Simple and Sensitive Method for Measurement of Metoclopramide in Cattle Plasma by LC-MS/MS Using a Multimode Chromatography

Kouko HAMAMOTO^{1)*}, Yasuharu MIZUNO¹⁾, Masaki KATO²⁾, Norio YAMAGISHI³⁾ and Kazuhisa FURUHAMA²⁾

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

²⁾Department of Veterinary Basic Medicine, Faculty of Agriculture, Iwate University, 3-18-8 Ueda, Morioka, Iwate 020-8550, Japan

³⁾Department of Veterinary Clinical Medicine, Faculty of Agriculture, Iwate University, 3-18-8 Ueda, Morioka, Iwate 020-8550, Japan

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ABSTRACT. A quantitative assay method using LC/ESI-MS/MS for simultaneous determination of MCP in cattle plasma was developed and validated. Chromatographic separation was carried out using a multimode column (2 × 150 mm, 3 μm) with gradient elution (0.05% formic acid/methanol with 0.05% formic acid). MCP and levosulpiride (internal standard) were analyzed in the precursor/product ion pair of m/z 300.1/226.9 and 342.0/112.0, respectively. Linear calibration curves were obtained in the range of 2.5–500 ng/ml ($R^2 > 0.999$) with a lower limit of quantification of 0.05 ng/ml. Mean recoveries were 96–103%, and the coefficient of variation was less than 6.5%. Plasma MCP concentrations after intravenous administration at 0.4 mg/kg to 12 cattle were determined by the LC-MS/MS method.

KEY WORDS: cattle, LC-MS/MS, metoclopramide, pharmacokinetics, plasma.

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Metoclopramide (MCP: 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, swines, cats and dogs in Japan. MCP's action results from antagonism at dopamine-2 (D_2) receptors [5] and serotonin (5-HT₃) receptors [7]. MCP also increases cholinergic receptor sensitivity to acetylcholine in the upper gastrointestinal (GI) tract [1]. MCP has efficacy in accelerated gastric emptying and reduced esophageal reflux. On the other hand, CNS side effects (shaking, muscle stiffness, etc.) have been reported in humans and goats after administration of MCP [2, 7, 8], as this agent readily crosses the blood-brain barrier [6]. Although there is a large amount a pharmacokinetic data available in humans [3, 4, 12–14, 16], there is little data about the MCP concentration in serum and urine of cattle [9–11]. According to the pharmacokinetic study in Angus cows reported by Jones *et al.* [11], MCP was administered intravenously at a dose of 4 mg/kg. In their study, the sensitivity of the HPLC-UV method adopted to determine plasma concentrations was 6 ng/ml, and the plasma concentration of MCP could only be determined until 4 hr after drug administration by this HPLC method. However, since the clinical recommended dose of MCP in Japan ranges from 0.1 to 0.4 mg/kg, the sensitivity of the analytical method needed to be lower than 6 ng/ml in order to determine plasma MCP levels in cattle over two days after intravenous administration of 0.4 mg/kg.

Analytical methods for the determination of MCP in blood

samples include high performance liquid chromatography with a UV detector (HPLC-UV) [4, 8, 9, 11, 13, 14], GC-MS [3, 10, 15] and LC-tandem mass spectroscopy (LC-MS/MS) [12, 16]. Of these methods, the LC-MS/MS method has a highest detection sensitivity in human plasma, e.g., 0.78 ng/ml (limit of quantitation: LOQ) according to Yan *et al.* [16]. However, no report is available on plasma MCP measurements in cattle by the LC-MS/MS method.

Here, a simple and sensitive analytical method utilizing LC-MS/MS was developed and validated to determine the MCP concentration in plasma in order to elucidate pharmacokinetics after an intravenous MCP administration at a clinical dose (0.4 mg/kg) to cattle.

Reagents and chemicals: MCP (100% purity) and levosulpiride, used as the internal standard (IS), were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and Sigma-Aldrich (St. Louis, MO, U.S.A.), respectively. The LC/MS grade of formic acid (about 99% purity) was purchased from Wako Pure Chemical Industries, Ltd. Other special grade reagents were also prepared.

Chromatographic conditions and instruments: The HPLC system consisted of a Nanospace SI-2/3202 degasser, a Nanospace SI-2/3201 dual pump, a Nanospace SI-2/3133 autosampler and a Nanospace SI-2/3004 column oven (Shiseido Co., Ltd., Tokyo, Japan). Samples were maintained at 4°C in the autosampler. The Tandem mass spectrometer used was the TSQ Quantum Discovery Max made by Thermo Fisher Scientific Inc. (Waltham, MA, U.S.A.). The analytic column was the TSKgel VMPak-25 (particle size: 3 μm, 2 × 150 mm, Tosoh, Co., Ltd., Tokyo, Japan). The column temperature was 40°C. The LC-MS/MS solvents were solvent A (0.05% formic acid in water, v/v) and solvent B (0.05% formic acid in methanol, v/v). The linear LC gradient for separation at a flow rate of 0.2 ml/min and the 14-min long LC gradient program included the following elution condi-

*CORRESPONDENCE TO: HAMAMOTO, K., National Veterinary Assay Laboratory, Ministry of Agriculture, Forestry and Fisheries, 1-15-1 Tokura, Kokubunji, Tokyo 185-8511, Japan.
e-mail: hamamok@nval.maff.go.jp

tions: 10–40% B in 10 min, 40–10% B in 1 min and 10% B for 3 min. The MS/MS conditions were as follows: positive mode of electrospray ionization (ESI), spray voltage of 3 kV, sheath gas pressure of 50 arbitrary units, aux gas pressure of 20 arbitrary units, capillary temperature of 300°C, capillary offset of 35 V, source CID of –5 V and argon collision gas at 1.2 mTorr. Quantification was performed using selected reaction monitoring (SRM) of the transitions observed after fragmentation: m/z 300.1 $[C_{14}H_{22}ClN_3O_2+H]^+ \rightarrow 226.9$ $[C_{10}H_{12}ClN_2O_2]^+$ (collision energy (CE)=18 eV) for MCP and m/z 342.0 $[C_{15}H_{23}N_3O_4S+H]^+ \rightarrow 112.0$ $[C_7H_{14}N]^+$ (CE=26 eV) for IS.

Preparation of calibration standards and quality control samples: The stock solution of MCP (10 $\mu g/ml$) was prepared by diluting the first MCP solution with acetonitrile. The secondary standard solution (1,000 ng/ml) and the working standard solutions were produced by diluting the secondary standard solutions with mobile phase. Nine calibration standards of MCP (final concentrations: 0.05, 0.5, 2.5, 5, 10, 25, 50, 100 and 500 ng/ml) were prepared independently at every analytical time point. The stock solutions were stored at –80°C, and the secondary solution was stored at –20°C until needed. The three quality control (QC) samples at concentrations of 5, 10 and 100 ng/ml were made by diluting the secondary solution with blank cattle plasma. The stock solution of IS (0.1 mg/ml) was prepared using acetonitrile. The working IS solution (100 ng/ml) was prepared by diluting the stock solution with acetonitrile and stored at –20°C.

Preparation of samples: Cattle plasma (500 μl) was pipetted into a clean glass tube and the working IS solution (100 μl) and acetonitrile (1 ml) were added. The mixture was vortex-mixed for 30 sec. After centrifugation at $2,073 \times g$ (3,000 rpm) for 10 min, the supernatant was evaporated to dryness at 35°C. The residue was reconstituted with the mobile phase (500 μl). Each sample was filtered using a 0.45 μm membrane filter (Ekicrodisk 13, Nippon Genetics Co., Ltd., Tokyo, Japan). The sample solution (4 μl) was injected into an LC-MS/MS system.

Validation of the method: The calibration curves of MCP were constructed from the peak area ratio of MCP/IS versus concentrations of MCP in the mobile phase. Linearity was determined using linear least-squares regression. The limit of detection (LOD) was defined as the plasma concentration that produced a signal-to-noise ratio (S/N) of 3. The LOQ was defined as the lowest plasma concentration that produced an S/N of 5 with a relative standard deviation (RSD) lower than 20% and accuracy between 80 and 120%. The matrix effect of co-extracted components in cattle plasma on the ionization of MCP was evaluated by comparing the peak areas of MCP and IS spiked in pre-extracted blank plasma with those of a relevant standard in reconstitution solvent at an equivalent concentration.

Three QC samples, in which the concentrations of MCP were 2.5, 10 and 100 ng/ml , were prepared to spike MCP in cattle blank plasma and measured by the same preparation method as plasma samples. The extraction recovery was estimated by comparing the peak area ratio of MCP to IS extracted from the QC samples ($n=6$). Seven samples, in which

the concentrations were 2.5, 5, 10, 25, 50, 100 and 500 ng/ml , were prepared to spike MCP in pre-extracted blank plasma. These samples were analyzed in each batch assay to determine intra-day and inter-day precision expressed as %RSD and accuracy (detected concentration/nominal concentration).

The stability of MCP in cattle plasma was tested using three QC samples ($n=6$) by storing at –20 and –80°C for 3 months. For stability of MCP in processed samples, the prepared QC samples ($n=6$) were placed in an autosampler at 5°C for 24 hr and then assayed for comparison.

Pharmacokinetic application: Twelve clinically healthy female Japanese Black cattle (0.9–5.2 years, 252–580 kg body weight) were used for MCP pharmacokinetics after intravenous administration of a 0.4 mg/kg dose of MCP, which is sold as a Primperan injection (Intervet K.K., Tokyo, Japan). The cattle were reared at the research farm of the Field Science Center, Iwate University (Morioka, Japan). Animals were maintained in a non-fasting state during this study. Venous blood samples (10 ml) were obtained before and at 0.25, 0.5, 0.75, 1, 2, 4, 6, 8, 12, 24 and 30 hr after MCP administration. The blood samples were immediately centrifuged, and the plasma samples were stored at –80°C until analysis. The pharmacokinetic parameters of MCP in plasma were estimated using the WinNonlin computer program (ver. 3.1, SCI Software, Lexington, KY, U.S.A.). All experimental procedures were performed in accordance with the Guidelines for Animal Experimentation issued by the Japanese Association for Laboratory Animal Science (1987) or approved by the Animal Experimental Ethics Committee of Iwate University.

LC-MS/MS method: As MCP is a basic substance ($pK_a=9.71$) [11], it was difficult to maintain in a reversed-phase HPLC column for separation. Therefore, we applied a multimode column, which can be used for reversed-phase separation and ion-exchange separation. Using this column, we separated MCP with mobile phase containing 0.05% formic acid and analyzed the MCP plasma concentrations.

We selected a gradient of 0.05% formic acid solution and methanol with 0.05% formic acid for the HPLC mobile phase in our method, because the retention times of MCP and IS in the case of using gradient mode for mobile phase was very short for determination of MCP. Selection of the IS was based on the conditions of the LC-MS/MS method reported by Lee *et al.* [12]. The SRM monitor ions selected in this study for the determination of MCP and IS were similar to those of Lee *et al.* [12].

Figure 1 shows a representative LC-MS/MS chromatogram of standard (100 ng/ml , A), blank plasma spiked with MCP, the plasma 15 min following a single intravenous administration of MCP and blank plasma. No interaction peak was observed in blank plasma under the present assay conditions. The retention time was 7.6 min for MCP and 3.8 min for IS.

The linearity of the standard calibration curves (R^2) was >0.999 for MCP levels of 0.05–500 ng/ml . The LOD and LOQ of our method were found to be 0.02 and 0.05 ng/ml , and the signal-to-noise ratios of the LOD and LOQ were 3

Fig. 1. Typical chromatograms of MCP and IS in cattle plasma. (A) standard (100 ng/ml), (B) blank plasma spiked with MCP (100 ng/ml), (C) the sample 15 min after i.v. administration at a dose of 0.4 mg/kg, (D) blank plasma.

and 10, respectively. The LOD and LOQ were more sensitive than other previous reports using LC-MS/MS (LOQ: 0.78 ng/ml [16], 2 ng/ml [12]).

Comparing the peak areas of MCP and IS spiked in pre-extracted blank plasma with those of relevant standards at equivalent concentrations, no significant increase or decrease (86–110%) was noted for either MCP or IS, indicating that the matrix effects of the endogenous plasma component on the ionization of the two analytes were negligible.

The precision and accuracy data for intra-day and inter-

day assays are shown in Table 1. This LC-MS/MS method possessed remarkable reproducibility with a precision of less than 6.5% and excellent accuracy ranging from 97.3% at 25 ng/ml to 110.0% at 500 ng/ml (n=6). Extraction recoveries of MCP were found to be satisfactory, with the average value ranging from 96.0 to 103.4% at the three QC concentrations (n=6).

The stability study showed that the concentration of MCP was not significantly changed in plasma stored at either –20 or –80°C for three months and in prepared plasma samples

Table 1. Recovery, precision and accuracy of MCP in plasma by the LC-MS/MS assay method (n=6)

Concentration in control plasma (ng/ml)	Mean recovery (%)	CV (%)
2.5	97.1	3.4
10	96.0	5.7
100	103.4	1.3

Concentration in pre-extracted control plasma (ng/ml)	Intra-day		Inter-day	
	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)
2.5	3.9	100.0	6.0	98.8
5	3.4	98.5	5.3	97.4
10	2.9	98.3	6.5	98.4
25	1.8	100.1	5.3	97.3
50	2.5	90.1	4.8	98.7
100	1.8	99.3	4.4	96.1
500	0.3	109.1	0.4	110.0

stored in an autosampler at 5°C for 24 hr. At a concentration of 2.5 ng/ml, the average remaining values (n=5) in these three states were 92.6, 96.8 and 98.7%. At a concentration of 10 ng/ml, the average remaining values (n=5) were 97.8, 98.0 and 98.1%. At a concentration of 100 ng/ml, the average remaining values (n=5) were 98.3, 103.6 and 97.2%. These results suggested that MCP had high stability under these three storage conditions.

Application: The present method was successfully applied to measure the plasma concentration of MCP up to 30 hr after MCP administration to cattle. The mean plasma concentration-time curve is illustrated in Fig. 2, and its estimated pharmacokinetic parameters are shown in Table 2. The pharmacokinetic parameters were characterized with a three-compartment model. The apparent volume of distribution (Vd) and the terminal half-life [half-life ($T_{1/2}$) of gamma phase] were 1.2 ± 1.1 l/kg and 8.4 ± 5.0 hr, respectively. The mean $T_{1/2}$ beta (1.3 hr) and mean $T_{1/2}$ alpha (0.3 hr) in our study were similar to the mean terminal half-life ($T_{1/2}$ beta: 0.9 hr) and the mean $T_{1/2}$ alpha (0.4 hr) reported by Jones *et al.* [11], which were estimated using a two-compartment model following intravenous administration to Angus cows at 4 mg/kg. On the other hand, the Vd obtained in our study was within the range of the Vd results (0.5 ± 0.2 l/kg following intravenous administration to Angus steers at 8 mg/kg and 2.1 ± 0.8 l/kg following intravenous administration to Angus cows at 4 mg/kg) reported by Jones *et al.* [11]. They also indicated that the plasma MCP concentration of Angus cows after intravenous administration (4 mg/kg) in their study became undetectable by the HPLC determination method (LOQ: 6 ng/ml) after more than 4 hr [11]. It is suggested that the difference between the results of Jones *et al.* [11] and those obtained by us may be caused by a difference in the dosage level, strain or length of the detectable period of MCP in the plasma used.

Fig. 2. Plasma concentration-time profile of MCP in cattle after i.v. administration at a dose of 0.4 mg MCP/kg (mean $\pm$ SD, n=12).Table 2. Pharmacokinetic parameters of plasma MCP in cattle after intravenous administration at a dose of 0.4 mg MCP/kg (mean $\pm$ SD, n=12)

PK parameter	Mean	SD	Unit
A	1170.12	1689.84	ng/ml
B	107.08	168.54	ng/ml
C	6.24	13.77	ng/ml
α	5.54	4.05	hr ⁻¹
β	0.81	0.68	hr ⁻¹
γ	0.11	0.07	hr ⁻¹
C _{max} ^{a)}	1247.57	1707.47	ng/ml
V _d	1.23	1.05	l/kg
K ₁₀ ^{b)}	3.06	2.75	hr ⁻¹
$T_{1/2}K_{10}$	0.41	0.32	hr
$T_{1/2}\alpha$	0.29	0.32	hr
$T_{1/2}\beta$	1.33	1.08	hr
$T_{1/2}\gamma$	8.36	4.94	hr
AUC _{0-inf} ^{c)}	313.97	287.61	ng·hr/ml
Cl _{total} ^{d)}	1.89	0.95	l/hr·kg
AUMC ^{e)}	637.3	419.31	ng·hr ² /ml
MRT ^{f)}	2.63	1.78	hr
V _{ss} ^{g)}	5.64	3.79	l/kg

Three-compartment open model, $C(t)=Ae^{-\alpha t}+Be^{-\beta t}+Ce^{-\gamma t}$; a) C_{max}, maximum plasma concentration; b) K₁₀, elimination rate; c) AUC, area under the concentration-time curve; d) Cl, total body clearance; e) AUMC, area under the first moment curve; f) MRT, mean residence time; g) V_{ss}, volume of distribution at steady state.

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