Title:

Formation of the Accumulative Human Metabolite and Human-specific Glutathione Conjugate of Diclofenac in TK-NOG Chimeric Mice with Humanized Livers

Hidetaka Kamimura, Satoshi Ito, Kohei Nozawa, Shota Nakamura, Hiroyuki Chijiwa, Shin-ichiro Nagatsuka, Miyuki Kuronuma, Yasuyuki Ohnishi, Hiroshi Suemizu, and Shin-ichi Ninomiya

ADME & Tox. Research Institute, Sekisui Medical Co., Ltd., Tokyo, Japan (H.K., S.I., K.N., S.Nak., H.C., S.Nag., S.Ni); Central Institute for Experimental Animals, Kawasaki, Kanagawa, Japan (M.K., Y.O., H.S.)

Running Title: Metabolism of diclofenac in humanized TK-NOG chimeric mice

Address Correspondence to: Dr. Hidetaka Kamimura, ADME & Tox. Research Institute, Sekisui

Medical Co. Ltd., 13-5, Nihonbashi 3-chome, Chuo-ku, Tokyo 103-0027 Japan.

E-mail: hidetaka.kamimura@sekisui.com,   Tel +81-3-3271-5634,   Fax +81-3-3272-0700

Text: 38 pages

Tables: 0

Figures: 8

References: 38

Abstract: 250 words (no more than 250 words)

Introduction: 750 words (no more than 750 words)

Discussion: 1,470 words (no more than 1,500 words)

ABBREVIATIONS: CID, collision-induced dissociation; k_{el}, elimination rate constant; k_{form}, formation rate constant; LC-MS/MS, liquid chromatography-tandem mass spectrometry; TK-NOG, NOD/Shi-scid IL2 receptor gamma null strain expressing a herpes simplex virus type-1 thymidine kinase transgene; T_{max}, time to the maximum plasma concentration.

DMD # 61689

ABSTRACT:

3′-Hydroxy-4′-methoxydiclofenac (VI) is a human specific metabolite and known to

accumulate in the plasma of patients after repeated administration of diclofenac sodium. Diclofenac

also produces glutathione-conjugated metabolites; some of which are human specific. In the present

study we investigated whether these metabolites could be generated in humanized chimeric mice

produced from TK-NOG mice. After single oral administration of diclofenac to humanized mice, the

unchanged drug in plasma peaked at 0.25 hour and then declined with a $t_{1/2}$ of 2.4

hours. 4′-Hydroxydiclofenac (II) and 3′-hydroxydiclofenac also peaked at 0.25 hour

and were undetectable within 24 hours. However, VI peaked at 8 hours and declined with a

$t_{1/2}$ of 13 hours. When diclofenac was given once per day, peak and trough levels of

VI reached plateau within 3 days. Studies with administration of II suggested VI was generated via II

as an intermediate. Among six reported glutathione-conjugated metabolites of diclofenac, M1 to M6,

we found three dichlorinated conjugates (M1, M2 and M3), and a single monochlorinated conjugate

(M4 or M5), in the bile of humanized chimeric mice. M4 and M5 are positional isomers and have

been previously reported as human specific in vitro metabolites likely generated via arene oxide and

quinone-imine type intermediates, respectively. The biliary monochlorinated metabolite exhibited

the same mass spectrum as those of M4 and M5, and we discuss whether this conjugate

corresponded to M4 or M5. Overall, humanized TK-NOG chimeric mice were considered to be a

3

functional tool for the study of drug metabolism of diclofenac in humans.

Introduction

Diclofenac sodium was the first approved nonsteroidal anti-inflammatory drug, and has been

widely used to treat pain or inflammation caused by arthritis or ankylosing spondylitis (Small, 1989).

Metabolism studies in animals and humans revealed that this drug undergoes almost complete

biotransformation by direct conjugation or oxidation of the aromatic rings followed by conjugation

(Stierlin et al., 1979; Stierlin and Faigle, 1979). In humans, 4&[prime]-hydroxydiclofenac (II),

5-hydroxydiclofenac (III), 3&[prime]-hydroxydiclofenac (IV) and 4&[prime], 5-

dihydroxydiclofenac (V) are excreted into urine as their free (minor) or conjugated forms. While

performing analyses on the concentrations of these metabolites in plasma samples from study

subjects, a new metabolite, 3&[prime]-hydroxy-4&[prime]-methoxydiclofenac (VI), was found. This

metabolite showed distinct pharmacokinetic behavior with a T_{max} and

$t_{1/2}$ of 12 and 80 hours respectively, after single oral administration with potential

accumulation after repeated dosing of diclofenac (Faigle et al., 1988; Degen et al., 1988). According

to the former report, unpublished data showed that VI was not found in mouse, rat or marmoset

plasma, but baboon plasma only. The cumulative urinary excretion (0-96 hours) of free and

conjugated VI in humans was very small (ca. 1% of dose), which might explain why VI had not been

found in the urine sample in the previous studies.

5

Formation of a reactive 1-O-acyl glucuronide by direct glucuronidation of diclofenac is

considered to be responsible for the major modification of proteins (Kretz-Rommel and Boelsterli,

1993). Evidence of this includes the detection of diclofenac-S-acyl-glutathione in rat bile treated

with high doses of diclofenac (Grillo et al., 2003). The formation of reactive metabolites of

diclofenac by various P450s, followed by their glutathione-dependent inactivation, has been

previously studied (Boelsterli, 2003; Tang, 2003). Tang et al. (1999) identified three glutathione

conjugates, namely, 5-hydroxy-4-(glutathion-S-yl)diclofenac (M1),

4&[prime]-hydroxy-3&[prime]-(glutathion-S-yl)diclofenac (M2) and

5-hydroxy-6-(glutathion-S-yl)diclofenac (M3), in rat bile and with in vitro incubations in rat liver

microsomes or human hepatocytes. This suggested the formation of quinone imine intermediates

during diclofenac metabolism. On the other hand, a novel glutathione conjugate,

2&[prime]-hydroxy-3&[prime]-(glutathion-S-yl)monoclofenac (M4) was reported to be generated

by incubation of diclofenac with human liver microsomes suggesting the existence of an arene oxide

intermediate as another route of bioactivation (Yan et al., 2005). Experiments using recombinant

enzymes and specific inhibitors demonstrated that CYP2C9 was involved in the formation of M4

and this metabolite was not detected in incubations with either rat or monkey liver microsomes.

Around the same time, Yu et al. (2005) reported another monochlorinated glutathione conjugate with

the same molecular weight, 4&[prime]-hydroxy-2&[prime]-(glutathion-S-yl)monoclofenac (M5),

6

followed by the discovery of the latest glutathione conjugate,

2&[prime]-(glutathion-S-yl)monoclofenac (M6) (Wen et al., 2008; Boerma et al., 2012). Although,

M4, M5 and M6 are generated in in vitro studies using recombinant enzymes or liver microsomes

fortified with glutathione, in vivo formation of these metabolites have not been previously reported.

Chimeric mice with humanized livers hold considerable advantages for more accurate prediction

of human metabolism over conventional in vivo animal or in vitro human systems (Yoshizato et al.,

2012; Grompe and Strom, 2013; Peltz, 2013). The ability to predict circulating human metabolites is

one of the characteristic features of this model which could satisfy regulatory requirements for the

safety testing of drug metabolites (Kamimura et al., 2010). Histological examination showing the

connection of human bile canaliculi to a mouse interlobular bile duct suggested easy access to

human bile samples unlike in clinical experiments (Kamimura et al., 2010). Following the

development of immunodeficient humanized mice with a urokinase-type plasminogen activator

(uPA) transgene (Mercer et al., 2001) or a disrupted gene encoding murine fumaryl acetoacetate

hydrolase (FAH) (Azuma et al., 2007), herpes simplex virus type-1 thymidine kinase (TK)-NOG

mice with humanized liver were generated (Hasegawa et al., 2011). Liver-specific expression of TK

by the albumin enhancer/promoter results in damage to murine hepatocytes following

administrations of a non-toxic dose of ganciclovir (GCV). This new humanized mouse model has

demonstrated formation of the human metabolites of debrisoqine (Hasegawa et al., 2011),

thalidomide including a glutathione conjugate of its 5-hydroxy form in plasma (Yamazaki et al.,

2012) and clemizole (Nishimura et al., 2013). These results, the application of this model in

pharmacokinetic modeling (Tsukada et al., 2013; Yamashita et al., 2014; Suemizu et al., 2014) and

the evaluation of drug-drug interactions (Nishimura et al., 2013; Yamazaki et al., 2013) prompted us

to conduct diclofenac metabolism studies using humanized TK-NOG mice. In this report, the

formation of the human-specific metabolites IV, VI and glutathione conjugate metabolites are

presented. Additionally, administration of a synthetic version of metabolite, II, was conducted to

elucidate the pathways of diclofenac metabolism in humans.

Materials and Methods

Materials. Diclofenac sodium (2-(2-(2,6-dichlorophenylamino)phenyl)acetic acid sodium salt)

was purchased from Sigma-Aldrich (St. Louis, MO). 4&[prime]-Hydroxydiclofenac (synthetic

version of metabolite II) was purchased from Becton, Dickinson and Company (Franklin Lakes, NJ).

4&[prime]-Hydroxydiclofenac-$^{13}C_6$, used as an internal standard (IS) was obtained from Toronto

Research Chemicals (Ontario, Canada). Other chemicals and solvents were of analytical grade.

Hepatocytes from a four year-old female donor (Lot 7F3063; LONZA Japan, Tokyo) or those from a

nine-year old female donor (Lot HC2-19; XenoTech, KS) were used for the transplantation.

Metabolic activities of CYP2C9 in the respective hepatocytes were 1.28 (4&[prime]-hydroxylation

of tolbutamide at 400 μM) or 467 (4&[prime]-hydroxylation of diclofenac at 100 μM) pmol/min/10^6

viable cells.

Reference incubation mixture containing 2&[prime]-(glutathion-S-yl)monoclofenac (M6) was

prepared at VU University Amsterdam as described by Boerma *et al.* (2014). Briefly, a final

concentration of 500 μM diclofenac was incubated with 100 nM horseradish peroxidase, 100 μM

glutathione and 8 μM human glutathione-S-transferase P1-1 for 1 hour at 37 °C. The reaction was

terminated by addition of methanol.

Animals, Dosing and Sample Collection. The humanized TK-NOG chimeric mice were

9

generated using the method of Hasegawa et al. (2011) with some modifications. For the plasma

concentration studies, 11 or 12 week old male TK-NOG mice were given intraperitoneal injections

of 6 mg/kg GCV, and then 6 and 50 mg/kg of GCV were given two and ten days later, respectively.

Eighteen days after the first GCV dose, mice with elevated serum ALT levels of more than 200

IU/liter received transplants of 1.1 or 1.5 x 10^6 of human hepatocytes by intrasplenic injection. For

the biliary excretion studies, 8 or 10 week old male TK-NOG mice were given intraperitoneal

injections of 10 mg/kg GCV and an additional 50 mg/kg GCV was given to a subset of these eight

days later. Eight to seventeen days after the first dose, mice with serum ALT levels of more than 240

IU/liter received transplants of 0.6 or 1.0 x 10^6 of human hepatocytes by intrasplenic injection.

Mouse serum levels of human albumin were measured and used as a marker for the hepatic

replacement ratio by human hepatocytes (Hasegawa et al., 2011). The hepatic replacement ratios

were calculated with respect to an in-house calibration curve, and those mice with a ratio greater

than 77% were used. TK-NOG mice whose serum ALT levels had not been elevated sufficiently by

the GCV treatments were used as control TK-NOG mice. Administration of drugs to and sample

collections from the mice were performed in compliance with protocols approved by the Sekisui

Medical Animal Care and Use Committee.

Control TK-NOG and humanized chimeric mice received a single oral administration of

diclofenac sodium (10 mg/kg as free form) or synthetic metabolite II (10 mg/kg), and blood samples

were collected for analyses at 15 and 30 minutes and 1, 4, 8, 24 and 48 hours. Three weeks later, a

group of the same humanized chimeric mice were administered the same dose of diclofenac sodium

orally once per day for fourteen days, and blood samples were taken at 8 and 24 hours after each

dose. Food and water were given ad libitum during the drug treatment. For the biliary excretion

study, a control NOG mouse and a humanized chimeric mouse, to which a catheter was implanted

into the whole part of the gall bladder, received diclofenac sodium (10 mg/kg as free form) or

synthetic metabolite II (10 mg/kg), and bile samples were collected for 0-24 hours on dry ice.

Analyses of Plasma Samples. Diclofenac, II, IV and VI were quantified by liquid

chromatography-tandem mass spectrometry (LC-MS/MS). To a 15 or 30 μl aliquot of control plasma,

the same volume of a standard working solution of diclofenac sodium or synthetic metabolite II was

added to prepare the standard sample. To a 15 or 30 μl aliquot of the sample plasma, the same

volume of 50% (v/v) methanol in water was added to prepare the analytical sample. To the analytical

and standard samples, 100 μl of IS solution (25 nmol/liter) was added. The tube was closed and

mixed vigorously for ca. 10 seconds using vortex-mixing and subsequently centrifuged (15,000×g,

4 °C, 10 minutes). The supernatant was pipetted into an autosampler vial and 50 μl of the sample

was injected into the column. The LC-MS/MS analysis was performed using the LC20AD HPLC

system (Simadzu, Kyoto, Japan) coupled with an API-4000 tandem mass spectrometer (AB Sciex,

Foster City, CA). The column (CAPCELL PAK MG C18 (250 X 4.6 mm i.d., 5 μm, Shiseido Japan))

was maintained at 40 °C and diclofenac and its metabolites were eluted with a linear gradient (flow

rate of 1.0 ml/min) that escalated from 15% B to 35% B by 15 minutes, maintained at 35% B for 15

minutes, then escalated again to 90% B over 5 minutes, and maintained at 90% B for 5 minutes [10

mmol/liter ammonium formate in distilled water (pH 5.0) (A) or acetonitrile (B)]. Analytes were

detected in the positive ion mode with electrospray ionization (collision energy of 50, 30, 30, and 45

eV for diclofenac, II, IV, and VI, respectively) using selected reaction monitoring: diclofenac,

$[M+H]^+$ m/z 296 → 214; II, $[M+H]^+$ m/z 312 → 231; IV, $[M+H]^+$ m/z 312 → 231; VI, $[M+H]^+$ m/z

342 → 260. The retention times of diclofenac, II, IV, VI, and 4&[prime]-hydroxydiclofenac-$^{13}C_6$

were 36, 25, 23, 25 and 25 minutes, respectively. The limits of quantification were 1 and 3 ng/ml in

plasma, and determination coefficients (R) were >0.993 and >0.983, for diclofenac and II,

respectively. Plasma concentrations of IV and VI were expressed as peak response since their

standard reference compounds were not available.

Analyses of Bile Samples. Diclofenac glutathione conjugates were analyzed by LC-MS^n using

the LC20AD HPLC system coupled with an LTQ Orbitrap XL mass spectrometer (Thermo Fisher

Scientific; Waltham MA). A 10 to 25 μl aliquot of bile sample was injected into the column

(CAPCELL Core ADME (150 X 2.1 mm i.d., 2.7 μm, Shiseido Japan)) without any pretreatment.

The column was maintained at 40 °C and chromatography was performed with a linear gradient

from 10% B to 30% B by 30 minutes at a flow rate of 0.2 ml/min [100 mmol/liter ammonium

formate in distilled water (pH 5.0) (A) or acetonitrile (B)]. After the analysis, the column was

washed by increasing the composition from 30% B to 100% B, and it was maintained at 100% B for

10 minutes and returned to 10% B. The electrospray ionization ion source was operated in positive

ion mode, and instrumental parameters were set as: spray voltage 4.5 kV, capillary temperature

330 °C, capillary voltage 3.0 V. Accurate mass chromatograms were obtained with resolution of

30,000 by monitoring: M1, M2 and M3, $[M+H]^+$ m/z 617.08394-617.09012; M4 or M5, $[M+H]^+$ m/z

583.12308-583.12892; M6 , $[M+H]^+$ m/z 567.12825-567.13393. The calculated high resolution mass

values as protonated ions for $C_{24}H_{27}Cl_2N_4O_9S^+$ (M1, M2 and M3), $C_{24}H_{28}ClN_4O_9S^+$ (M4 or M5) and

$C_{24}H_{28}ClN_4O_8S^+$ (M6) were m/z 617.08703, m/z 583.12600 and m/z 567.13109, respectively.

PK data analysis for VI. Metabolite VI was generated from diclofenac via more than two

sequential metabolic reactions and considered to exhibit complicated pharmacokinetics. However,

for a simplified simulation in the multiple-dose study, a one compartment open model was arbitrarily

applied to plasma concentration of VI after a single oral administration of diclofenac. Assuming that

both formation and elimination rates of VI follow first-order pharmacokinetics, pharmacokinetic

parameters, A, K_{el} and k_{form} defined by the following equation

were obtained.

$C(t) = A \times \{ \exp(-k_{el} \times t) - \exp(-k_{form} \times t)\}$

Where C(t) was plasma concentration of VI at time t.

13

DMD # 61689

Results

Unchanged drug and its metabolites after a single oral administration of diclofenac. After

oral administration of diclofenac sodium to control TK-NOG mice, plasma concentration of the

unchanged drug peaked at 0.25 hour and declined with a $t_{1/2}$ of 3.5 hours (Fig. 1).

Metabolite II also reached peak concentration at 0.25 hour. However, plasma level at this time point

was approximately 1/300 of the unchanged form. Although quantitative analyses could not be done

for IV and VI since authentic standards were not available (expressed as peak response), we could

not detect IV and only a trace amount of VI was found in the control mouse plasma.

The same dose of diclofenac sodium was given to TK-NOG chimeric mice with humanized

livers. Plasma levels of the unchanged drug were two to four times lower than those in the control

mice with a relatively shorter $t_{1/2}$ of 2.4 hours (Fig. 1). Oral clearance of diclofenac

(CL/F) in chimeric mice was 900 ml/hr/kg while in control TK-NOG mice it was 180 ml/hr/kg,

suggesting higher hepatic clearance in the humanized animal model for this drug. On the other hand,

plasma concentrations of II were three to four times higher than those in the control mice and

declined with a $t_{1/2}$ of 5.6 hours. Humanized chimeric mice also had IV in their

plasma which declined with a $t_{1/2}$ of 4.8 hours. With gradual decrease of II and IV,

metabolite VI peaked at 8 hours and declined with an apparent $t_{1/2}$ of 13.0 hours;

14

which allowed for detection of this metabolite even at 48 hours after dosing. Pharmacokinetic

parameters obtained by the curve fitting (A= 163 (peak response), = 0.19 hr^{-1}, k_{el}=

0.064 hr^{-1}) were used for the multiple-dose simulation described below.

Plasma profiles of II and VI after oral administration of II. To discern the metabolic

pathways of formation for the human-specific metabolite VI, 10 mg/kg of synthetic metabolite II

was orally administered to control TK-NOG and humanized chimeric mice. The plasma

concentration of unchanged II peaked at 0.25 hour and declined rapidly in both the animals (Fig. 2).

The metabolic product, VI, in the humanized chimeric mouse exhibited a similar plasma profile to

that obtained after administration of diclofenac. Unexpectedly, we could also detect the plasma

profile of VI in the control TK-NOG mouse. However, the levels of VI were relatively low, and the

AUC value was 7% of that in humanized chimeric mice.

Plasma levels of VI after repeated administrations of diclofenac. Diclofenac sodium was

given orally to three TK-NOG chimeric mice with humanized livers once per day, and peak and

trough levels were plotted with a simulated concentration-time curve assuming that the same

pharmacokinetic conditions and compartment model for the single dose experiment could be applied

(Fig. 3). Plasma levels of VI at 8 and 24 hours gradually increased and reached plateau within three

days. On the 7th day, one animal died and the average values of the remaining two animals tended to

decrease after the 9th day. However, data did not greatly diverge from the simulation curve.

15

Although the reason for the death was not clear, hepatic toxicity (Bort et al., 1999; Thanagari et al., 2012) or induction of intestinal injury (Zhu and Zhang 2012) by diclofenac (LD_{50} of 95 mg/kg in mice; Thanagari et al., 2012) could be responsible in part for this phenomenon.

Biliary glutathione conjugates with MH[+] ion of *m/z* 617 (M1, M2 and M3). Three glutathione conjugated metabolites of diclofenac have reportedly been detected in the bile of rats by LC/MS/MS with constant neutral loss scanning that loses 129 Da (pyroglutamate) on collision-induced dissociation (CID) from the MH[+] ion of *m/z* 617 (Tang et al. 1999). In the present study, we monitored with integrated accurate mass detection to detect these glutathione conjugates specifically (*m/z* 617.08394-617.09012) and found no interfering peaks were observed in blank bile samples (data not shown). In the bile of control TK-NOG mice treated with diclofenac sodium, three peaks were observed with retention times of 20.7, 21.5 and 24.9 minutes under the HPLC conditions described in Materials and Methods. Mass spectra of the three HPLC peaks with a CID of *m/z* 617 gave the same product ions at *m/z* 542 ([MH-75][+], loss of glycine), *m/z* 488 ([MH-129][+], loss of pyroglutamate) and *m/z* 342 ([MH-275][+], loss of the thioether moiety of glutathione), which agreed with those reported by Tang et al. (1999). Based on the similarity of the HPLC conditions and the pattern of chromatograms obtained to those of Tang et al. (1999), we considered that the respective peaks corresponded to M1, M2 and M3 (Fig. 4, A). After oral administration of diclofenac to humanized chimeric mice, a similar chromatogram with three peaks was obtained (Fig. 5, A).

16

Following oral administration of synthetic metabolite II to control TK-NOG and humanized

chimeric mice, a single peak at a retention time of 22 minutes was observed in the bile samples from

both the animals (Fig. 6, *m/z* 617). When the sample from the humanized chimeric mouse (Fig. 6, B)

was mixed with the bile obtained from the humanized mouse treated with diclofenac (Fig. 5, A) and

then injected into the HPLC column, the single peak of the former sample at *m/z* 617 co-eluted with

the second peak of the latter sample. Therefore, this single peak was considered as M2, as the

5-hydroxy analogues, M1 and M3, could simply not be generated from II.

Biliary glutathione conjugate with MH$^+$ ion of *m/z* 583 (M4 or M5). Two in vitro metabolites

with MH$^+$ ions of *m/z* 583 have been separately reported as human-specific glutathione conjugate

metabolites of diclofenac. M4 was previously generated by incubation of diclofenac with human

liver microsomes or recombinant CYP2C9 fortified with glutathione (Yan et al., 2005). The chemical

structure of M4 was proposed as a 2&[prime]-hydroxy-3&[prime]-glutathionyl analogue based on

the assumption that this monochlorinated metabolite was generated via diclofenac-2&[prime],

3&[prime]-oxide, because M4 had not been detected in the incubation mixture of II and

NADPH-fortified human liver microsomes. On the other hand, 4&[prime]-hydroxy-

2&[prime]-(glutathion-S-yl)monoclofenac was proposed by Yu et al. (2005), and designated as M5

later (Boerma et al., 2014). Similar to M4, M5 was generated in an incubation mixture of diclofenac

and human liver microsomes fortified with glutathione, and its chemical structural assignment was

17

obtained via comparison of mass and ^{1}H-NMR spectra with those of a chemically synthesized

authentic standard. In the present study, no monitored peaks were found in the bile of the control

TK-NOG mouse dosed with diclofenac at m/z 583 (m/z 583.12308-583.12892) (Fig. 4, B). However,

bile from the humanized chimeric mouse gave a single peak presumably produced by the

transplanted human hepatocytes (Fig. 5, B). When the ion at m/z 583 was subjected to CID (Fig. 7,

A), the product ions at m/z 508 ([MH-75]$^+$), m/z 454 ([MH-129]$^+$) and m/z 436 ([MH-147]$^+$) were

obtained as separately reported by Yan et al. (2005) for M4, and Yu et al. (2005) for M5. To define

that this biliary metabolite is M4 or M5, further studies including isolation of the metabolite from the

bile sample and subsequent NMR analyses are inevitable.

Oral administration of synthetic metabolite II to a humanized chimeric mouse also gave a single

peak at m/z 583 (Fig. 6, B) and CID produced the same product ions as those after diclofenac

treatment (Fig. 7, B). This conjugate was considered to be M5, based on the chemical structure of II,

and co-eluted with the corresponding peak of the bile sample from chimeric mice dosed with

diclofenac (Fig. 5, B). However, M4 and M5 are positional isomers and possible coincidence in the

HPLC retention times cannot be ruled out. Although M5 (and M4) had been considered human

specific, the same peak was unexpectedly found in the bile sample from a control TK-NOG mouse

dosed with II (Fig. 6, A). However, it was much smaller than that in the humanized chimeric mouse,

and was not observed in the control mouse when dosed with diclofenac (Fig.4, B).

18

Biliary glutathione conjugate with MH$^+$ ion of *m/z* 567 (M6).

2&[prime]-(Glutathion-S-yl)monoclofenac (we designated this conjugate "M6") was found first

in incubation mixtures of diclofenac with human liver microsomes (Wen et al., 2008). In vitro

studies using various recombinant human P450s fortified with glutathione and human

glutathione-S-transferase P1-1 demonstrated that all of the 14 tested P450 isozymes were capable of

forming M6 (Boerma et al., 2014). Furthermore, this reaction was NADPH independent with M6

produced to some extent by flavin-containing monooxygenase or even horseradish peroxidase

suggesting the involvement of one-electron oxidation. In the present study, a large single peak at *m/z*

567 (*m/z* 567.12825-567.13393) was obtained in the bile sample from a control TK-NOG mouse

(Fig. 4, C). This metabolite gave, on CID of *m/z* 567, product ions at *m/z* 492 ([MH-75]$^+$) and *m/z*

438 ([MH-129]$^+$) as reported by Boerma et al. (2012). Moreover, this single peak at *m/z* 567

co-eluted on HPLC with an in vitro reference product of M6 provided by Prof. Commandeur of VU

University (see Materials and Methods). Although this metabolite was reportedly generated in in

vitro studies using various human specimens, the peak of M6 in the bile of a humanized chimeric

mouse was not found (Fig. 5, C).

Discussion

Potential pathways of diclofenac metabolism are illustrated in Fig. 8. Hydroxylation at the

4&[prime]-position is one of the major metabolic routes of diclofenac and metabolite II has been

used as a marker of human CYP2C9 activity in humanized animal models. The in vitro assessment

using liver microsome preparations from humanized uPA/SCID chimeric mice with varying

replacement ratios showed that the formation of II correlated well with hepatic replacement ratio,

relative expression levels of *CYP2C9* mRNA and CYP2C9 protein content (Tateno et al., 2004;

Katoh et al., 2004). Recently, Hu et al., (2013) produced humanized TK-NOG chimeric mice using

hepatocytes from donors with different genetic backgrounds. After oral dosing of diclofenac to this

chimeric mouse model, plasma levels of II were extremely low in *CYP2C9* *2/*3, moderate in

CYP2C9 w/*2 and highest in *CYP2C9* w/w genotypes. More recently, Bateman et al. (2014)

introduced diclofenac as a suitable substrate for the in vivo evaluation of humanized uPA/SCID

chimeric mice with monitoring of II reaction product. In the present study, human hepatocytes

exhibiting significant CYP2C9 metabolic activity were transplanted into TK-NOG mice, and higher

plasma concentrations of II were observed in the humanized chimeric mice relative to control mice.

Hydroxylation at the 4&[prime]-position is also one of the major routes of metabolism in mice

(Sarda et al., 2012). However, it is possible that subsequent conjugation of II to its taurine,

glucuronic acid or glucose conjugates might have resulted in lower plasma concentrations of this

metabolite in the control mice.

On the other hand, hydroxylation of diclofenac at the 3&[prime]-position has been reported only

with non-human primates or humans (Stierlin et al., 1979; Stierlin and Faigle, 1979). After oral

dosing of diclofenac to humanized chimeric mice, significant levels of IV were observed while this

metabolite was completely missing in the plasma of control mice, suggesting generation of IV by the

transplanted human hepatocytes. Human-specific VI is a complex metabolite which may need both

3&[prime]- and 4&[prime]-hydroxylation followed by methylation at the 4&[prime]-position of

3&[prime],4&[prime]-dihydroxydiclofenac. In humanized chimeric mice, levels of VI in plasma

reached a maximum at 8 hours and then declined with a $t_{1/2}$ of 13.0 hours. These

parameters seem to be comparable to the respective values of 12 and 80 hours in humans (Faigle et

al., 1988), and could be used to retrospectively predict the distinct pharmacokinetic behavior of VI in

clinical practice. It had previously not been known whether VI was generated from diclofenac via II

or IV as an intermediate metabolite. We then administered synthetic metabolite II to humanized

chimeric mice and observed a plasma profile of VI similar to that after diclofenac dosing. Possible

formation of VI from IV remains not known since synthetic IV was not available.

Degen et al. (1988) reported that, following multiple b.i.d. dosing of a retard formulation of

diclofenac to patients, trough plasma concentrations of VI were about six times larger than the

21

unchanged drug. In the present study, diclofenac was given once per day to humanized chimeric

mice, with plasma peak and trough concentrations of VI reaching a plateau within three days of

treatment, and was retained at this level for a week. These data were not greatly different from the

simulated plasma profile based on a once-per day regimen. More frequent dosing or administration

of a delayed release formulation may, therefore, result in more significant accumulation of VI.

Metabolite VI showed about 300 times less activity than the parent compound in inhibiting

prostaglandin synthesis in vitro, and the anti-inflammatory effects in vivo were very weak (Faigle et

al., 1988). According to the MIST guidance by the FDA and the subsequent modified guidance by

the International Conference on Harmonization (ICH M3(R2); Food and Drug Administration, 2010),

safety of human-specific metabolites occurring at exposure greater than 10% of total drug-related

exposure should be evaluated before entering large scale clinical trials. Metabolite VI was one such

human-specific metabolites, and since it was generated through more than one metabolic reaction, it

could not be predicted by conventional in vitro systems such as human hepatocytes, hepatic S-9

fraction and microsomes (Dalvie et al., 2009). When $AUC_{0-\infty}$ values for each metabolite reported by

Degen et al. (1988) were included in the calculation, VI comprised 83% of total drug-related

exposure (diclofenac plus metabolites II-VI) since elimination of VI was slow. This metabolite was

largely pharmacologically inactive and deemed not to be a safety concern. However, if a

human-specific metabolite such as VI is found in a later stage of new drug development and exhibits

unexpected pharmacokinetic behavior, development of the new drug could be delayed. To address

this concern, chimeric mice with humanized livers are very useful for the prediction of circulating

human metabolites before entering first-in-man trials (Kamimura et al., 2010).

Toxicity of diclofenac to hepatocytes is reduced in vitro by the addition of cytochrome P450

inhibitors (Bort et al., 1999) and that to small intestine is ameliorated in intestinal

epithelium-specific P450 reductase knockout mice (Zhu and Zhang, 2012) suggesting involvement

of CYP mediated bioactivation. As evidence of bioactivation, three glutathione trapped metabolites,

M1, M2 and M3 were identified in the bile of rats (Tang et al., 1999). Among these metabolites, M1

and M3 were generated by oxidation of the phenyl acetic acid moiety followed by glutathione

conjugation, while formation of M2 was initiated by oxidation of the dichlorophenyl ring (Fig. 8). In

the present study, similar extracted ion chromatograms were obtained from the bile of the control

TK-NOG mice indicating the same reactions also occur in mouse species. In addition, M6, reported

to be generated in vitro by one-electron oxidation of diclofenac (Boerma et al., 2014), was also

detected in mouse bile. In metabolism studies using ^{3}H-labeled diclofenac, Sarda et al. (2012)

reported the major metabolites in mouse faeces were taurine conjugates of II and diclofenac, while

Bateman et al. (2014) reported the major metabolite in mouse bile was diclofenac acyl glucuronide.

Although their results did not agree well, both reports denied or did not mention formation of

glutathione conjugate metabolites of diclofenac. However, the present study suggested that

glutathione trapped metabolites of diclofenac were generated to some extent even in mice.

Metabolites M1, M2 and M3 were also detected in the bile of humanized chimeric mice which

suggested these glutathione conjugate metabolites were possibly generated at least in part by

transplanted human hepatocytes. Involvement of the human hepatocytes in the formation of M6, an

in vitro human metabolite, could not be demonstrated.

Monochlorinated metabolites M4 and M5 have been previously detected in incubation mixtures

of diclofenac with human liver microsomes fortified with glutathione (Yan et al., 2005; Yu et al.,

2005). Following oral administration of diclofenac to a control TK-NOG mouse, no glutathione

conjugates at m/z 583 were detected in the bile. On the other hand, a single peak producing a mass

spectrum identical to those of M4 and M5 was observed in the humanized chimeric mouse. This

result was puzzling, since it was uncertain if this in vivo metabolite corresponds to M4 or M5. If it is

assumed that this human-specific metabolite is M4, this metabolite was generated via

diclofenac-2&[prime],3&[prime]-oxide as proposed by Yan et al. (2005) (Fig.8). This hypothesis is

consistent with our finding that another possible product from the arene oxide, IV, was completely

missing in the plasma of control mice and considered to be human-specific. Bioactivation of

diclofenac to diclofenac-2&[prime],3&[prime]-oxide was mediated by human CYP2C9 (Yan et al.,

2005) and mice may lack this metabolic pathway. As supporting evidence in their report, incubation

of II with human liver microsomes did not produce M4. However, this result was inconsistent with

24

our finding that a peak at *m/z* 583 was also detected after administration of II to the humanized

chimeric mouse. If it is assumed that the human-specific metabolite is M5, detection of the peak in

the humanized chimeric mouse treated with II provides a rational basis for the proposed

diclofenac-1&[prime],4&[prime]-quinone imine intermediate for the formation of this metabolite

(Yu et al., 2005) (Fig.8). In this scenario, formation of M5 from the quinone imine intermediate was

either exclusively or predominantly a human metabolic reaction. Even a control TK-NOG mouse

dosed with 10 mg/kg of II gave a relatively small peak at *m/z* 583, probably because exposure of the

mouse to II was much higher than that after administration of the same dose of diclofenac. However,

further studies including NMR analysis of the biliary metabolite to distinguish the potential

positional isomers are necessary.

In conclusion, we could mimic the distinct plasma concentration profile of the human-specific

metabolite of diclofenac (VI) by TK-NOG mice with humanized livers. Glutathione conjugate

metabolites were also detected in the bile samples providing a new means of studying

glutathione-dependent inactivation of reactive human metabolites in vivo. As with the conventional

humanized animal models, humanized TK-NOG chimeric mice were considered to be a useful tool

for metabolism studies in new drug development.

Acknowledgements

Authorship Contributions

Participated in research design: Kamimura.

Conducted experiments: Ito, Nozawa, Nakamura, and Chijiwa.

Contributed animal new reagents or analytic tools: Kuronuma, Ohnishi, and Suemizu.

Performed data analysis: Nagatsuka.

Wrote or contributed to the writing of the manuscript: Kamimura, Ito, and Ninomiya.

References

Azuma H, Paulk N, Ranade A, Dorrell C, Al-Dhalimy M, Ellis E, Strom S, Kay MA, Finegold M, and Grompe M (2007) Robust expansion of human hepatocytes in Fah$^{-/-}$/Rag2$^{-/-}$/Il2rg$^{-/-}$ mice. *Nat Biotechnol* **25:**903-910.

Bateman TJ, Reddy VG, Kakuni M, Morikawa Y, and Kumar S (2014) Application of chimeric mice with humanized liver for study of human-specific drug metabolism. *Drug Metab Dispos* **42:**1055-1065.

Boelsterli UA (2003) Diclofenac-induced liver injury: a paradigm of idiosyncratic drug toxicity. *Toxicol Appl Pharmacol* **192:**307-322.

Boerma JS, Dragovic S, Vermeulen NP, and Commandeur JN (2012) Mass spectrometric characterization of protein adducts of multiple P450-dependent reactive intermediates of diclofenac to human glutathione-S-transferase P1-1. *Chem Res Toxicol* **25:**2532-2541.

Boerma JS, Vermeulen NP, and Commandeur JN (2014) One-electron oxidation of diclofenac by human cytochrome P450s as a potential bioactivation mechanism for formation of 2&[prime]-(glutathion-S-yl)-deschloro-diclofenac. *Chem Biol Interact* **207:**32-40.

Bort R, Ponsoda X, Jover R, Gómez-Lechón MJ, and Castell JV (1999) Diclofenac toxicity to hepatocytes: a role for drug metabolism in cell toxicity. *J Pharmacol Exp Ther* **288:**65-72.

Dalvie D, Obach RS, Kang P, Prakash C, Loi CM, Hurst S, Nedderman A, Goulet L, Smith E, Bu

HZ, and Smith DA. (2009) Assessment of three human in vitro systems in the generation of

major human excretory and circulating metabolites. *Chem Res Toxicol* **22:**357-368.

Degen PH, Dieterle W, Schneider W, Theobald W, and Sinterhauf U (1988) Pharmacokinetics of

diclofenac and five metabolites after single doses in healthy volunteers and after repeated doses

in patients. *Xenobiotica* **18:**1449-1455.

Faigle JW, Böttcher I, Godbillon J, Kriemler HP, Schlumpf E, Schneider W, Schweizer A, Stierlin H,

and Winkler T (1988) A new metabolite of diclofenac sodium in human plasma. *Xenobiotica*

18:1191-1197.

Food and Drug Administration (2010) Guidance for Industry: M3(R2) Nonclinical Safety Studies for

the Conduct of Human Clinical Trials and Marketing Authorization for Pharmaceuticals, FDA,

Rockville, MD.

Grillo MP, Knutson CG, Sanders PE, Waldon DJ, Hua F, and Ware JA (2003) Studies on the

chemical reactivity of diclofenac acyl glucuronide with glutathione: identification of

diclofenac-S-acyl-glutathione in rat bile. *Drug Metab Dispos* **31:**1327-1336.

Grompe M and Strom S (2013) Mice with human livers. *Gastroenterology* **145:**1209-1214.

Hasegawa M, Kawai K, Mitsui T, Taniguchi K, Monnai M, Wakui M, Ito M, Suematsu M, Peltz G,

Nakamura M, and Suemizu H (2011) The reconstituted 'humanized liver' in TK-NOG mice is

mature and functional. *Biochem Biophys Res Commun* **405:**405-410.

Hu Y, Wu M, Nishimura T, Zheng M, and Peltz G (2013) Human pharmacogenetic analysis in

chimeric mice with 'humanized livers'. *Pharmacogenet Genomics* **23:**78-83.

Kamimura H, Nakada N, Suzuki K, Mera A, Souda K, Murakami Y, Tanaka K, Iwatsubo T,

Kawamura A, and Usui T (2010) Assessment of chimeric mice with humanized liver as a tool

for predicting circulating human metabolites. *Drug Metab Pharmacokinet* **25:**223-235.

Katoh M, Matsui T, Nakajima M, Tateno C, Kataoka M, Soeno Y, Horie T, Iwasaki K, Yoshizato K,

and Yokoi T (2004) Expression of human cytochromes P450 in chimeric mice with humanized

liver. *Drug Metab Dispos* **32:**1402-1410.

Kretz-Rommel A and Boelsterli UA (1993) Diclofenac covalent protein binding is dependent on acyl

glucuronide formation and is inversely related to P450-mediated acute cell injury in cultured rat

hepatocytes. *Toxicol Appl Pharmacol* **120:**155-161.

Mercer DF, Schiller DE, Elliott JF, Douglas DN, Hao C, Rinfret A, Addison WR, Fischer KP,

Churchill TA, Lakey JR, Tyrrell DL, and Kneteman NM. (2001) Hepatitis C virus replication in

mice with chimeric human livers. *Nature Medicine* **7:**927-933.

Nishimura T, Hu Y, Wu M, Pham E, Suemizu H, Elazar M, Liu M, Idilman R, Yurdaydin C, Angus P,

Stedman C, Murphy B, Glenn J, Nakamura M, Nomura T, Chen Y, Zheng M, Fitch WL, and

Peltz G (2013) Using chimeric mice with humanized livers to predict human drug metabolism

and a drug-drug interaction. *J Pharmacol Exp Ther* **344:**388-396.

Peltz G (2013) Can 'humanized' mice improve drug development in the 21st century? *Trends Pharmacol Sci* **34:**255-260.

Sarda S, Page C, Pickup K, Schulz-Utermoehl T, and Wilson I (2012) Diclofenac metabolism in the mouse: novel in vivo metabolites identified by high performance liquid chromatography coupled to linear ion trap mass spectrometry. *Xenobiotica* **42:**179-194.

Small RE (1989) Diclofenac sodium. *Clin Pharm* **8:**545-558.

Stierlin H, Faigle JW, Sallmann A, Küng W, Richter WJ, Kriemler HP, Alt KO, and Winkler T (1979) Biotransformation of diclofenac sodium (Voltaren) in animals and in man. I. Isolation and identification of principal metabolites. *Xenobiotica* **9:**601-610.

Stierlin H and Faigle JW (1979) Biotransformation of diclofenac sodium (Voltaren) in animals and in man. II. Quantitative determination of the unchanged drug and principal phenolic metabolites, in urine and bile. *Xenobiotica* **9:**611-621.

Suemizu H, Sota S, Kuronuma M, Shimizu M, and Yamazaki H (2014) Pharmacokinetics and effects on serum cholinesterase activities of organophosphorus pesticides acephate and chlorpyrifos in chimeric mice transplanted with human hepatocytes. *Regul Toxicol Pharmacol* **70:**468-473.

Tang W (2003) The metabolism of diclofenac--enzymology and toxicology perspectives. *Curr Drug Metab* **4:**319-329.

Tang W, Stearns RA, Bandiera SM, Zhang Y, Raab C, Braun MP, Dean DC, Pang J, Leung KH, Doss GA, Strauss JR, Kwei GY, Rushmore TH, Chiu SH, and Baillie TA (1999) Studies on cytochrome P-450-mediated bioactivation of diclofenac in rats and in human hepatocytes: identification of glutathione conjugated metabolites. *Drug Metab Dispos* **27:**365-372.

Tateno C, Yoshizane Y, Saito N, Kataoka M, Utoh R, Yamasaki C, Tachibana A, Soeno Y, Asahina K, Hino H, Asahara T, Yokoi T, Furukawa T, and Yoshizato K (2004) Near completely humanized liver in mice shows human-type metabolic responses to drugs. *Am J Pathol* **165:**901-912.

Thanagari BS, Fefar DT, Prajapati KS, Jivani BM, Thakor KB, Patel JH, Ghodasara DJ, Joshi BP, and Undhad VV (2012) Haemato-biochemical alterations induced by diclofenac sodium toxicity in swiss albino mice. *Vet World* **5:**417-419.

Tsukada A, Suemizu H, Murayama N, Takano R, Shimizu M, Nakamura M, and Yamazaki H (2013) Plasma concentrations of melengestrol acetate in humans extrapolated from the pharmacokinetics established in in vivo experiments with rats and chimeric mice with humanized liver and physiologically based pharmacokinetic modeling. *Regul Toxicol Pharmacol* **65:**316-324.

Wen B, Ma L, Nelson SD, and Zhu M (2008) High-throughput screening and characterization of reactive metabolites using polarity switching of hybrid triple quadrupole linear ion trap mass spectrometry. *Anal Chem* **80:**1788-1799.

Yamashita M, Suemizu H, Murayama N, Nishiyama S, Shimizu M, and Yamazaki H (2014) Human

plasma concentrations of herbicidal carbamate molinate extrapolated from the pharmacokinetics

established in *in vivo* experiments with chimeric mice with humanized liver and physiologically

based pharmacokinetic modeling. *Regul Toxicol Pharmacol* **70:**214-221.

Yamazaki H, Suemizu H, Shimizu M, Igaya S, Shibata N, Nakamura M, Chowdhury G, and

Guengerich FP (2012) In vivo formation of dihydroxylated and glutathione conjugate

metabolites derived from thalidomide and 5-Hydroxythalidomide in humanized TK-NOG mice.

Chem Res Toxicol **25:**274-276.

Yamazaki H, Suemizu H, Murayama N, Utoh M, Shibata N, Nakamura M, and Guengerich FP

(2013) In vivo drug interactions of the teratogen thalidomide with midazolam: heterotropic

cooperativity of human cytochrome P450 in humanized TK-NOG mice. *Chem Res Toxicol*

26:486-489.

Yan Z, Li J, Huebert N, Caldwell GW, Du Y, and Zhong H (2005) Detection of a novel reactive

metabolite of diclofenac: evidence for CYP2C9-mediated bioactivation via arene oxides. *Drug

Metab Dispos* **33:**706-713.

Yoshizato K, Tateno C, and Utoh R (2012) Mice with liver composed of human hepatocytes as an

animal model for drug testing. *Curr Drug Discov Technol* **9:**63-76.

Yu LJ, Chen Y, Deninno MP, O'Connell TN, and Hop CE (2005) Identification of a novel glutathione

adduct of diclofenac, 4&[prime]-hydroxy-2&[prime]-glutathion-deschloro-diclofenac, upon

incubation with human liver microsomes. *Drug Metab Dispos* **33:**484-488.

Zhu Y and Zhang QY (2012) Role of intestinal cytochrome p450 enzymes in diclofenac-induced

toxicity in the small intestine. *J Pharmacol Exp Ther* **343:**362-370.

Footnotes:

Address correspondence to: Dr. Hidetaka Kamimura, ADME & Tox Research Institute,

Sekisui Medical Co., Ltd., 13-5, Nihonbashi 3-chome, Chuo-ku, Tokyo 103-0027 Japan.

E-mail: hidetaka.kamimura@sekisui.com

Figure Legends:

Fig. 1. Plasma concentration of diclofenac and its metabolites after oral administration of diclofenac

sodium (10 mg/kg as free form) to TK-NOG chimeric mice with humanized livers (— —) and

control TK-NOG mice (- -■- -). Mean±SD of three experiments.

Fig. 2. Plasma concentration of 4&[prime]-hydroxydiclofenac and

3&[prime]-hydroxy-4&[prime]-methoxydiclofenac after oral administration of 10 mg/kg

4&[prime]-hydroxydiclofenac to a TK-NOG chimeric mouse with humanized liver (— —) and a

control TK-NOG mouse (- -■- -).

Fig. 3. Plasma concentration of 3&[prime]-hydroxy-4&[prime]-methoxydiclofenac at 0 hour (●) and

8 hours (○) after repeated oral administration of diclofenac sodium (10 mg/kg as free form) to

TK-NOG chimeric mice with humanized livers. Each point represents mean±SD of three (1st to 7th

day) or mean of two (8th to 14th day) experiments.

Fig. 4. Glutathione conjugate metabolites in the bile by integrated high-resolution accurate mass

detection after oral administration of diclofenac sodium (10 mg/kg as free form) to a control

TK-NOG mouse. Each metabolite was monitored at corresponding mass number (A-C) and a mixed

chromatogram was shown in the upper panel.

Fig. 5. Glutathione conjugate metabolites in the bile by integrated high-resolution accurate mass

detection after oral administration of diclofenac sodium (10 mg/kg as free form) to a humanized

chimeric mouse. Each metabolite was monitored at corresponding mass number (A-C) and a mixed

chromatogram was shown in the upper panel.

Fig. 6. Glutathione conjugate metabolites in the bile by integrated high-resolution accurate mass

detection after oral administration of 10 mg/kg 4&[prime]-hydroxydiclofenac to control TK-NOG

(A) and humanized chimeric mice (B).

Fig. 7. Collision-induced dissociation spectra of product ion *m/z* 583 of the human-specific

metabolite (M4 or M5) in the bile of humanized chimeric mice treated with diclofenac sodium (A)

and 4&[prime]-hydroxydiclofenac (B).

Fig. 8. Proposed metabolic pathways of diclofenac in humans deduced from the metabolic studies

with TK-NOG chimeric mice with humanized livers.

37

VI was confirmed to be generated from II, but formation of VI from IV was not clear since synthetic

version of IV was not available. The biliary metabolite with the parent ion of *m/z* 583 may
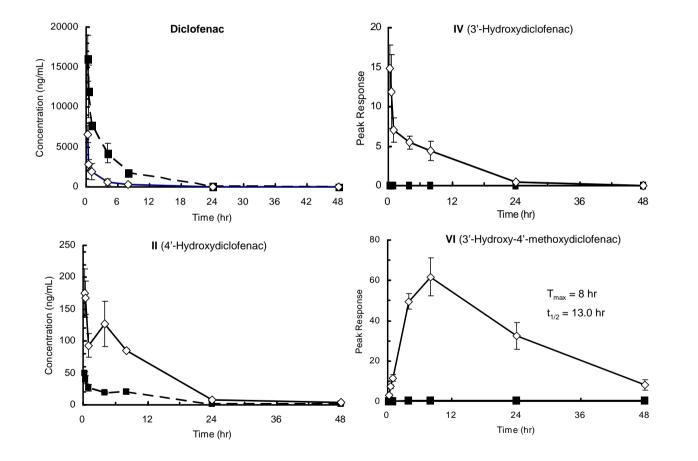
correspond to M4 (Yan et al., 2005) or M5 (Yu et al., 2005) proposed in separate studies using
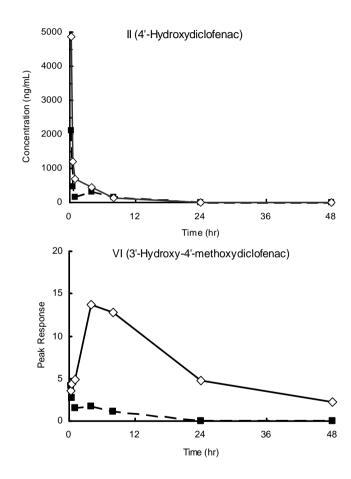
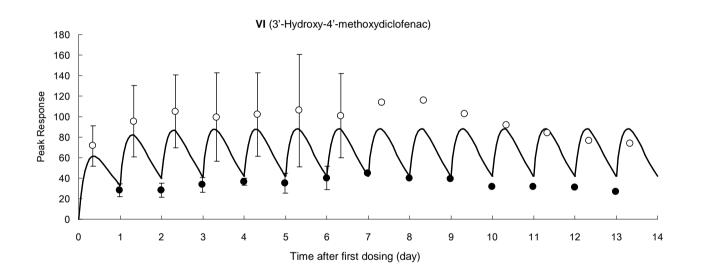human liver microsomes. Other abbreviations used are: III, 5-hydroxydiclofenac; V,

5,4&[prime]-dihydroxydiclofenac; M1, 5-hydroxy-4-(glutathion-S-yl)diclofenac; M3,

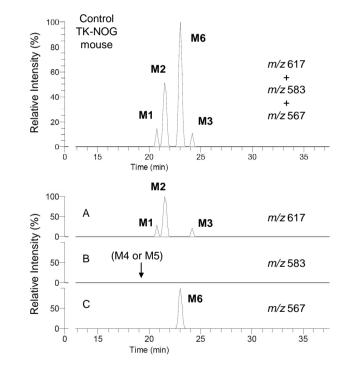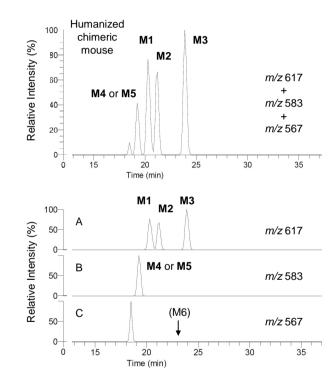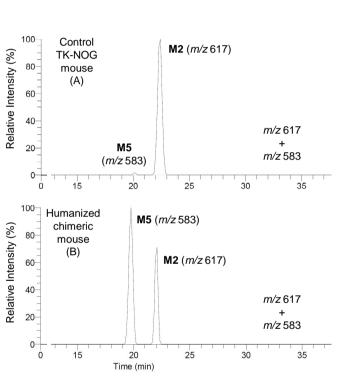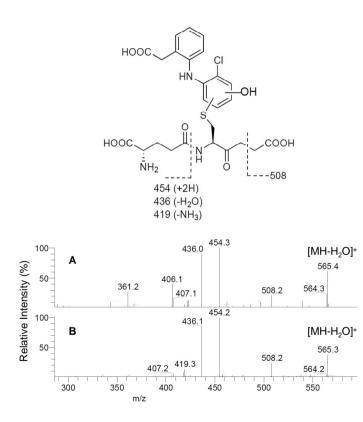5-hydroxy-6-(glutathion-S-yl)diclofenac.

FIGURE 1

FIGURE 2

FIGURE 3

VI (3'-Hydroxy-4'-methoxydiclofenac)

FIGURE 4

FIGURE 5

FIGURE 6

FIGURE 7

FIGURE 8

Modification of the phenylacetic acid ring

CYP3A4

III, V, M1, M3

Conjugation of the carboxyl group

UGT2B7 etc.

Diclofenac

One-electron oxidation

GST P1-1

M6 (2'-Glutathion-S-yl)monoclofenac)

CYP2C9

CYP2C9

II (4'-Hydroxydiclofenac)

IV (3'-Hydroxydiclofenac)

Diclofenac-2',3'-oxide

?

Diclofenac-1', 4'-quinone imine

VI (3'-Hydroxy-4'-mehtoxydiclofenac)

M2 (4'-Hydroxy-3'-(glutathion-S-yl)diclofenac)

M5 (4'-Hydroxy-2'-(glutathion-S-yl)monoclofenac)

A biliary metabolite found in the humanized chimeric mouse may correspond to M5 or M4.

M4 (2'-Hydroxy-3'-(glutathion-S-yl)monoclofenac)