Veterinary Science & Technology

Hamamoto, et al., J Veterinar Sci Techno 2016, 7:3
http://dx.doi.org/10.4172/2157-7579.1000318

Research Article Open Access

Cytochrome P450 2D14 (CYP2D14) Gene Deletion Variants in the Japanese Black Cattle and Characterization of their Effects on Metoclopramide Pharmacokinetics

Kouko Hamamoto[1*], Yasuharu Mizuno[1], Mayumi Kijima[1], Tsuyoshi Abe[2], Eiji Kobayashi[3], Masaki Kato[4], Norio Yamagishi[5] and Kazuhisa Furuhama[4]

[1]*National Veterinary Assay Laboratory, Ministry of Agriculture, Forestry and Fisheries, Tokura 1-15-1, Kokubunji, Tokyo, 185-8511, Japan*

[2]*Department of Technology, National Livestock Breeding Center, Odakurahara 1, Odakura, Nishigo, Fukushima, 961-8511, Japan*

[3]*Animal Breeding and Reproduction Research Division, National Institute of Livestock and Grassland Science, Ikenodai 2, Tsukuba, Ibaraki 305-0901, Japan*

[4]*Departments of Veterinary Basic Medicine, Faculty of Agriculture, Iwate University, Ueda 3-18-8, Morioka, Iwate 020-8550, Japan*

[5]*Veterinary Clinical Medicine, Faculty of Agriculture, Iwate University, Ueda 3-18-8, Morioka, Iwate 020-8550, Japan*

[*]**Corresponding author:** Kouko Hamamoto, National Veterinary Assay Laboratory, Ministry of Agriculture, Forestry and Fisheries, Tokura 1-15-1, Kokubunji, Tokyo, 185-8511, Japan, Tel: +81-042-321-1841; E-mail: koko_hamamoto180@maff.go.jp

Rec date: Apr 01, 2016; **Acc date:** Apr 18, 2016; **Pub date:** Apr 20, 2016

Abstract

The frequency of cytochrome P450 2D14 (CYP2D14) genetic polymorphisms and their effects on CYP2D14-mediated metabolism have not been reported in cattle. Genetic polymorphisms of the CYP2D14 gene in 57 Japanese Black (JB) cattle were determined by polymerase chain reaction (PCR)-direct sequencing. Two types of CYP2D14 gene deletion variants (GD), GD1 and GD2, were observed in this study. The CYP2D6 deletion reportedly results in delayed metabolism of metoclopramide in human. To investigate the effects of CYP2D14 gene deletion in JB cattle, the GD1, GD2 and wild-type (WT) cattle were administered metoclopramide by an intravenous or oral route. Plasma concentrations of metoclopramide in GD1 cattle were significantly higher 24 hour (h) after intravenous administration ($P<0.01$) and within 1 h after oral administration of metoclopramide compared to levels in GD2 and WT cattle ($P<0.01$). The half-life at γ-phase (γHL) of metoclopramide after intravenous administration was significantly higher in GD1 compared to WT cattle ($P<0.05$). Moreover, the elimination rate constant (K_{10}) of GD1 after oral administration of metoclopramide was significantly higher in GD1 compared to WT animals ($P<0.05$). These results suggest that the higher plasma concentration of metoclopramide in GD1 cattle is caused by delayed metabolism of metoclopramide, owing to CYP2D14 enzyme deficiency.

Keywords: CYP2D14 genetic polymorphism; Cattle; Pharmacokinetics; Metoclopramide

Introduction

As a member of the human cytochrome P450 (CYP450) superfamily, CYP450 2D6 (CYP2D6) is involved in endogenous biotransformation and the metabolism of many drugs, including antipsychotics, antidepressants, β-blockers, antiarrhythmics and opiates [1]. Approximately 40% of phase I metabolism processes in the human liver involve CYP2C9, CYP2C19 and CYP2D6 [2]. Various medications include substrates of CYP2D6, even though CYP2D6 accounts for only a small percentage of total hepatic CYPs (2-3%) [3].

More than 80 allelic variants and subvariants of the CYP2D6 gene have been reported [4-6]. Substrates of CYP2D6 are lipophilic bases with a protonatable atom [2]. In particular, CYP2D6*5 (gene deletion) is associated with altered or delayed drug metabolism [7,8] and the CYP2D6 polymorphisms can lead to adverse drug reactions and altered drug responses [2,9].

Metoclopramide (4-amino-5-chloro-N-(2-(diethylamino)ethyl)-2-methoxy-benzamide, CAS: 364-62-5) is widely used as a gastrointestinal prokinetic agent for humans, cattle, swine, cats and dogs in Japan. Metoclopramide exerts antagonistic effects on receptors for dopamine-2 [10,11] and serotonin [12]. Metoclopramide can cause side effects of the central nervous system (e.g., shaking, muscle stiffness, etc.), indicating that it crosses the blood-brain barrier [13-15].

Tsuneoka et al. [16] cloned, mapped and characterized CYP2D14 complementary deoxyribonucleic acid (cDNA) from the cattle liver, revealing 80% and 68% similarity to human CYP2D6 and rat CYP2D1, respectively. The CYP2D14 gene is located on bovine chromosome 5 [17]. Although the National Center for Biotechnology Information (NCBI) has indicated some sequence differences in cattle CYP2D14, functional effects of CYP2D14 gene polymorphisms have not been reported in cattle.

The aim of the present study was to reveal the effect of CYP2D14 gene deletion on metoclopramide pharmacokinetics after intravenous (IV) and oral administration of the drug to JB cattle. Furthermore, we considered abundance ratios of main metoclopramide metabolites after oral administration to gene deletion (GD) and wild-type (WT) cattle.

Materials and Methods

Chemical and reagents

The FastStart High-Fidelity PCR System, dNTpack (Roche Applied Science Inc., Mannheim, Germany) was used as a polymerase chain reaction (PCR) reagent. A monodeethyl metabolite of metoclopramide

(M3) was made in accordance with a previous knowledge on the metabolites of metoclopramide in cattle [18]. Metoclopramide (100% purity) and levosulpiride used as the internal standard (IS) were purchased from Wako Pure Chemical Industries, Ltd. for analysis of metoclopramide and its metabolites. The Western Breeze Chemiluminescent Westen Blot Immunodetection Kit (No. WB7105 (anti rabbit IgG as a secondary anti body), Invitrogen Corp., CA, USA) was used for the western blotting.

DNA samples and animals

Blood samples from 57 adult JB cattle reared on the farm of the Field Science Center of Iwate University (Morioka, Japan) were used to analyze DNA type by sequencing. Cattle DNA was extracted with the DNeasy Blood and Tissue kit (Quiagen Sciences Inc.), according to a standard protocol. Two types of gene deletion alleles, GD1 and GD2, were identified amongst the CYP2D14 gene polymorphisms. For the GD1 allele, DNA sequencing could not be performed from exon 1 to exon 9 of the CYP2D14 gene. For the GD2 allele, DNA sequencing could not be performed in exon 1. However, sequencing for the GD2 allele was possible in another part—an uncharacterized protein, LOC785824, located approximately 12 kbp downstream from the start codon of CYP2D14-by sequencing the primer for exon 4. The WT cattle had no polymorphisms in exons of CYP2D14.

PCR analysis

Sample DNA was PCR-amplified with PCR primers designed based on the CYP2D14 gene sequence from the NCBI Reference Sequence database (NW_174529) using primer 3 [19]. The NCBI Basic Local Alignment Search Tool was used to find regions of local similarity between sequences registered in the DNA database. Eleven PCR primers (Table 1) were used for CYP2D14 gene amplification and DNA sequencing.

The PCR amplification was performed in a reaction volume of 25 µL, which included 15.75 µL of water, 2.5 µL of reaction buffer with $MgCl_2$ (18 mmol), 0.5 µL of DMSO, 0.5 µL of nucleotide mix, 2.5 µL each of the R and L primer solutions (both 4 µmol), 0.25 µL of enzyme blend (5 units/µL) and 0.5 µL of DNA (10 ng). The PCR conditions were as follows: 95°C for 2 min, followed by 40 cycles of 30 s at 95°C, 30 s at melting temperature (Tm) (Table 1), 3.2 min at 72°C and a final extension for 5 min at 72°C.

Parts of CYP2D14 gene (length of PCR product)	Primer for PCR and DNA sequencing	Tm (°C)
Exon 1-4	1L: 5'-TCCCCATCATCAACCTTGTCCTC-3'	62
(2.5 kbp:1L+1R)	1R 5'-CCTCCGCTCCCTTTATACAGAGG-3'	
Exon 5-9	2L: 5'-CTGGTTTTGGGGAGACTGCTAAG-3'	61
(2.0 kbp:2L+2R)	2R: 5'- GACAACCTAGATCGGAAAGGCTG-3'	
Complementary strand		
Exon 1-4	3L: 5'-GCCCAGAAAGATAAACCTAAAATGT-3'	60
(2.5 kbp:3L+3R)	3R: 5'-ACTCTCCTTGACCCCTCTCTGTACT-3'	
Exon 5-9	4L: 5'-GTGGGAGTACTTCACTGCAAGG-3'	60
(2.0 kbp:4L+4R)	4R: 5'-AGACACTGGTTTATTGACCATCAG-3'	
Specific primer for DNA sequencing related to parts of polymorphism		
Complementary strand		
Exon 1	5L: 5'-AGTCTGTTTCCATGTTGTAAAAACC-3'	
Exon 3	6L: 5'-CGCACTAAGTTAAACTCTTCCTTCA-3'	
Exon 3	5R: 5'-CAAAATAAAAAGTGGAGCCAAGAC-3'	

Table 1: Primers for PCR and/or DNA sequencing of CYP2D14 gene in Japanese Black cattle.

Sequencing analysis

Before the sequencing analysis was performed, ExoSAP-IT (USB Corp.) was used to purify the PCR products. Purified samples were reacted according to the Big Dye Terminator Sequencing Protocol (Applied Biosystems). The PCR products were sequenced from exon 1 to exon 4 or from exon 5 to exon 9 with the BigDye Terminator ver. 3.1 Cycle Sequencing kit (Applied Biosystems) according to the recommended protocol. Three specific sequencing primers and PCR primers were used for DNA sequencing (Table 1). After the sequencing reactions were finished, the products were purified with the BigDye Xterminator Purification Kit (Applied Biosystems) and injected into the sequencer. Sequencing was performed on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems) with the POP-7 polymer and 50-cm capillary array.

Administration of metoclopramide

Eighteen healthy JB cattle (6 WT, 6 GD1 and 6 GD2; age: 0.8–5.2 years, body weight: 211–580 kg) were administered metoclopramide by

IV (Metoclo Injection 10 for animal use 'KMK', Kawasaki Mitaka Pharm Co., Ltd.) at a dose of 0.4 mg/kg. Six months later, four cattle in each group received a single oral administration of metoclopramide (Primperan for oral use, Serachem Co., Ltd.) at a dose of 0.4 mg/kg under the non-fasting condition.

Heparinized blood samples were collected from cattle at 0.25, 0.5, 0.75, 1, 2, 4, 6, 8, 12, 24 and 30 h after IV administration or at 0.5, 1, 2, 3, 4, 6, 8, 12, 24 and 30 h after oral administration of metoclopramide. Immediately after collection, the blood was centrifuged at 3,000 rpm for 10 min at 5 °C to separate the plasma, which was stored at -20 °C for approximately 1 week and then at -80 °C. Metoclopramide concentrations in plasma were analyzed by the liquid chromatography-tandem mass spectrometry method [20]. In order to detect major metoclopramide metabolites (M1, M3 and M4) shown in Figure 1, the LC-MS/MS operating conditions were given in Table 2. Ratio between two different metabolites was calculated as peak height ratio. Ratio of metabolite/metoclopramide was calculated as peak height ratio between each metabolite/IS and metoclopramide/IS in a same sample. M1, M3 and M4 were confirmed to detect a target ion and a qualifier ion of each metabolite referred to MS2 pattern of metabolites reported by Argikar et al. [21] and Livezey et al. [22]. All experimental procedures were performed in accordance with the Guidelines for Animal Experimentation issued by the Japanese Association for Laboratory Animal Science [23] or approved by the Animal Experimental Ethics Committee of Iwate University. Animal Ethics Committee approval numbers (dates) in the intravenous administration and the oral administration study are 006-MK-6 (24 November 2010) and 007-MK-7 (8 August 2011), respectively.

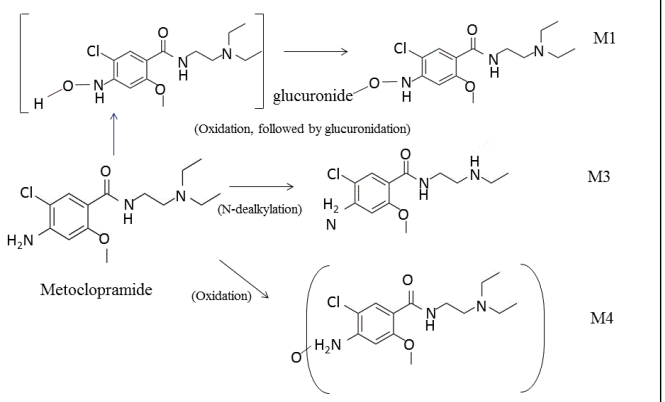

Figure 1: Chemical structural formulas of metoclopramide and three major metabolites (M1, M3 and M4).

Analyte	Precursor ion	Target ion	Qualifier ion	Collision energy	Polarity
M1	*m/z* 492	*m/z* 227	*m/z* 299	20 eV	Positive
M3	*m/z* 272	*m/z* 184	*m/z* 227	26 eV	Positive
M4	*m/z* 316	*m/z* 227	*m/z* 184	20 eV	Positive

Table 2: Selected reaction monitoring (SRM) parameters.

Pharmacokinetics of metoclopramide

Pharmacokinetic parameters were calculated from the plasma concentrations of metoclopramide after IV and oral administration by WINNONLIN ver. 6.2 (Pharsight Co. Ltd.). The following three-compartment open model was used for the IV administration study:

$$C(t) = Ae^{-\alpha t} + Be^{-\beta t} + Ce^{-\gamma t}$$

The following two-compartment model was used for the oral administration study:

$$C(t) = Ae^{-\alpha t} + Be^{-\beta t}$$

In these equation, C (t) is the plasma drug concentration at time (t) following a bolus dose; A, B and C represent intercept on the ordinate at time zero; and α, β and γ are the first order disposition rate constants. The area under the curve (AUC) of the plasma concentration versus time graph within 30 h after administration ($AUC_{0\text{-}30}$) was calculated by the linear trapezoidal method (linear interpolation). When appropriate, the terminal elimination phase of the Pharmacokinetics profile was estimated from the best fit (r^2) of at least the last three observed concentrations. The percent bioavailability (% of F) was calculated by dividing the dose-normalised extravascular plasma AUC by the dose-normalised IV plasma AUC times 100. The maximum plasma concentration (Cmax) and the time to peak concentration (Tmax) were observed values.

Statistical Analysis

Plasma concentrations and pharmacokinetic parameters were compared amongst the GD1, GD2 and WT cattle with the statistical function of Excel (Social Survey Research Information Co., Ltd.). One-way analysis of variance followed by Fisher's least significant difference (LSD) test ($P<0.05$ or $P<0.01$) was used to compare differences amongst means of groups of plasma concentrations at each time after IV or oral metoclopramide administration and pharmacokinetic parameters. Dunnett's test ($P<0.05$ or $P<0.01$) was used to perform multiple comparisons of the metabolite ratios in GD1 or GD2 cattle with these of WT cattle at 0.5 and 1h after oral metoclopramide administration.

Western blot analysis

Liver biopsies were collected from 4 WT, 4 GD1 and 4 GD2 cattle used in the metoclopramide pharmacokinetic studies after a lapse of more than a year from the pharmacokinetic studies. Liver microsomes were prepared from the biopsies according to the preparation method reported by Xia et al. [24]. In the preparation process, 0.1 mol/L potassium phosphate buffer (pH7.4) contained with protease inhibiter (cOmplete Mini EDTA free, Protease Inhibitor Cocktail Tablets provided in easy pack, Roche Applied Science Inc.) was used for homogenisation and RIPA buffer (Sigma-Aldrich Inc., MO, USA) contained with the protease inhibiter was used for resuspension of sediment. Protein contents of the liver microsome were adjusted 1 μg/μL with RIPA buffer contained with protease inhibiter. Cytochrome P450 2D6 Human Recombinant (20 μg/57 μL, ProSpec-Tany TechnoGene Ltd., Ness Ziona, Israel) was used as a positive standard. 15 μL of the liver microsome from the WT, GD1 and GD2 cattle and 15 μL of the human CYP2D6 (8.8–70.2 ng/μL concentrations) after reduction at 95°C for 5 min, and 5 μL of a protein marker (MagicMark XP western protein standard, Invitrogen Corp., CA, USA) were subjected to a 5-14% SDS-polyacrylamide gel (5-14% Q-page mini 1

mm, 10 well (TEFCO Co. Ltd., Tokyo, Japan)) electrophoresis at 200 V for 40 min and transferred to PVDF membrane (iBlot Gel Transfer Stack PVDF mini, Invitrogen Corp.) with the iBlot Gel Transfer Device (Invitrogen Corp.). The PVDF membrane was blocked with 10 mL of a PVDF blocking agent for Can Get Signal (Toyobo, Co. Ltd., Osaka, Japan) according to a standard protocol. The PVDF membrane after blocking was processed using a standard protocol of the Western Breeze Chemiluminescent Westen Blot Immunodetection Kit. A 1000-fold dilution of AB1251 antibody against human CYP2D6 (Millipore Corp., MA, USA) was used as a primary antibody solution in the western blotting method.

Results

In this study, the GD1 and GD2 polymorphisms were found in 17.5% and 12.3% of the 57 analysed JB cattle, respectively. All DNA samples of 57 JB cattle used in this study were able to be amplified and sequenced by using the primer set for sequencing of the CYP2E1 gene in our previous study (data not shown). This indicates that all DNA samples were sufficiently extracted. Moreover, the CYP2D14 gene deletions found in the DNA sequencing study were similarly observed when sequencing tests were performed with a double volume of the same PCR samples and when the PCR-sequencing test was repeated three times with the same method.

The cattle CYP2D protein (including CYP2D14) screened by using Western blot analysis show in Figure 2. The cattle CYP2D protein and the human CYP2D6 protein comigrated with a 50 kDa marker protein in the SDS- polyacrylamide gel electrophoresis. Nonspecific reaction by the RIPA buffer and other test reagents used in the western blot analysis were not detected (not shown). The chemiluminescent intensity of CYP2D protein in the WT cattle liver microsome was observed highest among three genetic types of cattle liver microsomes. Ratios (means±standard deviations) of chemiluminescent intensities of the CYP2D protein in the WT, GD1 and GD2 cattle (n=4) to the intensity of the human CYP2D6 protein (23.4 ng/μL) were 1.02 ± 0.08, 0.55 ± 0.14, 0.57 ± 0.07, respectively.

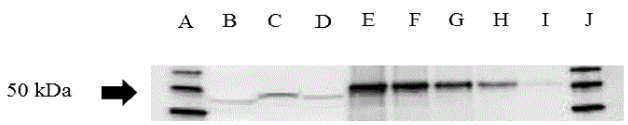

Figure 2: Western blot analysis of CYP2D protein in liver microsomes of the WT, GD1 and GD2 cattle. A and J: protein marker (MagicMark XP Western Protein Standard), B: GD1 cattle, C: WT cattle, D: GD2 cattle, E: human CYP2D6 (70.2 ng/μL), F: human CYP2D6 (35.1 ng/μL), G: human CYP2D6 (26.4 ng/μL), H: human CYP2D6 (17.6 ng/μL), I: human CYP2D6 (8.8 ng/μL).

Figure 3 and Table 3 show the plasma concentration vs. time curves and pharmacokinetic parameters for WT, GD1 and GD2 cattle after IV administration of metoclopramide at 0.4 mg/kg. At 24 h after IV administration, the mean plasma concentration of metoclopramide in GD1 cattle was 3.5 and 4.2 times higher than that in GD2 and WT cattle, respectively (both $P<0.01$). The distribution rate constant (α), transfer constant from deep peripheral (3) to central (1) (K_{31}) and half-life at γ-phase (γHL) in GD1 cattle after IV administration of metoclopramide were higher than those in WT cattle ($P<0.05$), but no significant difference was observed between GD2 and WT cattle.

Pharmacokinetic Parameter	GD1		GD2		WT		Fisher's LSD	
	Mean	SD	Mean	SD	Mean	SD	*:P<0.05	Unit
A	825.94	800.32	1,311.49	1,277.36	1,028.76	1,887.75		ng/mL
B	76.1	28.67	62.32	23.79	151.84	217.96		ng/mL
C	2.62	1.69	1.98	1.76	10.5	17.56		ng/mL
α	8.06	2.22	7.53	4.13	3.55	2.25	GD1-WT*	h^{-1}
β	0.66	0.27	0.76	0.36	0.86	0.85		h^{-1}
γ	0.07	0.03	0.08	0.04	0.13	0.08		h^{-1}
Cmax	2,404.66	3,505.73	1,305.17	1,301.15	1,189.97	1,909.29		ng/mL
Vd[a]	0.99	0.95	0.85	0.54	1.6	1.21		L/kg
K_{21}[b]	2.03	1.45	1	0.22	1.29	1.13		h^{-1}
K_{31}[c]	0.08	0.04	0.09	0.05	0.3	0.27	GD1-WT*	h^{-1}
K_{10}	4.05	2.7	4.1	3.12	2.02	1.42		h^{-1}
K_{12}[b]	2.19	1.28	1.69	1.3	0.75	0.86		h^{-1}
K_{13}[c]	0.44	0.41	0.27	0.11	0.28	0.36		h^{-1}
K_{10}HL	0.28	0.15	0.28	0.25	0.53	0.3		h

αHL	0.09	0.02	0.25	0.35	0.34	0.24		h
βHL	1.17	0.31	1.07	0.28	1.6	1.38		h
γHL	12.62	5.73	10.8	5.09	5.91	2.63	GD1-WT*	h
AUC_{0-30}	202.98	86.59	152.55	42.73	281.97	199.37		ng.h/mL
AUC_{0-infd}	392.98	329.5	236.67	86.61	391.27	363.6		ng.h/mL
Cl[e]	2.63	2.46	1.89	0.61	1.89	1.13		L/h.kg
AUMC[f]	955.03	532.59	687.71	446.66	586.88	343.14		$ng.h^2/mL$
MRT[g]	3.02	1.41	3.2	2.15	2.06	0.72		h
Vss[h]	5.15	3.45	6.28	4.23	5.01	2.77		L/kg

Table 3: Pharmacokinetic parameters of plasma metoclopramide in gene deletion 1 (GD1), gene deletion 2 (GD2) and wild –type (WT) cattle after IV administration of metoclopramide at 0.4 mg/kg (mean ± standard deviation (SD), n=6). $_aV_d$: Volume of the central compartment. $_bK_{12}$ and K_{21}: Transfer constant from central (1) to peripheral (2) and from 2 to 1, respectively. $_cK_{13}$ and K_{31}: Transfer constant from central (1) to deep peripheral (3) and from 3 to 1, respectively. $_dAUC_{0-inf}$: Estimated area under the concentration-time curve from 0 to infinity. $_e$Cl: Total body clearance. $_f$AUMC: Area under the first moment curve. gMRT: Mean residence time. $_h$Vdss: Volume of distribution at steady-state.

Pharmacokinetic parameter	GD1		GD2		WT		Fisher's LSD	
	Mean	SD	Mean	SD	Mean	SD	*:P<0.05 **:P<0.01	Unit
A	278.32	414.5	24.47	43.03	112.6	222.38		ng/mL
B	6.45	1.15	6.43	2.66	5.24	1.42		ng/mL
α	2.07	1.65	2.45	1.98	2.05	2.65		h^{-1}
β	0.06	0.01	0.06	0.02	0.06	0.02		h^{-1}
Cmax	9.2	1.76	6.12	1.62	4.09	0.35	GD1-GD2* GD1-WT**	ng/mL
Tmax	1.13	0.63	1.63	1.11	1.25	1.19		h
Vb[a]	11.13	8.81	25.04	12.35	21.11	11.94		L/kg
K_{21}[b]	0.82	0.6	1.83	1.76	0.94	0.62		h^{-1}
K_{01}[c]	2.82	1.51	5.04	6.41	4.42	2.21		h^{-1}
K_{10}[d]	0.16	0.04	0.08	0	0.1	0.04	GD1-GD2** GD1-WT*	h^{-1}
K_{12}[b]	1.15	1.08	0.59	0.58	1.07	2.05		h^{-1}
K_{01}HL	0.35	0.27	0.33	0.24	0.21	0.14		h
K_{10}HL	4.13	0.8	8.77	0.49	7.99	3.83	GD1-GD2*	h
αHL	0.55	0.38	0.45	0.3	0.81	0.59		h
βHL	11.11	1.95	12.49	4.11	11.92	3.3		h
AUC_{0-30}	98.23	26.89	87.79	21.01	72.13	16.68		ng.h/mL
AUC_{0-inf}[e]	107.36	30.16	100.31	18.68	86.72	21.10		ng.h/mL
Cl[f]	3.90	1.31	4.00	0.70	4.73	1.39		L/h.kg
AUMC[g]	1462.74	478.61	1564.32	307.97	1336.17	629.19		$ng.h^2/mL$
MRT[h]	13.46	1.54	15.96	3.97	14.79	3.88		h
F_{0-30}	61.19	28.11	66.99	24.02	45.4	29.23		%

$F_{0\text{-}inf}$[j]	38.87	23.82	48.46	20.27	44.37	25.65		%

Table 4: Pharmacokinetic parameters of plasma metoclopramide in gene deletion 1 (GD1), gene deletion 2 (GD2) and wild-type (WT) cattle after oral administration of metoclopramide at 0.4 mg/kg (mean ± standard deviation (SD), n=4). $_aVd$: Volume of the central compartment. $_bK_{12}$ and K_{21}: Transfer constant from central (1) to peripheral (2) and from 2 to 1, respectively. $_cK_{01}$: Absorption rate. $_dK_{10}$: Elimination rate. $_eAUC_{0\text{-}inf}$: Estimated area under the concentration-time curve from 0 to infinity. fCl: Total body clearance. $_gAUMC$: Area under the first moment curve. $_hMRT$: Mean residence time. $_iF_{0\text{-}inf}$: Bioavailability from 0 to infinity.

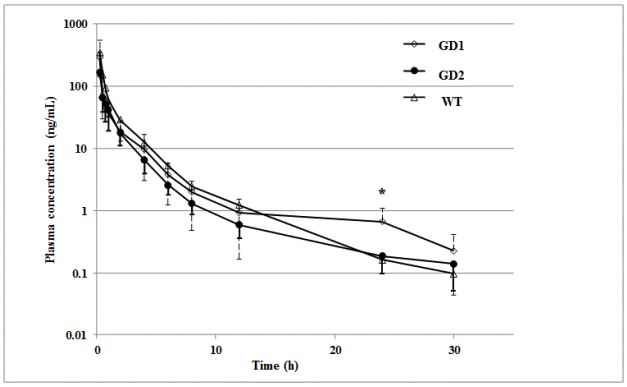

Figure 3: Plasma concentration-time profile of metoclopramide in gene deletion 1 (GD1), gene deletion 2 (GD2) and wild type (WT) cattle after IV administration of metoclopramide at 0.4 mg/kg (mean+/- standard deviation, n=6, *: significant difference between GD1 and other types (GD2 and WT) (P<0.01)).

Figure 4 and Table 4 show the plasma concentration vs. time curves and pharmacokinetic parameters, respectively, for WT, GD1 and GD2 cattle after oral administration of metoclopramide at 0.4 mg/kg. At 0.5 and 1 h after oral administration of metoclopramide, the plasma concentrations of metoclopramide were different between GD1 and WT cattle, and between GD1 and GD2 cattle (P<0.01). The Cmax in GD1 cattle were higher than those in WT (P<0.01) or GD2 cattle (P<0.05) and elimination rate constant (K_{10}) in GD1 cattle were higher than those in WT (P<0.05) or GD2 cattle (P<0.01). The half-life calculated from K_{10} (K_{10} HL) in GD1 cattle was significantly lower than that in GD2 cattle (P<0.05); however, half-life at β phase (βHL) was not significantly different amongst the three groups. In GD1, GD2 and WT cattle, the mean values of bioavailability from 0 to 30 h ($F_{0\text{-}30}$) were 61.19%, 66.99% and 45.40%, respectively.

Typical chromatograms of metoclopramide metabolites and IS in GD1and WT cattle plasma at 1 h after oral administration of metoclopramide and blank cattle plasma spiked with IS are shown in Figure 5. Metabolite ratios in GD1, GD2 and WT cattle plasma at 0.5 and 1 h after oral administration of metoclopramide to GD1, GD2 and WT cattle were shown in Table 5. M4c metabolite reported by Livezey et al. [22] was measured as a typical M4 metabolite in this study because it is mainly detectable metabolite in the cattle plasma samples. The M1, M3 and M4 were able to detect specifically; however, other metabolites (M2 and M5) reported by Argikar et al. [21] were not detected sufficiently in this study. Ratio of M3/M1 at 1 h after oral administration was statistically significant differences between GD1 and WT cattle (P<0.01). Likewise, ratio of M4/M1 at 1 h after oral administration was statistically significant differences between GD1 and WT cattle (P<0.05).

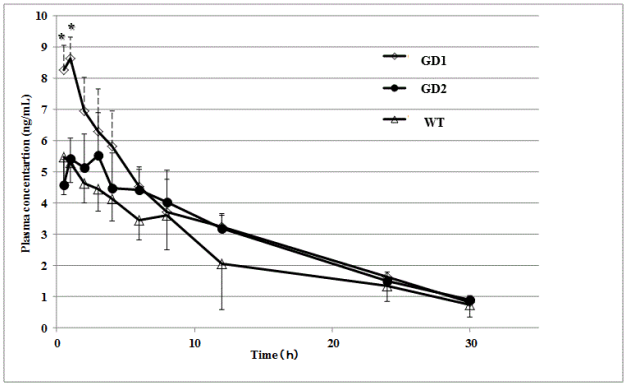

Figure 4: Plasma concentration-time profile of metoclopramide in gene deletion 1 (GD1), gene deletion 2 (GD2) and wild-type (WT) cattle after oral administration at a dose of 0.4 mg metoclopramide /kg （mean +/- standard deviation, n=4, *: significant difference between GD1 and other types (GD2 and WT) (P<0.01).

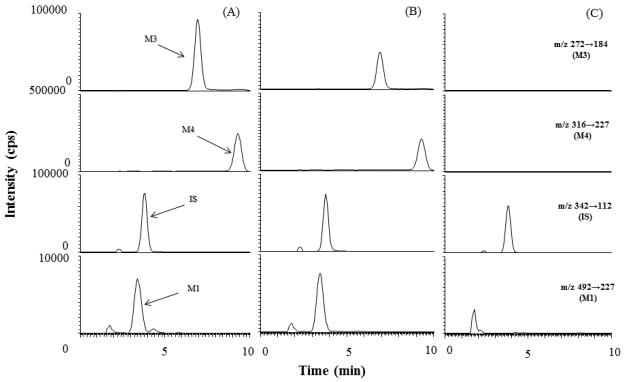

Figure 5: Typical chromatograms of metoclopramide metabolites and internal standard (IS) in gene deletion 1 cattle plasma (A) and in wild-type cattle plasma (B) spiked with IS at 1 h after oral administration at a dose of 0.4 mg/kg and in blank plasma spiked with IS (C).

Sampling time (h)	0.5			**Dunnett's test**	1			**Dunnett's test**
Experimental Group	GD1	GD2	WT		GD1	GD2	WT	
Peak height ratio of metabolite/ metoclopramide	Mean (SD)	Mean (SD)	Mean (SD)		Mean (SD)	Mean (SD)	Mean (SD)	
M3/metoclopramide	0.39 ± 0.24	0.58 ± 0.33	0.49 ± 0.24	NS	0.47 ± 0.23	0.57 ± 0.28	0.39 ± 0.16	NS
M4/metoclopramide	1.51 ± 0.54	2.72 ± 1.45	2.88 ± 1.03	NS	1.91 ± 0.21	2.54 ± 0.98	2.22 ± 0.34	NS
M1/metoclopramide	0.03 ± 0.01	0.07 ± 0.04	0.05 ± 0.02	NS	0.04 ± 0.01	0.08 ± 0.04	0.07 ± 0.02	NS
Sampling time (h) Experimental group	0.5			**Dunnett's test**	1			**Dunnett's test**
	GD1	GD2	WT		GD1	GD2	WT	
Peak height ratio between 2 different metabolites	Mean (SD)	Mean (SD)	Mean (SD)		Mean (SD)	Mean (SD)	Mean (SD)	
M3/M4	0.24 ± 0.08	0.21 ± 0.09	0.16 ± 0.04	NS	0.24 ± 0.11	0.26 ± 0.07	0.17 ± 0.05	NS
M3/M1	14.51 ± 2.77	8.08 ± 3.18	10.71 ± 0.79	NS	10.81 ± 3.24	7.06 ± 0.75	5.25 ± 1.35	GD1>WT**
M4/M1	63.29 ± 20.57	38.48 ± 8.93	67.82 ± 10.33	WT>GD2*	47.33 ± 14.1	28.38 ± 7.42	30.83 ± 5.1	GD1>WT*

Table 5: Peak height ratio between each metabolite and metoclopramide and between 2 different metabolites in MS chromatograms at 0.5 and 1h after oral administration of metoclopramide to gene deletion 1 (GD1), gene deletion 2 (GD2) and wild-type (WT) cattle (mean ± standard deviation (SD), n=4). NS: non-significant. *: P<0.05. **: P<0.01.

Discussion

Although the CYP450 family members present in different animals and their roles in veterinary drug interactions have been reviewed [25], little is known about the CYP450 types in cattle. This paper represents the first examination of the relationship between CYP2D14 gene deletion and the pharmacokinetics of metoclopramide in JB cattle. The AA sequences of CYP2D subfamily members show high similarity between human and cattle [16], and CYP2D14 is an important phase I drug-metabolizing enzyme for veterinary medicine in cattle.

The CYP2D protein in the GD1 and GD2 cattle liver microsomes were detected; however, those were lower concentrations than the CYP2D protein in the WT cattle liver microsome. It is suggested that the expression levels of CYP2D protein in the GD1 and GD2 cattle livers are lower than those in the WT cattle livers.

Therefore, we examined the metoclopramide pharmacokinetics in CTP2D14 gene deletion and WT cattle, to determine whether this genetic polymorphism had an effect. As in our previous report [20], we used a three-compartment model of pharmacokinetics after IV administration of metoclopramide to cattle. Other pharmacokinetic studies after oral administration of metoclopramide to human subjects [26] and after IV administration of metoclopramide to patients with chronic renal failure (CRF) [27,28] utilised a three-compartment model with the central, hepatoportal and peripheral compartments.

In the IV administration study, the mean plasma concentration at 24 h, mean HL of the elimination phase and mean K_{31} in GD1 cattle were significantly higher than those in GD2 or WT cattle. The mean terminal HL of WT cattle observed after IV administration in this study (5.91 h) was similar to values previously reported in human (5.1–6 h, Harrington et al. [29]) and steers (7 h, Jones et al. [30]). Magueur et al. [31] observed that the plasma concentration and HL of metoclopramide in patients with liver cirrhosis were greater than those in normal subjects, as a consequence of a 50% lower clearance in the patients. These results suggest that the longer elimination HL and K_{31} values in GD1 cattle compared to GD2 and WT cattle are due to an enzyme deficiency of the GD1 cattle.

In Bateman et al. [27], total clearance after IV administration of metoclopramide to patients with CRF was approximately 30% of that in normal subjects and the terminal HL was prolonged. However, renal clearance was approximately 20% of the total clearance in both CRF patients and normal subjects. They concluded that the reduced elimination rate of metoclopramide in CRF was due to either impaired metabolism or alteration of the enterohepatic circulation. The latter mechanism may be responsible for the high plasma concentration at 24 h in GD1 cattle in our study. The higher α and plasma

concentration at 24 h after IV administration in GD1 cattle compared to WT cattle may cause an increase in the enterohepatic [28,32] or gasteroenteric circulation of metoclopramide [33].

When metoclopramide was administered orally to cattle, the plasma concentrations up to 1 h, Cmax, mean K_{10} and mean K_{10} HL in GD1 cattle had significantly larger values compared to those in GD2 and WT cattle. Taken together, these results suggest that GD1 cattle are unable to metabolize metoclopramide in the liver after its absorption because of a deficiency or absence of the CYP2D14 enzyme in metoclopramide metabolism. The first-pass effect was shown to eliminate 25–40% of the volume of oral metoclopramide administered to humans [34]. In human, CYP2D6 mainly metabolizes metoclopramide to a monodeethyl metabolite [21,35]. Jones et al. [18] reported that a major metabolite of metoclopramide in cattle is the monodeethyl metabolite, which suggests that cattle and human have the same major metabolite (M3).

From these findings, it can be supposed that metoclopramide is metabolized by the cattle CYP2D subfamily, similar to human. Van der Padt et al. [36] found that CYP2D6*4/*4 and *4/*5 caused delayed metabolism of metoclopramide in human. Similarly, Pass et al. [37] reported that the CYP2D6 gene deletion delayed metabolism of cyclophosphamide in human. Furthermore, the HLs of metoclopramide in liver cirrhosis patients administered IV and oral metoclopramide were longer than those in normal subjects [31]. We conclude from these reports and our results that the higher plasma concentration and delayed metabolism of metoclopramide in GD1 cattle may be caused by deletion of the CYP2D14 enzyme.

It is known that the M3 metabolite is produced by CYP2D6, CYP1A2, CYP3A4, CYP2C9 and CYP2D19, on the other hand, the M4 metabolite is mainly produced by CYP2D6 and 1A2 in metoclopramide metabolism of human [22]. Ratio of M4/metoclopramide in WT cattle was higher than these in GD1 cattle, and besides, ratio of M3/M4 in WT cattle was lower than that in GD1 cattle. Ratios of M3/M1 and M4/M1 at 1 h after oral administration in GD1 cattle had significant higher than those in WT cattle ($P<0.01$, $P<0.05$, respectively). Furthermore, ratio of M1/metoclopramide in GD1 cattle was lower than these in WT cattle. It is believed that synthesis of the oxidative metabolite (M4) and the glucuronide metabolite (M1) through oxidative metabolism of metoclopramide in GD1 cattle is especially lower than that in WT cattle due to CYP2D14 gene deletion. Statistical difference between M4/M1 ratio of GD2 cattle and that of WT cattle was observed only at 0.5 h ($P<0.05$). This might be due to a trend of both high M1 and low M4 plasma concentration observed only at an early stage after administration of metoclopramide in GD2 cattle.

Conclusions

The data presented in this study suggest that the higher plasma concentration of metoclopramide in the gene deletion (type 1) cattle is caused by delayed metabolism of metoclopramide, owing to CYP2D14 enzyme deficiency.

Acknowledgements

We would like to thank Dr. Ikuko Sagawa (Support Center for Advanced Medical Sciences, Institute of Health Biosciences, University of Tokushima Graduate School) for cooperation in the cattle CYP2D detection study.

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