FACTORS INFLUENCING PHARMAKOKINETIC PARAMETERS

PhD THESIS

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Abbreviations

ADP: adenosine 5'- diphosphate

APS: adenosine 5'-phosphopsulfate

ATP: adenosine- triphosphate

AUC: area under the curve

CYP: cytochrome P-450 enzyme system

MDR1: Multidrug resistance protein 1

n: number of experiments

PAP: 3- phosphoadenosine- 5'- phosphate (adenosine 3' 5'- biphosphate)

PAPS: 3- phosphoadenosine 5'-phosphosulfate'

PEP: p-ethylphenol

PNP: p-nitrophenol

PNP-G: p-nitrophenol glucuronide

PNP-S: p-nitrophenol sulfate

PP1: pirophosphate

S.E.: standard error

STZ: streptozotocin

Tm: transport maximum

UDP: uridindiphosphate

UDPGA: uridine-diphospho-glucuronic acid

UGT: uridine-glucuronyltransferase

1. Introduction – Aim of the experiments

1.1. Importance of pharmacokinetics in the administration and action of drugs

Pharmacokinetics means basically the fate of drug molecules in the body of patiens: absorption, distribution, metabolism and excretion. These steps of pharmacokinetics will determine the active concentrations of drugs in the blood and in various organs and tissues.

1.2. Role of pharmacokinetic parameters after oral drug administration: first pass effect, bioavailability

The majority of drugs are administered orally. Only a given fraction of drugs can reach the site of action and produce pharmacological effects (biovailability). Drug molecules after oral administration can be metabolized and excreted in the intestinal tract and in the liver (first pass effect) before they reach the systemic blood circulation (presystemic elimination).

1.3. Fate of phenolic drugs in the gastrointestinal tract, p-nitrophenol (PNP) as a model compound

In these experiments a jejunal loop was prepared in vivo and it was perfused with isotonic medium containing different concentrations of PNP, this experimental arrangement was similar to the oral drug administration. Samples were obtained from the perfusion solution at various time during the intestinal perfusion and the concentration of PNP and its metabolites was determined.

PNP was used as a model compound because many drugs contain phenolic structure and hydroxyl group, therefore the metabolism of these drugs is similar to that of PNP. Moreover, it is well known that PNP is metabolized almost exlusively by conjugations and forms two metabolites: p-nitrophenol glucuronide (PNP-G) and p-nitrophenol sulfate (PNP-S). There are different methods for the analysis of these metabolites which were modified in our experiments. The glucuronide formation is catalyzed by glucuronyltransferase (UGT) and PNP-G is hydrolyzed by beta-glucuronidase enzyme. Sulfatation of PNP is catalyzed by sulfotransferase and PNP-S is hydrolyzed by arylsulfatase.

1.4. The following aspects of pharmacokinetic parameters were investigated:

1.4.1. Effect of dose of the drug
1.4.2. Effect of pathological changes (hyperglycemia)
1.4.3. Differences of drug metabolism in various segments of small intestine

It is known that several factors can influence the pharmacokinetic parameters. In these experiments three of them have been investigated, which are important from practical and pharmacotherapeutic points of wiew.

1.4.1. Dose of the drug

The amount of the drug (dose) is one of the most important parameters, which basically influences and determines the pharmacological and therapeutic effects. By

the elevation of the dose the pharmacolgical effect can be stimulated, howerer, at the same time the pharmacokinetic parameters will also be modified. The effects of drugs will be determined by the type of administration (oral, parenteral) and the dose of the drug.

The carrier-mediated transport processes and enzyme reactions can be saturated and by the further elevation of the dose of drugs the formation of metabolites can not be increased, which produces a suddenly and extremely high concentration of the mother compound with very strong pharmacological or toxic effects and consequences. Therefore in these experiments the changes of intestinal drug elimination and the metabolic activity of small intestine (formation and luminal appearence of PNP-G and PNP-S) were intestigated at the elevation of the dose of PNP.

1.4.2. Pathological changes (hyperglycemia)

Pathological circumstances and diseases can modify the pharmacokinetic parameters and the elimination of drugs. Hyperglycemia is one of the most characteristic clinical signs of diabetes, which means a complex disturbance of carbohydrate metabolism with hormonal and transport changes. Diabetes can also influence the pharmacokinetics of drugs. In these experiments the effect of hyperglycemia provoked by continous glucose infusion has been investigated on drug metabolism, especially on the biotransformation of PNP in the small intestine. It should be mentioned, however, that the hyperglycemia is only one factor of diabetes, but is not identical in all respects with the clinical or experimental diabetes.

1.4.3. Differences of drug metabolism in various segments of small intestine

Some data show that there are differences in physiological absorption processes and in the intestinal elimination of drugs in various segments of intestinal tracts. It is known that different diseases influence specifically the function of various segments of gastrointestinal tract: e.g. gastric- or duodenal ulcer, enteritis, chronic inflammation of large intestine (colitis ulcerosa, Crohn-disease). Specific intestinal segments are removed by some surgical operations and in these cases different functions can be changed. Therefore it is very important and interesting to study the relative importance of various segments of small intestine (proximal, distal jejunum and terminal ileum) in the biotransformation and elimination of drugs.

2. Methods: Experimental models- Analytical investigations

2.1. Materials, chemicals for analysis

P-nitrofenol, its glucuronide, the monopotassium salt of p-nitrophenol sulfate were obtained from the Sigma Aldrich Company (Budapest, Hungary). All other chemicals and reagents were analytical or HPLC-grade. The standard isotonic perfusion medium had the following compositions (mmol/l): NaCl 96.4, KCl 7.0, $CaCl_2$ 3.0, $MgSO_4$ 1.0, sodium phosphate buffer (pH 7.4) 0.9, TRIS Buffer 29.5, glucose 14.0, mannitol 14.0.

2.2. Animals and experimental procedure

Male Wistar rats weighing 220-250 g were used. The animals were anesthetized with urethane (1.2 g/kg i.p.). The abdomen was opened by a mid-line incision and in different groups of rats a proximal and distal jejunol loop or a segment of terminal ileum (length about 10 cm) were cannulated in vivo. The lumen of various segments was gently flushed with warmed isotonic solution to remove digesta and food residues and then blown empty with 4-5 ml air. Perfusion through the lumen of various segments of small intestine with isotonic medium containing PNP was carried out at rate of 13 ml/min in a reciculation mode for 90 minutes. In control rats the lumen of intestinal segments was perfused with isotonic medium without PNP. The volume of samples obtained from the perfusion medium coming out from the cannulated segments was 250µl, the initial perfusion volume was 15 ml.The temperature of perfusion medium was maintained constant at 37°C. The animals ware fasted 16-20 hours prior to the experiment, water was given ad libitum.

Hyperglycemia was maintained by a continous i.v. infusion of glucose in different concentrations (10-20-30 %) after the administration of a priming dose of glucose infusion solution enabling a high blood glucose level (15-50 µM) to be reached right at the start of continous glucose infusion.

For the investigation of biliary flow the bile duct was cannulated with a polyethylene tube (PE-10) and the bile was collected in 15-min periods.

2.3. Analytical conditions, instrumentations, determinations

The samples were analyzed and quantified by the HPLC-methods which have been develepod and used in our related experiments.

Summarized shortly: the mobile phase consisted of methanol and distilled water (50:50, V/V) containing 0.01 M tetrabutyl ammonium bromid to determine the metabolites in the perfusates. The perfusates were vortexed and centrifuged at 3000 g for 10 minutes before separation. The flow rate of the eluent was 1.2 ml/min. The volume of samples was 20 µl and the detection was effected at 290 nm, because this wavelength was optimal for the simultaneous determination of for PNP, PNP-S and PNP-G. Before the analysis the temperature of samples was allowed to rise to ambient temperature.

The HPLC system consisted of a Varian 2010 pump, a Rheodyne 7725 i injection valve, an UV-Detector 308 with data collection and integration using a Power Chrom 280 data module and sowftware. A Nucleosil 100 C_{18} reversed phase column (250 mm x 4.6 mm I.D, 10 µm particle size) was employed for the separation of metabolites.

The blood glucose levels were measured by Acuh-Check® digital blood glucose meter (Roche).

3. Calculations, statistical analysis

The luminal appearences of PNP-G and PNP-S were calculated on the base of their luminal concentrations and the actual volume of perfusion solution. The original volume of perfusion medium was 15 ml, which was modified by the obtained samples and the absorptive and resorptive activity of the perfused jejunal loop or the intestinal segment. Therefore the actual volume of perfusion solution was calculated and corrected by these changes. Data show the mean values ± S.E. (n: number of experiments or determinations).

Significant differences were calculated by the Student's t-test.

4. Results

4.1. Metabolic activity of the small intestine: formation of glucuronide and sulfate metabolites during the intestinal perfusion of PNP

It was shown in previous investigations that the metabolites of PNP (PNP-G and PNP-S) appearance in the small intestine of guinea pig relatively rapidly and efficiently during the intestinal perfusion of PNP. The major metabolite in the guinea pig was PNP-S, which amounted to about 50 % of administered PNP (perfused in a concentration of 500 μM) during the perfusion time (90 minutes).

4.1.1. Luminal appearence of metabolites in the small intestine of the rat during the intestinal perfusion of PNP: investigation of dose - dependency

In these experiments the intestinal metabolism of PNP has been investigated during the luminal perfusion of different concentrations (20-100-500-1,000 μM) of PNP. It was found that - in contrast with the findig in guinea pigs – the major metabolite in rats was PNP-G and PNP-S appeared in the intestinal lumen at a definitely slower rate. Other authors found also differences in the activity of UDP-glucuronyltransferase in various animal species.

Our experimental data show clearly that the amount of PNP was rapidly and continously decreased in the perfusion medium and at the end of experiments only about 10-15 % of the original amount of PNP were detected in the perfusion solution. However, PNP-G appeared simultaneously in the perfusate and showed an increasing tendency: at the end of the experiments 21.4; 16.1; 5.66 and 3.33% of the perfused PNP were detected in glucuronide form at the perfusion of PNP in a concentration of 20, 100, 500 and 1,000 μM, respectively. These results show a saturability of the intestinal appearence of PNP-G at the elevation of the dose (perfused concentration) of PNP.

At the perfusion of lower concentrations (20-100 μM) of PNP, the appearance of PNP-S was very low and at higher PNP concentrations (500-1,000 μM) the luminal appearance of sulfate conjugate of PNP was significatly lower than that of PNP-G. These results indicate that the luminal excretion of PNP-G exceeded that of PNP-S at all PNP concentrations investigated.

The luminal appearance of PNP-G was elevated by the increase of the dose of PNP, however, a tendency for saturation was observed : e.g. the 5-times higher PNP concentration (100 μM) produced a 3.8-times greater elevation in the luminal appearence of PNP-G compared to the value measured at 20 μM PNP concentration. Further increase of the PNP concentration (from 100 up to 500 μM) produced only 76 % elevation of intestinal excretion of PNP-G.

4.1.2. Effect of hyperglycemia on the intestinal metabolism of PNP

Hyperglycemia was provoked and mainained in these experiments by a continous i.v. glucose infusion after the administration of a priming dose of glucose enabling a high blood glucose level (20-40 mM) to be reached right at the start of glucose infusion. This range of the blood glucose corresponds to the hyperglycemic level of experimental diabetic rats produced by streptozotocin (STZ).

It is known that the hyperglycemia is one of the most characteristic symptoms of diabetes and it can produce itself changes in the functions and transport processes

of different organs. In our experiments the bile glucose level was increased parallel with glucose cocentration in the blood, howerer, the biliary flow was decreased.

The expereimental data of our investigations indicate that the biliary flow was definitely depressed in hyperglycemic rats, the magnitude of these changes seem to be parallel but in the opposite direction with that of the elevation of blood glucose level.

The luminal appearances of PNP-G were unchanged in hyperglycemic rats compared to the values of control animals, significant difference was detected only at 15-min perfusion period between the values of control and hyperglycemic rats.

However, the luminal appearance of PNP-S was significantly greater in hyperglycemic rats than in controls. On the basis of these changes, the total amount of metabolites showed a decreasing tendency, but the change did not reach the statistically significant level, because the appearance rate of the predominant metabolite (PNP-G) remaind basically unchanged.

4.1.3. Differences in drug metabolism of various segments of small intestine of the rat

Drug metabolism in various segments of small intestine (proximal-, distal jejunum and terminal ileum) was investigated in these experiments.

It was found that different segments of small intestine of rats were able to metabolize PNP rapidly and efficiently and to transport the conjugates (PNP-G and PNP-S) into the intestinal lumen. However, great differences were observed in the conjugating capacity in various segments of small intestine. PNP-G seemed to be the major metabolite in the small intestine at all PNP concentrations and a definite gradient activity could be detected in the formation and in the luminal appearence of PNP-glucuronide from proximal to distal segments: greatest amount of PNP-G was measured in the proximal jejunum, smaller amount in the distal jejunum and the lowest luminal appearence of PNP-G was detected in the terminal ileum: e.g. only 23 % of the appeared PNP-G in the proximal jejunum were measured in the terminal ileum when PNP was perfused in a concentration of 500 μM.

Sulfate conjugate of PNP appeared at significantly lower rate than PNP-G in all investigated segments of small intestine. It is interesting that, in contrast to the luminal appearance of PNP-G, no depression was found in the sulfatation of PNP, even a slight elevation was observed from the proximal to distal segments of small intestine: cumulative luminal appearance of PNP-S did not show decreasing tendency from proximal to distal direction, howerer, a slight increase was detected in distal segments (distal jejunum and terminal ileum).

The appearance of PNP-G in the intestinal lumen depended on the substrate (PNP) concentration of the luminal perfusion medium and tended to saturability: e.g. the 10-fold higher PNP-concentration (1,000 μM) produced only 2.2 – fold increase (525 nmol PNP-G) compared to the value measured at 100 μm PNP-concentration (242 nmol PNP-G). The values show a dose-dependent increase in the luminal appearance of PNP-G, however, a clear tendency for saturation could be observed with the elevation of the concentration of perfused PNP: e.g. 2 – fold higher PNP concentration (from 500 μM to 1,000 μM) produced only 24 %, 27 % and 23 % increase in the luminal appearence of PNP-G in the proximal-, distal jejunum and in the terminal ileum, respectively.

5. Discussion – Conclusions

In these experiments the role of some factors in the pharmacokinetics, more preciously the importance of some pharmacokinetic parameters influencing the intestinal drug elimination have been investigated during the intestinal perfusion of PNP.

Nowdays generic drugs are more widely used and in these cases the study a pharmacokinetic parameters is especially important.

Different steps of pharmacokinetics can be influenced by several factors, e.g. species -, genetic differences, diseases and drug interactions.

In our investigations the following aspects of pharmacokinetic parameters were studied: change of dosage of drugs, pathological alterations (e.g. hyperglycemia as a characteristic symptom of diabetes) and the role of various segments of small intestine in the drug metabolism.

The role of the intestinal tract in the elimination of drugs was investigated because some newer experimental data show that extrahepatic (e.g. intestinal) biotransformation and excretion can be also important in the elimination of xenobiotics. The enterocytes are able to metabolize the drugs and to excrete the metabolites into the intestinal lumen and to eliminate them with the faeces. A special and important aspect of the intestinal drug metabolism is the location of this system at the site of entry of exogenous compounds, because the intestinal biotransformation and excretion of xenobiotics can be an important factor of biovailability of drugs at oral drug administration.

Different metabolic reactions are important in the hepatic and intestinal biotransformation of drugs, e.g. the conjugation reactions (conjugation with glucuronic acid, glutathion, amino acids, sulfate….). Several enzymes are expressed in the intestinal tract and in the liver e.g. UDP-glucuronyltransferases, sulfotransferases, which produce glucuronide and sulfate conjugates of drugs.

In our experiments PNP was used as a model compound because it is metabolized almost exclusively by conjugation with glucuronic acid and sulfate and different methods are available to determine and quantify these metabolites. Furthermore the method of in vivo luminal perfusion of PNP in small intestine is very similar to the oral drug administration.

The small intestine of rats metabolized PNP rapidly and efficiently and the major metabolite was PNP-G, this finding is different from the data obtained in guinea pigs, because in guinea pigs the major metabolite was PNP-S. The luminal appearance of PNP-G showed an increasing tendency at the elevation of the dose (perfused concentration) of PNP, however, a saturation could be observed, which mean, that capacity of the intestinal elimination of PNP is limited.

Diabetes is caused basically by hormonal changes (insulin, glucagon), one of the most characteristic changes is hyperglycemia. Only a few data are available about the metabolism of xenobiotics in diabetes or hyperglycemia. Hyperglycemia is a very specific and important symptom of diabetes, however, in chronic diabetes many other metabolic changes occur simultaneously with some compensatory reactions. On the other hard, hyperglycemia can be produced in acute experiments, as well, e.g. by glucose infusion. In these experiments the effect of hyperglycemia was investigated and it was found that the glucose level in the bile changed parallel with the blood glucose concentration. In contrast to these parallelism, the biliary flow was depressed by hyperglycemia (cholestatic effect).

No significant changes were found in the luminal appearance of PNP-G in hyperglycemic rats compared to the controls, except the first 15- min period. However, the luminal appearance of PNP-S was enhanced by hyperglycemia. The sum of metabolites (PNP-G + PNP-S) in the intestinal lumen was not changed by high blood sugar level. A slight elevation tendency of the luminal appearance of PNP-S was similarly observed in experimental diabetic

rats, as well. It is intersting that the luminal appearance of PNP-G in STZ-diabetic rats was signififantly elevated, which means a difference between the reactions of the acute hyperglycemic and chronic diabetic rats. Our results indicate that hypergylcemia itself is able to produce some changes in the bile production and in the elimination of xenobiotics, however, differences can also be found in the effects produced by acute hyperglycemia and experimental diabetes, respectively.

Drug metabolism in various segments of small intestine (proximal-, distal jejunum and terminal ileum) was also investigated. It was found that different segments of small intestine of rats were able to metabolize PNP rapidly and efficiently and to transport the conjugates (PNP-G and PNP-S) into the intestinal lumen. However, great differences were observed in the conjugating capacity in various segments of small intestine. PNP-G seemed to be the major metabolite in the small intestine and a definite gradient activity could be detected in the formation and in the luminal appearance of PNP-glucuronide in the small intestine from proximal to distal segments: the greatest amount of PNP-G was measured in the proximal jejunum, smaller amount in the distal jejunum and the lowest luminal appearance of PNP-G was detected in the terminal ileum. Similar decreasing tendency in the second phase metabolic reactions from the stomach to large intestine was also published by other authors, too. It is interesting that in contrast with the decreased luminal appearance of PNP-G, no depression was found in the sulfatation of PNP, even a slight elevation of luminal appearance of PNP-S was observed from the proximal to distal segments of small intestine.

6. Summary and new results

1. Our experimental results indicate that in the elimination of drugs the small intestine may play an important role.

2. The small intestine metabolizes PNP rapidly and efficiently, the metabolites appear and can be detected in the intestinal lumen after the luminal perfusion of PNP.

3. The major metabolite of PNP is PNP-G, only a smaller amount of PNP-S appeared in the intestinal lumen of rats.

4. The luminal appearance of PNP-G in the proximal jejunum is dose-dependent, however, this process can be saturated.

5. Hyperglycemia itself can also produce important changes, e.g. depression in the biliary flow and increase in the luminal appearance of PNP-S, however, no alteration was found in the glucuronidation of PNP and in the luminal appearance of the sum of metabolites.

6. The dose-dependent luminal appearance of PNP-G could be observed in the distal segments of small intestine, as well.

7. A clear gradient was found in the intestinal elimination of PNP-G from proximal to distal segment of small intestine: the luminal appearance of PNP-G was significantly decreased in this direction.

8. In contrast to the decreared luminal appearance of PNP-G from proximal to distal segments of small intestine, the intestinal elimination of PNP-S was not decreased, even a slight elevation was observed: PNP-S appeared at a higher rate in the distal segments, than in the proximal jejunum.

These results indicate the importance of the small intestine in the elimination of drugs especially after oral administration of xenobiotics. Changes of this activity of small intestine should be considered at different pharmacological, physiological and pathophysiological conditions, as well (e.g. in determination and modification of bioavailability of drugs at different interactions, intestinal surgical interventions and intestinal diseases). Further investigations are needed to clarify the mechanism of the presented changes and differences and the role of other factors which can be important at oral drug administration.

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