EFFECT OF EXPERIMENTAL DIABETES ON THE ELIMINATION ACTIVITY OF SMALL INTESTINE AND LIVER

PhD thesis

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Pécs, 2013.

1. Introduction – Aim of experiments

1.1. Introduction

After the oral administration of drugs, the drug molecules reach the gastrointestinal tract first, and after the absorption they reach the liver through the *vena portae*, and only a fraction of the administered drug reach the site of action by the systemic blood circulation (different organs, tissues, receptors, enzymes, etc.). During these processes the drug molecules can be chemically altered (metabolism, biotransformation) and excreted into the intestinal lumen or into the bile. The function of these organs have a major influence on the bioavailability of drugs. The intestine has a special significance because of its location, and only the intestinally non-metabolized and non – eliminated fraction of the administered drug molecules can enter into the liver and the systemic blood circulation.

In the biotransformation of xenobiotics various enzyme reactions, phase I, (or functionalization) and phase II (conjugation)) are involved. The phenolic compounds are metabolized predominantly with (phase II) conjugation reactions.

The formation of the glucuronides is catalysed by the UDP – glucuronyltransferase (UGT), while the sulfate formation is catalysed by the sulfotransferase (SULT). The β – glucuronidase and arylsulfatase enzymes take part in the degradation of the metabolites by hydrolysis. The above mentioned enzymes can be found in the liver and in the mucosa of the intestinal tract, as well.

In our studies p – nitrophenol (PNP) was used as a model compound, because it is known that the PNP is matabolised almost exclusively with conjugation reactions: they form PNP – glucuronide with glucuronidation (PNP –G) and PNP – sulfate (PNP – S) with sulfation.

For the transport of the compounds in the liver cells and enterocytes, the multidrug resistent transporters (MDR) and the multidrug resistant proteins (MRP) are responsible.

The metabolised compound are excreted into the bile from the hepatocytes through the canalicular membran and thereafter the molecules reach the intestinal lumen, while from the enterocytes the drug molecules excreted directly to the intestinal lumen. The activity or the change of activity of the different transporters might sufficiently influence the degree of excretion rate of the drugs.

The elimination of xenobiotics can be influenced by several factors, e.g. interactions, food intake disorders, pathological changes, diseases, e.g. diabetes. Diabetes causes a number of important changes in the metabolic and hormonal processes and it produces different effects in various organs. Diabetes influences the tissue glucose uptake and utilization, in diabetes several changes can occur in protein synthesis, and in the amount enzymes and transport proteins as well.

The two most characteristic changes in diabetes are the insulin deficiency and hyperglycaemia, therefore the insulin replacement is frequently used in experimental diabetes. However, the strategy or methodology of insulin administration may be different, e.g. insulin can be given continuously during the experimental diabetic period to determine whether the slow developing diabetic effects can be prevented or not. Another approach is the addition of a single dose of insulin to investigate its acute effects.

1.2. Aims of experiment

The aim of our experiments was to investigate the conjugative metabolic activities in the small intestine and liver in control and experimental diabetic animals, because these two organs play a fundamental role in the elimination processes and their metabolising and excretory functions have an outstanding importance at the *per os* administration of drugs.

In our experiments the intestinal and hepatic excretion and the phase II biotransformation reactions (conjugation reactions with glucuronic acid and sulfate) have been investigated.

It is unclear, is there any direct connection between the changes of the activity of enzymes of the conjugation reactions and those of the excretion precesses.

Therefore, parallel experiments were performed to investigate the intestinal and hepatic enzyme activities and the intestinal and biliary excretion of metabolites, furthermore their relations were observed in control, experimental diabetic animals without or with insulin replacement. The experimental diabetes was induced by streptozotocin (STZ), and the experiments were performed one week after the STZ administration.

Some authors published significant differences in the biliary excretion of drugs. The extrahepatic metabolism, for example the intestinal elimination in control animals was rarely studied and there are only a few information about the changes of the intestinal elimination in diabetes. Therefore, our experiments were designed to study and to compare the eliminating activity and their changes in the liver and small intestine in experimental diabetes.

The effect of insulin on the changes provoked experimental diabetes was also investigated by administration of rapid – acting insulin preparation.

The rapid-acting insulin was administered in a single dose immediately before the intestinal perfusion experiment one week after STZ administration.

To draw adequate conclusions, the accurate, reliable, reproducible and large-scale determination of the PNP – G and PNP – S metabolites from the perfusion fluid and the bile is essentially important. Therefore, our experiments were also designed to develop new HPLC methods for the determination of PNP and its metabolites, in these biological samples.

Our study was mainly focused on the following three questions:

1. Development of a HPLC method to separate and analyse the PNP and its metabolites (PNP – G , PNP – S) from biological samples (intestinal perfusate, bile);
2. Investigation of the correlation between the activity of the metabolising enzymes and the intestinal and hepatic excretion rate of metabolites in control animals;
3. Effect of the experimental diabetes and insulin replacement on the drug elimination activity of the small intestine and liver.

2. Methods: Materials, animal experiments, analytical investigations

In these experiments we used a method and experimental arrangement which correspond to the *per os* drug administration. The *in vivo* isolated jejunal loops remained in connection with the bood circulation, the connection between the intestinal segment and the body was maintained.

Male Wistar rats (weighing 220-250 g) were used in the animal experiments. The animals were anesthetized with urethane (1.2 g/kg. i.p.). The abdominal wall was opened on the midline with a longitudinal incision, and a 10 cm long jejunal segment just after the duodenum was cannulated *in vivo*. The segment was flushed with warm (37°C) isotonic buffer solution to remove the food residues, then it was made empty by blowing through 4 – 5 ml air. The jejunal segment was perfused with the isotonic perfusion medium in a recirculation mode with a speed of 13 ml/min. The perfusion medium contained PNP in a previously determined concentration (generally 500 µM). In other experiments various PNP concentrations (20 – 1000 µM) were used as well, altought the 500 µM PNP was found to be optimal to analyze reliably the PNP metabolites and compare the intestinal and biliary excretions. The solution coming out from distal end of the isolated intestinal segment was

collected in a reservoir, from where it was continously recirculated to the intestinal lumen with a peristaltic pump. Samples (250 ml each) were obtained from the perfusion medium coming out from the jejunal loop. The initial perfusion volume was 15 ml and the experiment lasted 90 minutes. The temperature of the perfusion medium was maintained constant at 37 °C. When the biliary excretion was investigated, the bile duct was cannulated with a PE-10 tubing and the bile collected in 15 min periods. The samples were stored in refrigerator (-20 °C) until analysis. Experimental diabetes was induced by i.v. administration of streptozotocin (STZ) in a dose of 65 mg/kg and the experiments were made one week after the STZ treatment. The rapid – acting insulin (1 NE / kg i.v.) was adminstered immediately before the experiments.

To measure the parent compound and its conjugation metabolites in numerous samples, a new HPLC method was developed. The PNP (pK_a = 7,19) having different acid – base character than its metabolites PNP – G ($pK_a \approx$ 3,0-3,4), PNP – S ($pK_a \leq 4$), and reversed phase HPLC column was used to separate them (Nucleosil 100, RP-C18 (250 mm x 4,6 mm, 10µm)). The retention times of the two acidic metabolites were increased with the use of tetrabutyl ammonium bromide ion-pair forming reagent. At the analysis of the small intestinal perfusate 0.01 M tetrabutyl ammonium bromide 50 : 50 (v/v/%) methanol : water mixture was used as eluent.

The within day and day to day reproducibility of the retention times and the area under the curve values were characterised with the relative standard deviation (RSD).

At the measurement of the bile samples an internal standard (4 – ethylphenol) and a guard column was used. Due to the complex composition of the bile, the eluent was modified to 0.03 M tetrabutyl ammonium bromide 53:47 (v/v/%) methanol: 0.01 M citrate buffer pH 6.2.

The measurement of the activities of the UDPGT, SULT, β – glucuronidase and arylsulfatase enzymes was performed from small intestinal and liver homogenate contained 1 mg protein, with a spectrophotometric method after adding a specific substrate to the homogenates.

The blood sugar level was measured by Accu - Check® blood glucose meter (Roche).

3. Calculations, statistical analysis

Data in our experiments represent the mean of the values and the standard error (SE) $\left(S.E. = \frac{S.D.}{\sqrt{n}}\right)$or the standard deviation (SD), n is the number of rats of measurements. The significance was calculated with the one-tailed Student's t – test and significant differences from the control were indicated with: * P <0.05, ** p <0.01. To show the significance between the values of diabetic animals in the presence and absence of insulin the following symbols are used: # p <0.05, # # p <0.01. The corresponding data pairs are indicated.

4. Results

4.1. Development of HPLC method for analysis of PNP and its metabolites

An isocratic reversed phase HPLC method with UV detection has been developed on the base of ion – pair formation for the simultaneous analysis of PNP and its metabolites. This method provided acceptable results in the target quantitation range of PNP, PNP – G and PNP – S for within day precision (repeatiblity), and day to day precision (reproducibility) and linearity. The method has proved to be simple and easy to perform determination of PNP, PNP – G and PNP – S in intestinal (luminal perfusion solution) and bile samples in the rat. For the analysis of bile samples a guard column and internal standard (4 – ethylphenol) were also used. In our developed method the retention times became shorter (about 6 – 15 min) , which means adventage, especially in the analysis of a great number of biological samples.

4.2. Enzyme activities of conjugation reactions, intestinal and biliary excretion of metabolites of PNP in control rats.

It was found that the activity of UDP – glucuronyltransferase was about three times higher in the liver than in the small intestine. It is interesting, that the activity of β – glucuronidase was even higher, about six times greater in the liver than in the small intestine. This finding – at least partly – can explain our observation, that in spite of the increased hepatic UDP – glucuronyltransferase activity, no significant difference was found between the luminal appearance and the biliary excretion rate of PNP – G.

Similar tendency was observed in the activities of hepatic and intestinal metabolic enzymes which are important in the production of the sulfate conjugate of PNP. The activity of the sulfotransferase in the small intestine was about three times lower than in the liver, furthermore the intestinal activity of arylsulfatase was about seven times lower than the hepatic activity of this enzyme. However, on the contrary of the ratio of intestinal and biliary excretion values of PNP – G, the biliary excretion rate of the PNP – S was consequently and significantly greater than the intestinal appearance of the sulfate conjugate of PNP. It should also be mentioned that the absolute values of the activity of sulfotransferase and arylsulfatase were definitely (with about two or three order of magnitude) lower than those of UDP – glucuronyltransferase and β – glucuronidase in the liver and in the small intestine, as well.

4.3. Changes of the intestinal and hepatic drug eleimination in experimental diabetes

Experimental procedure was performed after one week of STZ treatment which represents basically a short term diabetes. Rapid – acting insulin was administered i.v. just before starting the experiment.

4.3.1. Effect of experimental diabetes on the intestinal drug elmination

Our experimental data demonstrate a significant increase in the luminal appearance of PNP – G in experimental diabetes, which was paralell with the change of blood sugar level and could be completely compensated by insulin administration. There was also found an elevation in the activity of UDP – glucuronyltransferase and β – glucuronidase and these changes were also compensated – at least partly – by insulin treatment. The increase in the activity of UDP – glucuronyltransferase was about two times higher, however the activity value of β – glucuronidase was about four times higher in diabetic rats than in the controls. The increase of the intestinal appearance of PNP – G was completely antagonised by insulin, however, the increase of the enzyme activities of glucuronide production was only partially compensated by insulin.

No significant changes were found in the luminal appearance of PNP –S and in the activity of sulfotransferase and arylsulfatase in diabetic rats in the absence or in the presence of insulin administration, respectively. These findings show great differences from the results of intestinal elimination of PNP – G, which were obtained in experimental diabetes.

4.3.2. Effect of experimental diabetes on the hepatic drug elimination

The biliary excretion of PNP – G decreased definitely in diabetic rats, which shows a sharp contrast with the change of luminal appearance of this metabolite. It is interesting, however, that insulin was able to antagonize the depression of the biliary excretion and the elevation of the luminal appearance of the PNP – G, as well. Activities of UDP – glucuronyltransferase and β – glucuronidase decreased in the liver, however, an elevation was found in these activities in the small intestine in diabetic rats. The elevation of the activity of UDP – glucuronyltransferase could be compensated by insulin, but the activity of the β – glucuronidase remained unchanged after insulin administration.

Activity of sulfortransferase and arylsulfatase did not change in the liver in diabetic rats and insulin had no effect on the enzyme activities. However the biliary excretion of PNP – S was decreased and insulin had no influence on this depressive effect.

5. Discussion – Conclusions

The ion – pair reversed phase HPLC method with UV detection provided acceptable results in the target quantitation range of PNP, PNP – G, and PNP – S for within day precision (repeatibility), day to day precision (reproducibility) and linearity.

The method has proved to be simple and easy to perform for simultaneous determination of PNP, PNP – G and PNP – S in intestinal (luminal perfusion) and bile samples of the rat.

In our developed method the retention times became shorter, which means adventages, especially at the analysis of great number of biological samples.

Our experimental data obtained in control rats show that in the luminal appearance and in the biliary excretion of PNP – metabolites the activity of metabolic enzymes play an important role. However, no direct correlation was found between the activity of metabolic enzymes and the drug excretion in the small intestine and in the liver. The luminal appearance and biliary excretion of drugs and their metabolites can be determined by several other factors (e.g. substrate concentrations, dosages, species differences, transport processes), not exclusively by the activities of the metabolic enzymes or their expression and capacity.

At the interpretation of changes produced by diabetes many factors can be important, e.g. the duration of experimental diabetes. During the long term diabetes many changes can occur, e.g. in the body weight, in the mass of the liver, in the protein synthesis, in the volume of distribution, furthermore some compensation reactions can also be observed. There are

differences in the administration of insulin, e.g. differences in the dosages and in the duration of administration or in the type of insulin preparations (rapid – acting, long – acting). From our experimental data can be drawn the conclusion that the experimental diabetes can provoke different relatively selective effects on the activity of metabolic enzymes or on the transport processes of xenobiotics. Typical changes have been observed first of all in the glucuronidation of PNP and in the intestinal appearance of PNP – G, because of these changes are connected with the glucose metabolism. We have found differences between the changes of the activity of metabolic enzymes (UDP – glucuronyltransferase, β – glucuronidase) and the intestinal excretion of PNP – G. Furthermore, great differences and variations have been found in experimental diabetes between the formations of glucuronide and sulfate metabolites and in the intestinal appearance of metabolites, as well.

Experimental data clearly show that opposite changes can be observed in drug elimination of diabetic rats: the increase is the typical change in the small intestine, however, the elimination in the liver was depressed. Insulin was able to compensate both (intestinal and hepatic) changes in diabetic rats. Selective changes were found in the glucuronide formation and in the glucuronide transport which are in connection with the glucose metabolism. Ont he other hand, the effects of diabetes had no influence on the formation of PNP – S and the biliary excretion of PNP – S. The biliary excretion of the free (unconjugated) form of PNP and the PNP – S could not be influenced by insulin. Other authors have found similar results and published similar conclusions obtained from various experimental arrangements. Our data suggest that no direct correlation exists between the changes of the activities of metabolic enzymes and the changes of the biliary excretion of metabolites in experimental diabetes.

Changes of enzyme activities which are connected with glucuronide formation were completely or partially compensated by insulin in the liver and small intestine of diabetic rats.

In spite of these findings the differences caused by diabetes in the excretion of glucuronides were completely eliminated by insulin both in the liver and in the small intestine. Activities of enzymes of sulfate formation (sulfotransferase, arylsulfatase) were not changed by experimental diabetes. However, the biliary excretion of PNP – S was decreased and this changes could not be influenced by insulin. These changes indicate the role of specific alterations connected with glucose metabolism and support our conclusion that no direct correlation exist between the changes of enzyme activities and transport (excretory) processes in diabetic rats.

6. Summary and new results

1. A new ion – pair reversed phase HPLC method with UV detection has been developed for simultaneous analysis of PNP, PNP – G and PNP – S. The method has proved to be simple and easy to perform for determination of PNP and its metabolites in intestinal perfusates and bile samples.
2. The major metabolite of PNP in rats is the PNP – G, both in the small intestine and in the bile.
3. The activity of UDP – glucuronyltransferase and β – glucuronidase was significantly greater than that of sulfotransferase and arylsulfatase.
4. No direct correlation was found between the activities of metabolic enzymes and the luminal and biliary excretion of metabolites.
5. Intestinal excretion of PNP – G was stimulated, however, the biliary excretion of PNP – G was decreased in diabetic rats.
6. Activity of UDP – glucuronyltransferase was enhanced in the liver, but it decreased in the small intestine, however, the activity of β - glucuronidase was decreased in the liver, but it increased in the small intestine in diabetic rats.
7. Changes of intestinal and biliary excretion of PNP – G were completely reversed by insulin, however, the alterations in enzyme activities of glucuronide formation were only partially compensated by insulin in diabetic rats.
8. Biliary excretion of PNP – S was decreased in the liver, but no change was found in the intestinal appearance of PNP – S in diabetic rats.
9. Activity of sulfotransferase and arylsulfatase was not influenced by experimental diabetes.
10. Insulin had no effect on the intestinal and biliary excretion of PNP – S, the activity of sulfotransferase and arylsulfatase remained unchanged after insulin administration in diabetic rats.
11. Free (unconjugated) form of PNP is also excreted into the bile, which was inhibited by diabetes and this depression was not influenced by rapid – acting insulin.

In conclusion, our results demonstrate that experimental diabetes provokes changes in the intestinal and hepatic elimination of drugs. These changes are specific: first of all alterations occure in connection with the glucose metabolism. No correlation was found

between the changes of activity od metabolic enzymes and alterations of excretory processes. Changes provoked by experimental diabetes in the intestinal and hepatic drug elimination could be compensated partly by insulin.

7. Acknowledgements

I would like to express my thanks to my program leader, Professor Emil Fischer and my project leaders, Professor Emil Fischer and Professor Pál Perjési for their guidance and continous support during my expreimental and scientific work.

I woluld like to thank Professor Loránd Barthó for the possibility to perform my research activity at the Institute of Pharmacology and Pharmacotherapy and at the Pharmacological Sciences of Doctoral School.

I am very graetful to Reiszné Mária Horváth for her expert and tireless technical assistance in the laboratory work and to Péter Kovács for his technical help during the writing of the dissertation.

Finally, I express my thanks to my family, first of all to my parents for their support during my studies and scientific work.

8. Publications

1. Almási A, Fischer E, Perjési P: A simple and rapid ion-pair HPLC method for simultaneous quantitation of 4-nitrophenol and its glucuronide and sulfate conjugates. J. Biochem. Biophys. Methods 69, 43-50 (2006) (IF: 1,403)

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5. Almási A, Bojcsev S, Fischer T, Simon H, Perjési P, Fischer E: Effect of experimental diabetes and insulin replacement on the intestinal metabolism and excretion of p-nitrophenol in the rat. Submitted for publication.

6. Fischer E ,Almási A, Bojcsev S, Fischer T, Simon H, Perjési P: Changes in the metabolic enzyme activities and hepatic elimination of p-nitrophenol in streptozotocin-induced diabetes with or without insulin replacement in the rat. Submitted publication.

9. Scientific presentations

9.1. Presentations in english

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