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PHARMACOKINETICS AND DISPOSITION

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The role of *CYP2C9* genotype in the metabolism of diclofenac in vivo and in vitro

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Abstract *Introduction*: The polymorphic cytochrome P_{450} enzyme 2C9 (CYP2C9) catalyses the metabolism of many drugs including *S*-warfarin, acenocoumarol, phenytoin, tolbutamide, losartan and most of the non-steroidal anti-inflammatory drugs. Diclofenac is metabolised to 4′-hydroxy (OH), the major diclofenac metabolite, 3′-OH and 3′-OH-4′-methoxy metabolites by CYP2C9. The aim of the present study was to clarify the impact of the CYP2C9 polymorphism on the metabolism of diclofenac both in vivo and in vitro.
Subjects, materials and methods: Twenty healthy volunteers with different *CYP2C9* genotypes [i.e. *CYP2C9*1/*1* ($n=6$), **1/*2* ($n=3$), **1/*3* ($n=5$), **2/*3* ($n=4$), **2/*2* ($n=1$), **3/*3* ($n=1$)] received a single 50-mg oral dose of diclofenac. Plasma pharmacokinetics [peak plasma concentration (C_{max}), half-life ($t_{1/2}$) and area under the plasma concentration–time curve (AUC_{total})] and urinary recovery of diclofenac and its metabolites were compared between the genotypes. Diclofenac 4′-hydroxylation was also analysed in vitro in 16 different samples of genotyped [i.e. *CYP2C9*1/*1* ($n=7$), **1/*2* ($n=2$), **1/*3* ($n=2$), **2/*3* ($n=2$), **2/*2* ($n=2$), **3/*3* ($n=1$)] human liver microsomes.
Results: Within each genotype group, a high variability was observed in kinetic parameters for diclofenac and 4′-OH-diclofenac (6- and 20-fold, respectively). No significant differences were found between the different genotypes either in vivo or in human liver microsomes. No correlation was found between the plasma AUC ratio of diclofenac/4′-OH-diclofenac and that of losartan/E-3174, previously determined in the same subjects.
Conclusion: No relationship was found between the *CYP2C9* genotype and the 4′-hydroxylation of diclofenac either in vivo or in vitro. This, together with the lack of correlation between losartan oxidation and diclofenac hydroxylation in vivo raises the question about the usefulness of diclofenac as a CYP2C9 probe.

Keywords CYP2C9 · Diclofenac · Phenotyping

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Introduction

Diclofenac is a widely used phenylacetic acid non-steroidal anti-inflammatory drug (NSAID). It undergoes extensive phase-I and phase-II metabolism [1]. The major metabolite, 4′-hydroxy(OH)-diclofenac, and the minor metabolites, 3′-OH-diclofenac and 3′-OH-4′-methoxy-diclofenac, are formed by the hepatic cytochrome P_{450} 2C9 (CYP2C9) [2, 3]. However, the formation of 5-OH-diclofenac is catalysed by several CYP enzymes including CYP3A4, CYP2C8, CYP2C18, CYP2C19 and CYP2B6 [4, 5]. The hydroxy metabolites are further conjugated and excreted in urine and bile [6].

Apart from diclofenac, CYP2C9 metabolises most of the NSAIDs, including flurbiprofen, naproxen, indomethacin, ibuprofen, lornoxicam, tenoxicam, piroxicam and celecoxib, as well as *S*-warfarin, acenocoumarol, phenytoin, tolbutamide, torsemide, sildenafil, sulphamethoxazole and losartan [7, 8, 9, 10, 11, 12, 13, 14, 15, 16]. CYP2C9 is polymorphic and, to date, at least six different variants of the *CYP2C9* gene have been designated and can be found at http://www.imm.ki.se/CYPalleles/cyp2c9.htm [17, 18, 19, 20, 21]. The frequencies of *CYP2C9*2* and *CYP2C9*3* variant alleles giving rise to decreased enzyme activity are higher in Caucasian (0.08–0.12 and 0.03–0.08, respectively) than in Oriental (0 and 0.02–0.03, respectively) or Black populations (0.01–0.04 and 0.005–0.02, respectively) ([22, 23] and

references therein). The *CYP2C9*4* allele refers to a point mutation in exon 7 [21]. To date, only one individual with this variant allele, a Japanese subject, has been reported [21]. Another point mutation in exon 7 has recently been identified in an African-American population [24].

In a recent in vitro study, the catalytic activity of the *CYP2C9*3* variant relative to *CYP2C9*1* expressed in yeast was studied [25]. A 3- to 34-fold decreased metabolite formation was observed for *CYP2C9*3* depending on which of seven different CYP2C9 substrates was used [25]. Diclofenac 4′-hydroxylation has been widely used as an in vitro assay for CYP2C9 activity [26, 27, 28, 29, 30]. However, in a recent in vivo study, there was no difference in diclofenac pharmacokinetics between the *CYP2C9*1/*3* and *CYP2C9*1/*1* genotypes [31]. The effect of the other major *CYP2C9* genotypes (i.e. *CYP2C9*1/*2, *2/*2*, **3/*3* and **2/*3*) on diclofenac 4′-hydroxylation in vivo has not been evaluated. The aim of the present study was to clarify the impact of the *CYP2C9* genotype in the metabolism of diclofenac both in vivo and in vitro in human liver microsomes. The pharmacokinetics of diclofenac in relation to *CYP2C9* genotype were also compared with losartan, another CYP2C9 substrate, previously given to the same subjects [32].

Materials and methods

Subjects

Eleven female and nine male Swedish healthy volunteers (age 25–54 years, body weight 52–91 kg) were recruited among subjects previously genotyped for the *CYP2C9* polymorphism [22]. The same subjects participated in a recent study on losartan pharmacokinetics in relation to *CYP2C9* genotype [32]. Subjects with the combinations of **1*, **2* and **3* alleles, i.e. *CYP2C9*1/*1* (six subjects), **1/*3* (five subjects), **1/*2* (three subjects), **2/*3* (four subjects), **2/*2* (one subject) and **3/*3* (one subject), were included in the study. All subjects were considered to be healthy on the basis of medical history, physical examination and clinical laboratory test results. Written informed consent was obtained from the volunteers. The study was conducted in accordance with the Declaration of Helsinki and approved by the local ethics committee at Huddinge University Hospital, Stockholm, Sweden.

Study design

A single 50-mg oral dose of diclofenac (Voltaren, Novartis) was administered after an overnight fast. Subjects had not taken any medication for at least 2 weeks before diclofenac administration. Venous blood samples (10 ml) were obtained before and at 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, 12 and 24 h after drug intake for the determination of diclofenac and its 4′-OH and 3′-OH-4′-methoxy metabolite concentrations. The plasma was separated and stored at –20°C until analysis. Urine samples were obtained before diclofenac administration and as 0- to 4-h, 4- to 8-h, 8- to 12-h and 12- to 24-h fractions. The volume of urine voided was noted in order to be able to calculate the urinary recovery. Following a 4-h fast after drug intake, a standard lunch was served. During the study, all side effects reported by the subjects were recorded.

Preparation and characterisation of human liver microsomes

Microsomes of 16 livers with *CYP2C9*1/*1* ($n=7$), **1/*2* ($n=2$), **1/*3* ($n=2$), **2/*3* ($n=2$), **2/*2* ($n=2$) and **3/*3* ($n=1$) genotypes were prepared from the human liver bank (approved by the ethics committee at Huddinge University Hospital) established at the Department of Clinical Pharmacology in Huddinge University Hospital, as described previously [33]. The protein content was estimated according to the method of Lowry et al. [34]. The microsomes were stored in potassium phosphate buffer (50 mM, pH 7.4) at –80°C until use. The total P_{450} content in microsomes was determined by measuring the reduced carbon monoxide spectrum [35]. A QIAamp Tissue DNA preparation kit (Qiagen, Hilden, Germany) was used to isolate genomic DNA. After polymerase chain reaction (PCR) amplification, performed as described previously [22] using the primers from Sullivan-Klose et al. [19], the products were digested using the endonucleases *Ava*II and *Nsi*I for the *CYP2C9*2* and *CYP2C9*3* genotypes, respectively. Immunoblotting of CYP2C9 apoprotein was performed as described previously [36] using CYP2C9 specific patient sera *PIJ*, kindly provided by P. Beaune, Paris.

Analysis of enzyme kinetics for diclofenac 4′-hydroxylation in human liver microsomes

Human liver microsomes, corresponding to 1 mg protein, were incubated at 37°C in 50 mM potassium phosphate buffer (pH 7.4) with nine different concentrations of diclofenac (1–200 μM). Reactions were started by the addition of reduced nicotinamide adenine dinucleotide phosphate (NADPH; Sigma) at 1 mM final concentration and terminated after 15 min by freezing on dry ice. The incubation conditions were chosen based on experiments showing that the formation of 4′-OH-diclofenac was linear in the range from 5 min to 30 min of incubation time and from 0.25 mg to 2 mg of microsomal protein.

Drug analysis

Diclofenac, 3′-OH-diclofenac, 4′-OH-diclofenac, 5-OH-diclofenac and 3′-OH-4′-methoxydiclofenac were kindly supplied by Novartis (Basel, Switzerland). Plasma and urine samples were analysed for diclofenac and its 4′-OH and 3′-OH-4′-methoxy metabolites, while microsomal incubation samples were analysed for 4′-OH-diclofenac. Plasma (1 ml) and microsomal (0.5 ml) incubation samples were acidified with 1 ml 0.5 M phosphoric acid, and 10 μg ibuprofen (Sigma) and 30 μg losartan (Merck Sharp Dohme) were added as internal standards for plasma and microsomal samples, respectively. The samples were extracted with 4 ml dichloromethane and thereafter re-extracted to 500 μl di-sodium hydrogen phosphate (40 mM). An aliquot of this extract was acidified with 0.5 M phosphoric acid (acid/sample, 1/10, v/v). Urine samples were pre-treated with β-glucuronidase (4/1, urine/β-glucuronidase, v/v; Roche Diagnostics GmbH, Mannheim, Germany) at 46°C for 2 h. After centrifugation, urine samples were acidified with 25 mM potassium phosphate (pH 1.8).

Acidified plasma extracts (25 μl) and urine samples (10 μl) were injected into a Hewlett Packard high-performance liquid chromatography (HPLC) ChemStation with ultraviolet detection at 270 nm. Chromatographic separation was provided by a Zorbax SB-C18 (75×4.6 mm) column connected to a pre-column at 30°C. The flow rate was 1 ml/min for the first 8 min with a mobile phase consisting of acetonitrile, ammonium dihydrogen phosphate (10 mM) and methanol (0.3/45/54.7, v/v/v). A gradient was applied from the eighth to the sixteenth minute with a flow rate of 1.5 ml/min to reach the final concentration of the mobile phase (12/43.5/44.5, v/v/v). Total run time was 20 min. The retention times of 3′-OH-4′-methoxy and 4′-OH metabolites and diclofenac were 4.6 min, 5.4 min and 10.8 min, respectively. The retention times of two other metabolites, 3′-OH-diclofenac (4.0 min) and 5-OH-diclofenac (6.3 min), were also determined to exclude any interference, but these metabolites were not quantified. Standard curves were prepared in the range of 0.15–5 μM for diclofenac and 0.05–2 μM for 4′-OH-diclofenac and 3′-OH-4′-methoxy-diclofenac in plasma. Quality control samples, 0.5 μM diclofenac and 0.25 μM

4′-OH-diclofenac and 3′-OH-4′-methoxy-diclofenac in plasma were included in each analytical run. For urine samples, standard curves were prepared in the range of 0.5–200 μM for both diclofenac and 4′-OH-diclofenac. The quality controls were 6 μM and 60 μM for both diclofenac and 4′-OH-diclofenac. The coefficients of variation were less than 10% for the parent compound and the metabolites. The limit of detection was 0.05 μM in plasma and 0.5 μM in urine for both the parent compound and the metabolites. The recoveries of diclofenac, 4′-OH-diclofenac and 3′-OH-4′-methoxy-diclofenac after extraction were 73%, 82% and 86%, respectively.

Pharmacokinetic and statistical analysis

The maximum plasma concentration (C_{max}) and the time to reach C_{max} (t_{max}) of diclofenac, 4′-OH-diclofenac and 3′-OH-4′-methoxy-diclofenac were determined from the concentration–time data. The total area under the plasma concentration versus time curve (AUC_{total}) was calculated for diclofenac and 4′-OH-diclofenac using the trapezoidal rule and extrapolation to infinity using the terminal linear part of the curve. For 3′-OH-4′-methoxy-diclofenac, the 0–24 h trapezoidal AUC ($AUC_{0-24\ h}$) was determined. The apparent terminal elimination half-life ($t_{1/2}$) of diclofenac and 4′-OH-diclofenac was calculated using linear regression analysis from the terminal linear part of the plasma concentration versus time curves. The recoveries (% of dose) of diclofenac, 4′-OH-diclofenac and 3′-OH-4′-methoxy-diclofenac in urine were calculated based on the molar amounts excreted during 0- to 8-h and 0- to 24-h periods after drug intake.

The pharmacokinetic parameters C_{max}, $t_{1/2}$, AUC, the ratios between the plasma AUCs of diclofenac and its metabolites and diclofenac/4′-OH-diclofenac urinary recovery ratios were log-transformed and analysed by means of one-way analysis of variance (ANOVA). The plasma AUC ratios between losartan and its E-3174 metabolite were taken from our previous study of losartan pharmacokinetics in the same subjects [32] and compared with the diclofenac plasma ratios with Spearman rank correlation test. Statistical analysis was done using STATISTICA 4.3 software (StatSoft. Inc.). *P* values of less than 0.05 were regarded as statistically significant.

Kinetic data from microsomal incubations were applied to a one-enzyme Michaelis-Menten kinetic model in GraFit 4.03 (Erithacus Software Limited, Surrey, UK), a curve-fitting software based on non-linear regression analysis, in order to estimate the apparent Michaelis constant (K_m) and maximal velocity (V_{max}) for diclofenac 4′-hydroxylation.

Results

All 20 subjects completed the study. Only one subject, with a *CYP2C9*2/*2* genotype, reported an adverse event – nose bleeding after intake of diclofenac. This subject had experienced spontaneous nose bleeding frequently before.

The plasma levels of diclofenac, 4′-OH-diclofenac and 3′-OH-4′-methoxy-diclofenac in six subjects (mean ± SD) with the *CYP2C9*1/*1* genotype as well as in the single subjects with *CYP2C9*2/*2* or *CYP2C9*3/*3* genotypes are shown in Fig. 1. Up to 6- and 20-fold intra-group variations were observed in the pharmacokinetic parameters of diclofenac and 4′-OH-diclofenac, respectively (Table 1). No significant differences were found between the genotype groups.

Table 2 summarises the recoveries of diclofenac and 4′-OH-diclofenac in urine collected up to 24 h. On average, 10% of a single 50-mg oral dose of diclofenac was recovered as diclofenac and 4′-OH-diclofenac in the *CYP2C9*1/*1* genotype group. There was, however, a large inter-individual variability in the total recovery, from 4.4% to 30.3% for different **1/*1* subjects. No significant differences were found in the recovery or the diclofenac/4′-OH-diclofenac ratio (either 0- to 8-h or 0- to 24-h urine) between the **1/*1*, **1/*2*, **1/*3* and **2/*3* genotype groups (Table 2). The recovery of 4′-OH-diclofenac in the **3/*3* subject was among the lowest and the diclofenac/4′-OH-diclofenac ratio among the highest of all the subjects studied. However, as only one subject with the **3/*3* genotype was included, no statistical calculation could be performed.

The ratio between the AUC_{total} of diclofenac and its metabolite ($AUC_{diclofenac}/AUC_{4'\text{-}OH\text{-}D}$) was used as a measure of the CYP2C9 dependent metabolism of diclofenac. This plasma ratio did not differ between the different genotypes including single *CYP2C9*2/*2* and *CYP2C9*3/*3* subjects. There was no correlation between plasma and urinary ratios of diclofenac/4′-OH-diclofenac among the 20 study subjects ($r_s = 0.1$, $P = 0.5$) and neither was there any correlation between the plasma diclofenac/4′-OH-diclofenac AUC_{total} ratios and the losartan/E-3174 AUC_{total} ratios ($r_s = 0.1$, $P = 0.8$; Fig. 2).

The total P_{450} content of the microsomal preparations from the 16 human livers varied from 321 pmol/mg to 965 pmol/mg protein and was at an intermediate

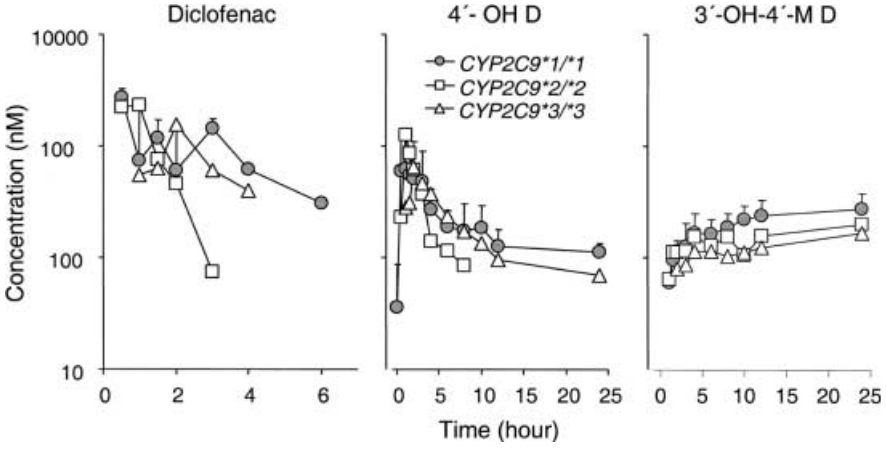

Fig. 1 The plasma concentration versus time curves of diclofenac, 4′-OH diclofenac (4′-OH D) and 3′-OH-4′-methoxy diclofenac (3′-OH-4′-M D) in the *CYP2C9*1/*1* genotype group (mean ± SD of six subjects) and the *CYP2C9*2/*2* and *CYP2C9*3/*3* subjects after a single 50-mg oral dose of diclofenac

Table 1 Pharmacokinetic parameters (mean ± SD with range in parentheses) of diclofenac, 4′-OH diclofenac and 3′-OH-4′-methoxy diclofenac in volunteers with different genotypes of *CYP2C9* after a single oral dose of 50 mg diclofenac. C_{max} peak plasma concentration, $t_{1/2}$ half-life, *AUC* area under plasma concentration–time curve

CYP2C9 genotype No. of subjects	**1/*1* 6	**1/*2* 3	**1/*3* 5	**2/*2* 1	**3/*3* 1	**2/*3* 4
Diclofenac						
C_{max} (μM)	3.4 ± 1.9 (1.5–6.2)	4.0 ± 1.8 (2.2–5.7)	3.8 ± 1.9 (1.4–6.6)	2.3	1.5	4.9 ± 1.1 (3.8–6.0)
$t_{1/2}$ (h)	0.8 ± 0.4 (0.4–1.4)	1.3 ± 0.5 (0.9–1.8)	1.5 ± 0.6 (0.7–2.4)	0.4	1.0	1.8 ± 1.7 (0.7–4.4)
AUC_{total} (μmol/h/l)	3.4 ± 1.2 (1.3–4.6)	5.5 ± 2.1 (3.5–7.7)	5.0 ± 2.7 (2.2–9.6)	3.0	2.5	6.5 ± 3.2 (3.4–10.2)
4′OH diclofenac						
C_{max} (μM)	1.1 ± 0.4 (0.4–1.5)	1.6 ± 0.5 (1.3–2.1)	1.0 ± 1.1 (0.2–3.0)	1.2	0.6	2.3 ± 2.2 (0.5–5.3)
$t_{1/2}$ (h)	11.4 ± 6.6 (3.2–17.8)	8.2 ± 6.3 (3.7–15.4)	10.7 ± 8.5 (4.5–17.8)	5.6	4.8	7.4 ± 4.5 (3.8–12.4)
AUC_{total} (μmol/h/l)	3.4 ± 2.1 (0.9–6.4)	8.1 ± 7.0 (3.2–16.1)	4.1 ± 3.6 (0.8–10.0)	2.5	2.9	7.8 ± 7.0 (0.7–14.8)
3′OH 4 methoxy diclofenac						
$C_{24\ h}$ (μM)	0.3 ± 0.1 (0.2–0.4)	0.4 ± 0.1 (0.3–0.5)	0.2 ± 0.1 (0.1–0.3)	0.2	0.2	0.3 ± 0.1 (0.2–0.4)
$AUC_{0–24\ h}$ (μmol/h/l)	4.8 ± 1.7 (3.6–7.1)	7.8 ± 2.1 (5.6–9.7)	4.2 ± 1.5 (1.9–5.9)	3.5	2.8	5.3 ± 3.1 (1.4–7.9)
$AUC_D/AUC_{4'OH\ D}$	1.3 ± 0.8	0.9 ± 0.4	1.6 ± 0.7	1.2	0.9	1.9 ± 2.0
$AUC_D/AUC_{3'OH4'M\ D}$	0.8 ± 0.4	0.7 ± 0.1	1.2 ± 0.3	0.9	0.9	2.4 ± 3.1
$AUC_D/AUC_{4'OH\ D\ +\ 3'OH4'M\ D}$	0.5 ± 0.2	0.4 ± 0.1	0.6 ± 0.1	0.5	0.4	0.5 ± 0.1

Table 2 The 24-h urinary recoveries of diclofenac and 4′-OH diclofenac (4′-OH D) and the diclofenac/4′-OH D recovery ratio in the different *CYP2C9* genotype groups (mean ± SD and range in parentheses)

CYP2C9 genotype	*n*	Recovery % of dose			Diclofenac/4′-OH D in urine	
		Diclofenac	4′-OH D	Total	0–8 h	0–24 h
**1/*1*	6	4.7 ± 4.9 (2.1–14.5)	5.7 ± 5.1 (2.8–15.9)	10.4 ± 9.9 (4.4–30.3)	1.1 ± 0.1 (0.9–1.3)	0.9 ± 0.1 (0.7–1.1)
**1/*2*	3	2.9 ± 1.1 (1.7–3.8)	5.3 ± 2.3 (3.8–8.0)	8.2 ± 3.3 (5.5–11.8)	1.0 ± 0.1 (0.9–1.0)	0.6 ± 0.2 (0.5–0.9)
**1/*3*	5	3.2 ± 1.4 (1.6–4.8)	2.9 ± 0.8 (2.1–4.1)	6.1 ± 2.2 (3.7–9.0)	1.4 ± 0.2 (1.2–1.7)	1.2 ± 0.2 (0.9–1.5)
**2/*2*	1	4.0	4.9	8.9	1.0	1.0
**3/*3*	1	3.3	2.2	5.5	1.8	1.7
**2/*3*	4	4.1 ± 2.0 (1.8–6.7)	4.6 ± 1.8 (3.4–7.2)	8.6 ± 3.7 (5.2–13.9)	1.4 ± 0.3 (1.2–1.8)	1.0 ± 0.3 (0.6–1.4)

level in all microsomes with **2* and **3* alleles (Table 3). The CYP2C9 apoprotein levels were similar for all microsomes tested. The kinetic parameters of 4′-OH-diclofenac formation are summarised in Table 3. Even though the V_{max} of microsomes with the *CYP2C9*3/*3* genotype was among the lowest, the intrinsic clearance (V_{max}/K_m) was in the same range as in the other microsomes studied. No significant correlation was found between the CYP2C9 apoprotein levels and the intrinsic clearance for diclofenac 4′-hydroxylation ($r_s = 0.47$, $P = 0.07$). Differences in K_m or V_{max} were apparently not related to genotype.

Discussion

The purpose of the present study was to clarify the role of the *CYP2C9* genotype in the metabolism and pharmacokinetics of diclofenac in vivo in healthy volunteers and in vitro in human liver microsomes. Our aim was to include subjects with all the possible genotypes of *CYP2C9*. However, we were able to recruit only single subjects and single human liver samples with the *CYP2C9*3/*3* and **2/*2* genotypes because of their low frequency, about 1%. Obviously, this is a drawback in our study, as no statistical calculations could be performed with respect to these genotypes. Still, together with the data from heterozygous individuals, our findings from both in vivo and in vitro experiments indicate that the impact of the *CYP2C9*2* and *CYP2C9*3* alleles on diclofenac 4′-hydroxylation seems to be small. Previous studies have been performed mostly in subjects or human liver microsomes heterozygous for *CYP2C9*2* or *CYP2C9*3*. No impact of *CYP2C9*3* on single-dose kinetics of diclofenac was found in Japanese healthy volunteers when comparing the *CYP2C9*1/*3* genotype

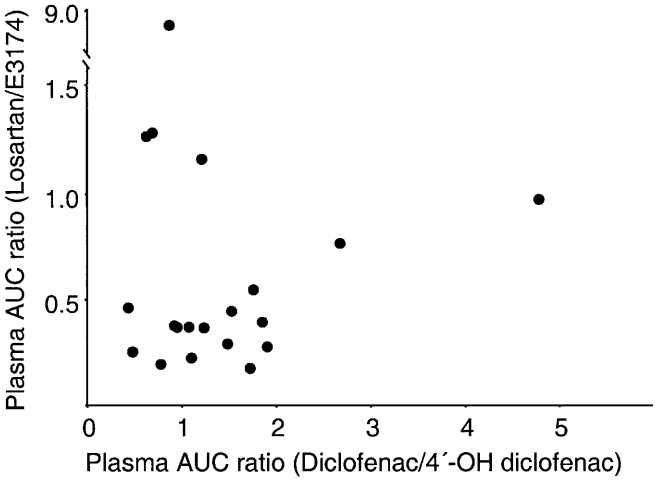

Fig. 2 The relationship between the plasma ratios $AUC_{losartan}/AUC_{E\text{-}3174}$ and $AUC_D/AUC_{4'\text{-}OH\ D}$ among 20 subjects with different genotypes of *CYP2C9* ($r_s = 0.1$, $P = 0.8$)

to **1/*1* [31]. In another study, apparent metabolic clearance of diclofenac to 4′-OH-diclofenac tended to be lower in subjects heterozygous for *CYP2C9*3* than those homozygous for *CYP2C9*1* [37]. However, the difference was not statistically significant, possibly due to the low number of subjects studied and a large inter-individual variation within the *CYP2C9*1/*1* group [37]. The authors related this negative finding to the galenic form of diclofenac (enteric-coated) used, giving a highly variable intestinal absorption rate of diclofenac [37], and suggested that another galenic form of diclofenac might be a potential probe to quantify CYP2C9 activity. Furthermore, the subjects did not fast before drug intake, which was suggested as another possible confounding factor for the large inter-individual variation [37]. Our results, however, confirm the lack of significant genotype differences in diclofenac 4′-hydroxylation despite the use of a non-coated tablet and despite the fact that all subjects had fasted before drug intake.

The C_{max}, $t_{1/2}$ and AUC values of diclofenac, 4′-OH-diclofenac and 3′-OH-4′-methoxy-diclofenac, as well as the urinary recoveries of diclofenac and 4′-OH-diclofenac after a single oral dose of 50 mg diclofenac were all in the same range as previously reported ([1] and references therein). Our primary aim was to analyse the conversion of diclofenac to 4′-OH-diclofenac as a measure of CYP2C9 activity. According to Bort et al., the formation of the 3′-OH-4′-methoxy metabolite is also dependent on CYP2C9 [3]. We therefore also analysed this metabolite in plasma. However, the 24-h blood sampling was not sufficient to allow calculation of the proper pharmacokinetic parameters of this metabolite because of its long $t_{1/2}$. In the literature, the $t_{1/2}$ of 3′-OH-4′-methoxy-diclofenac was reported to be around 12 h, which is much longer than that of diclofenac or 4′-OH-diclofenac [1]. There were no differences between the genotype groups with respect to the $AUC_{0-24\ h}$ of 3′-OH-4′-methoxy-diclofenac or the $AUC_{diclofenac}/AUC_{3'\text{-}OH\text{-}4'\text{-}methoxy\text{-}diclofenac}$ ratio.

No differences were detected in the urinary ratios between the different genotypes, which is in line with the plasma data. Another observation was the lack of correlation ($r_s = 0.1$, $P = 0.5$) between the plasma and urinary diclofenac/4′-OH-diclofenac ratios. This is in contrast to the losartan/E-3174 ratio, which correlated highly with the corresponding plasma ratios in the same subjects [32].

Recently, we have shown an approximately 20- and 30- fold decreased activity in losartan oxidation for the *CYP2C9*3/*3* genotype relative to that of **1/*1* in vitro

Table 3 Total P_{450} content (pmol/mg protein), CYP2C9 apoprotein levels (arbitrary units), K_m (μM), V_{max} (pmol/mg protein/min) and V_{max}/K_m ratio of 4′OH diclofenac formation from diclofenac in human liver microsomes with different *CYP2C9* genotypes

CYP2C9 Genotype	Liver	Total P_{450}	CYP2C9 apoprotein	K_m	V_{max}	V_{max}/K_m
**1/*1*	HL 17	613	13.2	27.6	1584	57.4
	HL 19	857	4.8	19.8	1555	78.5
	HL 57	359	4.1	9.2	217	23.6
	HL 58	349	2.5	24.1	967	40.1
	HL 60	729	4.2	37.6	715	19.0
	HL 63	376	2.1	44.1	781	17.7
	HL 67	530	6.5	66.9	4781	71.5
	Median	530	4.2	27.6	967	40.1
**1/*2*	HL 39	610	5.2	43.7	2213	50.6
	HL 49	417	7.7	46.7	1602	34.3
**1/*3*	HL 37	465	3.2	31.6	1233	39.0
	HL 55	321	3.5	40.9	1789	43.7
**2/*3*	HL 18	544	6.3	26.8	902	33.7
	HL 65	644	5.3	44.9	1869	41.6
**2/*2*	HL 20	965	4.4	31.0	1130	36.5
	HL 40	426	4.0	9.9	252	25.4
**3/*3*	HL 7	516	3.1	6.0	156	26.0

[36] and in vivo [32], respectively. Significant differences were also found between subjects heterozygous for **3* and those homozygous for **1* [32, 36]. The contribution of CYP3A4 was minimal at clinically relevant losartan concentrations [36]. In the present study, the same subjects were recruited and the same human liver microsomes were used. Interestingly, 4′-OH-diclofenac formation did not differ between the *CYP2C9* genotypes and the losartan and diclofenac parent drug/metabolite plasma ratios did not correlate with each other. There was no correlation between 4′-hydroxylation of diclofenac and the CYP2C9 apoprotein levels which also varied about sixfold within the *CYP2C9*1/*1* genotype group. A similar lack of correlation between CYP2C9 apoprotein levels and the metabolism of different CYP2C9 substrates has been reported previously [36, 38, 39]. Furthermore, the variations in diclofenac pharmacokinetics within each genotype group were large, up to 20-fold. These findings suggest that diclofenac 4′-hydroxylation is to a large degree affected by other factors than *CYP2C9* genotype. The presence of alternate metabolic pathways is one possible explanation. Although 4′-hydroxylation is the main metabolic pathway of diclofenac, there are several other metabolites formed (i.e. 3′-OH, 3′-OH-4′-methoxy and 5-OH metabolites) [3, 4, 5]. Additionally, there are conjugated metabolites formed from these hydroxy metabolites as well as acyl glucuronides independent of these hydroxy metabolites [4, 6, 40]. Thus, the 4′-OH-diclofenac formed is further metabolised in vivo. These factors may explain the large variation in the parameters used as measures of 4′-hydroxylation of diclofenac even in genetically homogenous *CYP2C9* groups. The lack of differences in 4′-hydroxylation of diclofenac in human liver microsomes with different *CYP2C9* genotypes suggests a major role of phase-I enzymes rather than phase-II enzymes as an explanation to this variability (as the microsomal incubations did not include co-factors required for phase-II reactions).

We found an up to 20-fold difference in V_{max} of diclofenac 4′-hydroxylation between different human liver microsomes encoded by *CYP2C9*1/*1*. There was also a great variation in K_m and intrinsic clearance (V_{max}/K_m) of 4′-OH-diclofenac formation within the *CYP2C9*1/*1* group. In a recent in vitro study, the role of *CYP2C9*1/*2* and *CYP2C9*1/*3* genotypes in diclofenac 4′-hydroxylation was studied in human liver microsomes [41]. An approximate fivefold lower V_{max} for the 4′-hydroxylation of diclofenac was reported in microsomes encoded by *CYP2C9*1/*3* ($n=4$) than the median in the **1/*1* group ($n=4$) [41]. In our study, the V_{max} in a single *CYP2C9*3/*3* liver was among the lowest, although still within the range observed in the **1/*1* group. The K_m for diclofenac 4′-hydroxylation was two- to fivefold lower in the published studies than the present study, which might be explained by differences in the experimental conditions used [2, 41].

Our aim was to evaluate the contribution of the *CYP2C9* genotype in diclofenac metabolism. Diclofenac has been suggested as a possible phenotyping probe in vivo for CYP2C9 activity because single doses of diclofenac are widely used and relatively well tolerated. It has also been used as an in vitro probe for CYP2C9 activity [26, 27, 28, 29, 30]. However, in the present study, the parameters used as measures of CYP2C9 activity did not differ between the genotypes, either in vitro or in vivo. Taking the large inter-individual variability in the pharmacokinetic parameters within each genotype group into consideration, it can be calculated that approximately 100 subjects are required to show a difference between heterozygous mutated and CYP2C9*1/*1 subjects with a power of 80%. Thus, further population studies in a larger number of individuals are required before it can be established whether diclofenac is a suitable probe for CYP2C9 activity.

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