Ph.D. Thesis

Effect of experimental diabetes on the elimination of phenolic and arylpropionic acid derivatives in the small intestine and liver

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2019

1. Introduction

The primary function of the small intestine is to absorb nutrients and water that are vital to the body, coupled with its ability to biotransform and excrete orally administered compounds. For a long time, science considered only the liver to be the metabolizing organ, as this is where the amount and activity of metabolizing enzymes is greatest. It is now known that other organs also play an important role in the biotransformation and excretion of compounds. Of these, the small intestine is noteworthy, as orally administered compounds are absorbed from the gastrointestinal tract into the systemic circulation. However, not only the absorption but also the metabolism of foreign compounds and the excretion of the parent compound and its metabolites take place in the small intestine. Compounds secreted into the bloodstream are transported through the *vena portae* to the liver, where they can undergo further metabolic transformation. The compounds enter the systemic circulation from the liver through the *hepatic vein* and are transported to the site of action by the bloodstream. A number of transporters are involved in the excretion of the compounds from epithelial cells as well as hepatocytes.

The chemical processes involved in the biotransformation of the compounds (Phase I and Phase II reactions) are catalyzed by various enzymes. There are members of four major enzyme families involved in the processes of the topics of this doctoral dissertation. Among these, the dissertation focused on the activaty of the UDP-glucuronyltransferase (UGT) and sulfotransferase (SULT) enzymes, which catalyze conjugation reactions. Members of the UGT and SULT family of enzymes are localized in the endoplasmic reticulum membrane of the liver, intestinal epithelial cells, and some other extrahepatic tissues, and the cytosol of the cells. These enzymes

catalyze the biosynthesis of glucuronic acid and sulfate conjugates of many endogenous as well as exogenous compounds. The formed conjugates are polar, water-soluble compounds that are more difficult to bind to proteins and are therefore more easily excreted from the body.

Hyperglycemia and diabetes are pathological conditions that involve changes in many physiological functions. These affect the body's uptake and utilization of glucose, hormonal changes, protein synthesis, the amount and activity of enzymes, and thus may alter the pharmacokinetics of pharmacons.

The structure and function of the intestinal tract of the rat used in our experiments show many similarities with the human intestinal system, unlike other species closer to it in strain development. The small intestine is the longest section of the gastrointestinal tract and contains a number of surface-enhancing structures that are important for both absorption and elimination. The expression and activity of each enzyme varies at different stages of the intestinal tract, so the metabolism of drugs may also differ significantly in each segment. In all cases, our experiments were performed by perfusing the proximal section of the jejunum.

2. Aims

Hyperglycemia and diabetes affect a large proportion of the world's population, influencing a number of physiologically important reactions. Ibuprofen (IBP) can be used by many patients with diabetes to relieve pain of various origins. For this reason, we consider it important to study the effect of these pathological conditions on the elimination of the compound, as we do not yet have sufficient information about it. *p*-Nitrophenol (PNP) was chosen

because of the previous study of the intestinal elimination of the compound under similar conditions and the almost exclusive route of its biotransformation *via* glucuronic acid and sulfate conjugation.

In our studies, we set the following goals:

- ✓ Development of an RP-HPLC-UV-Vis analytical method for identification and quantification of IBP in rat small intestine perfusates,
- ✓ Development of an RP-HPLC-UV-Vis analytical method for identification and quantification of IBP and IBP-β-D-glucuronide in rat bile samples,
- ✓ Investigation of the role of the small intestine and liver in the first-pass effect during intestinal perfusion similar to oral administration of ibuprofen in *in vivo* animal experiments,
- ✓ Effect of experimental diabetes on small intestinal and hepatic elimination activity in *in vivo* animal experiments and
- ✓ Comparison of the role of the small intestine and the liver in elimination of ibuprofen and *p*-nitrophenol.

3. Methods

Male Wistar rats (weighing 250-300 g) were used. The animals were anesthetized with urethane. The abdomen was opened by a mid-line incision, and a jejunal loop of about about 10 cm length was isolated and cannulated. The lumen of the jejunal loop was gently flushed with warmed (37 ^{o}C) isotonic solution to remove digesta and food residues and then blown empty with air. The isolated a jejunal loop was cannulated both at the proximal and the distal ends. Perfusion through the lumen of the jejunal loop with isotonic medium containing 250 μM IBP or 500 μM PNP was carried out at a rate of 13 ml/min in a recirculation mode for 90

minutes. The volume of the samples obtained from the perfusion medium flowing out of the jejunal loop was 250 µl; the initial volume of the perfusate was 15 ml. Samples were collected at specified times from the perfusion medium coming out from the isolated intestinal segment. Body temperature was maintained at 37 °C using a heating pad. The samples were refrigerated (-20 °C) until analysis.

For investigation of the biliary excretion rate, the bile duct was cannulated with a polyethylene tubing and the bile collected in 15-min-periods. The samples were refrigerated (-20 °C) until analysis.

Experimental diabetes was induced by *i.v.* administration of streptozotocin (STZ) freshly dissolved in citrate buffer. The experiments were performed on day 7 after STZ administration. Blood glucose levels and the development of hyperglycemia were monitored with an Accu-Chek® (Roche) digital glucose meter.

Chromatographic separation and quantification of ibuprofen and its formed metabolite was performed with an integrated Agilent 1100 Series HPLC instrument consisting of a solvent reservoir, degasser, quaternary pump, autosampler, injector, thermostatable column section, and UV-Vis detector. In the analytical measurements, a Zorbax SB C18 (4.6 mm x 150 mm, 5 µm) column was used, a Teknochrome TR-C-160K1 front column to prevent possible contamination from biological samples.

Chromatographic separation and quantification of PNP and its metabolites was performed by passing through a Nucleosil 100 reversed phase C18 column (250 mm x 4.6 mm, 10 µm) and a TR-C-160K1 precursor column. The device consists of the following units: Varian 2010 pump, Rheodyne 7725i injector, UV-Detector 308 detector and PowerChrom 280 data processing unit. A Pye Unicam

(Philips) PU 8800 UV-Vis spectrophotometer was used for the UV-Vis measurements.

Student's t-test was used to calculate significance. The significant difference was marked as * $p < 0.05$, ** $p < 0.01$.

4. Results and Conclusions

4.1. Development and validation of an HPLC-UV-Vis chromatographic method for the analysis of small intestinal perfusate (Method I) and bile samples (Method II).

For the analysis of samples collected during the small intestine perfusion, an RP-HPLC-UV-Vis chromatographic method was developed for the separation and quantification of racemic IBP and diclofenac (DICL; as internal standard) (Method I). In order to investigate and compare the simultaneous elimination activity of the small intestine and liver, bile samples were taken during the experiments also analyzed. Method I was not suitable for the analysis of the bile samples, so a new method (Method II) was developed for this purpose. To validate the developed chromatographic methods, the following parameters were tested: selectivity, linearity, system suitability, accuracy, and the minimum detectable and quantifiable values.

During the study of the selectivity parameter, the compounds were well separated from each other, no interfering signs were observed in the biological samples (intestine and bile) during the time interval in question. Retention times (t_R) were as follows: IBP in the intestinal perfusate: 6.11 min., DICL: 5.21 min., in bile, 10.92 min. and 10.08 min. respectively. IBP-glucuronide (IBP-β-D-G) could be only identified in the bile, where the retention time of the metabolite was 4.74 min.

For the Method I linearity study, a series of IBP and DICL solutions of known concentration were prepared, in which the compounds were dissolved in the control intestinal perfusate in the concentration range of 5-250 μM. In Method II, the IBP, IBP-G and DICL standards were dissolved in control bile. In this case, the concentrations of IBP and IBP-G in the calibration solution series ranged from 7.5 to 150 μM. The calibration line was obtained by plotting the relative areas under peaks as a function of the concentration of the known compound. The calibration line for ibuprofen in the perfusate was $y = 0.0092x + 0.1131$ ($R^2 = 0.9942$); in bile, for IBP $y = 0.0062x + 0.0013$ ($R^2 = 1.000$), and for IBP-G $y = 0.0049x – 0.0008$ ($R^2 = 0.9999$).

Examination of the Method I accuracy parameter was characterized by determining intraday repeatability as well as inter-day reproducibility. The RSD% of the retention times ranged from 0.53 to 0.67, and the RSD% of the areas under the curve ranged from 3.20 to 12.91. Based on the determination of inter-day repeatability, the RSD% values of the retention times fell in the range of 1.83-2.17, while the RSD% values of the areas under the peak fell in the range of 1.58-10.40. According to Method II, the inter-day repeatability of the retention time of IBP standard solutions of different concentrations fell in the range of 0.22-1.08 RSD%, while similar values in the areas below the peak fell in the range of 3.79-8.76 RSD%. For IBP-G, the retention time was similar in the range of 1.05–2.45 RSD%, while the areas under the curve fell in the range of 4.68–8.33 RSD%.

Data on the suitability of the chromatographic system were derived from chromatograms of ibuprofen and ibuprofen-β-D-G standard solutions dissolved in the control small intestine and control bile.

The lower limit of detection (LOD) was determined using the formula LOD=$3xRMSE/m$, the limit of quantification (LOQ) was determined using the formula LOQ=$10xRMSE/m$. Based on these calculations, LOD = 0.15 µM, and LOQ = 0.51 µM were obtained for ibuprofen in the small intestinal perfusate. In bile, IBP had a LOD concentration of 1.33 µM, a LOQ of 4.42 µM, while IBP-G-LOD had a concentration of 3.09 µM and a LOQ of 10.3 µM.

Based on the obtained results, the developed RP-HPLC-UV-Vis assay method was suitable for the accurate, easy, and fast analysis of a large number of biological samples. It is suitable for the identification of all the three compounds (IBP, IBP-β-D-G, and DICL) and the quantification of IBP and IBP-β-D-G in the samples collected from the small intestine lumen and the bile.

4.2. The role of the small intestine in the first-pass effect during oral administration of ibuprofen

Samples collected during animal experiments were analyzed by the developed and validated Method I chromatography method. Plotting the results, we obtained a curve of the disappearance of IBP from the small intestinal perfusate, which showed that the amount of the compound was less in the control group compared to the hyperglycemic group of animals at almost each measurement time. This difference became statistically significant by the end of the experiment; at the 75th minute *p <0.05, while at the last sampling time *p <0.01 there was a difference between the two study groups. The amount of ibuprofen in the samples taken at the last time point approaches the limit of detection.

The observed decrease may be the common consequence of the absorption and metabolism of the compound, as there is an abundance of literature on the study of UGT and SULT enzymes

expressed at the gastrointestinal tract. Several metabolic enzymes are expressed in the small intestine, and intestinal metabolic transformations have been previously described for other compounds.

As a result of the large disappearance of ibuprofen, we expected the appearance of the glucuronide conjugate of the compound in the perfusate, but we couldn't identify the conjugated metabolite in our studies. In an earlier paper, we examined the modification of the pharmacokinetics of PNP under similar experimental conditions when conjugated metabolites of the compound (glucuronide and sulfate conjugate) appeared in the intestinal perfusate. There are several possible explanations for the observed difference: on the one hand, glucuronic acid conjugation of carboxyl and phenolic compounds is catalyzed by members of different UGT families, on the other hand, the stability of the metabolites formed is different, which is why they are hydrolyzed to different degrees by glucuronidase enzymes present in the intestinal tract.

4.3. Examination of hepatic elimination activity of bile ibuprofen and its conjugate metabolite in untreated and experimental diabetic animals

Samples collected during animal experiments were analyzed using the Method II analytical procedure. Based on the data obtained and the equations of the calibration lines, the concentration and cumulative excretion of IBP and the conjugated metabolite IBP-G in the two study groups (control and hyperglycemic) were calculated. We found that both compounds, ibuprofen and its conjugated metabolite, were excreted into the bile. The biliary excretion of both the unconverted ibuprofen and its glucuronide conjugate was

decreased by hyperglycemia (*p <0.01 for IBP and *p <0.05 for IBP-G). During the experiments, we also observed that the glucuronide conjugate was excreted into bile in much greater amounts than the unconverted ibuprofen itself.

4.4. The role of small intestine and liver in elimination of *p*-nitrophenol

Samples collected during animal experiments were analyzed using Method III. During the experiments, the initial concentration of PNP in the small intestinal perfusate gradually decreased. However, the difference between the values of PNP disappearance from the perfusate did not prove to be statistically significant between the two groups. PNP was extensively metabolized during the experimental time interval. PNP and the formed metabolites were excreted into the bile. Excretion of PNP-G was found to be higher than that of PNP-S. During the experiments, the control group excreted PNP-G and PNP-S to a greater extent than the group of diabetic animals.

4.5. Effect of hyperglycemia on the elimination activity of the major organs, intestines and liver involved in the first-pass effect during oral administration of *p*-nitrophenol and ibuprofen

For our experiments, we selected compounds from two groups, ibuprofen (a non-steroidal anti-inflammatory drug) from the 2-arylpropionic acid derivatives and *p*-nitrophenol from the group of phenolic compounds. The hyperglycemic state negatively affected the elimination of both compounds from the intestinal perfusate. In the hyperglycemic state, the amount of P-gp in the small intestine is decreased, as a result of which the efflux of the compounds from the cells decreased, thereby increasing their absorption from the small

intestine. Given this experimental experience, it was likely that hyperglycemia would increase the disappearance of the compounds from the intestinal perfusate and improve their absorption from the small intestine. However, in our experiments, higher concentrations of IBP and PNP were detected in the circulating perfusate compared to the control groups. Due to the weakly acidic properties of the compounds, they may be able to cross cells under physiological conditions without the aid of transporters and the decrease in P-gp expression does not significantly affect the pharmacokinetics of the compounds at the small intestinal level.

The effect of hyperglycemia on the biliary excretion of the compounds had a negative effect. This pathological condition reduced the biliary excretion of both the glucuronide and sulfate conjugates for both of the compounds we tested. But the excretion of not only metabolites but also the parent compounds (PNP and IBP) showed a decreasing trend as a result of the experimental diabetes. The changes in excretion we experienced can be explained by changes in the amount of the relevant transport proteins. In a hyperglycemic state, the expression of P-gp (P-glycoprotein transporter) and MRP2 (multidrug resistance protein 2) in liver cells decreases, as a result of which the excretion of compounds from tissues and cells into the blood decreases, so less compounds are excreted in the bile and small intestine. The conjugation reaction with glucuronic acid after pretreatment with STZ resulted in increased glucuronide formation in the hepatocytes. In hyperglycemia and experimental diabetes, glucose utilization occurs in the insulin-intensive pathway, resulting in a number of processes that are vital to the body. In this case, as a result of a multi-step process, UDP-glucuronic acid (UDPGA) is formed from UDP-glucose by the dehydrogenase enzyme, and the conjugation reaction

in the cells may increase due to the increased amount of glucuronic acid supply and the presence of UGT enzymes.

Based on all this, it can be concluded that hyperglycemia and experimental diabetes can modify the pharmacokinetics of the compounds.

5. Summary of results

- ✓ The developed HPLC-UV-Vis analytical methods (Method I and Method II) are selective, accurate and suitable for the analysis of large quantities of biological samples.
- ✓ The HPLC-UV-Vis isocratic analytical method (Method I) developed for the analysis of samples obtained from intestinal perfusate allowed the identification and quantification of IBP
- ✓ The HPLC-UV-Vis gradient analytical method (Method II) developed for the analysis of bile samples allowed the simultaneous separation and quantification of IBP, IBP-β-D-G.
- ✓ The experimental diabetes created by STZ treatment provides an opportunity to study the changes in biotransformation and transport processes caused by diabetes.
- ✓ The applied experimental protocol can be used to study the biotransformation and transport processes of the small intestine and liver simultaneously.
- ✓ The hyperglycemic condition negatively affects the disappearance of both IBP and PNP from the intestinal lumen.
- ✓ No ibuprofen glucuronide was detected in the small intestinal perfusate during the ibuprofen study.
- ✓ In the liver, in an UGT-catalyzed bioptransformation reaction of ibuprofen IBP-β-D-G is formed, which is excreted into the bile.

- ✓ The hyperglycemic state significantly reduces the biliary excretion of both IBP and its conjugated metabolite, as well as that of PNP and its formed metabolites.
- ✓ During investigation of PNP, PNP-G and PNP-S metabolites could be identified from small intestinal perfusate samples.
- ✓ Experimental diabetes produced by STZ treatment affects the metabolism and transport processes of both PNP and IBP.

6. Acknowledgment

I am grateful to **Professor Dr. Pál Perjési**, my program and former supervisor, who supported and encouraged me throughout, provided me with the opportunity to do scientific work and stimulated me throughout my Ph.D.

I am grateful to **Professor Dr. Emil Fischer**, my supervisor, whose helpfulness, guidance, and patience helped me to carry out the experiments successfully and to present the data in a summary of the thesis.

I am grateful to my current supervisor, **Dr. Attila Almási**, assistant professor, whose helpfulness, advice and spiritual support have fundamentally contributed to the realization of my experimental work and the successful presentation of the obtained data in the framework of my dissertation.

I am grateful to **Professor Dr. Erika Pintér**, head of the Doctoral School of Pharmaceutical Sciences, for providing me with the opportunity to pursue my Ph.D. activities within the framework of the Doctoral School.

I am grateful to **Professor Dr. Emese Sipos**, at the University of Medicine and Pharmacy in Târgu Mureș, who set me on this journey and supported me from home throughout my work.

I would like to express my gratitude to the assistant **Mária Reiszné Horváth**, from whom I received valuable information and assistance in carrying out animal experiments.
I would like to thank **Dr. Vancea Szende**, Assistant Professor at the University of Medicine and Pharmacy in Târgu Mureş, who helped me with my work in Târgu Mureş.
I am grateful to the management of the **Transylvanian Museum Society**, who contributed financially to my scientific work.
Last but not least, I am grateful to **my family**, **my husband** who has been patient and supportive throughout my work.

7. Publications and Congress Presentations

7a. Publications

1. Fischer, Emil; Almási, Attila; Bojcsev, Sztojan; Fischer, Tamás; Kovács, Noémi Piroska; Perjési, Pál. Effect of experimental diabetes and insulin replacement on intestinal metabolism and excretion of 4-nitrophenol in rats, *Can. J. Physiol. Pharmacol.*, 93, 459–464 (2015).
Q-rank: Q2
IF (2015): 1.704
2. Almási, Attila; Pinto, ÉILN; Kovács, Noémi Piroska; Fischer, Tamás; Markovics, Zoltán; Fischer, Emil; Perjési, Pál. Changes in hepatic metabolic enzyme activities and biliary excretion of 4-nitrophenol in streptozotocin induced diabetic rats, *Braz. J. Pharm. Sci*., 54, e17347 (2018).
Q-rank: Q2
IF(2018): 0.512
3. Kovács, Noémi Piroska; Almási, Attila; Garai, Kitti; Kuzma, Mónika; Vancea, Szende; Fischer, Emil; Perjési, Pál. Investigation of

intestinal elimination and biliary excretion of ibuprofen in control and hyperglycemic rats, *Can. J. Physiol. Pharmacol*., 97, 1080-1089 (2019).
Q-rank: Q2
IF(2019): 1.946

7b. Congress Presentations

1a. Székely, Noémi Piroska; Almási, Attila; Kuzma, Mónika; Fischer, Emil; Perjési, Pál. Az ibuprofen felszívódásának és kiválasztásának vizsgálata *in vivo* állatkísérletes modellen, Congressus Pharmaceuticus Hungaricus XV., Budapest (2014).

1b. Székely, Noémi Piroska; Almási, Attila; Kuzma, Mónika; Fischer, Emil; Perjési, Pál. Az ibuprofén felszívódásának és kiválasztásának vizsgálata *in vivo* állatkísérletes modellen, *Gyógyszerészet*, 58 Suppl. 1., S78-S78 (2014).

2. Almási, Attila; Kovács, Noémi Piroska; Szabó, Anett; Szente, Lajos; Fischer, Emil; Perjési, Pál. Investigation of absorption and metabolism of ibuprofen in bile and small intestinal perfusate. 7th BBBB International Conference on Pharmaceutical Sciences, Balatonfüred (2014).

3. Székely Noémi-Piroska; Kuzma Mónika; Almási Attila; Vancea Szende; Sipos Emese; Fischer Emil; Perjési Pál. Az ibuprofén felszívódásának és kiválasztásának összehasonlító vizsgálata *in vivo* állatkísérletekben, Erdélyi Múzeum Egyesület,

Orvos- és Gyógyszerésztudományi Szakosztály, XXIV. Tudományos Ülésszak, Marosvásárhely (2014).

4. Almási, Attila; Kovács, Noémi Piroska; Fischer, Tamás; Kuzma, Mónika; Mayer, Mátyás; Fischer, Emil; Perjési, Pál. Az ibuprofén felszívódásának és kiválasztásának vizsgálata vékonybél-perfuzátumban és epében, 45. Membrán-Transzport konferencia, Sümeg, Magyarország (2015).

5. Almási, Attila; Szabó, Anett; Kovács, Noémi Piroska; Mayer, Mátyás; Fischer, Tamás; Fischer, Emil; Perjési, Pál. Az ibuprofén oxidatív metabolitjainak és felszívódásának vizsgálata a vékonybélben és az epében fiziológiás és diabéteszes körülmények között. 46. Membrán-transzport konferencia, Sümeg, (2016).

6. Fischer, Emil; Almási, Attila; Bojcsev, Sztojan; Kovács, Noémi Piroska; Fischer, Tamás; Perjési, Pál; Simon Higin. A máj eliminációs funkciójának változása Crohn-betegség modellben, 48. Membrán-transzport konferencia, Sümeg, 2018.