Specificity of procaine and ester hydrolysis by human, minipig and rat skin and liver

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Running Title:
Ester hydrolysis by skin and liver subcellular fractions

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Abbreviations: BNPP, bis-nitrophenylphosphate; CES, carboxylesterase; hCE1, human carboxylesterase-1; hCE2, human carboxylesterase-2; hCE3, human carboxylesterase-3; PV, phenyl valerate; PA, phenyl acetate; NPA, para-nitrophenyl acetate; MUA, 4-methylumbelliferyl acetate; PMSF phenylmethyl sulfonyl fluoride
Abstract

The capacity of human, minipig, and rat, skin and liver subcellular fractions to hydrolyse the anaesthetic ester procaine was compared to carboxylesterase substrates 4-methylumbelliferyl-acetate, phenylvalerate, para-nitrophenylacetate, and the arylesterase substrate, phenylacetate. Rates of procaine hydrolysis by minipig and human skin microsomal and cytosolic fractions were similar, with rat displaying higher activity. Loperamide inhibited procaine hydrolysis by human skin suggesting involvement of hCE2. The esterase activity and inhibition profiles in the skin were similar for minipig and human, whereas rat had a higher capacity to metabolise esters and a different inhibition profile. Minipig and human, liver and skin esterase activity was inhibited principally by paraoxon and bis-nitrophenyl phosphate, classical carboxylesterase inhibitors. Rat skin and liver esterase activity was inhibited additionally by phenylmethylsulfonyl fluoride and the arylesterase inhibitor mercuric chloride, indicating a different esterase profile. These results have highlighted the potential of skin to hydrolyse procaine following topical application, which possibly limits its pharmacological effect. Skin from minipig used as an animal model for assessing transdermal drug preparations had similar capacity to hydrolyse esters to human skin.
Introduction

Understanding the nature of skin xenobiotic metabolising enzymes is important in assessing the fate of absorbed xenobiotics and drugs applied to the skin. Following absorption through the stratum corneum, most metabolism in the skin occurs in the basal keratinocytes of the epidermis (Hotchkiss, 1992). Despite the importance of esterases in the hydrolysis of topically applied drugs and pro-drugs, investigation of skin esterases has been limited.

Esterases are members of the hydrolase family of enzymes which primarily hydrolyse endogenous and exogenous esters with substrate specificity overlapping with lipases (Williams 1985, Mentlein et al., 1988). They are ubiquitously expressed in mammalian liver, blood, and extra-hepatic tissues including skin, kidney, intestines, testes, brain, central nervous system, and lung (Satoh and Hosokawa 1998). Carboxylesterases (E.C. 3.1.1.1) are present in both the endoplasmic reticulum and cytosol (McCracken et al., 1993). The nature of microsomal esterases in human liver and gut have been most extensively studied (Huang et al., 1996) because of their role in hydrolysing orally ingested drugs. Satoh and Hosokawa (2006) have attempted to rationalise the classification of carboxylesterases. Three human carboxylesterase (CES) isoforms, hCE1 and hCE2 and hCE3 have been identified in microsomes with hCE1 highly expressed in liver but not the gut, and hCE2 expressed extra-hepatically, especially in the gut and at lower levels in the liver (Imai et al., 2006, Sanghani et al., 2004, Satoh and Hosokawa et al., 2006). Carboxylesterases are also found in the cytosol but they have not been classified. Cloning of hCE1, hCE2 and hCE3 has allowed ranking of the specificity for hydrolysis of drugs; Procaine, aspirin and irinotecan had specificity for hCE2 (Wu et al.,
2004) and methylphenidate and meperidine had specificity for hCE1 (Sun et al., 2004). hCE3 which is mainly expressed in the brain and gut had low affinity for these substrates (Sanghani et al., 2004). The rat expresses up to six carboxylesterases which are classified as CES1 type and or CES2 type (Sanghani et al., 2002). Two carboxylesterases have been identified in the pig and have been classified as CES1 and CES. (Satoh and Hosokawa, 2006). David et al., (1998) reported a pig intestinal carboxylesterase that showed high similarity with that of rat and human intestinal carboxylesterases.

Clark et al., (1993) and Hewitt et al., (2000) showed that esters applied to the skin surface were hydrolysed during dermal absorption using skin in vitro in a diffusion cell. Ester drugs and chemicals applied to the skin surface undergo hydrolysis if they are substrates for the carboxylesterase isoforms expressed in the skin. This might influence the efficacy of drugs such as procaine, used as a local anaesthetic both transdermally and subcutaneously. It was hypothesized that differing esterase isozyme profiles and affinity of skin esterases would influence systemic availability. Development of an ester pro-drug increases lipophilicity and promotes passage through the stratum corneum. If absorbed pro-drugs are converted to the active form of the drug by dermal esterases, this form passes directly into the systemic circulation (Liederer and Borchardt, 2006). To allow successful pro-drug development for dermal absorption, the esterase isoforms in human skin need to be identified and their substrate specificity defined. The primary focus of this study was to investigate the hydrolysis of the local anaesthetic procaine, specific for hCE2, by the skin of human, minipig and rat. The hydrolysis of procaine was compared to less specific esters. The secondary aim was to assess the suitability of minipig as a model for studying local hydrolysis of dermally applied drugs.
Methods

Materials

All chemicals used were purchased from Sigma Chemicals Co. (Poole, Dorset, UK).

Tissue preparation

Human skin was obtained following breast reduction surgery of healthy female individuals. Patients gave informed consent and ethical approval was obtained from Durham University Hospital, U.K. Human liver was from the liver bank of the Toxicology Unit, University of Newcastle upon Tyne, UK. Tissues were stored at -70°C. Four 28 day-old Male Wistar rats were killed by cervical dislocation, clipped of fur, and dorsal skin and livers stored at -70°C. Liver and skin from 3 male minipigs (weight 9±1.1kg) were obtained from Pfizer, US and were stored at -70°C.

Human, minipig and rat skin were cut with a dermatome to 350µm to obtain the epidermis and minimal thickness of upper dermis. Tissue was weighed, minced, pulverised with liquid nitrogen with a mortar and pestle and added to 1ml ice-cold KCl/phosphate buffer (150 mM KCl, 0.1M K₂HP0₄, pH 7.4) per 100 mg tissue. Tissue was homogenised with an Ultra-turrax homogeniser with 3 x 10 s bursts. The homogenate was centrifuged for 10 min at 750g followed by ultra-centrifugation of the supernatant for 10 min at 10,000g to remove mitochondria, nuclei and cell debris. Further centrifugation of the supernatant for 70 min at 100,000g separated the microsomes from the cytosol. Cytosol was retained and stored at -70°C. The microsomal pellet was resuspended with phosphate buffer and centrifuged at 100,000g for 70 min and the pellet
resuspended in glycerol buffer (10% glycerol, 0.05 M Tris, 0.1 mM KCl, 250 mM sucrose, pH7.4) and stored at -70°C for analysis.

Liver samples were minced and added to 1ml ice-cold KCl/phosphate buffer per 100 mg tissue. Liver was homogenised (4°C) with 2 x 10 s bursts with an Ultra-turrax. The same procedure for homogenisation and centrifugation was repeated as for the skin. Microsomal and cytosolic samples were stored at -70°C until required.

**Enzyme Assays**

Due to the small size of available tissue samples it was not possible to determine the full kinetic profiles for hydrolysis of the substrates used. Therefore substrate concentrations reported in the published literature which gave hydrolysis rates approaching the maximal rate were used.

**Procaine hydrolysis**

Procaine (100 µM) (Wu et al., 2004) in phosphate buffer (100 mM, pH 8.0) was incubated with microsomal or cytosolic protein (200 µg skin or 10 µg liver) in a reaction volume of 250 µl for 30 min at 37°C. Procainamide (50 µM) was used as an internal standard and the reaction was stopped by 250 µl of acetonitrile:methanol:phosphoric acid (80:19.2:0.8). Less protein was required for minipig liver microsomes to maintain a linear reaction. The reaction mixture was centrifuged for 10 min at 3000g and the supernatant removed to HPLC vials for analysis (10 µl on column). The hydrolysis product para-amino benzoic acid was quantified using a HPLC (Varian Prostar), with a C18 Gemini™ microbore column (5 µm, 250 mm x 2 mm, Phenomenex) maintained at 40°C, and detection at 280 nm. Separation was achieved using a flow-rate of 0.4 ml/min and gradient from 100% phosphate buffer (25 mM, pH 2.6) changing linearly to 15%
acetonitrile, 25% methanol and 60% phosphate buffer over 3 min maintained for 10 min and returned to 100% phosphate buffer (25 mM, pH 2.6) and re-equilibrated for 10 min. Retention times were procainamide at 6.1 min, procaine at 6.8 minutes and p-aminobenzoic acid at 7.4 min (Figure 2). A calibration curve of peak area ratio (metabolite versus internal standard) against concentration was constructed for p-aminobenzoic acid. Lower limit of detection was 1 ng on column. The substrate was at sufficient concentration to achieve maximum rate of hydrolysis and the reaction was linear with time over the assay period (data not shown).

**4-Methylumbelliferyl Acetate hydrolysis**

Hydrolysis of 4-methylumbelliferyl acetate (MUA) to 4-methylumbelliferone was determined in a 96-well blackened fluorescence plate. Each well contained 1 µg microsomal protein or 10 µg of cytosolic protein, MUA (1 mM) and bovine serum albumin (1.2 mg/ml) in phosphate buffer (100 mM, pH 7.4) in a final volume of 200 µl. Reaction was started by addition of 1 mM MUA and fluorescence was recorded for 15 min using excitation wavelength of 355 nm and emission 460 nm. Spontaneous hydrolysis of MUA was measured in wells in absence of protein under the same conditions. A standard curve of 4-methylumbelliferone versus fluorescence at 355/460 nm in the presence of BSA was used to calculate product formed during the reaction. MUA at 1 mM was at a sufficient concentration for the maximum rate of hydrolysis (Wadkins et al., 2005). The rate of hydrolysis was linear with time.

**Phenylvalerate and phenylacetate hydrolysis**

Hydrolysis of phenyl valerate (PV) (carboxylesterase substrate) or phenyl acetate (PA) (aryl esterase substrate) to phenol was determined in a 96-well plate by a modification of
the method used by Mutch (1998). To each well was added 1 µg of microsomal protein or 10 µg of cytosolic protein, 4-aminoantipyrine (2.5 mM) and potassium ferricyanide (5 mM) in 200 µl tris buffer (50 mM, pH 8.0) 37°C. The reaction was started by addition of PV or PA (3 mM), and increase in absorption followed at 510 nm for 15 min. Spontaneous hydrolysis was measured in the absence of protein. A phenol standard curve (range 0.3 to 2 µg/well) was prepared in parallel to determine phenol formation. Phenol liberated by hydrolysis was converted to the coloured ferricyanide complex. Substrate was at 3 mM (Mutch, 1998) and sufficient to give linearity and maximum rate of reaction over the assay period.

**p-Nitrophenyl acetate hydrolysis**

Hydrolysis of p-nitrophenyl acetate NPA (carboxylesterase and arylesterase substrate) to p-nitrophenol was determined using a 96 well plate method. Microsomal protein (1 µg) or cytosolic protein (10 µg) in Tris buffer 200 µl (50 mM, pH 8.0) 37°C was added to each well of a 96 well plate and NPA (0.5 mM) added to start the reaction and absorption measured at 406 nm for 6 min. Spontaneous hydrolysis of NPA was measured in the absence of protein. A standard curve of p-nitrophenol (0.1 to 1 µg/well) was used to quantitate formation of product. Substrate concentration was 0.5 mM (Prusakiewicz et al., 2006) and was sufficient to achieve maximum rate of reaction and linearity with time over the assay period.

**Inhibition Assays**

The carboxylesterase inhibitors bis-nitrophenylphosphate (BNPP, stock solution of 1mM made up in DMSO) (Yoshigae et al., 1999), paraoxon (stock solution of 1mM made up in ethanol) (Imai et al., 2003), and phenylmethylsulfonyl fluoride (PMSF, stock solution of
1mM made up in DMSO) (Yoshigae et al., 1999) were added at a final concentration of 1 µM. Additionally, to determine the presence of cholinesterases, the acetyl/butyryl cholinesterase inhibitor eserine (stock solution of 1mM made up in DMSO) (Hewitt et al., 2000), the specific acetylcholinesterase inhibitor bis-(4-allyl dimethyl ammonium phenyl)-pentane-3-one-dibromide (Bw284c51, stock solution of 1mM made up in water), and the specific butyrylcholinesterase inhibitors IsoOMPA (tetraisopropylpyrophosphoramidate, stock solution of 1mM made up in ethanol) were used at 1µM. Bw284c51 and IsoOMPA were shown to completely inhibit acetylcholinesterase and cholinesterase activity at 1µM (data not shown). To determine whether arylesterase (paraoxonase) contributed to the hydrolysis of ester substrates, mercuric chloride (stock solution of 1mM made up in water), which completely inhibited arylesterase activity was added at a final concentration 1 µM (McCracken et al., 1993). All inhibitors were added to microsomes and cytosol fractions from human, minipig and rat, skin and liver in the presence of procaine, MUA PV, PA or NPA. Loperamide, an inhibitor of hCE2, was added to incubations with procaine and phenylvalerate at 20 µM (stock solution of 20mM made up in DMSO), the concentration used by Quinney et al., 2005. All inhibitors were added to incubations for 5 minutes and maintained at 37°C prior to addition of substrate. Solvent vehicles alone, at the concentrations used, had no effect on esterase activity.

**Protein Assay**

Protein concentration in microsomal and cytosolic fractions was measured using bicinchoninic acid, by the method of Smith et al., (1985) and using bovine serum albumin as the protein standard (200mg/ml; Sigma).
Statistical Analysis

Enzyme activities were expressed as µmol product/min/mg protein (mean ± SEM) and µmol/min/g tissue (mean ± SEM). Activities were compared by ANOVA followed by Dunnett’s post-hoc test using Prism, GraphPad Inc, San Diego, USA. Activities in the presence of inhibitors were expressed as a percentage of the control.
Results

Enzyme Assays

Procaine was hydrolysed to p-aminobenzoic acid by skin microsomes and cytosol from human, minipig and rat at similar rates when expressed in terms of mg protein (Table 1). Procaine hydrolysis by minipig liver microsomes was about 10 fold higher than rat and about 70 fold higher than human (P<0.001). For liver cytosolic fractions, procaine hydrolysis by minipig was about 40 times higher than rat and 370 times higher than human (P<0.001) (Table 1). When expressed per gram of tissue, skin cytosolic hydrolysis of procaine was significantly higher than microsomal fractions for all species (P<0.01) (Table 1). Rates of procaine hydrolysis were slower (pmol/min) than all other ester substrates (µmol/min rates).

MUA, a non-specific carboxylesterase substrate (Barker and Clothier 1997), was hydrolysed by skin microsomes and cytosol from minipig and human, but no hydrolysis was detected with rat skin fractions (Table 2). Rates of MUA hydrolysis by skin fractions were lower than by liver fractions (P<0.01). Twenty and twenty six times higher hydrolysis rates for MUA were seen with minipig liver microsomes compared to rat or human liver microsomes respectively (P<0.001). For liver cytosol, MUA hydrolysis by minipig was only 3 times higher than rat and 4 times higher than human (P<0.001). However, when cytosolic hydrolysis was expressed per gram wet weight tissue, hydrolysis of MUA by rat was 4 times higher than minipig and 16 times higher than human (P<0.001).
Skin cytosolic PV hydrolysis was higher than microsomal when expressed per mg protein for all species but not significantly different (Table 3). Minipig liver microsomal and cytosolic fractions showed the highest rate of PV hydrolysis ($P<0.001$). Rat skin cytosol hydrolysis of PA was similar to human cytosolic activity, and although minipig cytosolic PA hydrolysis was higher the difference was not significant (Table 4). PA hydrolysis was lower with liver cytosol than microsomes for all species, though only significantly for human and rat ($P<0.001$).

Rat skin had the highest hydrolase activity with NPA as substrate ($P<0.001$) (Table 5). Human skin had the lowest microsomal NPA activity, though not significantly lower than minipig ($P>0.05$). Minipig had the lowest cytosolic NPA activity, but only significantly lower than rat ($P<0.001$). Rates of NPA hydrolysis by skin cytosolic fractions were greater than with the skin microsomal fraction for all species, in contrast to the liver, which showed higher microsomal activity towards NPA than cytosol.

The rate of NPA hydrolysis by rat and human liver microsomes and cytosol was more rapid than all other substrates ($P<0.01$). Human liver microsomes showed the highest microsomal NPA hydrolysis and rat liver cytosol the highest cytosolic NPA hydrolysis rates. For minipig liver microsomes, the substrate with the highest rate of hydrolysis was MUA, being significantly higher than procaine, PA and NPA ($P<0.01$), but not PV. The activities were expressed in relation to the wet weight of tissue as well as protein to define the relative contributions of microsomes and cytosol within the liver or skin. The cytosolic and microsomal protein content of minipig, human and rat, liver and skin microsomal and cytosolic fractions are detailed in table 6. However it is difficult and perhaps inappropriate to make direct comparisons between liver and dermatomed skin.
(containing epidermis and some dermis) on the basis of wet weight as the enzyme activity in skin is predominantly from the basal layer of the epidermis, whereas it is more widely distributed in the hepatocytes in liver tissue.

**Effect of Inhibitors on hydrolysis of procaine**

Procaine hydrolysis was completely inhibited by BNPP and paraoxon (1 µM) confirming the involvement of carboxylesterases (data not shown). Loperamide inhibited procaine hydrolysis by skin and liver, microsomal and cytosolic fractions from human, minipig and rat to varying degrees (figure 3). Procaine hydrolysis by human liver microsomes and cytosol was completely inhibited by loperamide, whereas 4.0% activity remained with minipig liver microsomes and 20.6% with rat microsomes, and with similar effects on the cytosolic fractions. Procaine hydrolysis by skin of all species was less sensitive to inhibition by loperamide than the liver. Procaine hydrolysis by rat skin cytosol was inhibited by loperamide more than by microsomes (41.7% and 65.3% activity remaining respectively). Procaine hydrolysis by minipig skin was the least inhibited by loperamide with 69.6% activity remaining for microsomal and 77.9% for cytosolic fractions. Of the three species, procaine hydrolysis by human skin showed the greatest sensitivity to loperamide, with a reduction of hydrolysis down to 26.9%.

**Effects of inhibitors on hydrolysis of MUA, PV, PA and PNA**

A concentration of 1 µM was selected for comparative studies looking at sensitivity of hydrolysis of substrates to an inhibitor. The results for minipig, human and rat are shown in figures 4, 5 and 6. Paraoxon at 1 µM, inhibited NPA hydrolysis by microsomal and cytosolic, liver and skin fractions for all species ($P<0.001$). Paraoxon inhibited hydrolysis of all substrates by minipig and human microsomes ($P<0.01$) but was less effective with
cytosolic fractions, not significantly inhibiting minipig skin microsomal PV, PA or MUA hydrolysis or minipig skin cytosolic PA hydrolysis. In human skin, paraoxon inhibited hydrolysis of all substrates ($P<0.001$) except for cytosolic PA hydrolysis. In rat liver, paraoxon did not significantly inhibit microsomal PV hydrolysis or cytosolic PV or MUA hydrolysis. In rat skin cytosolic fractions, paraoxon did not significantly inhibit PV hydrolysis.

BNPP at $1 \mu$M significantly inhibited hydrolysis of all ester substrates by liver fractions from all species ($P<0.01$). However, BNPP did not inhibit minipig skin hydrolysis of any substrate except PV by the cytosolic fraction ($P<0.001$). With human skin BNPP inhibited microsomal hydrolysis of all substrates and only cytosolic PV hydrolysis. In rat, BNPP significantly inhibited hydrolysis of all substrates by skin fractions ($P<0.01$) except for skin cytosolic PA hydrolysis.

PMSF at $1 \mu$M, was not an effective inhibitor of ester hydrolysis by human or minipig liver and only inhibited minipig liver microsomal NPA and PV hydrolysis, liver cytosolic PV hydrolysis and skin microsomal NPA hydrolysis ($P<0.001$). In human, only liver and skin microsomal NPA was inhibited ($P<0.001$). In rat, PMSF significantly inhibited liver microsomal and cytosolic NPA hydrolysis ($P<0.05$) and PA and MUA hydrolysis ($P<0.001$). Hydrolysis by rat skin was inhibited similarly except for microsomal NPA hydrolysis which was not inhibited.

Mercuric chloride at $1 \mu$M was also a poor inhibitor of minipig and human liver and skin ester hydrolysis. Only minipig liver microsomal NPA hydrolysis and human liver microsomal NPA and PA hydrolysis were inhibited ($P<0.001$). In rat, mercuric chloride inhibited liver microsomal and cytosolic NPA, PA and MUA hydrolysis ($P<0.01$), skin
microsomal NPA and PA hydrolysis and skin cytosolic NPA and PV hydrolysis ($P<0.001$).

Eserine and Bw284c51, inhibitors of acetylcholinesterase and IsoOMPA, a cholinesterase inhibitor, at 1 µM had little effect on hydrolysis of any of the substrates by liver microsomal or cytosolic fractions of any species (data not shown).
Discussion

Carboxylesterase activity with PV, PNA, and procaine as substrate, expressed in terms of mg protein for skin microsomes and cytosol, was lower than liver for human, minipig and rat. The interspecies profile of activity in the skin was not predicted by that in the liver suggesting differing expression of the carboxylesterase isoymes. Procaine, specific for hCE2 in the gut and recombinant hCE2 enzyme, was most efficiently hydrolysed by minipig liver and least by human liver. Skin hydrolysis of procaine was greatest with rat but at similar levels for minipig and human. The activity in human skin suggested expression of hCE2 and the equivalent isoforms in minipig and rat skin remain to be identified. Hydrolysis of hCE2 specific substrates has not previously been studied with skin but Zhu et al., (2005) showed that hCE2 protein was highly expressed in human skin whereas hCE1 was weakly expressed or not detectable. The capacity of skin microsomes and cytosol to metabolise procaine suggests that skin has similarities to other extrahepatic tissues such as in the gut (Sanghani et al., 2003) and lung (Mutch et al., 2006). Procaine administered transdermally would be a substrate for these esterase isoforms locally in the skin, limiting local anaesthetic effects and contributing to its low potency. Loperamide has been shown to specifically inhibit hCE2 in gut and liver (Quinney et al., 2005). This study has shown that loperamide specifically inhibited procaine hydrolysis in human skin microsomes and cytosol by 73%, rat skin microsomes and cytosol by 25% and 60% respectively, but had less effect on minipig skin where hydrolysis was only reduced by 30% for microsomes and 20% for cytosol. By comparison loperamide had little effect on the hydrolysis of the less specific substrates. Lack of hydrolysis of MUA by rat skin
indicated that esterases had low affinity for MUA but were able to hydrolyze PV and NPA.

Studies in which a range of ester substrates were incubated with liver microsomes have previously shown that the sizes of the alcohol and acyl leaving groups influence the specificity for hCE1 and hCE2. hCE1 metabolised molecules with a small alcohol group and large acyl group (e.g. methyl cocaine); conversely, hCE2 metabolised substrates with a small acyl and large alcohol group (e.g. procaine). For homologous series of esters with increasing sizes of acyl group the specificity changed from hCE2 to hCE1 (Yoshigae et al., 1999, Imai et al., 2006, Satoh and Hosokawa 2006). Inspection of the formulae (figure1) of the substrates used indicate that PV and NPA have small alcohol and acyl groups and may be non-specific substrates for carboxylesterase isoforms. MUA has a large umbelliferyl alcohol group contributing to a different activity profile from the other substrates, possibly having some affinity for hCE2. Procaine has a large alcohol group was and this would be consistent with preferential hydrolysis by hCE2. PA which has previously been shown to have a greater affinity for arylesterase (cysteine active site) was also hydrolysed by skin carboxylesterases.

This is the first time that minipig skin esterase activity has been compared to human using procaine as a substrate in comparison with less specific substrates. Prusakiewicz et al., (2006) reported hydrolysis of NPA by minipig skin. Generally there is limited literature on metabolism of drug esters by minipig skin despite the use of minipigs as an in vivo model for determination of the kinetics of transdermal drug preparations. In this study we have included results for dorsal minipig skin, the site at which ester prodrugs would generally be applied.
Previously published investigations have concentrated on the identity and activity of microsomal carboxylesterases, but these results indicate the importance of cytosolic esterase activity in the skin and that the inhibition profile was similar to the microsomes. Both microsomal and cytosolic carboxylesterases could potentially hydrolyse an ester drug during percutaneous penetration. A diffusing ester which enters the keratinocyte would first come into contact with cytosolic enzymes before uptake into the lumen of the endoplasmic reticulum, where microsomal esterases are located. These studies have been conducted with subcellular fractions, however, it is very important to study whole tissue to indicate the actual importance of esterases during absorption. Also inter-individual variability in the expression of isoforms in the skin may influence the efficacy of transdermal ester pro-drugs as has been suggested for irinotecan hydrolysis by hCE2 in the gut (Marsh et al., 2004).

In conclusion, ester hydrolysis by human skin was similar to minipig and both were lower than rat. These results have highlighted the potential of skin to hydrolyse procaine which is topically applied and loperamide inhibition indicated involvement of hCE-2. This knowledge will aid in advancing transdermal pro-drug development.
References


DMD #15727


Legends for figures.

Figure 1
Structures of substrates

Figure 2
HPLC chromatogram showing separation and retention times of procainamide (6.1 minutes), procaine (6.8 minutes) and $p$-amino benzoic acid (7.4 minutes) with 200 ng of each compound injected on column.

Figure 3
Effect of the inhibitor loperamide (20 µM) on the hydrolysis of procaine (100 µM) with minipig, human and rat liver and skin microsomal and cytosolic fractions. Control had solvent vehicle only. Activity expressed as a percentage of control. (mean ± SEM, n = 4 for rat, n = 3 for human and minipig). (ND = Not Detected) (* = $P<0.05$, ** = $P<0.01$, *** = $P<0.001$).

Figure 4
Effect of the inhibitors (1 µM) HgCl$_2$, bis-nitrophenyl phosphate (BNPP), paraoxon and phenylmethylsulfonyl fluoride (PMSF) on the hydrolysis of the substrates para-nitrophenyl phosphate (PNPA, 0.5 mM), phenyl valerate (PV, 3 mM), phenyl acetate (PA, 3 mM) and methylumbelliferyl acetate (MUA, 1 mM) with minipig liver and skin microsomal and cytosolic fractions. Controls were treated with solvent vehicle only.
Activity expressed as a percentage of control. (mean ± SEM, n = 3) (* = P<0.05, ** = P<0.01, *** = P<0.001).

Figure 5
Effect of the inhibitors (1 µM) HgCl₂, bis-nitrophenyl phosphate (BNPP), paraoxon and phenylmethylsulfonyl fluoride (PMSF) on the hydrolysis of the substrates para-nitrophenyl phosphate (PNPA, 0.5 mM), phenyl valerate (PV, 3 mM), phenyl acetate (PA, 3 mM) and methylumbelliferyl acetate (MUA, 1 mM) with human liver and skin microsomal and cytosolic fractions. Controls were treated with solvent vehicle only. Activity expressed as a percentage of control. (mean ± SEM, n = 3) (* = P<0.05, ** = P<0.01, *** = P<0.001).

Figure 6
Effect of the inhibitors (1 µM) HgCl₂, bis-nitrophenyl phosphate (BNPP), paraoxon and phenylmethylsulfonyl fluoride (PMSF) on the hydrolysis of the substrates para-nitrophenyl phosphate (PNPA, 0.5 mM), phenyl valerate (PV, 3 mM), phenyl acetate (PA, 3 mM) and methylumbelliferyl acetate (MUA, 1 mM) with rat liver and skin microsomal and cytosolic fractions. Activity expressed as a percentage of control. (mean ± SEM, n = 4) (* = P<0.05, ** = P<0.01, *** = P<0.001).
Table 1. Rates of procaine (100 µM) hydrolysis in liver and skin microsomal and cytosolic fractions expressed as pmol/min/mg (pmol/min/g wet weight tissue in italics) (mean ± SEM, n = 3 for minipig and human n = 4 for rat).

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<td>Minipig</td>
<td>Human</td>
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<tr>
<td>Microsomes</td>
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<td></td>
<td>5703 ± 1085</td>
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<td>Cytosol</td>
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Table 2. Rates of 4-methylumbelliferyl acetate (1 mM) hydrolysis in liver and skin microsomal and cytosolic fractions expressed as µmol/min/mg (µmol/min/g wet weight tissue in italics) (mean ± SEM, n = 3 for minipig, n = 4 for human and rat) ND = Not Detectable.

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<td>25.9 ± 0.52</td>
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Table 3. Rates of phenyl valerate (3 mM) hydrolysis in liver and skin microsomal and cytosolic fractions expressed as µmol/min/mg (µmol/min/g wet weight tissue in italics) (mean ± SEM, n = 4 for rat and human, n = 3 for minipig).

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<td></td>
<td>129 ± 4</td>
<td>51.30 ± 5.61</td>
</tr>
<tr>
<td>Cytosol</td>
<td>1.04 ± 0.05</td>
<td>0.200 ± 0.090</td>
</tr>
<tr>
<td></td>
<td>108 ± 12</td>
<td>20.0 ± 9.0</td>
</tr>
</tbody>
</table>
Table 4. Rates of phenyl acetate (3 mM) hydrolysis in liver and skin microsomal and cytosolic fractions expressed as µmol/min/mg (µmol/min/g wet weight tissue in italics) (mean ± SEM, n = 3 for minipig, n = 4 for human and rat).

<table>
<thead>
<tr>
<th></th>
<th>Liver</th>
<th>Skin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Minipig</td>
<td>Human</td>
</tr>
<tr>
<td></td>
<td>Microsomes</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.49 ± 0.13</td>
<td>3.15 ± 0.71</td>
</tr>
<tr>
<td></td>
<td>41.1 ± 1.09</td>
<td>31.5 ± 7.1</td>
</tr>
<tr>
<td>Cytosol</td>
<td>0.364 ± 0.013</td>
<td>0.082 ± 0.019</td>
</tr>
<tr>
<td></td>
<td>37.8 ± 3.38</td>
<td>8.20 ± 1.90</td>
</tr>
<tr>
<td></td>
<td>0.023 ± 0.004</td>
<td>0.0145 ± 0.012</td>
</tr>
<tr>
<td></td>
<td>0.029 ± 0.003</td>
<td>0.286 ± 0.034</td>
</tr>
<tr>
<td></td>
<td>0.062 ± 0.022</td>
<td>0.111 ± 0.010</td>
</tr>
<tr>
<td></td>
<td>0.977 ± 0.330</td>
<td>2.58 ± 0.31</td>
</tr>
</tbody>
</table>
Table 5. Rates of \( p \)-nitrophenyl acetate (0.5 mM) hydrolysis in liver and skin microsomal and cytosolic fractions expressed as µmol/min/mg (µmol/min/g wet weight tissue in italics) (mean ± SEM, \( n = 3 \) for minipig, \( n = 4 \) for human and rat).

<table>
<thead>
<tr>
<th></th>
<th>Liver</th>
<th>Skin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Minipig</td>
<td>Human</td>
</tr>
<tr>
<td>Microsomes</td>
<td>4.07 ± 0.33</td>
<td>18.2 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>47.8 ± 2.7</td>
<td>182 ± 11</td>
</tr>
<tr>
<td>Cytosol</td>
<td>0.412 ± 0.009</td>
<td>1.68 ± 0.32</td>
</tr>
<tr>
<td></td>
<td>42.8 ± 2.34</td>
<td>168 ± 32</td>
</tr>
</tbody>
</table>
Table 6. Recovery of subcellular protein from liver and skin of minipig, human and rat. (mean ± SEM, n = 3 for minipig, n = 4 for human and rat). § Recovery data for samples not determined, data provided as an approximation based on previous studies.

<table>
<thead>
<tr>
<th>Recovered Subcellular Protein (mg/g tissue)</th>
<th>Minipig</th>
<th>Human</th>
<th>Rat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver Microsomes</td>
<td>11.8 ± 1.26</td>
<td>10 § ± -</td>
<td>9.84 ± 1.03</td>
</tr>
<tr>
<td>Liver Cytosol</td>
<td>104 ± 7.09</td>
<td>100 § ± -</td>
<td>126 ± 1.62</td>
</tr>
<tr>
<td>Skin Microsomes</td>
<td>1.40 ± 0.17</td>
<td>1.93 ± 0.11</td>
<td>4.02 ± 0.15</td>
</tr>
<tr>
<td>Skin Cytosol</td>
<td>16.1 ± 0.74</td>
<td>22.9 ± 1.39</td>
<td>35.3 ± 0.77</td>
</tr>
</tbody>
</table>
Figure 1.

- **p-nitrophenyl acetate (NPA)**
- **4-methylumbelliferyl acetate (MUA)**
- **phenyl valerate (PV)**
- **procaine**
- **phenyl acetate (PA)**
Figure 3.

Loperamide Inhibition of Procaine Hydrolysis

Activity as % control

Minipig | Human | Rat

Liver Mic | Liver Cyt | Skin Mic | Skin Cyt

*** | *** | *** | ***
Figure 4.

Minipig liver microsomal Activity

Minipig liver cytosol Activity

Minipig skin microsomal Activity

Minipig skin cytosol Activity
Figure 5.
Figure 6.