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Presystemic Metabolism and Intestinal Absorption of Antipsoriatic Fumaric Acid Esters

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ABSTRACT: Psoriasis is a chronic inflammatory skin disease. Its treatment is based on the inhibition of proliferation of epidermal cells and interference in the inflammatory process. A new systemic antipsoriasis drug, which consists of dimethylfumarate and ethylhydrogenfumarate in the form of their calcium, magnesium and zinc salts has been introduced in Europe with successful results. In the present study, a homologous series of mono- and diesters of fumaric acid has been studied with respect to the sites and kinetics of presystemic ester degradation using pancreas extract, intestinal perfusate, intestinal homogenate and liver S9 fraction. In addition, intestinal permeability has been determined using isolated intestinal mucosa as well as Caco-2 cell monolayers, in order to obtain estimates of the fraction of the dose absorbed for these compounds. Relationships between the physicochemical properties of the fumaric acid esters and their biological responses were investigated. The uncharged diester dimethylfumarate displayed a high presystemic metabolic lability in all metabolism models. It also showed the highest permeability in the Caco-2 cell model. However, in permeation experiments with intestinal mucosa in Ussing-type chambers, no undegraded DMF was found on the receiver side, indicating complete metabolism in the intestinal tissue. The intestinal permeability of the monoesters methyl hydrogen fumarate, ethyl hydrogen fumarate, *n*-propylhydrogen fumarate and *n*-pentyl hydrogen fumarate increased with an increase in their lipophilicity, however, their presystemic metabolism rates likewise increased with increasing ester chain length. It is concluded that for fumarates, an increase in intestinal permeability of the more lipophilic derivatives is counterbalanced by an increase in first-pass extraction.

Key words: first-pass extraction; prodrug; permeability; degradation; bioavailability

Introduction

The antipsoriatic fumaric acid therapy is based on the peroral administration of dimethyl fumarate and various salts of ethyl hydrogen fumarate [1]. The diester dimethyl fumarate turned out to be more effective than the monoester ethyl hydrogen fumarate, although it was shown that dimethyl fumarate is rapidly cleaved to methyl hydrogen fumarate by hydrolysis in the circulation [2, 3]. Successful absorption of esters into the systemic circulation not only requires a sufficiently high permeability of the active species across the intestinal mucosa but also stability against intestinal and hepatic hydrolysis.

The presystemic metabolic barrier of the intestine is responsible for the rapid disappearance of the ester from the absorption site due to cleavage of the ester bond by esterases associated with the intestinal lumen and the mucosa.

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Introduction

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Considerable esterase activity is also localized in the hepatic system [4]. There are, however, indications that in the intestine dimethyl fumarate is completely biotransformed to its metabolite, methyl hydrogen fumarate, before reaching the liver [3].

In the present work, the metabolic activity of the intestinal mucosa as a barrier for the absorption of various fumaric acid esters (fumarates) with different chain lengths has been investigated. Additionally, the effect of pancreatic and hepatic enzymes on the ester stability has been studied. The effect of chain length on metabolic stability and permeability was investigated with fumarates with different acyl chain lengths. In 1982, Chang and Lee showed in albino rabbits that the ocular hydrolysis of a series of α- and β-naphthyl esters increased with increasing chain length of the acyl moiety. Several causes have been suggested for this increase in hydrolysis rate with chain length. An increase of the acyl residue is accompanied by an increase in lipophilicity which itself has an impact on its metabolic disposition [5–7], principally because the compound must first partition into the active site of the enzyme being considered as a hydrophobic pocket. On this premise, the acyl chain length of an ester, via its influence on lipophilicity, may significantly influence the rate at which an ester would be hydrolyzed by esterases. Chang and Lee as well as other investigators [8–11] found a parabolic relationship between the rate of hydrolysis and the acyl chain length. A linear increase in hydrolytic rate with chain length, however, has been noted by Hofstee [12], and a reduction was reported by Jordan [13].

Esterases are classified in three groups based on their interaction with organophosphates: A-esterases hydrolyse organophosphates, B-esterases are inhibited by organophosphates and include cholinesterase (EC 3.1.1.8) and carboxylesterase (EC 3.1.1.1) and finally, C-esterases which do not interact with organophosphates [14]. In order to classify the esterases involved in the metabolism of fumarates, studies in the presence of well known esterase inhibitors (organophosphates and others) are presented.

The lipophilicity of a compound, in addition to its degradation by esterases, will also influence its intestinal permeability, the second important prerequisite for bioavailability by the peroral route of administration. In general, an increase in the lipophilicity of a solute is accompanied by an increased ability to passively enter and permeate cell membranes. However, there appears to be an upper limit of the absorption rate as a function of lipophilicity [15], i.e. the rate limiting step shifts from membrane-controlled to unstirred water layer-controlled permeability [16–18]. By studying acetyl-, propionyl- and butyrylsalicyclic acid, Kimura *et al*. [19] reported an increase in permeability with increasing acyl chain length.

In the present study, both cell culture and excised tissue permeability models were used in order to determine the permeability coefficients of dimethyl, methyl hydrogen, ethyl hydrogen, *n*-propyl hydrogen and *n*-pentyl hydrogen fumarate. As an *in vitro* animal model, excised porcine intestinal mucosa was employed, providing full physiological intestinal cell heterogeneity, mucus layer and residuals of luminal enzymes, all of which may affect the transport of permeants [20]. As a second model, the well established Caco-2 cell culture model, derived from human colon adenocarcinoma and characterized by cell homogeneity was used.

In the past, only limited attention has been paid to the intestinal permeability of fumarates, although transport studies of fumarate across porcine intestinal brush-border membrane were performed by Wolffram *et al*. [21]. Using isolated brush-border membrane vesicles, the authors observed Na^+-dependent saturable transport of fumarate consistent with secondary active Na^+/di(tri)carboxylate co-transport. The uptake was reported to be mediated by a single saturable transport process and to a much smaller extent by diffusional uptake.

Materials and Methods

Metabolism studies

Chemicals. Dimethyl fumarate (DMF), calcium methyl fumarate (CaMF), calcium ethyl fumarate (CaEF), calcium *n*-propyl fumarate (Ca n-PrPF) and calcium *n*-pentyl fumarate (Ca n-PeF) were a gift from Fumapharm AG (Muri, CH). Phenyl-

methylsulfonyl fluoride (PMSF), diethyl *p*-nitrophenyl phosphate (paraoxon), bis-nitrophenol phosphate (BNPP) and physostigmine (eserine) were purchased from Sigma Chemicals (Buchs, CH). Mercuric chloride was obtained from Fluka Chemie AG (Buchs, CH) and ethylene-diaminetetraacetate (ETDA) from Siegfried Handels AG (Zofingen, CH). All other chemicals were of analytical purity and purchased from Fluka Chemie AG (Buchs, CH) or Merck Schweiz (Dietikon, CH).

Pancreatic enzymes. Crude porcine pancreas extract (Eurobiol® Interdelta, Fribourg, CH) with standardized activities of lipase (1350 U), amylase (1350 U), trypsin (33 U) and chymotrypsin (450 U) at a concentration of 1 mg protein ml^{-1} was used [22]. The concentration of the protein was determined by means of the Bio-Rad protein assay kit (Bio-Rad Laboratories, Glattbrugg, CH) with bovine plasma gamma globulin as a standard. Eurobiol® was suspended in prewarmed (37°C) Krebs' phosphate buffer containing 120.8 mM sodium chloride, 4.8 mM potassium chloride, 1.2 mM magnesium sulfate, 16.5 mM dibasic sodium phosphate and 1.3 mM calcium chloride, adjusted to pH 6.5 with hydrochloric acid. Control samples were obtained by incubating the esters with pancreas extract which had been boiled for 15 min.

Intestinal metabolism. Perfusate and homogenate were prepared from porcine small intestine (jejunum) obtained from the local slaughterhouse immediately after slaughter. Fresh parts of jejunum with a length of 50 cm were perfused with 50 ml Krebs' phosphate buffer pH 6.5. The perfusate was kept at 4°C and transported to the laboratory within 30 min. The perfused jejunum was stored in 0.9% sodium chloride solution at 4°C and also transported to the laboratory for preparation of intestinal homogenate.

Small intestine perfusate. Immediately after arrival in the laboratory the perfusate was centrifuged at 3000 **g** for 10 min at 4°C (Sorvall® RC-5B refrigerated superspeed centrifuge, Du Pont Instruments, Digitana AG, Horgen, CH). Thereafter the amount of protein was determined (Bio-Rad protein assay kit, Bio-Rad Laboratories, Glattbrugg, CH) and the perfusate was stored at –80°C.

Small intestine homogenate. Homogenates were obtained by scraping off the mucosa with a glass slide at 4°C. The scraped material was diluted 1:10 in ice-cold Krebs' phosphate buffer pH 6.5 and homogenized with a Polytron® PT 3000 homogenizer (Kinematica AG, Luzern, CH) at 16 000 rpm for 2–4 min. The protein content was determined and the homogenate was stored at –80°C.

Hepatic metabolism. Fresh porcine liver was obtained from the local slaughterhouse immediately after slaughter and was placed into ice-cold 1.15% KCl in 0.1 M phosphate buffer pH 7.4, and transported to the laboratory within 30 min. All subsequent procedures were carried out at 4°C according to Lake [23]. After washing the liver twice with fresh phosphate buffer, 3 ml of 1.15% KCl in 0.1 M phosphate buffer pH 7.4 per g wet tissue was added. The liver was chopped with scissors and then homogenized using a Polytron® PT 3000 homogenizer (Kinematica AG, Luzern, CH) at 16 000 rpm for 4 min. During homogenization the vessel containing the homogenate was kept in ice water to keep the temperature at 4°C. The homogenate was centrifuged at 9000 **g** for 10 min in a high-speed centrifuge at 4°C (Sorvall® RC-5B). After decanting the supernatant and determination of the protein concentration, the decanted supernatant (S9 fraction) was stored at –80°C.

Incubation studies. Solubilized pancreatic extract, jejunal perfusate, jejunal homogenate or liver S9 fraction, each standardized to a protein concentration of 1 mg ml^{-1}, were preincubated at 37°C for 20 min. Dimethyl fumarate and calcium salts of methyl, ethyl, *n*-propyl and *n*-pentyl fumarate were added to yield final concentrations of 0.05 mM, 0.1 mM, 0.5 mM and 1.0 mM, respectively. The concentration range was similar to the venous plasma concentrations found in pigs after oral and intravenous administration of dimethyl fumarate and calcium salts of methyl, ethyl, *n*-propyl and *n*-pentyl fumarate at doses of 10 and 20 mg kg^{-1} bodyweight. Incubation experiments with liver S9 fractions were also performed in the

presence of 1.0 mM NADPH as cofactor for cytochrome P450 mediated metabolism. Samples were taken at 0, 5, 10, 15, 20, 30, 45, 60, 90 and 120 min after the addition of the fumarate and diluted in an equal volume of chilled 1.0 M perchloric acid to stop the reaction. After maintaining the sample >15 min in ice-water the samples were centrifuged for 4 min at 10 280 **g** (Hettich EBA 12, Andreas Hettich AG, Bäch, CH). The supernatant was analysed by HPLC.

Inhibition studies. Pancreatic extract, jejunal perfusate, jejunal homogenate or liver S9 fraction (protein concentration 1 mg ml^{-1}) were preincubated at 37°C with 0.2 mM PMSF (in 100% isopropanol, the final isopropanol concentration was 2% in the incubate) and 5 mM EDTA, 0.1 mM paraoxon, 0.1 mM BNPP, 0.1 mM eserine and 0.1 mM mercuric chloride, respectively, for 5 min prior to the addition of fumarates. Final concentrations of fumarates were 0.05 mM, 0.1 mM, 0.5 mM or 1.0 mM, respectively. The rate of fumarate hydrolysis was measured as described under incubation studies.

HPLC assay. Fumarate. The HPLC system used consisted of a L-6200A pump, an AS 2000 autosampler, a SP8773XR UV detector (Spectra-Physics, Basel, CH) and a D-2500 Chromato-Integrator (Merck-Hitachi, Darmstadt, D). Analyses of dimethyl, methyl hydrogen, ethyl hydrogen, *n*-propyl hydrogen and *n*-pentyl hydrogen fumarate in the supernatant were performed by using a Li-Chrospher 100 RP-8 (5 µm) column of 25 cm length and 4 mm in diameter (Merck, Dietikon, CH). The mobile phase consisted of a mixture of 0.05 M monobasic sodium phosphate and acetonitrile (65:35, v/v) adjusted to pH 3.2 with ortho-phosphoric acid (85%). The flow rate was 1.0 ml min^{-1} and detection was at 230 nm. Calibration curves were determined in aqueous 0.05 M monobasic sodium phosphate and covered the full range of the expected concentrations in the samples. One batch of three samples of high (1.0 mM) and three samples of low concentration (0.05 mM) was prepared and analyzed, together with a calibration curve, in order to determine precision. The limit of quantification was at 0.1 mg l^{-1}. As expected the retention time of the monoesters rose with increasing chain length (Table 1), due to the more intense interaction between the more lipophilic monoesters with the lipophilic stationary phase.

Table 1. Elemental compositions, molecular weights and retention times of fumarates

Compound	Formula	Molecular weight	Retention time[a]
Dimethyl fumarate	$C_6H_8O_4$	144	6.5–6.7
Methyl hydrogen fumarate	$C_5H_6O_4$	130	3.2–3.3
Ethyl hydrogen fumarate	$C_6H_8O_4$	144	4.2–4.6
n-Propyl hydrogen fumarate	$C_7H_{10}O_4$	158	6.0–6.1
n-Pentyl hydrogen fumarate	$C_9H_{14}O_4$	186	15.7–16.0

[a] HPLC assay: Li-Chrospher 100 RP-8 column (5 µm particle size; 4 × 250 mm) (Merck, Dietikon, CH); mobile phase: 0.05 mM monobasic sodium phosphate and acetonitrile (65:35, v/v) adjusted to pH 3.2 with ortho-phosphoric acid (85%), flow rate 1.0 ml min^{-1}, UV 230 nm.

Atenolol/propranolol. The HPLC system used consisted of a L-6200A pump, an AS 4000 autosampler, a L-4250 UV-VIS detector, a D-6000A interface and a D-7000 HPLC System Manager (Merck-Hitachi, Darmstadt, D). Analyses of atenolol in the supernatant were performed by using a Li-Chrospher 100 RP-8 (5 µm) column of 25 cm length and 4 mm in diameter (Merck, Dietikon, CH); analyses of propranolol were performed by using a Li-Chrospher 60-5 selected B column of 25 cm length and 4 mm in diameter (Macherey-Nagel AG, CH). The mobile phase A consisted of 0.02 M KH_2PO_4, pH 4.7 and the mobile phase B of acetonitrile. Elution of atenolol and propranolol was accomplished by changing the mobile phases, A and B, according to a gradient running from 10% to 20% of solution B in 10 min applying a flow rate of 1.4 ml min^{-1}. Detection was by UV absorbance at 280 nm in case of atenolol and at 220 nm in case of propranolol.

Data processing. Degradation half-lives and metabolic turnover rates. Degradation half-lives were calculated according to the following equation:

$$t_{1/2} = \frac{0.693}{K}$$

with K as the degradation rate constant.

Statistical analysis. Results are presented as mean ± SD of n=3–4 degradation determina-

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