

PhD thesis

***In vitro* and *in vivo* examinations of metabolic transformation of
capsaicinoids and salicylates**

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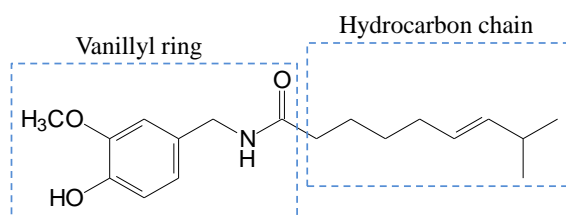
I. Introduction

I.1. Capsaicinoids

Capsaicinoids is the collective name of several structurally related compounds responsible for the pungency of capsicum fruits. Among capsaicinoids, capsaicin and dihydrocapsaicin are the most important in terms of abundance and pungency.

Capsaicinoids are used not only as a flavoring agent but also display several biological activities. It was earlier demonstrated that capsaicinoids display analgesic action, protect the mucosa against gastrointestinal adverse effects of drugs and alcohol and have anti-inflammatory properties as well as anti-tumor and antioxidant potential.

Structural characteristics of capsaicinoids are responsible for their spicy flavor and biological activities are associated with the presence of an amide bond connecting a vanillyl ring (3-hydroxy-4-methoxybenzylamide) and an acyl chain. Capsaicinoids differ from each other in the side chain moiety, including saturation of the carbon-carbon double bond, deletion of a methyl group, and the length of the hydrocarbon chain (1).



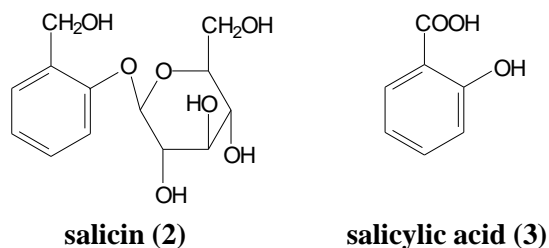
capsaicin (1)

In vivo biotransformation of capsaicinoids is not well known. Several research groups confirmed that after oral administration capsaicinoids are absorbed and hydrolyzed in the gastrointestinal tract. In the consequence of the significant first pass effect the level of capsaicinoids is very low in the general circulation. The metabolites do not have those effects which are characteristic for capsaicinoids.

I.2. Derivatives of salicylic acid, salicylates

Analgesic and anti-inflammatory effect of the extract prepared from the bark and leaves of *Salix alba* (white willow) has been known since antiquity.

Initially it was thought that the active extract of the powdered bark is salicin (2). Later it was found that the transformation of salicin in the body results in the formation of salicylic acid (3), so the active compound of the willow bark is the salicylic acid.



In the 70-80s of the 19th century, salicylic acid was the most effective drug in the treatment of rheuma, but the administered high dose (4-6 g/day) was accompanied by serious side effects, for example gastritis and tinnitus. Acetylsalicylic acid synthesized by the chemical modification of salicylic acid has fewer side effects. Biological activities of acetylsalicylic acid are associated with the presence of acetyl group and salicylate part, furthermore its primary metabolite (salicylic acid).

Salicylates inhibit enzyme cyclooxygenase (COX) which play role in the biosynthesis of prostaglandines. As the consequence of the inhibition of COX system, salicylates have some very important therapeutic effects, for example antipyretic, analgesic and anti-inflammatory effects (additionally acetylsalicylic acid has antiplatelet effect). However, the inhibition of COX-enzyme is responsible for the side effects of salicylates, such as gastrointestinal bleeding and nephrotoxicity.

Salicylic acid and acetylsalicylic acid can be characterized by acidic properties ($pK_a(\text{SA})=3,00$; $pK_a(\text{ASA})=3,50$). Their absorption from the stomach is carried out by passive diffusion. In the small intestine (pH 2,0-7,0) the ionic form of salicylates is the dominant, nonetheless absorption of salicylates from the small intestine is very significant. It can be explained by the better solubility of salicylates and the bigger surface area (100-200 m²) of duodenum and jejunum due to the highest concentration of villi and microvilli in these regions. Specific carrier-mediated transport mechanisms also play important role in the absorption of several monocarboxylic acids such as salicylic acid.

In the body, acetylsalicylic acid is rapidly deacetylated to form salicylic acid, this compound is the primary metabolite of aspirin and responsible for many of its biological actions. Salicylic acid is metabolized in the liver microsomes by oxidation or by conjugation, with either glycine (70-75%) or glucuronic acid (15%). In the reaction catalyzed by UDP-glucuronyl transferase acyl and phenolic glucuronide conjugates are also be formed.

II. Aims

Development of drug containing acetylsalicylic acid and a low-dose capsaicinoid has been started at 2006 at the University of Pécs in the support of MEDIPOLISZ to prevent gastric mucosal damage of NSAIDs (nonsteroidal anti-inflammatory drugs). Our main goal was to develop analytical methods for the determination of capsaicin and dihydrocapsaicin content of capsaicinoid containing drugs and plasma samples derived from preclinical animal experiments.

In the course of the analysis of samples derived from preclinical animal experiments unexpected result was found, such as capsaicin and dihydrocapsaicin could not be detected in plasma after *per os* administration of capsaicinoids. Based on these results assuming presystemic transformation, *in vivo* absorption and extrahepatic metabolism of capsaicinoids were also be examined.

Biotransformation of acetylsalicylic acid and its primer metabolite (salicylic acid) in the liver is proven and well-known, but intestinal metabolism may also significantly influence the disposition of salicylates.

Salicylic acid has antioxidant effect (scavenger effect), for example in patients suffering from rheumatoid arthritis after oral administration of acetylsalicylic acid, significant increase in the level of 2,3-dihydroxybenzoic acid (formed in only non-enzymatic reactions) can be observed in the plasma. However, salicylic acid administered in higher dose is able to disconnect oxidative phosphorylation resulting in increased oxygen uptake, thereby enhances the probability of the formation of reactive oxygen species.

The presence of reactive oxygen species and salicylate metabolites (formed by non-enzymatic transformation) in the body raises several questions, for example they are responsible for the appearance of the side effects attributed to salicylates or they play role in still uncleared mechanism of action of salicylates.

Our main goals were the followings:

- Development and application of validated high performance liquid chromatography (HPLC) method for pharmacopoeial qualification of Capsicum extracts to be used in pharmaceutical formulations.
- Development of sample preparation procedure and validated HPLC method for determination of capsaicin and dihydrocapsaicin content of plasma samples derived from beagle dogs treated by capsaicinoid extract qualified according to United States Pharmacopoeia.

- Analysis of intestinal absorption and metabolism of capsaicinoids in the rat, identification of the metabolites.
- Development of validated HPLC method for the separation of salicylic acid and its hydroxylated metabolites (2,3-dihydroxybenzoic acid and 2,5-dihydroxybenzoic acid).
- Analysis of intestinal absorption of salicylic acid in the rat.
- Analysis of intestinal metabolism of salicylic acid in the rat, identification of the metabolites.
- *In vitro* oxidative metabolism of salicylic acid under the condition of Fenton- and Udenfriend-incubation.

III. Methods and results

III.1. Development and validation of a HPLC-DAD analysis for determination of capsaicinoids from industrial capsicum extracts

An isocratic, easy-to-perform reversed-phase HPLC method coupled with DAD detection ($\lambda=281$ nm) was developed with the specific aim to qualify the capsaicinoid content of industrial Capsicum extracts according to the Capsaicin monograph of United States Pharmacopoeia (USP 29).

USP Capsaicin is a Capsicum extract that can be used by the pharmaceutical industry to prepare different pharmaceutical formulations. The main capsaicinoids of the extracts are capsaicin and dihydrocapsaicin. Since the USP requirements of the Capsicum extracts set not only the amount of capsaicin and dihydrocapsaicin but that of the total capsaicinoids as well, a Capsaicin natural extract (Fluka) was used as secondary standard and standardized against USP Capsaicin and USP Dihydrocapsaicin reference standard.

The developed and validated HPLC-DAD method has been applied for determination of capsaicin, dihydrocapsaicin and total capsaicinoid content of an industrial Capsicum extract and the Fluka Capsaicin natural standard sample. Composition of both extracts fulfilled the USP 29 requirements.

III.2. Development of sample preparation procedure and validated HPLC method for determination of capsaicin and dihydrocapsaicin content of plasma samples after oral administration of capsaicinoid extract qualified according to United States Pharmacopoeia

Preclinical-toxicological examinations needed for drug development of capsaicinoids were performed at the Research Centre of Toxicology at Veszprém. Treatments were carried out on beagle dogs applying qualified capsaicinoid extract dissolved in PEG 400, in the dose of 0-1200 µg/body weight, once a day, for 28 consecutive days. Blood samples were collected EDTA-containing tubes at different times (0 h; 0,25 h; 0,5 h; 1 h; 2 h; 3 h; 4 h). After sample collection plasma was immediately separated by centrifugation, then it was stored in ultra freezer until analysis.

After protein precipitation and solid phase extraction of the plasma samples HPLC method based on fluorescent detection ($\lambda_{\text{ex}} = 230 \text{ nm}$; $\lambda_{\text{em}} = 323 \text{ nm}$) provided very high sensitivity because of the strong fluorescent activity of hydroxymethoxybenzyl group of capsaicinoids.

In the course of the analysis of the samples unexpected result was found, such as capsaicin and dihydrocapsaicin could not be detected in plasma after *per os* administration of capsaicinoids. These results raise several questions in connection with the pharmacokinetics of capsaicinoids.

III.3. Analysis of intestinal absorption and metabolism of capsaicinoids in the rat, identification of the metabolites

Our experiments were designed to study the biotransformation of capsaicinoids in the small intestine in the rat. Perfusion through the lumen of small intestine with isotonic medium containing 100 µM Capsaicin natural was carried out for 90 minutes.

The developed HPLC-FLD was revalidated for the analysis of the capsaicin and dihydrocapsaicin content of the perfusates, furthermore the luminal appearance of metabolites.

We could prove that capsaicinoids are easily absorbed from the gastrointestinal tract, due to their lipophilicity. Capsaicin and dihydrocapsaicin content decreased exponentially in the perfusate, approximately 90% of the initial capsaicinoid concentration was absorbed until the end of the perfusion experiment.

It was found that the jejunal loop (length about 10 cm) of small intestine of the rats was able to metabolize capsaicinoids rapidly and efficiently and to transport the metabolites into

the intestinal lumen. The exact structure of the two metabolites was identified by mass spectrometry. The metabolites have been proven to be the glucuronide ethers of the original capsaicinoids.

The results indicate that the small intestine of the rat has a significant metabolic activity. This recognition can be one of the explanations of our earlier experience that capsaicinoids can not be detected in plasma after *per os* administration of low-dose Capsaicin natural.

III.4. Analysis of intestinal absorption of salicylic acid in the rat

In the small intestine (pH 2,0-7,0) the ionic form of salicylates is the dominant, nonetheless absorption of salicylates from the small intestine is very significant. In addition to the passive diffusion specific carrier-mediated transport mechanisms (e.g. organic anion transporter-1) also play important role in the absorption of salicylic acid.

We could prove that the examined sodium salicylate was absorbed from the gastrointestinal tract. Approximately half of the initial salicylate concentration can be measured at the 90th minutes of the perfusion period.

III.5. Analysis of intestinal metabolism of salicylic acid in the rat, identification of the metabolites

Our experiments were designed to study the biotransformation of salicylic acid in the small intestine in the rat. Perfusion through the lumen of small intestine with isotonic medium containing 250 μM sodium salicylate was carried out for 90 minutes.

Since only half of the administered salicylate concentration (250 μM) absorbed from the perfusate, a rather low concentration of transferred metabolites can be expected in the perfusate. For the detection and identification of metabolites in the perfusates, an optimized LC-DAD-MS method combined with liquid-liquid extraction was developed. The developed HPLC method provided separation of salicylic acid and their two hydroxylated derivatives (2,3-dihydroxybenzoic acid and 2,5-dihydroxybenzoic acid), which could be applied to observation of luminal appearance of the two metabolites. The used liquid-liquid extraction combined with salting out technique ensured appropriate purity of the perfusates reducing the baseline noise therefore promoting the increase of the limit of quantification for the hydroxylated metabolites.

The salicylate in the administered concentration (250 μM) may have caused the increased activity of myeloperoxidase in the intestinal mucosa resulting in the generation of oxygen-

derived species. As the consequence of the hydroxyl radical scavenger effect of the salicylic acid, its aromatic hydroxylation can occur preventing the onset of intestinal damage of reactive oxygen species.

III.6. *In vitro* oxidative metabolism of salicylic acid

A large number of drug molecules (e.g. nonsteroidal anti-inflammatory agents) containing aromatic rings are administered in different disorders in which involvement of oxidative damage has been suggested. Hydroxylation is one of the most common types of metabolic transformation of aromatic rings resulting in phenolic compounds by enzyme-catalyzed or non-enzymatic reactions (e.g. Fenton-reaction, Udenfriend-reaction).

Examination of salicylic acid (as a model compound) in Fenton- and Udenfriend-incubation is well-known, but these examinations in the most cases were performed under optimal conditions (e.g. pH-optimum of Fenton-reaction is in acidic condition). The effect of the reaction parameters for the quantity and quality of the products were investigated, furthermore the hydroxylation mechanism of the two similar reactions were compared.

Transformation of salicylic acid in Fenton-incubation

Transformation of salicylic acid under the condition of Fenton-incubation is determined by the equilibrium of three parallel reactions: (1) the Fenton-reaction, (2) complex formation of Fe(III)-ions (derived from Fenton-reaction) and salicylic acid and (3) substitution reaction of salicylic acid with hydroxyl radicals (derived from Fenton-reaction). Quantity of the products is influenced by the applied hydrogen peroxide concentration, the Fe(II)/H₂O₂ ratio, the pH and the duration of the incubation.

Under acidic conditions (1 mM H₂O₂ és 3 mM Fe(II)-ions) as the consequence of non-region selective substitution, the hydroxylated metabolites (2,3-DHB, 2,4-DHB and 2,5-DHB) of salicylic acid were identified in the incubate. Using higher peroxide concentration (1 mM → 10 mM) salicylates were not detected. Decrease in the concentration of Fe(II) (3 mM → 300 μM) caused the change in the ratio of the formed dihydroxybenzoic acids.

Under physiological conditions (pH 7.2-7.4) 2,3-dihydroxybenzoic acid and 2,5-dihydroxybenzoic acid were identified from the incubate and the ratio of the two metabolites was shifted towards 2,5-dihydroxybenzoic acid.

Transformation of salicylic acid in Udenfriend-incubation

Udenfriend and his colleagues described a model system consisting of ascorbic acid, molecular oxygen and a transition metal ion in which phenolic compounds are produced due to hydroxylation of aromatic rings. In this system salicylic acid → gentisic acid transformation was observed.

In our laboratory we used this model system (Udenfriend's system) for investigation of the non-enzyme-catalyzed hydroxylation of salicylic acid. It was found that Udenfriend's incubation of salicylic acid results in formation of 2,3-, 2,4- and 2,5-dihydroxybenzoic acids.

IV. Conclusions, novel findings

- A validated high performance liquid chromatography (HPLC) method was developed for pharmacopoeial qualification of Capsicum extracts to be used in pharmaceutical formulations.
- A sample preparation procedure (solid phase extraction) and validated HPLC method were developed for determination of capsaicin and dihydrocapsaicin content of plasma samples derived from beagle dogs after oral administration of qualified capsaicinoid extract.
- Intestinal absorption and metabolism of capsaicinoids were investigated in the rat. We could prove that capsaicinoids are easily absorbed from the gastrointestinal tract, furthermore the jejunal loop of small intestine was able to metabolize capsaicinoids rapidly and efficiently and to transport the metabolites into the intestinal lumen. The exact structure of the two metabolites was identified. The metabolites have been proven to be the glucuronide ethers of the original capsaicinoids.
- Intestinal absorption and metabolism of sodium salicylate were investigated in the rat. We could prove that approximately the half of the initial salicylate concentration is absorbed from the gastrointestinal tract, furthermore two hydroxylated metabolites (2,3- and 2,5-dihydroxybenzoic acid) of salicylic acid were identified from the perfusate. The salicylate in the administered concentration (250 μ M) may have caused the increased activity of myeloperoxidase in the intestinal mucosa resulting in the generation of oxygen-derived species. As the consequence of the hydroxyl radical scavenger effect of the salicylic acid, its aromatic hydroxylation can occur preventing the onset of intestinal damage of reactive oxygen species.

- Non-enzymatic oxidative transformation of salicylic acid was investigated in the course of *in vitro* experiments using Fenton- and Udenfriend-models. In both reactions Fe(II) has significant role resulting in the formation of common metabolites. In the non-enzymatic biotransformation of salicylic acid the role of Fenton- and Udenfriend-reactions in the formation of dihydroxybenzoic acids can not be excluded.

V. List of publications

Publications related to the present PhD thesis

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