit of detection of paracetamol in the concentration range of 10-100 μM was 5.84 μM. The limit of detection for paracetamol β-D-glucuronide in the concentration range of 2–10 μM (this interval was used for all other metabolites) was 0.61 μM, for paracetamol sulfate it was 0.29 μM, for paracetamol cysteine it was 0.35 μM and for paracetamol mercapturate it was 0.13 μM. Based on the calculation of the root mean square error (RMSE) of the concentration, the limit of quantification for paracetamol β-D-glucuronide in the concentration range of 2–10 μM (this interval was used for all other metabolites) was 2.02 μM. The limit of quantification for paracetamol sulfate was 0.96 μM, for paracetamol cysteine it was 1.17 μM and for paracetamol mercapturate it was 0.42 μM.

Absorption of 250 and 500 μM paracetamol from the small intestinal lumen in control and experimental diabetic animals

The amount of paracetamol in intestinal perfusate decreased continuously in case of 250 and 500 μM paracetamol perfusion. No significant difference was observed between the control and STZ-treated groups for either concentration. In the case of 250 μM paracetamol perfusion, the rate of paracetamol absorption was consistently lower in the control animals than in the experimental diabetic group, while this trend was reversed in the case of 500 μM paracetamol perfusion.

Metabolism of 250 and 500 μM paracetamol in the small intestinal lumen in control and experimental diabetic animals

The paracetamol perfused at the two different concentrations was metabolized in large amounts during the experimental time interval (90 minutes). The largest amount of paracetamol β-D-glucuronide was excreted into the small intestinal lumen, and smaller amounts of paracetamol sulfate, paracetamol cysteine, and paracetamol mercapturate. In the case of 250 μM paracetamol perfusion, the experimental diabetic group excreted significantly higher amounts of paracetamol β-D-glucuronide, paracetamol sulfate, and paracetamol mercapturate than the untreated group. In the case of 500 μM paracetamol perfusion, the STZ-treated group excreted significantly higher amounts of paracetamol β-D-glucuronide, paracetamol cysteine, and paracetamol mercapturate.

Determination of enzyme activities in small intestinal homogenate

1. Determination of CYP3A4 enzyme activity in small intestinal homogenate

The enzyme activity of CYP3A4, which is involved in the oxidative metabolism of paracetamol, was determined in small intestinal homogenate in paracetamol-free control and diabetic rats. The CYP3A4 enzyme inhibitor ketoconazole was used as a positive control. The activity of CYP3A4 in the proximal segment of the small intestine was expressed as the amount of oxidative metabolite formed in the small intestine (μmol) by the enzyme, per 1 mg of protein and per minute. The activity of CYP3A4 was significantly reduced in STZ-induced diabetic rats.

2. Determination of CYP2E1 enzyme activity in small intestine homogenate

The enzyme activity of CYP2E1, another enzyme involved in the oxidative metabolism of paracetamol, was determined in small intestine homogenate in paracetamol-free control and diabetic rats by HPLC UV-Vis method.

Using the areas under the curve, the amount of converted substrate and the activity of the enzyme were determined with the help of a calibration line: expressed as the amount of 4-nitrocatechol metabolite (nmol), per 1 mg of protein and per minute.

There was no significant difference in the CYP2E1 enzyme activity between the STZ-treated and control groups.

3. Determination of COX enzyme activity in small intestine homogenate

COX activity was also determined in the proximal segment of the small intestine, expressed as the amount of oxidative metabolite (TMPD) formed in the small intestine by the enzyme (μmol), per 1 mg protein and 0.5 min. COX activity was significantly increased in STZ-induced diabetic rats. These samples were from animals that did not participate in the paracetamol perfusion experiment.

Determination of glutathione and cysteine in small intestine homogenate

In animals treated with streptozotocin without paracetamol perfusion, the concentrations of glutathione and cysteine were also significantly lower. In the case of paracetamol perfusion (250 μM**),** the concentration of glutathione was significantly lower in the STZ-treated group; the concentration of cysteine showed a significant difference in both the control and STZ-treated groups compared to the empty control group. Comparing the results of paracetamol perfusion control with the STZ-treated rats, the concentration of glutathione was significantly lower, while there was no significant difference in cysteine between the two groups.

Determination of the amount of oxidized and reduced glutathione in small intestinal homogenates

Pretreatment with steptrozotocin and paracetamol perfusion (500 μM) did not show a significant difference in the amount of glutathione in small intestinal homogenates using this method. Oxidized glutathione was detected in STZ-pretreated animals (0.1539 ± 0.06 μM) and in 500 μM paracetamol perfusion and STZ pretreatment (0.6686 ± 0.12 μM). There was a significant difference between the two groups, so paracetamol perfusion further increased oxidized glutathione levels.

SUMMARY OF RESULTS

1. The developed reversed-phase HPLC UV-Vis analytical method for the determination of paracetamol and its metabolites from small intestinal perfusate enables selective, accurate, rapid and simultaneous qualitative and quantitative determination during the measurement of a large number of biological samples.
2. Both streptozotocin-induced experimental diabetes and paracetamol perfusion affect the permeability of the small intestine, which thus significantly affects the absorption of paracetamol and its metabolites and their return to the small intestinal lumen.
3. In the case of 250 μM and 500 μM paracetamol perfusion, four metabolites could be identified with the developed reversed-phase HPLC method: paracetamol β-D-glucuronide, paracetamol sulfate, paracetamol cysteine, paracetamol mercapturate. Overall, it can be said that experimental diabetes induced by streptozotocin increases the amount of paracetamol metabolites secreted into the small intestine both in the case of 250 μM and 500 μM paracetamol perfusion: paracetamol β-D-glucuronide and paracetamol mercapturate increased significantly in the case of both applied paracetamol concentrations, paracetamol sulfate significantly after 250 μM, while paracetamol cysteine showed a significant difference after 500 μM paracetamol perfusion compared to the control group.
4. In the case of the enzymes involved in the metabolism of paracetamol, which were examined during my doctoral work, experimental diabetes reduces the enzyme activity in the case of CYP3A4, increases the enzyme activity in the case of COX enzyme, while no significant difference was detected in the case of CYP2E1.
5. STZ pretreatment causes increased oxidative stress in the metabolizing cells, which also affects the transformation of the xenobiotic. Both experimental diabetes and paracetamol perfusion significantly affect the body's defense system against exogenous compounds, including the levels of oxidized and reduced glutathione and cysteine.

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