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# Inter-individual variability in esterases in human liver

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ABSTRACT

Human liver has numerous hydrolytic enzymes involved in metabolism of endogenous and exogenous esters. Of these enzymes, carboxylesterases (EC 3.1.1.1) form an important group which hydrolyses many diverse ester substrates, including pro-ester drugs. Carboxylesterase activity was investigated in liver subcellular fractions from 22 individuals using the general carboxylesterase substrate phenylvalerate and the homologous series of esters methyl-, ethyl-, propyl-, butyl- and benzylparaben. The intra- and inter-individual variation in phenylvalerate and paraben metabolism was compared. Rates of hydrolysis were higher in microsomal fractions than cytosolic fractions for all compounds. The rate of paraben hydrolysis varied depending on the size of the paraben alcohol leaving group, showing a decrease with increasing leaving group size. Comparisons showed that individuals with high rates of hydrolysis towards methyl paraben also showed high rates of hydrolysis to the other parabens and phenylvalerate. Phenylvalerate as a non-specific carboxylesterase substrate was hydrolysed mainly by hCE1 in human livers and there was good correlation with small alcohol leaving group parabens, suggesting hCE1 involvement. Lower correlations with larger alcohol leaving group parabens are consistent with more hCE2 involvement.



## 1. Introduction

In the human liver, hydrolysis of esters leads to the formation of more polar compounds that may be more easily eliminated from the body [1]. Carboxylesterase enzymes (EC 3.1.1.1) have a protective function by inactivating drugs via hydrolysis and eliminating xenobiotics avoiding toxicity [2]. Clinically carboxylesterase activity is also essential for activation of ester pro-drugs (such as irinotecan) and has been utilised during drug development [3–5]. Understanding expression levels of human carboxylesterases and their distribution profiles is paramount in predicting fate of drugs and pro-drugs [2,5].

Esterases catalyse the hydrolysis of esters. Carboxylesterases form part of the B class of esterases that are inhibited by organophosphates and are present in the endoplasmic reticulum and cytosol of cells [6,7]. Satoh and Hosokawa [8] developed a classification scheme for carboxylesterase enzymes and to date three fundamental groups have been described: human carboxylesterase 1 (hCE1, CES1), human carboxylesterase 2 (hCE2, CES2, hiCE) and human carboxylesterase 3 (hCE3, CES3). Of the three groups, hCE1 is highly expressed in human liver microsomes [9,10]. hCE2 is more highly expressed in the colon and possibly other extra hepatic tissues [11]. hCE1 and hCE2 have inter-related substrate specificity but may be distinguished through their different activities with selected substrates [12]. For example it is known that meperidine and methylphenidate are substrates for the carboxylesterase enzyme hCE1 but not hCE2 [12,13]. Recent studies have indicated that hCE1 preferentially cleaves the ester linkage with large acyl group and small alcohol group

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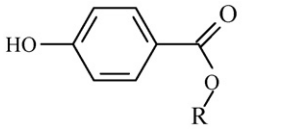


Methylparaben R= -$CH_3$
Ethylparaben R= -$CH_2CH_3$
Propylparaben R= -$CH_2CH_2CH_3$
Butylparaben R= -$CH_2CH_2CH_2CH_3$


4-Hydroxybenzoic acid R = -H

**Fig. 1 – Structures of paraben esters and metabolite hydroxybenzoic acid.**

[13], whereas substrates with large alcohol group and small acyl group are more often hydrolysed by hCE2 [14]. The anti-cancer pro-drug irinotecan (CPT11) and the local anaesthetic procaine are specific substrates for hCE2 [15]. Knowledge of substrates selective for hCE3 is limited and this subgroup of carboxylesterase enzymes requires further research [13]. The nature of cytosolic carboxylesterases is less well understood with available information being minimal [24,25].

Paraben esters act as anti-microbial agents and have been widely accepted for use as preservatives in cosmetics, foods and medicines due to their safety profile and low toxicity [16,17]. Chemically, parabens are highly lipophilic and have a simple structure of 4-hydroxybenzoic esters with varying alcohol chain lengths [17] (Fig. 1). Recent studies have suggested that parabens may accumulate in breast tissue and due to oestrogenic activity, increase the risk of breast cancer [18]. In vitro studies have indicated that parabens increased toxicity in hepatocytes by reducing ATP levels and impairment of mitochondrial function, and that paraben exposed HaCat cells have increased susceptibility to UV induced oxidative stress [19,20]. Parabens are hydrolysed rapidly after oral intake during first pass metabolism by the liver although the carboxylesterase isoforms involved have not been identified.

Carboxylesterase activity may show inter-individual variations due to both genetic polymorphisms [21] and environmental factors and these may influence toxicity induced by parent ester [22,23]. The aim of this study was to determine the capacity of human liver carboxylesterases to hydrolyse parabens, define inter-individual variation and the nature of the carboxylesterases involved.

## 2. Materials and methods

### 2.1. Chemicals

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

### 2.2. Tissue preparation

Human liver samples were obtained from healthy individuals prior to transplantation and stored in the toxicology unit liver bank. Human livers were obtained with ethical approval and were shown by histological investigation not to have disease. These human liver samples had been previously used for investigating the metabolism of diazinon, chlorpyrifos and parathion by multiple cytochromes P450 [26,27], details of these livers, i.e. age, sex and smoking status are referred to in these papers. Thawed liver was minced and homogenised with an Ultraturex homogeniser and centrifuged (Sorvall RC-5B) for 10 min at 750 × *g*. The supernatant was then ultra-centrifuged (Sorvall OTD65B) for 10 min at 10,000 × *g* to remove unwanted organelles. To separate microsomes from cytosol the supernatant was centrifuged for a further 70 min at 100,000 × *g*. The cytosol was retained and the microsomal pellet was re-suspended with buffer and re-centrifuged for 70 min at 100,000 × *g*. The pellet was re-suspended in glycerol buffer (10% glycerol, 0.1 M $K_2HPO_4$, 0.1 mM KCL, and 250 mM sucrose, pH 7.4). The samples were frozen at −80 °C ready for analysis. Samples were found to be stable at −80 °C for at least 6 months with repeat analysis giving consistent results during this period.

### 2.3. Phenylvalerate assay

The phenylvalerate assay used was a modified version of that used by Mutch [28]. The hydrolysis of phenylvalerate to phenol was analysed in a 96 well plate assay. Each well contained 0.5 μg liver microsomal or 5 μg liver cytosolic protein to which 200 μl of freshly prepared substrate solution was added containing phenylvalerate (3 mM), amino anti-pyrene (AAP) (2.5 mM), $K_3Fe(CN)_6$ (5 mM) in Tris buffer (50 mM pH 8.0). Phenol production was identified through colour change following conversion to a ferricyanide complex. The samples were continuously monitored on a plate reader at 510 nm for 6 min at 37 °C. Background (spontaneous) hydrolysis of substrate was measured in the presence of all components with the absence of protein and this rate was subtracted from all samples. A phenol standard curve (range 0–1.4 μg/ml) was prepared to calculate phenol produced during the reaction.

### 2.4. Incubation of paraben compounds with human liver microsomes and cytosol

Paraben hydrolysis in liver fractions was undertaken by incubating methyl-, ethyl-, propyl-, butyl- or benzylparaben (100 μM) for 30 min at 37 °C in phosphate buffer (100 mM, pH 8.0) with liver microsomes (final concentration 5 μg/ml) or liver cytosol (5 μg/ml). Background (spontaneous) hydrolysis of substrate was measured in the presence of all components with the absence of protein and this rate was subtracted from all samples. The reaction was terminated by adding one volume of acetonitrile:methanol:phosphoric acid (49.1:49.1:0.8) which contained 40 μg/ml 2,4-dihydroxybenzoic acid as an internal standard for HPLC. The samples were centrifuged for 15 min at 3000 × *g*, after which the supernatant was removed and analysed by HPLC. Parabens were found to be stable in solution and did not spontaneously hydrolyse. The reaction was linear with time and substrate concentration at saturation for maximum enzyme kinetics. There was less than 10% conversion of parent to metabolite during reaction.

### 2.5. Inhibition studies

Microsomal and cytosolic fractions were incubated with paraoxon (1 μM) or bis-nitrophenylphosphate (1 μM) or loperamide (20 μM) for 5 min prior to addition of either phenylvalerate or parabens. Incubation conditions and analysis were as described earlier.

### 2.6. HPLC measurement of 4-hydroxybenzoic acid

4-Hydroxybenzoic acid was measured using a C18 gemini microbore column (5 μm, 250 mm × 2 mm, Phenomenex) on a Varian Prostar HPLC. Separation was achieved with an initial gradient of 25% methanol and 75% phosphate buffer (25 mM, pH 3.6) gradually converting to 25% methanol, 40% acetonitrile and 35% phosphate buffer over a period of 3 min. This was then maintained for the duration of the 10 min run. The temperature of the column was controlled at 40 °C, the flow rate of the mobile phase solution at 0.4 ml/min and UV detection at 254 nm. Injection volume was 10 μl and between each run an equilibration time of 10 min was used. A calibration curve was constructed for 4-hydroxybenzoic acid (1–100 μM) versus dihydroxybenzoic acid (internal standard). The auto-sampler had a cooled sample tray set to 4 °C.

### 2.7. Protein assay

The protein concentration of each of the human tissue samples was measured by the bicinchoninic acid (BCA) method using a bovine serum albumin protein as standard (200 mg/ml from Sigma) [29].

### 2.8. Statistical analysis

Enzyme activities were expressed as nmol/min/mg protein in microsomal and cytosolic subcellular fractions. Activities of phenylvalerate or paraben hydrolysis were compared by one-way ANOVA followed by Tukey post hoc test using the statistical package, Prism 3.03, Graphpad Inc, San Diego, USA. Correlations between substrates were determined by linear regression. Significance was determined from *t*-tables (tabulated value at $p < 0.05$ or less) using a two-tailed test and $(n - 2)$ degrees of freedom.

## 3. Results

### 3.1. Phenylvalerate hydrolysis

Phenylvalerate, a substrate for non-specific carboxylesterase activity, was hydrolysed to phenol by human liver microsomes ($n = 22$) and human liver cytosol ($n = 20$) samples. Individual results for the rates of hydrolysis are shown in Fig. 2. Liver microsomes had 10-fold greater capacity for hydrolysis of phenylvalerate (3 mM) than cytosol (2150 ± 140 compared to 190 ± 10 nmol phenol/min/mg, mean ± S.E.M.). Hydrolysis of phenylvalerate by microsomes varied 2.5× between individuals (1.30 to 3.56 μmol phenol/min/mg) and 3× for cytosolic activity (0.09 μmol to 0.31 μmol phenol/min/mg. (Fig. 2).

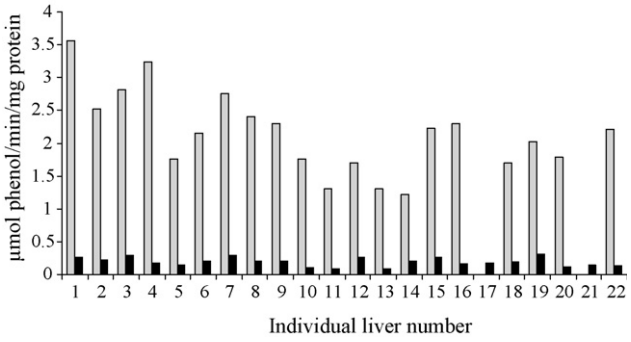


**Fig. 2 – Inter-individual variation in phenylvalerate hydrolysis by human liver microsomal (grey bars) and cytosolic (black bars) fractions expressed as μmol/min/mg protein. Each sample a mean of two determinations. Microsomal samples numbers 17 and 21 were not measured.**

### 3.2. Paraben hydrolysis

Methyl-, ethyl-, propyl-, butyl- and benzyl paraben were hydrolysed by human liver microsomal and cytosolic subcellular fractions. The rate of hydrolysis (at 100 μM) by microsomes was greatest with methylparaben and ethylparaben (255 ± 27 and 213 ± 37 nmol/min/mg) and was lower with propyl-, butyl- and benzylparaben (53 ± 5, 31 ± 4, 24 ± 3 nmol/min/mg, respectively) (Fig. 3a and Table 1).

Similarly the highest 4-hydroxybenzoic acid production with liver cytosol was observed with methylparaben as substrate (23 ± 2 nmol/min/mg) and activity decreased as the length of the paraben alcohol group increased (Fig. 3b and Table 1). Fig. 3a and b illustrate the rates of hydrolysis of each of the parabens for each individual by microsomal and cytosolic fractions. Fig. 4a and b are scatter plots that illustrate the variability in paraben hydrolysis in the sample population for each of the parabens.

It has been previously shown that using our method for preparation of human liver subcellular fractions the protein yield for human liver microsomes was approximately 10 mg/g wet weight liver, and 10× greater for cytosol at 100 mg/g liver. Therefore, when the rates of hydrolysis for the paraben compounds were expressed in terms of wet weight of liver tissue, as would occur in the cell, the relative contribution of cytosolic carboxylesterase increased 10-fold (e.g. for methylparaben 2550 nmol/min/g tissue and 2300 nmol/min/g tissue for microsomal and cytosolic protein, respectively).

The inter-individual variation for paraben hydrolysis by microsomal and cytosolic fractions from human livers was of a similar order to phenylvalerate hydrolysis. There were significant correlations between hydrolysis of individual paraben esters and phenylvalerate (Table 2). Fig. 5 illustrates the correlation between phenylvalerate with methylparaben and for phenylvalerate with benzylparaben for microsomes and cytosol, for all other parabens, graphical representation is not shown, but $r^2$ values are presented in Table 2.

For microsomal and cytosolic fractions, the correlations for hydrolysis of the various parabens with each other are shown in Table 3. Correlations were generally higher with

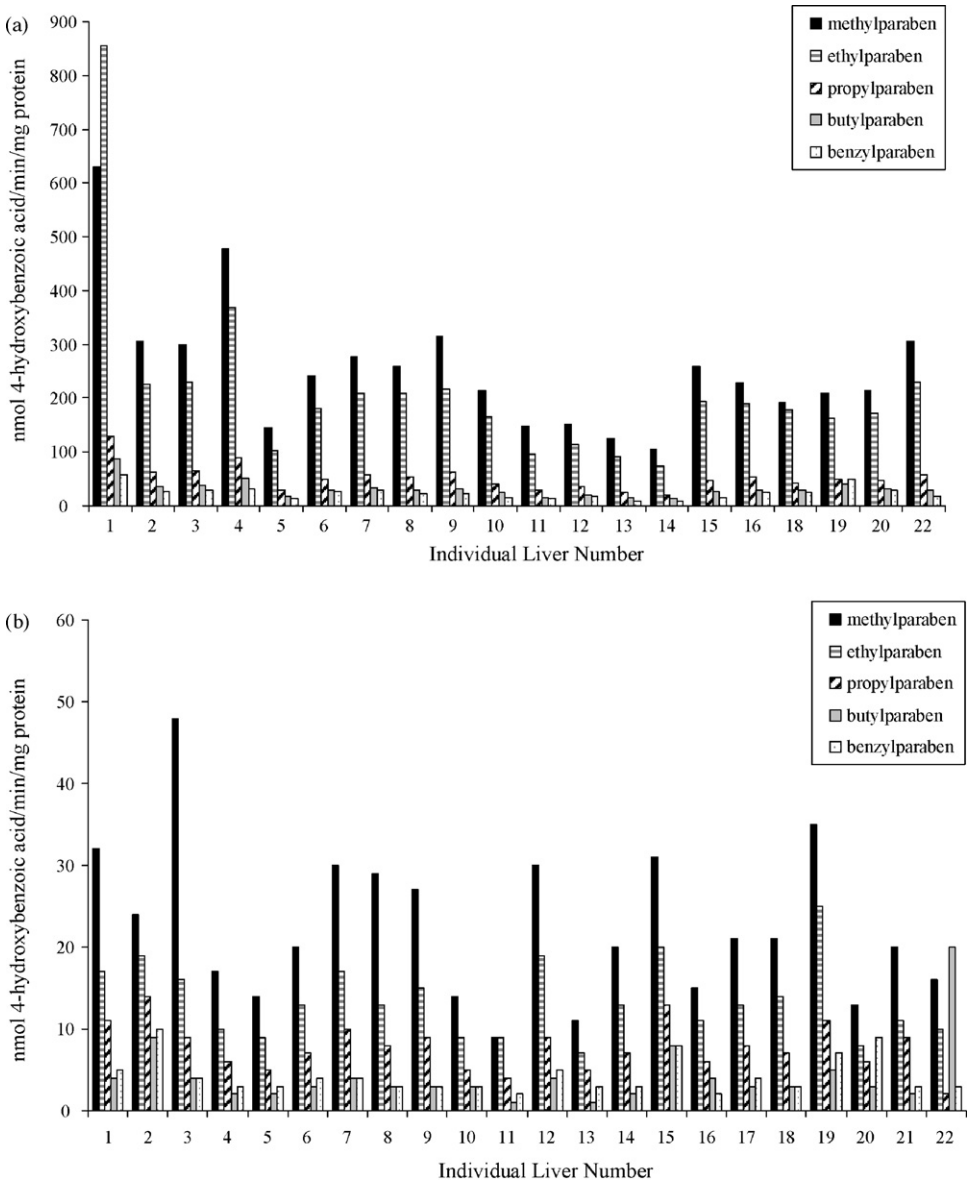


**Fig. 3 – Paraben hydrolysis by human liver microsomes. Methyl-, ethyl-, propyl-, butyl- and benzylparaben (100 μM) hydrolysis to 4-hydroxybenzoic acid by (a) human liver microsomes and (b) human liver cytosol. Activity expressed as nmol/min/mg protein. Each bar is an average of two determinations.**

**Table 1 – Rates of hydrolysis of methyl-, ethyl-, propyl-, butyl- and benzylparaben and phenylvalerate by human liver microsomal and cytosolic subcellular fractions expressed as nmol/min/mg protein**

| | Microsomal protein (nmol/min/mg, $n = 20$) | | Cytosolic protein (nmol/min/mg, $n = 22$) | |
|---|---|---|---|---|
| Substrate | Mean ± S.E.M. | Range | Mean ± S.E.M. | Range |
| Methylparaben | 255 ± 27 | 104–629 | 23 ± 2 | 9–48 |
| Ethylparaben | 213 ± 37 | 72–855 | 14 ± 1 | 7–25 |
| Propylparaben | 53 ± 5 | 20–130 | 8 ± 1 | 2–14 |
| Butylparaben | 31 ± 4 | 14–86 | 4 ± 1 | 1–20 |
| Benzylparaben | 24 ± 3 | 10–59 | 4 ± 0.4 | 0.2–10 |
| Phenylvalerate | 2150 ± 140 | 1214–3556 | 190 ± 10 | 90–310 |

Mean ± S.E.M., $n = 20$ for microsomes, $n = 22$ for cytosol.

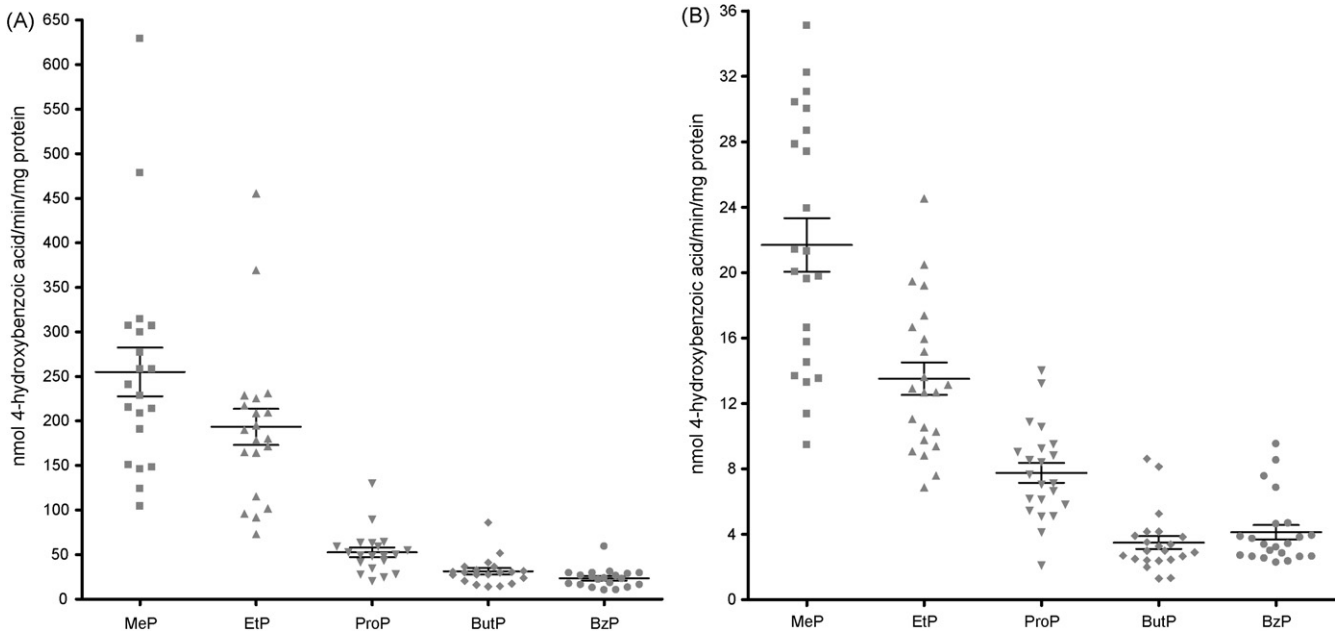


**Fig. 4 – Scatter plot showing the variability in rates of paraben hydrolysis between individual human liver (a) microsomal samples and (b) cytosolic samples. MeP = methylparaben, EtP = ethylparaben, ProP = propylparaben, ButP = butylparaben and BzP = benzylparaben. Bars indicate mean ± S.E.M. ($n$ = 20 for microsomes and $n$ = 22 for cytosol).**

microsomal fractions than with the cytosolic fractions. Correlations were also higher when comparing parabens with similar size alcohol groups. For example, methylparaben with ethylparaben ($r^2$ = 0.968) as opposed to methylparaben with benzylparaben ($r^2$ = 0.512).

The range of rates of hydrolysis of parabens were generally around 3-fold for both microsomal and cytosolic fractions, but also contained a few outliers going above this range. The microsomal outliers were from livers 1 and 4 with all parabens (Fig. 3a) and with cytosol from liver 22 for butylparaben and 2 and 20 for benzylparaben (Fig. 3b). The range of rates of hydrolysis for phenylvalerate were slightly higher with microsomes than paraben hydrolysis at around 4-fold, whereas for cytosol was similar to parabens at 3-fold.

### 3.3. *Inhibition studies*

Paraoxon (1 μM) and bis-nitrophenylphosphate (1 μM) completely inhibited the ability of microsomal and cytosolic fractions to hydrolyse phenylvalerate or parabens. Loperamide (20 μM), a hCE2 specific inhibitor, did not inhibit hydrolysis of phenylvalerate or parabens.

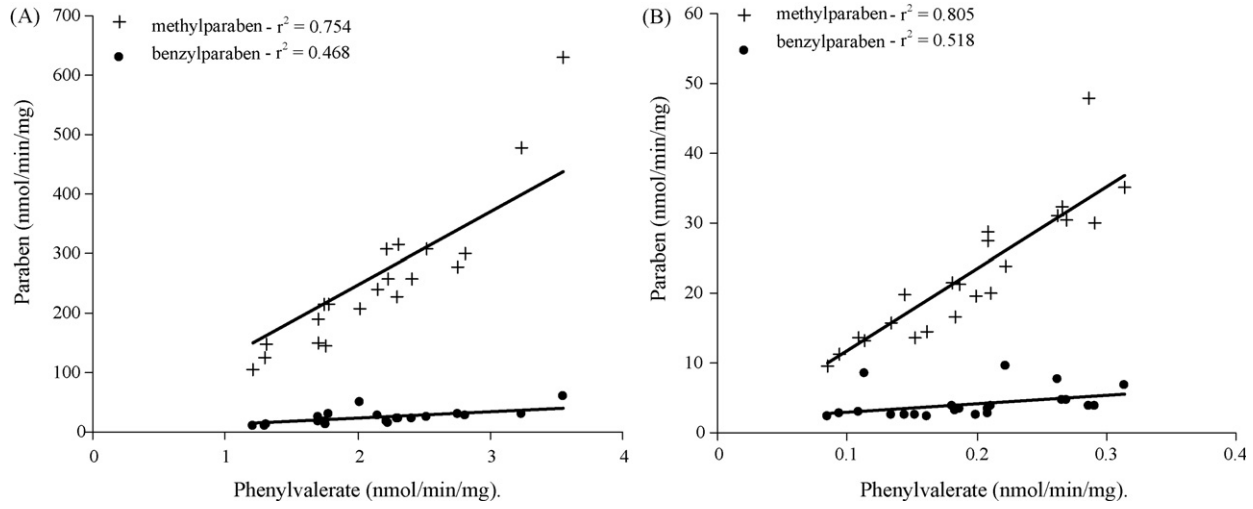


**Fig. 5 – The relationship between rates of hydrolysis of phenylvalerate with methylparaben or butylparaben (nmol/min/mg protein) for (a) human liver microsomal fractions ($n$ = 20) and (b) human liver cytosolic fractions ($n$ = 22). $r^2$ Showing best fit of linearity through data points.**

**Table 2 – $r^2$ Least square linear fit correlation values comparing paraben hydrolysis for each ester with phenylvalerate for microsomal fractions ($n = 20$) and cytosol fractions ($n = 22$) of 22 human livers**

| | Liver microsomes ($n = 20$) | | Liver cytosol ($n = 22$) | |
|---|---|---|---|---|
| Substrate | $r^2$ Value | $P$ | $r^2$ Value | $P$ |
| Methylparaben | 0.754 | <0.001 | 0.805 | <0.001 |
| Ethylparaben | 0.471 | <0.05 | 0.807 | <0.001 |
| Propylparaben | 0.775 | <0.001 | 0.511 | <0.05 |
| Butylparaben | 0.657 | <0.01 | 0.555 | <0.05 |
| Benzylparaben | 0.468 | <0.05 | 0.518 | <0.05 |

Significance determined from $t$-tables (tabulated value at $P < 0.01$ or less) using a two-tailed test and $(n - 2)$ degrees of freedom.

## 4. Discussion

Inter-individual variation in phenylvalerate and paraben hydrolysis was observed between human livers for both microsomal and cytosolic fractions. The degree of inter-individual variation for hydrolysis reflected the different capacities of individuals to hydrolyse these substrates. This may be due to varying levels of enzyme expression, resulting in a varied ability to metabolise substrates or genetic polymorphisms in the carboxylesterase isoforms [9,13].

This study investigated the variability in carboxylesterase activity between human livers using phenylvalerate and various parabens. Hepatic hydrolysis of phenylvalerate and parabens was shown here to be greater in microsomal than cytosolic fractions. The structures of these substrates would suggest that they are substrates for hCE1, as they have small alcohol leaving groups [34]. hCE1 is highly expressed in liver and hCE2 is expressed to a much lesser degree [34]. Though greater activity of carboxylesterases in microsomes was seen compared to cytosol, they had a similar profile. This was similar to the profile seen for the hydrolysis of pyrethroids by human liver microsomes and cytosol [35], where additionally hCE1 was shown to be a carboxylesterase of liver cytosol as well as microsomes.

Phenylvalerate is mainly hydrolysed by hCE1 as indicated by inhibition studies. Complete inhibition of hydrolysis with paraoxon (1 μM) and bis-nitrophenylphosphate (1 μM) are consistent with carboxylesterase involvement, and loperamide (20 μM) a specific inhibitor for hCE2 [30] had no effect on phenylvalerate hydrolysis. There was good correlation between phenylvalerate hydrolysis, a non-specific carboxylesterase substrate, and hydrolysis of the parabens. Loperamide did not inhibit paraben hydrolysis by human liver fractions. In contrast, it has been shown that loperamide inhibits propyl-, butyl-, and benzylparaben hydrolysis with skin carboxylesterases, indicating hCE2 involvement in skin (Jewell et al., manuscript in preparation).

Hydrolysis rates progressively decreased from methyl- to benzylparaben. This may largely be due to an increased difficulty in the cleavage of the larger alcohol functional groups from the parabens (such as the benzyl ring of benzylparaben, opposed to the methyl group of methylparaben) [16]. Previous studies have suggested that hCE1 and hCE2 preferentially cleave compounds with large and small acyl group, respectively [13,14]. Therefore it follows that hCE1 is most likely to be the isozyme involved in the hydrolysis of phenylvalerate and parabens, though hCE2 may have some involvement with the larger alcohol leaving groups of butyl- and benzylparaben. This would correlate with previous findings on cocaine hydrolysis, in which the methyl ester was hydrolysed by hCE1 whereas the benzyl ester was hydrolysed by hCE2 [10,13,31]. In addition, Imai et al., recently reported a study of paraben hydrolysis by pooled human liver microsomes and recombinant hCE1 and hCE2. This study showed that methylparaben was hydrolysed preferentially by hCE1 and butylparaben by hCE2 [32]. Substrate preferences of carboxylesterase isoforms however require further study [33]. This also highlights the need for a specific hCE1 inhibitor in order to elucidate the specificity of hCE1 substrates.

Hydrolysis of the parabens to the metabolite 4-hydroxybenzoic acid at concentrations less than 10 μM was below the limit of detection for the HPLC assay used. Preliminary experiments with one liver preparation have shown that at 100 μM the rate of hydrolysis was maximal and similar to activity at 10 μM (data not shown). The liver samples we had available were from biopsies and were small and it was not possible to define the kinetics or determine whether there was a high affinity component(s).

**Table 3 – $r^2$ Least square linear fit correlation values comparing paraben hydrolysis for each of the paraben esters with all other paraben esters for microsomal fractions ($n = 20$) and cytosol fractions ($n = 22$) of 22 human livers**

| | Microsomes ($n = 20$) | | Cytosol ($n = 22$) | |
|---|---|---|---|---|
| | $r^2$ Value | $P$ | $r^2$ Value | $P$ |
| Methylparaben vs. ethylparaben | 0.968 | <0.001 | 0.846 | <0.001 |
| Methylparaben vs. propylparaben | 0.977 | <0.001 | 0.801 | <0.001 |
| Methylparaben vs. butylparaben | 0.869 | <0.001 | 0.474 | <0.05 |
| Methylparaben vs. benzylparaben | 0.512 | <0.05 | 0.568 | <0.01 |
| Ethylparaben vs. propylparaben | 0.955 | <0.001 | 0.689 | <0.001 |
| Ethylparaben vs. butylparaben | 0.927 | <0.001 | 0.644 | <0.01 |
| Ethylparaben vs. benzylparaben | 0.556 | <0.05 | 0.761 | <0.001 |
| Propylparaben vs. butylparaben | 0.942 | <0.001 | 0.723 | <0.001 |
| Propylparaben vs. benzylparaben | 0.842 | <0.001 | 0.705 | <0.001 |
| Butylparaben vs. benzylparaben | 0.917 | <0.001 | 0.855 | <0.001 |

Significance determined from $t$-tables (tabulated value at $P < 0.01$ or less) using a two-tailed test and $(n - 2)$ degrees of freedom.

The individual livers studied therefore showed notable involvement of hCE1 in phenylvalerate and paraben hydrolysis, especially for methyl-, ethyl- and propylparaben, which correlated with phenylvalerate hydrolysis. The lower correlation of phenylvalerate to butylparaben and benzylparaben hydrolysis was consistent with the smaller leaving group of phenylvalerate being more hCE1 specific than that of butyl and benzyl parabens, which are possibly more specific for hCE2. The study by Imai et al. [32] supports this. There was overall 3-fold range in variability between most individuals measured was seen, however, there were a few outliers from this range. Individuals 1 and 4 showed high rates of microsomal hydrolysis of all parabens. The high hydrolysis activity recorded for butylparaben with liver cytosol from one individual (liver number 22) could potentially be due to a polymorphism in hCE2 which preferentially cleaves large paraben side chains, and is known to undergo alternative splicing [14,25]. Marsh et al. [22] found 16 single-nucleotide polymorphisms in hCE1 and 11 in hCE2 in multiple populations. They also found an association of reduced expression of hCE2 mRNA with the incidence of colorectal tumours. No single-nucleotide polymorphisms were shown to influence hCE1 expression. A more detailed study of how single-nucleotide polymorphisms affect carboxylesterase functionality is still required. Environmental factors may also affect inter-individual expression and therefore activity of carboxylesterase enzymes, as exposure to some foods and/or drugs can induce or inhibit enzyme activity [25]. The relatively small number of human liver samples tested here limits any potential to observe individuals that fall outside the average range or to identify polymorphisms. A higher number of samples would identify the frequency of higher or lower rates of ester hydrolysis and the potential to correlate them with lifestyle factors such as smoking or drug use. In addition, though parabens are generally regarded as not being toxic, as they are rapidly metabolised, identifying individuals with low levels of carboxylesterase activity would highlight the potential for these compounds to exert a toxic effect. Therefore, defining the metabolic activity, inhibition profiles and inter-individual variation in expression of carboxylesterases is of continuing interest.

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