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Aromatic Hydroxylation of Salicylic Acid and Aspirin by Human Cytochromes P450

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Abstract

Aspirin (acetylsalicylic acid) is a well-known and widely-used analgesic. It is rapidly deacetylated to salicylic acid, which forms two hippuric acids—salicyluric acid and gentisuric acid—and two glucuronides. The oxidation of aspirin and salicylic acid has been reported with human liver microsomes, but data on individual cytochromes P450 involved in oxidation is lacking. In this study we monitored oxidation of these compounds by human liver microsomes and cytochrome P450 (P450) using UPLC with fluorescence detection. Microsomal oxidation of salicylic acid was much faster than aspirin. The two oxidation products were 2,5-dihydroxybenzoic acid (gentisic acid, documented by its UV and mass spectrum) and 2,3-dihydroxybenzoic acid. Formation of neither product was inhibited by desferrioxamine, suggesting a lack of contribution of oxygen radicals under these conditions. Although more liphophilic, aspirin was oxidized less efficiently, primarily to the 2,5-dihydroxy product. Recombinant human P450s 2C8, 2C9, 2C19, 2D6, 2E1, and 3A4 all catalyzed the 5-hydroxylation of salicylic acid. Inhibitor studies with human liver microsomes indicated that all six of the previously mentioned P450s could contribute to both the 5- and 3-hydroxylation of salicylic acid and that P450s 2A6 and 2B6 have contributions to 5-hydroxylation. Inhibitor studies indicated that the major human P450 involved in both 3- and 5-hydroxylation of salicylic acid is P450 2E1.

Keywords

Aspirin (Acetylsalicylic acid); Salicylic acid; Cytochromes P450; Aromatic hydroxylation

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6. Disclosures

The authors declare no conflicts of interest.

1. Introduction

Aspirin (acetylsalicylic acid) is a commonly used analgesic and can be used to treat arthritis as well as inflammation. Aspirin is also used as antithrombotic to reduce the risk of heart attacks. Although Charles Frederich von Gerhardt synthesized aspirin in 1853 (Gerhardt, 1853) and Felix Hoffman developed it as a drug in 1897 (Chemical Heritage Foundation, 2014), our knowledge of how aspirin is metabolized in humans is still not completely understood. The acetyl group is quickly hydrolyzed (enzymatically and non-enzymatically) after oral ingestion to form salicylic acid in the body (Trnavsky and Zachar, 1975). Humans further metabolize salicylic acid in a number of different ways (Fig. 1), or the compound can be directly excreted (1–31%) (Hutt et al., 1986).

The major route of metabolism is conjugation with glycine to form salicyluric acid, accounting for 20–65% of the products (Hutt et al., 1986). The other major metabolites result from conjugation to glucuronides (ester and ether, 1–42%) (Hutt et al., 1986). Glucuronidation involves a wide array of uridine 5′-diphosphoglucuronosyltransferases (UGTs), including the 1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15, and 2B17 enzymes (Kuehl et al., 2006). Aspirin metabolism can vary, depending on the UGTs in different animals. For instance, cats show increased toxicity to aspirin due to a lack of UGT and have a prolonged half-life of aspirin (22 h) compared to dogs (7.5 h) (Parton et al., 2000). The half-life of aspirin has been reported to vary from 1 to 38 h in various animals, being ~ 6 h in humans (Trnavsky and Zachar, 1975).

Two minor metabolites of salicylic acid are 2,3-dihydroxybenzoic acid (2,3-DHBA) (Crabtree et al., 1958; Grootveld and Halliwell, 1986) and 2,5-dihydroxybenzoic acid (2,5-DHBA) (Hutt et al., 1986; Reidl, 1983). These products can be attributed to oxidation by cytochrome P450 (P450) enzymes, as well as non-enzymatic Fenton-type reactions. Further conjugation of 2,5-DHBA with glycine yields gentisuric acid (Wilson et al., 1978) (which in principle could also occur by hydroxylation of salicyluric acid) (Fig. 1). The evidence for P450 oxidation may also be supported by studies on aspirin sensitivity due to polymorphisms in P450 2C9, an enzyme that has been proposed to oxidize aspirin. One study reported that the variant *rs4918758* had significant association with aspirin-intolerant urticaria (Palikhe et al., 2011). Attack by hydroxyl radicals has been reported to form the 2,3-DHBA product and is speculated to form some of the 2,5-DHBA product as well) (Grootveld and Halliwell, 1986, 1988; Coudray et al., 1995; Ghiselli et al., 1992; Ingelman-Sundberg et al., 1991).

The objective of this work was to determine the rates of aromatic hydroxylation and contributions of individual P450 enzymes involved in metabolism of aspirin and salicylic acid.

2. Materials and methods

2.1. Chemicals and enzymes

Acetylsalicylic acid, salicylic acid, and 2,3- and 2,5-DHBA were obtained from SigmaAldrich (St. Louis, MO). Chelex 100® resin was from Bio-Rad (Hercules, CA). Ten

individual human liver samples from a stock (Schadt et al., 2008) in our laboratory were pooled (equal weights) to prepare human liver microsomes (Guengerich, 2014). *Escherichia coli* recombinant human P450s 2C8 (Shimada et al., 2001), 2C9 (Sandhu et al., 1993), 2C19 (Komatsu et al., 2000), 2D6 (Gillam et al., 1995; Hanna et al., 2001), 2E1 (Gillam et al., 1994), and 3A4 (Gillam et al., 1993; Hosea et al., 2000) were prepared and purified as described in the indicated references. *E. coli* recombinant rat NADPH-P450 reductase (Hanna et al., 1998) and *E. coli* recombinant human cytochrome b_5 (Guengerich, 2005; Shimada et al., 1986) were prepared as described.

2.2. Human liver microsome incubations

Incubations were conducted at 37 °C in 0.1 or 1.0 ml incubation mixtures containing 5 μM P450 in 50 mM potassium phosphate buffer (pH 7.4), an NADPH-generating system (10 mM glucose 6-phosphate, 0.5 mg/ml $NADP^+$, and 2 μg/ml yeast glucose 6-phosphate dehydrogenase) (Guengerich, 2014), and the reaction substrate (0.5 mM aspirin/salicylic acid if not otherwise stated). Greater volume of incubations was used for aspirin (1.0 ml) because lower rates of hydroxylation were observed when compared to salicylic acid (incubation volume of 0.1 ml). Incubations were performed generally for 30 minutes with or without the NADPH-generating system in a 37 °C reciprocal shaking water bath. Reactions were quenched using 6 M NaOH to bring the pH to 13. The aspirin samples stood for 15 minutes to deacetylate the acetylsalicylic acid. $HClO_4$ (6 M) was added to precipitate protein and to acidify (pH 1) the products for extraction with $(C_2H_5)_2O$ (aspirin) or CH_2Cl_2 (salicylic acid). Extraction was done twice with 3 ml of $(C_2H_5)_2O$ or CH_2Cl_2, followed with centrifugation (3000*g*, 10 minutes) to separate the organic and aqueous layers.

The organic layer was concentrated under a stream of nitrogen, and the residue was dissolved in 30 μl of CH_3CN-H_2O (1:9, v/v) for LC analysis. 2,3-DHBA and 2,5-DHBA were separated by UPLC on a Waters Acquity octadecylsilane (C_{18}) column (2.1 mm × 100 mm, 1.7 μm) and quantified using UV (photodiode array) and fluorescence detectors (Acquity, Waters, Milford, MA). CH_3CN-H_2O (5:95, with 0.1% CH_3CO_2H, v/v) was used as mobile phase A and CH_3CN-H_2O (40:60, with 0.1% CH_3CO_2H (v/v)) as mobile phase B. The following gradient program was used with a flow rate of 300 μl/min: starting point of 100% (v/v) A, held at 100% to 2 min and raised linearly to 70% B (v/v) at 13 min. The column was re-equilibrated to 100% A at 15 min and held for 2 min more.

2.3. Mass spectrometry analysis

The incubation products were quenched and extracted as described above. Prior to evaporating the $(C_2H_5)_2O$ under a stream of nitrogen, the reaction products were reacted with diazomethane (prepared by alkaline treatment of Diazald® (SigmaAldrich) in 2-(2-ethoxyethoxy)ethanol) to esterify the carboxylic acids to the corresponding methyl esters. The diazomethane solution was added until a yellow color persisted in the solution. The $(C_2H_5)_2O$ was evaporated under a stream of nitrogen, and the dried product was dissolved in 30 μl of a mixture of CH_3CN-H_2O (1:9, v/v). The products were analyzed using LC-MS by introduction into a Waters Acquity UPLC system (Waters, Milford, MA) interfaced to a Thermo-Finnigan LTQ mass spectrometer (Thermo Scientific Corp., San Jose, CA). Chromatographic separation was achieved with a Macherey-Nagel HILIC column (4.6 mm

× 100 mm, 5 μm). The LC conditions used were an isocratic solution of 97% CH_3CN:3% 5 mM $NH_4CH_3CO_2$ in H_2O (v/v) at 0.3 ml/min. Mass spectrometry analysis was performed using an atmospheric pressure chemical ionization source (negative ion) with a full scan range of *m/z* 50–400. The source current was set at 10 μA, the capillary temperature at 300 °C, and the vaporizer temperature at 350 °C. The sheath gas flow rate was 30 and the auxiliary gas flow rate was 5. MS fragmentation analysis was performed with a collision energy of 35%, 2 *m/z* isolation width, activation Q of 0.25, and an activation time of 30 ms. Data was acquired using a Finnigan Xcalibur software package.

2.4. P450 incubations

Incubations were done at 37 °C in 0.10 ml incubation mixtures containing 50 mM potassium phosphate buffer (pH 7.4), an NADPH-generating system (10 mM glucose 6-phosphate, 0.5 mM $NADP^+$, and 2 μg/ml yeast glucose 6-phosphate dehydrogenase) (Guengerich, 2014), and 5 mM substrate (salicylic acid or aspirin). Each P450 enzyme system used in the incubations included 0.25 μM of each *E. coli* recombinant human P450, 0.5 μM *E. coli* recombinant rat NADPH-P450 reductase, 0.5 μM *E. coli* recombinant human cytochrome b_5, and a lipid mixture (L-*α*-1,2-dioleoyl-*sn*-glycero-3-phosphocholine, L-*α*-1,2- dilauroyl-*sn*-glycero-3-phosphocholine, and bovine brain phosphatidylserine in a ratio of 1:1:1 (w/w/w), 40 μg/ml) (with 0.5 mM sodium cholate only for P450 3A4) (Imaoka et al., 1998). Incubations proceeded for 30 minutes with or without the NADPH-generating system. Incubation samples were quenched, extracted, and analyzed as shown above in the human liver microsomal incubations.

2.5. Inhibition studies

Salicylic acid (0.5 mM) was incubated with pooled human liver microsomes in the presence of selective P450 inhibitors. *α*-Naphthoflavone (for P450 1A2, 1 μM), methoxalen (for P450 2A6, 2 μM), ticlopidin (for P450 2B6, 5 μM), quercetin (for P450 2C8, 50 μM), sulfaphenazole (for P450 2C9, 5 μM), fluconazole (for P450 2C19, 10 μM), ketoconazole (for P450 3A4, 2 μM), quinidine (for P450 2D6, 2 μM), and 4-methylpyrazole (for P450 2E1, 100 μM) (Correia, 2005) were individually added at inhibiting concentrations to the incubation mixture by adding 0.1 μl of the inhibitor (dissolved in CH_3CN with the exception of 4-methylpyrazole, in H_2O). Inhibition assays were done in triplicate with control CH_3CN and H_2O blanks. Concentration-dependent inhibition of P450 2E1 was examined by incubating salicylic acid in the presence of 0, 10, 20, 50, and 100 μM concentrations of 4-methylpyrazole with human liver microsomes. Statistical analysis was done using a *t*-test and a one-way ANOVA in Prism 6 (GraphPad Software, San Diego, CA).

3. Results

3.1. Detection and separation of oxidized metabolites

Previous studies have utilized direct HPLC electrochemical measurements (Coudray et al., 1995; Ghiselli et al., 1992; Grootveld and Halliwell, 1986, 1988; Reidl, 1983) for the separation and analysis of the oxidation metabolites of aspirin. Chromatography of the products is problematic due to their hydrophilicity. Derivatization methods to dansylate or to form an acetonide proved to be unsuccessful. Esterification (with diazomethane) facilitated

separation of the standards, but the assays were not sensitive enough for quantitation (data not shown).

2,5-DHBA has an absorbance maximum at 328 nm (Fig. 2A). This wavelength was used for fluorescence excitation, and the maximum fluorescence emission was recorded at 422 nm (Fig. 2C). 2,3-DHBA has an absorbance maximum at 313 nm (Fig. 2B) and a maximum fluorescence emission at 432 nm (Fig. 2D). A UPLC separation method developed by (Sucharitakul et al., 2013) was used to separate 2,3-DHBA from 2,5-DHBA using an increasing gradient of CH_3CN in H_2O (with 0.1% HCO_2H, v/v). Using this protocol, the t_R for 2,5-DHBA was 4.1 min (Fig. 2E), and 2,3-DHBA eluted at 5.9 min (Fig. 2F). The differences in t_R and spectral properties allowed us to quantify the formation of both 2,5-DHBA and 2,3-DHBA.

3.2. Dependence of product formation on NADPH

Product formation from salicylic acid was not detected with microsomes for 2,3-DHBA or 2,5-DHBA in the absence of NADPH (Fig. 3A, 3C). With NADPH, both 2,5-DHBA and 2,3-DHBA were detected by fluorescence (Fig. 3B). In the aspirin incubation, 2,5-DHBA was detected but 2,3-DHBA was not (after the cleavage of the acetyl group in the post-incubation analysis) (Fig. 3D).

3.3. Confirmation of 2,5-DHBA by mass spectrometry

Aspirin was incubated in the presence of human liver microsomes and NADPH, and the hydrolyzed products were extracted into $(C_2H_5)_2O$ and reacted with diazomethane. Products were injected onto an LC-MS system for mass analysis. Standard methyl 2,5-DHBA (t_R 2.29 minutes) (Fig. 4A) yielded ions at *m/z* 135, 152, and 167 (Fig. 4B), with the aspirin incubation product (t_R 2.19 minutes) (Fig. 4C) also yielded ions at *m/z* 135, 152, and 167 (Fig. 4D).

3.4. Effect of desferrioxamine

Assays with the substrates, salicylic acid and aspirin, were performed in the presence and absence of the iron chelator desferrioxamine (using Chelex 100®-treated buffers) (Fig. 5) to address the contribution of oxygen radicals in non-enzymatic formation of the observed products. The presence of the chelating agent did not inhibit but instead increased the formation of both 2,3- and 2,5-DHBA.

3.5. Time dependence

In a kinetic assay was with salicylic acid, a 30-minute time point was selected for further studies for the two products (Fig. 6).

3.6. Rates of oxidation

Formation of both 2,3- and 2,5-DHBA from salicylic acid was concentration-dependent (Fig. 7). With salicylic acid, 2,3-DHBA was the major product formed, compared to 2,5-DHBA. With aspirin only 2,5-DHBA was found (after deacetylation). The maximum rate of

5-hydroxylation was approximately five times lower with aspirin than salicylic acid (data not shown).

3.7. Catalytic activity of individual cytochrome P450s

Recombinant P450 enzymes were incubated with salicylic acid (Fig. 8). Because of the limit of detection (more sensitive for 2,5-DHBA, Fig. 2) in these assays, only 2,5-DHBA was quantified. P450 2C9 showed the highest product formation of 2,5-DHBA, followed by 2D6, 2C8, 3A4, 2E1, and 2C19 in respective order. With aspirin as the substrate, concentrations of the products were too low to quantitate.

3.8. Inhibition studies

In order to determine which P450 enzymes are responsible for the metabolism in liver microsomes, inhibition studies were performed (Fig. 9A and 9B). Selective inhibitors for P450s 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 3A4, and 2E1 were individually added to reaction incubation mixtures, either in CH_3CN or H_2O (only for 4-methylpyrazole, for P450 2E1). In these assays, it was found that P450s 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 3A4, and 2E1 all had significant contribution ($p < 0.05$) to the 5-hydroxylation reaction (Fig. 9A). For 3-hydroxylation, P450s 2C8, 2C9, 2C19, 2D6, and 2E1 had significant contribution ($p < 0.05$) to the enzymatic activity (Fig. 9B). When aspirin was used as substrate, there were also decreases in the oxidation rates in the presence of inhibitors, although none of the individual enzymes reached statistical significance ($p < 0.05$) (Fig. 9C).

For P450 2E1, which showed a major role in Fig. 9A, the concentration dependence was examined for the selective inhibitor 4-methylpyrazole. 4-Methylpyrazole decreased the formation of both 2,3- and 2,5-DHBA in human liver microsomal incubations (Fig. 10).

4. Discussion

Previous studies involved HPLC for separation of 2,3- and 2,5-DHBA using sodium citrate-acetate buffers and CH_3OH, with electrochemical detection of the catechol and hydroquinone (Coudray et al., 1995; Ghiselli et al., 1992; Grootveld and Halliwell, 1986, 1988). Electrochemical detectors are no longer generally used, and their use is restricted to a few electrochemically active molecules. We utilized acidic H_2O/CH_3CN mixtures, based on other previous literature (Sucharitakul et al., 2013). CH_3CN was used instead of CH_3OH to limit UV background absorbance during analysis. Major differences in the fluorescence of the products allowed further distinction and quantitation of these compounds.

In human liver microsomes fortified with NADPH, salicylic acid reproducibly generated 2,3- and 2,5-DHBA but acetylsalicylic acid generated only 2,5-DHBA (Fig. 3). The results vary with some previous results with rat and rabbit liver microsomes, which catalyzed the formation of 2,5- but not 2,3-DHBA (Ingelman-Sundberg et al., 1991). The differences may result from variance in the P450s in the different species. Our limits of detection were much higher for 2,3-DHBA (30 nM) than 2,5-DHBA (3 nM). This could have lead to the inability to quantify 2,3-DHBA in earlier studies although it is not clear how the sensitivity compares with an electrochemical detector.

Product formation was time-dependent (Fig. 6) and concentration-dependent (Fig. 7). Mass spectrometry was used to confirm the formation of 2,5-DHBA in aspirin incubation. Both hydroxylations of salicylic acid were not saturating (Fig. 7), and the results can be presented as estimates of catalytic efficiency (V_{max}/K_m), ≈ 0.50 min^{-1} M^{-1} for 2,5-DHBA, 0.71 min^{-1} M^{-1} for 2,3-DHBA) but V_{max} and K_m cannot be calculated individually. This result may be attributed to multiple P450 enzymes having a role in the oxidation, e.g. (Figs. 8, 9), or simply reflect the kinetic behavior of one or more P450s with the substrate. Although we originally hypothesized that aspirin would be a better substrate than salicylic acid (due to consideration of the hydrophilicity of salicylic acid), the opposite was found to be the case. The oxidation of salicylic acid is probably more relevant, in light of the known rapid hydrolysis of aspirin (Trnavsky and Zachar, 1975).

Incubations done in the presence of the iron chelator desferrioxamine showed that the formation of 2,5-DHBA was catalyzed by P450 instead of oxygen radicals formed in solution (Fig. 5). Inhibition assays with selective P450 inhibitors showed roles for multiple P450 enzymes, i.e. 2C8, 2C9, 2C19, 2D6, 3A4, and 2E1 (Fig. 9). These results (for 2,5-DHBA) were confirmed by performing incubations in the presence of recombinant P450 enzymes (Fig. 8).

The low *in vitro* rates of formation of 2,3- and 2,5-DHBA can explain why the oxidized products are minor metabolites of salicylic acid. The formation of low levels of products may be important, in the context of aspirin sensitivity in people with polymorphisms in P450 2C9 (*vide infra*).

Although 3-hydroxylation of salicylic acid has been suggested to be a marker of hydroxyl radicals (Grootveld and Halliwell, 1986), our results with desferrioxamine (Fig. 5) did not show a decrease in activity. This conclusion is in contrast to a report with rabbit and rat liver microsomes (Ingelman-Sundberg et al., 1991). We are unsure what the differences are, but we used Chelex 100®-treated buffers in these experiments, and we routinely prepare liver microsomes in the presence of EDTA and butylated hydroxytoluene to prevent lipid peroxidation artifacts (Guengerich, 2014; van der Hoeven and Coon, 1974). We have not specifically prepared microsomes according to the procedure of Ingelman-Sundberg et al. (Eliasson et al., 1988; Ingelman-Sundberg and Johansson, 1984; Ingelman-Sundberg et al., 1991) (lower EDTA concentration, no butylated hydroxytoluene (Eliasson et al., 1988)), nor have we examined these animal species in this work.

Although P450 oxidation is a minor pathway of metabolism (Fig. 1) and low-dose aspirin is often used in cardioprotective dose, it should be considered that aspirin is a high-dose nonsteroidal anti-inflammatory drug (typical recommended dose of 2,700 to 6,000 mg/day for humans) (1999) and that the oxidation products (2,3- and 2,5-DHBA) are *o*-and *p*-dihydroxyphenolic compounds (i.e., a catechol and hydroquinone, respectively) and are capable of oxidation to quinones, potentially toxic chemicals. However, we are unaware of any evidence that this is an issue in any toxicity related to aspirin use.

Aspirin has also been reported to induce P450s. A low dose (50 mg/day for 14 days) was reported to increase P450 2C19 and, to a lesser extent, P450 3A4, as judged by in vivo

assays (mephenytion and midazolam oxidations) (Chen et al., 2003). Thus, in considering Figs. 8 and 9, aspirin might be expected to increase its own metabolism.

We found that P450 2C9 had catalytic activity in oxidizing salicylic acid (Figs. 8, 9) and possibly aspirin itself (Fig. 9C). A major role for P450 2C9 in aspirin metabolism has been proposed (Agundez et al., 2009; Palikhe et al., 2011). However, this conclusion appears to be based only on association studies of side effects (urticaria) of aspirin and some *CYP2C9* alleles (Palikhe et al., 2011). However, another report showed a similar association with other non-steroidal anti-inflammatory drugs (Carbonell et al., 2010), suggesting that the issue may be pharmacodynamic (and possibly the result of linked genes, not *CYP2C9*). Our evidence (Figs. 7, 8) indicates that P450 2C9 is not dominant, being only one of several P450s involved (Figs. 8, 9) similar to the promiscuity observed with UGTs in the glucuronidation reactions (Kuehl et al., 2006). The evidence favors P450 2E1 as having the largest single role among P450s (Figs. 9, 10), which is not surprising in light of the small size of the active site of that P450 (Porubsky et al., 2008).

5. Conclusion

We found that human liver microsomes catalyzed the formation of 2,3- and 2,5-DHBA from salicylic acid and 2,5-DHBA from aspirin. This formation was NADPH-, time-, and concentration-dependent and was confirmed by mass spectrometry. Formation of these metabolites was shown not to be due to oxygen radicals (Fig. 5). Individual P450 incubations and inhibitor assays were used to determine the enzymes that have activity in these reactions, including P450s 2C8, 2C9, 2C19, 2D6, 3A4, and 2E1. The contribution of multiple P450s and UGTs (Kuehl et al., 2006) in metabolism is consistent with a lack of issues regarding enzyme polymorphisms with this widely used drug.

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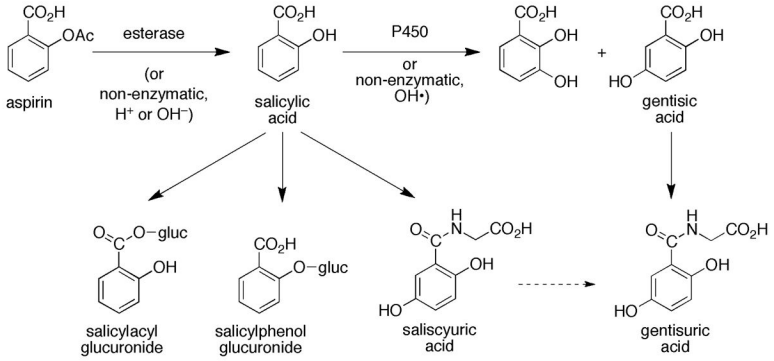

Fig. 1.

Aspirin metabolism. Aspirin is readily hydrolyzed to salicylic acid, both enzymatically and non-enzymatically. Salicylic acid undergoes conjugation reactions generating the major metabolites salicyluric acid (apparently catalyzed by an acyl-CoA *N*-acyltransferase) and glucuronides (gluc) (catalyzed by UGTs). Products of oxidation of salicylic acid have been attributed to catalytic activity of P450s as well as non-enzymatic Fenton-type reactions (2,3-DHBA). Gentisic acid (2,5-DHBA) can also be conjugated by an acyl-CoA *N*-acyltransferase to form gentisuric acid (Wilson et al., 1978). The conjugation reaction shown with the broken line is hypothetical.

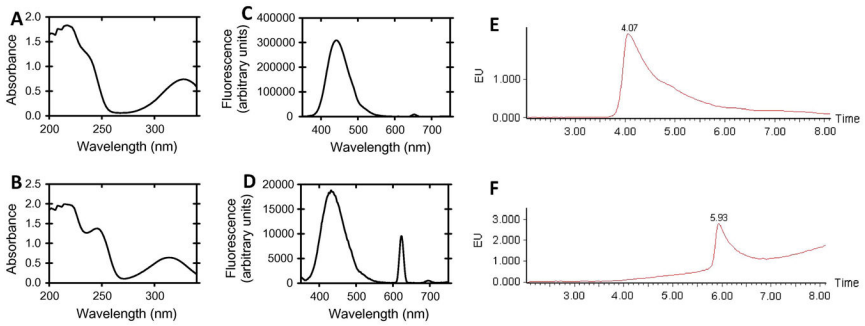

Fig. 2.

Separation and detection of oxidized products of aspirin and salicylic acid. 2,5-DHBA showed absorbance maxima at 239 and 328 nm (A). Maximum fluorescence emission was observed at $\lambda_{excitation}$ 328 nm and $\lambda_{emmission}$ 422 nm (C); the 422 nm emission was used for UPLC detection (*t*R 4.1 min, E). 2,3-DHBA showed absorbance maxima at 246 and 313 nm (B). Maximum fluorescence emission was observed at $\lambda_{excitation}$ 313 nm and $\lambda_{emmission}$ 432 nm (D); the 432 nm emission was used for UPLC detection (*t*R 4.1 min, F).

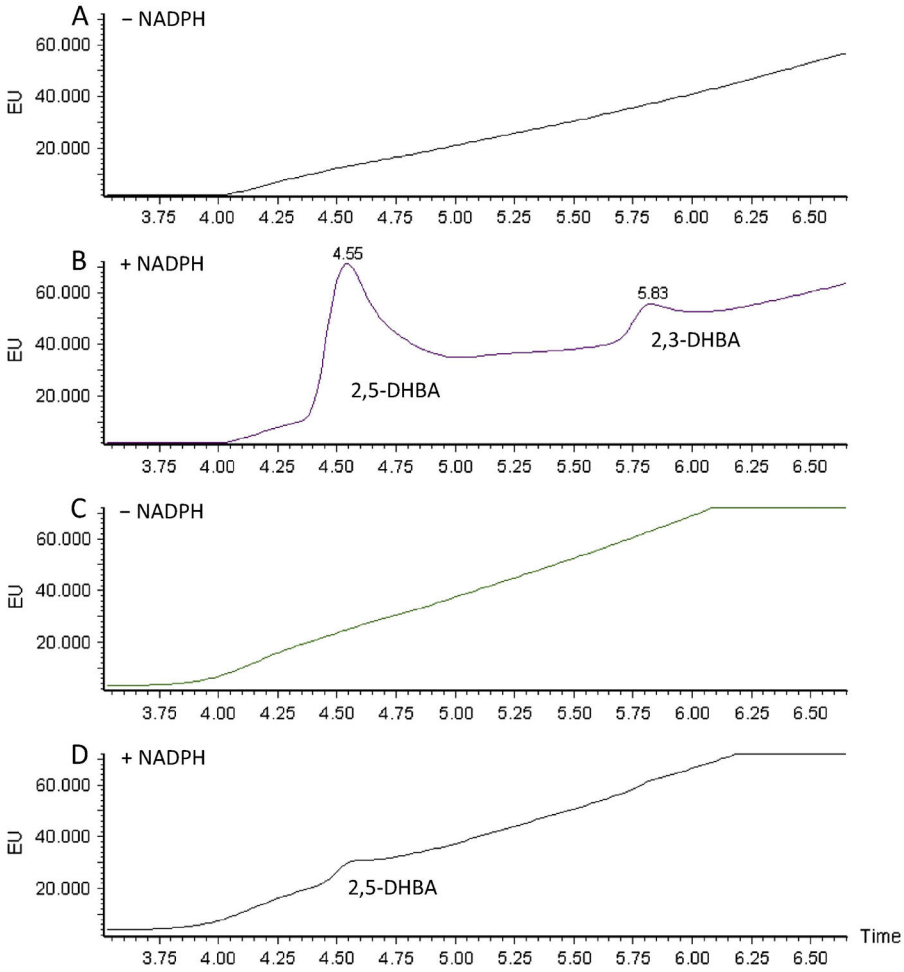

Fig. 3.

NADPH-dependent formation of 2,5- and 2,3-dihydroxybenzoic acid (DHBA). To determine P450 dependent metabolism, salicylic acid and aspirin were incubated in presence and absence of NADPH. Salicylic acid (1 mM final concentration) was incubated with human liver microsomes (0.5 nmol P450), and NADPH-dependent generation of 2,5- and 2,3-DHBA was observed (A,B) indicating P450-mediated oxidation of salicylic acid. When aspirin (1.5 mM final concentration) was incubated with human liver microsomes under these conditions, NADPH-dependent formation of 5-hydroxy-2-acetylsalicylic acid (detected as 2,5-DHBA after hydrolysis) was observed (D). No product formation was observed without NADPH (C) indicating P450 dependent hydroxylation of aspirin.

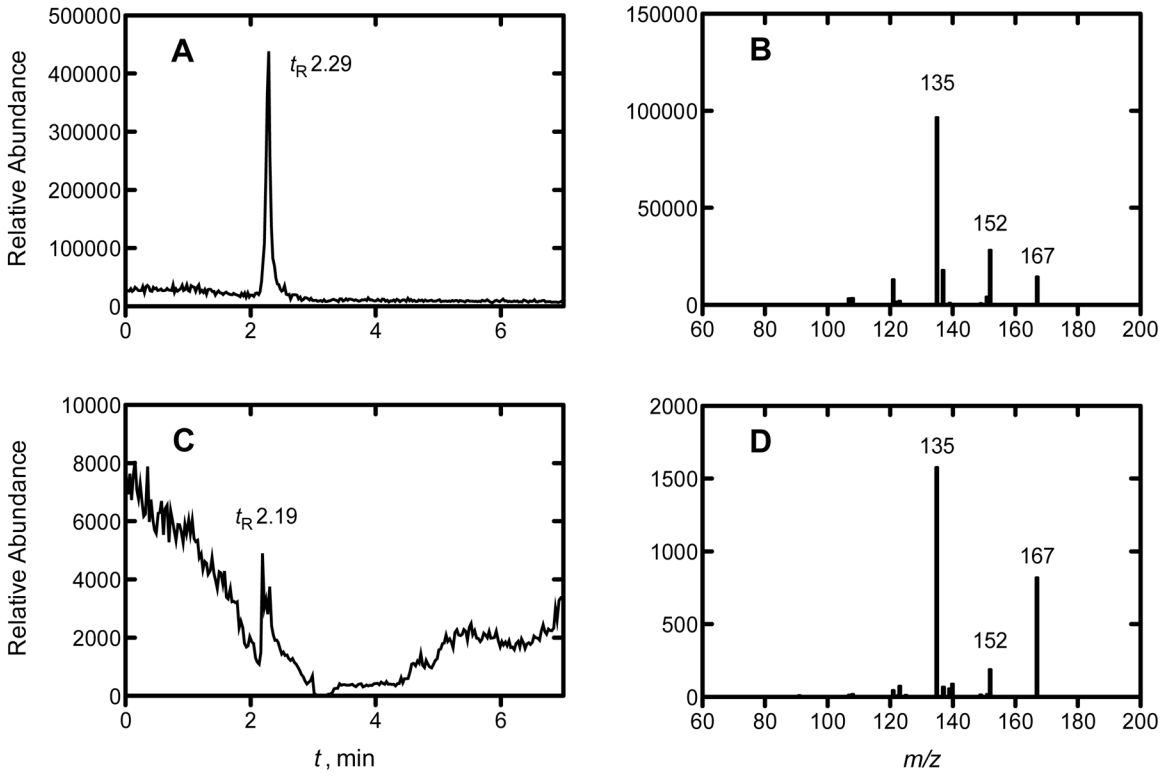

Fig. 4.

Confirmation of 5-hydroxylation of aspirin by mass spectrometry. To confirm that aspirin can be directly oxidized, the hydroxylated product was hydrolyzed with perchloric acid, neutralized, and methylated with diazomethane. The UPLC trace (A) and mass spectrum (B) of standard 2,5-DHBA corresponded to the hydrolyzed and subsequently esterified aspirin product (C and D, respectively) after incubation with human liver microsomes (0.7 nmol P450).

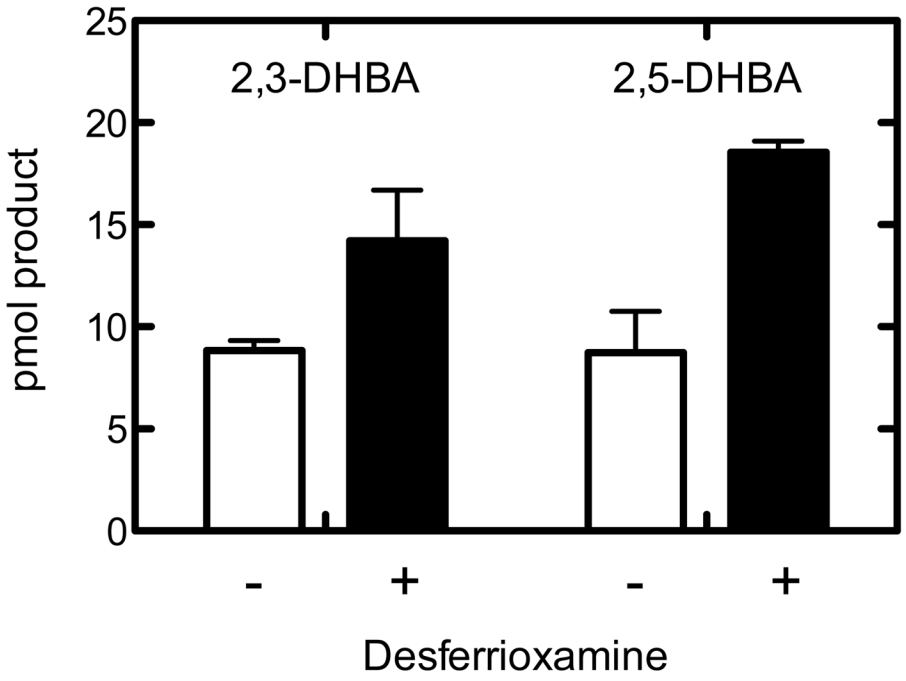

Fig. 5.

Effect of desferrioxamine on P450 oxidation of salicylic acid using human liver microsomes. To determine if hydroxylation of salicylic acid is result of Fenton-type reactions, incubations were performed in presence and absence of 100 μM desferrioxamine with 0.5 nmol microsomal P450, incubated for 30 minutes. Under these conditions reactive oxygen species production should be inhibited due to chelation of iron with desferrioxamine. However, no inhibition in production of 2,3 and 2,5- DHBA was observed, indicating P450-dependent enzymatic hydroxylation of salicylic acid.

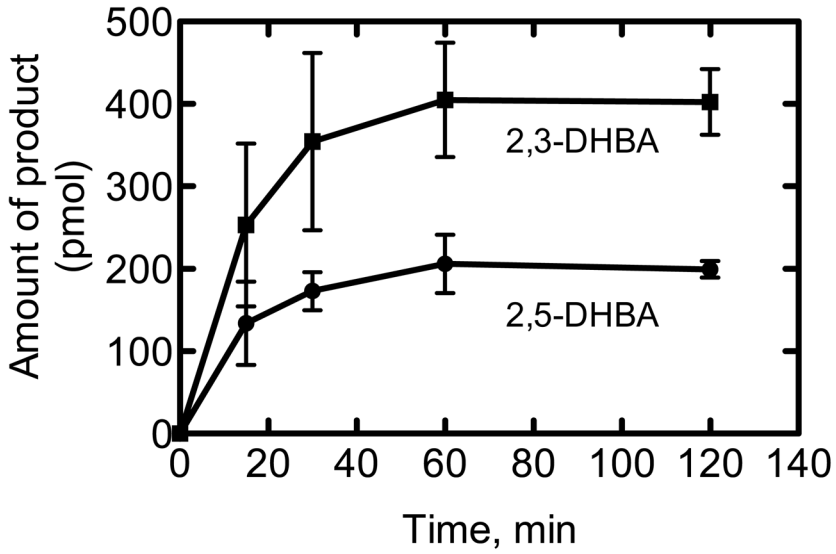

Fig. 6.

Time dependence of salicylic acid oxidation. If the hydroxylation of salicylic acid is metabolism dependent it should show time dependence as well. To confirm this incubations were performed for 15, 30, 60, and 120 minutes. The incubation reaction was done with 0.5 nmol of microsomal P450. A 30 min incubation time was selected for further kinetic and inhibition assays.

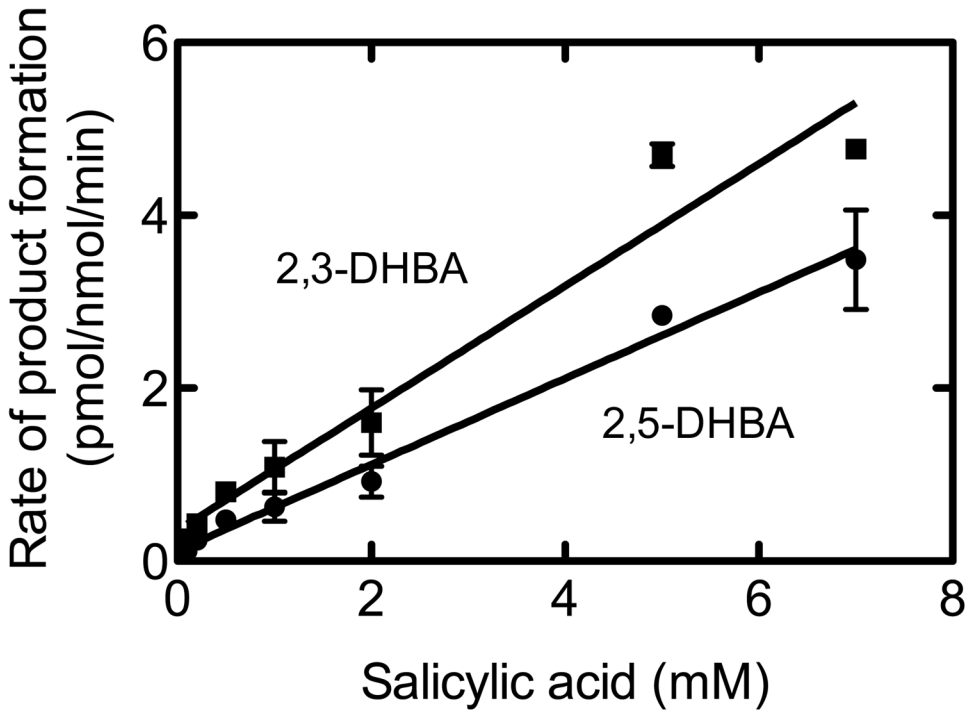

Fig. 7.

Rates of microsomal oxidation of salicylic acid in human liver microsomes (0.5 nmol P450). Data points were fit to a linear equation (linear regression analysis). Due to limits of solubility of salicylic acid enzyme saturation (V_{max}) cannot be achieved and consequently K_m determined. Under these conditions the slope is an upper limit of catalytic efficiency (V_{max}/K_m), ≈ 0.50 min^{-1} M^{-1} for 2,5-DHBA, 0.71 min^{-1} M^{-1} for 2,3-DHBA, based on total P450).

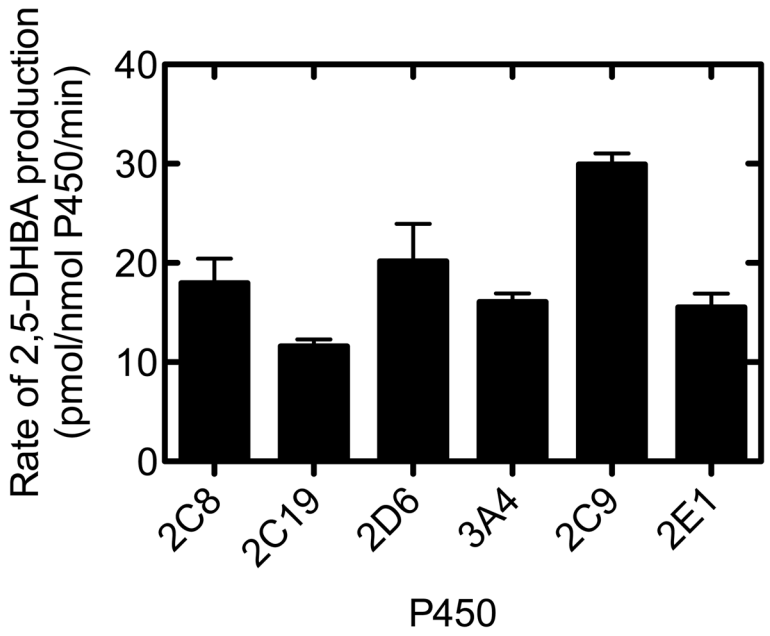

Fig. 8.

Rates of 2,5-DHBA production by individual recombinant P450s. Salicylic acid (0.5 mM) was incubated with 25 pmol of each P450 enzyme for 30 minutes. Low hydroxylation rates were observed (11 – 30 pmol/nmol/min).

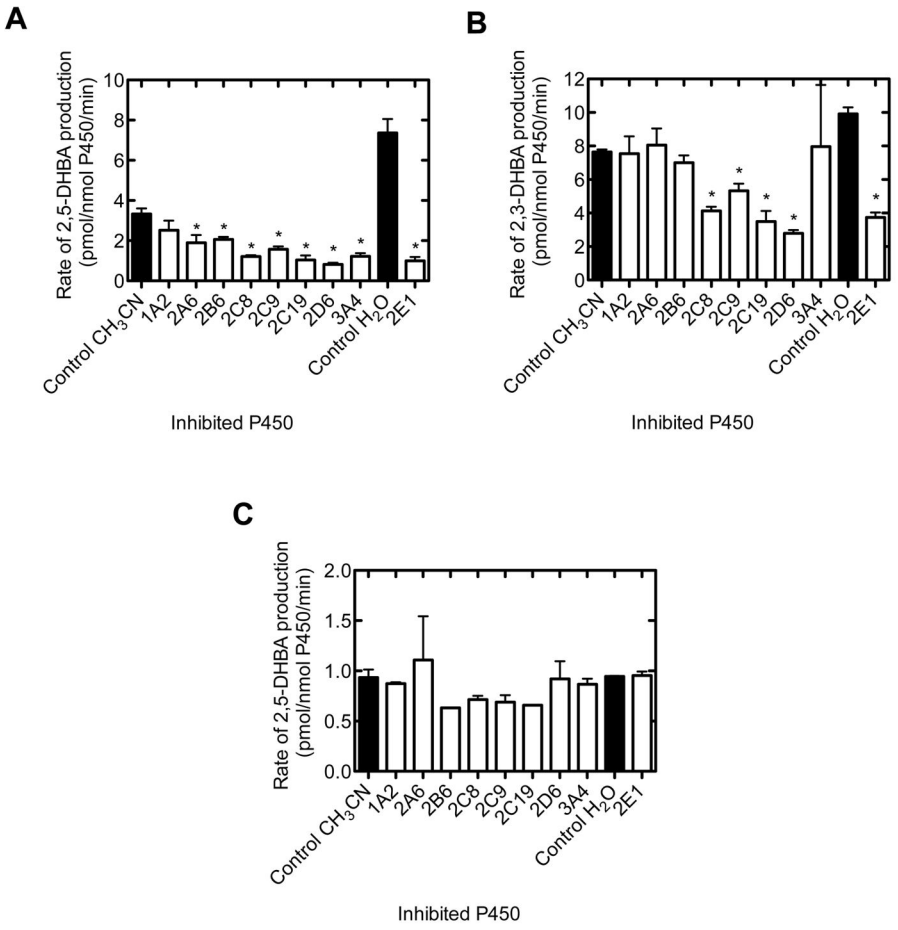

Fig. 9.

Analysis of P450s responsible for oxidation of salicylic acid (A, B) and aspirin (C) using selective P450 inhibitors on human liver microsomes (*see Materials and Methods*). Statistically significant changes ($p < 0.05$, *) in the rate of salicylic acid (0.5 mM) oxidation using 0.5 nmol human liver microsomes were observed when P450s 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 3A4, and 2E1 were inhibited. Solvent controls were performed using CH3CN (used for P450s 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, and 3A4) and H2O (used for P450 2E1) to examine solvent inhibition.

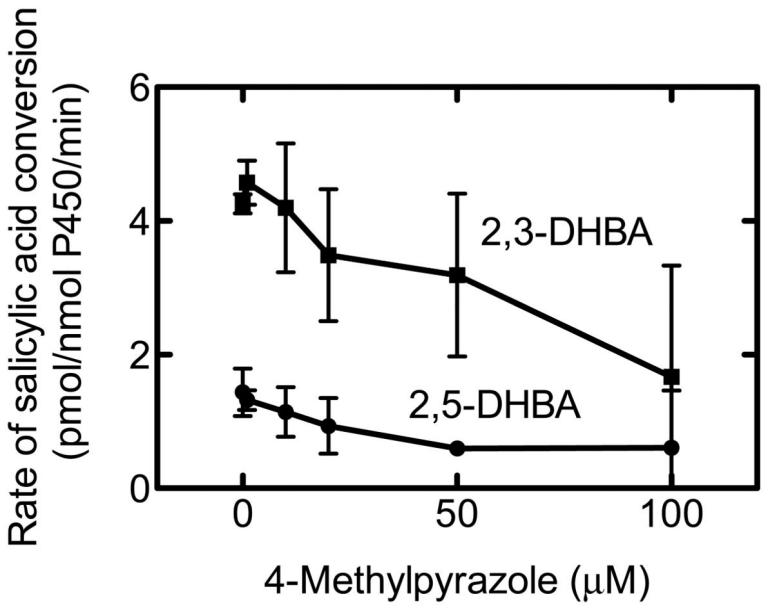

Fig. 10.

P450 2E1 inhibitor titration. The contribution of P450 2E1 to the oxidation of salicylic acid (0.5 mM) to 2,5- and 2,3-dihydroxybenzoic acid (DHBA) was measured using different concentrations of the selective P450 2E1 inhibitor 4-methylpyrazole (0, 1, 10, 20, 50, 100 μM) in incubations with human liver microsomes (0.5 nmol P450, different pool of microsomes than used in Fig. 9). As production of dihydroxybenzoic acids was dependent on the inhibitor concentration, hydroxylation of salicylic acid can be attributed to metabolic activity of P450 2E1.