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Liquid chromatography–tandem mass spectrometric method for determination of mosapride citrate in equine tissues

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Abstract

A simple method for determination of mosapride citrate and its metabolite, des-*p*-fluorobenzyl mosapride (M-1), in equine muscle, liver, kidney, adipose tissue and intestine by liquid chromatography–tandem mass spectrometry has been developed. (±)-4-Amino-5-chloro-2-ethoxy-*N*-[[4-(2-chlorobenzyl)morpholinyl]methyl]benzamide was used as an internal standard. The analytes and internal standard were spiked and extracted from tissues by acetonitrile. The chromatographic separation was performed on a reversed-phase TSK-GEL SUPER ODS column with a mobile phase of acetonitrile–0.05% (v/v) formic acid containing 5 mmol/L nonafluoropentanoic acid (2:3, v/v). The method exhibited a large linear range from 0.0005 to 0.2 μg/mL for both mosapride citrate and M-1 ($r > 0.9976$). In the intra-day assay ($n = 5$), the relative standard deviations (RSDs) ranged from 1.1 to 7.8% for mosapride citrate and 1.6 to 7.2% for M-1. In the inter-day assay ($n = 3$), the RSDs ranged from 1.0 to 13% for mosapride citrate and 0.8 to 11% for M-1. The extraction recovery at 1.28 μg/g of mosapride citrate from equine tissues ranged from 97 to 107%. The lower limit of quantification for mosapride citrate was found to be 0.004 μg/g. Stability studies were carried out at different storage conditions. The method reported is reliable, precise, and accurate and it has the capacity to be used for determination of mosapride citrate and its metabolite in tissue samples.

Keywords: Mosapride; Equine tissues; LC/MS/MS

1. Introduction

Mosapride citrate is a benzamide derivative that possesses a gastrointestinal prokinetic activity [1]. Mosapride citrate does not antagonize the dopamine D2 receptors [2], but selectively acts as an agonist at the serotonin (5-HT_4) receptors [3,4], enhancing gastrointestinal motility by accelerating acetylcholine release from neurons in the gastrointestinal tract [5]. The enhancing effect on gastric motility and emptying does not have influence on autonomic nervous activity, suggesting that mosapride citrate may be very useful for elderly patients or patients with cardiac complications [6]. Recently, mosapride citrate has been reported to improve symptoms in patients with gastroesophageal reflux disease [7,8] and to ameliorate constipation and response fluctuations in Parkinsonian patients [9,10]. In addition, mosapride citrate has beneficial effects on glycemic control in patients with Type 2 diabetes mellitus by improving insulin sensitivity [11]. Considering these reports that indicated the efficacy of mosapride citrate on human, this compound has a potential to be used in the field of veterinary medicine and animal science.

Recently, it was demonstrated that mosapride promoted motility in the small intestine and cecum of horses and that the optimal orally administered dosage was 1.5 to 2 mg/kg, suggesting that mosapride is useful for treatment of equines with gastrointestinal tract dysfunction [12]. Because people eat horse meat in Belgium, France, and Japan, this report suggests that horse meat, which is from a horse treated with mosapride, will go on the market in the future. In addition, pet food industries produce a variety of pet foods that contain horse meat in a wider

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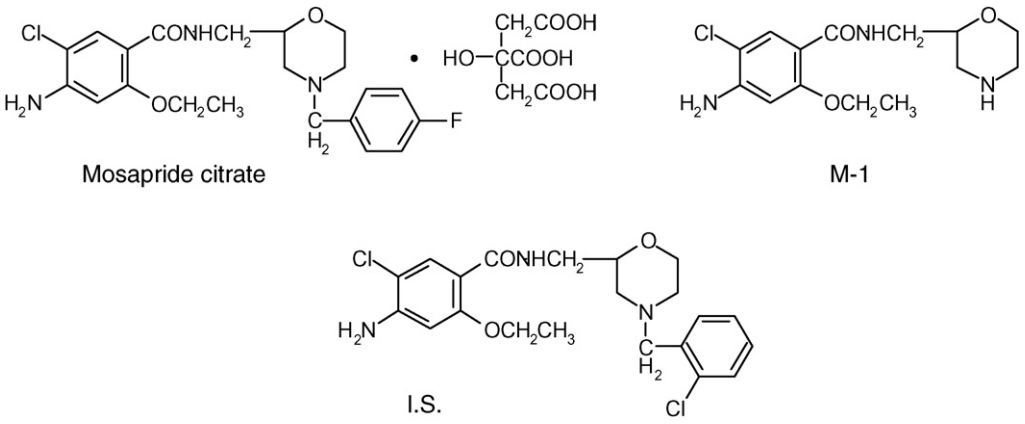

Fig. 1. Structures of mosapride citrate ((±)-4-amino-5-chloro-2-ethoxy-*N*-[[4-(4-fluorobenzyl)-2-morpholinyl]methyl]benzamide citrate), M-1 (des-*p*-fluorobenzyl mosapride; (±)-4-amino-5-chloro-2-ethoxy-*N*-[(2-morpholinyl)methyl]benzamide), and I.S. ((±)-4-amino-5-chloro-2-ethoxy-*N*-[[4-(2-chlorobenzyl)morpholinyl]methyl]benzamide).

selection of countries. To keep the safety of horse meat, it is necessary to determine mosapride contents in horse meat. For this purpose, developing a method for determination of mosapride in horse meat is a prerequisite step.

Several methods are available to determine mosapride citrate. High-performance liquid chromatography (HPLC) with UV detection was used to determine mosapride in bulk drugs or pharmaceutical preparations [13–15]. The enantioseparation and enantioselective determination by HPLC with UV detection [16,17] or HPLC with fluorometric detection [18] were also reported. Pharmacokinetic profiles of mosapride in rats [19], dogs, monkeys [20], and healthy human subjects [21] have been well characterized by HPLC with UV detection. Recently, liquid chromatography–tandem mass spectrometry (LC/MS/MS) was introduced to detect a polar impurity in the bulk drugs of mosapride citrate [22]. LC/MS/MS was further applied to the determination of mosapride in human plasma [23]. However, a through literature search showed that there were no reports that described the determination of mosapride in human or animal tissue samples, except for the tissue distribution analyses of ^{14}C-labeled mosapride in rats, dogs, and monkeys [24,25].

Among a number of methods for determination of mosapride, LC/MS/MS approaches have apparent advantages with respect to their high sensitivity and specificity when biological samples are analyzed. Thus, in the present study, an LC/MS/MS method for determination of mosapride citrate in equine tissues was developed. Moreover, an attempt to apply the present method to simultaneous determination of mosapride and its metabolite in equine tissues was also carried out.

2. Experimental

2.1. Materials and reagents

((±)-4-Amino-5-chloro-2-ethoxy-*N*-[[4-(4-fluorobenzyl)-2-morpholinyl]methyl]benzamide citrate), des-*p*-fluorobenzyl mosapride ((±)-4-amino-5-chloro-2-ethoxy-*N*-[(2-morpholinyl)methyl]benzamide; M-1), and (±)-4-amino-5-chloro-2-ethoxy-*N*-[[4-(2-chlorobenzyl)morpholinyl]methyl]benzamide (I.S.) (Fig. 1) were obtained from Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan). Nonafluoropentanoic acid was purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). Methanol (HPLC grade) and acetonitrile (HPLC grade) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Other reagents were of reagent or HPLC grade available from commercial sources.

2.2. Standard solutions

A stock standard solution mixture of mosapride citrate and M-1 (100 μg/mL each) was prepared by dissolution in methanol and diluted with methanol to 10 μg/mL. Mosapride citrate was provided as a dihydrate form, but for preparation of the standard solution, it was calculated as an anhydrate form. Internal standard solution was also prepared by dissolution of I.S. in acetonitrile (100 μg/mL) and diluted with acetonitrile to 10 μg/mL. The standard solution mixture of mosapride citrate and M-1 (10 μg/mL) was diluted with the blank extraction solution to 0.001, 0.002, 0.01, 0.02, 0.1, 0.2, and 0.4 μg/mL. The blank extraction solution was prepared by extraction of 5.0 g of a frozen blank tissue sample with 20 mL of acetonitrile in the presence of 0.5 g of NaCl and 0.4 μg of I.S. without addition of mosapride citrate and M-1 (see Section 2.3). The diluted standard solution mixtures and 0.05% (v/v) formic acid containing 5 mmol/L nonafluoropentanoic acid were mixed by 1:1 (v/v) ratio to prepare 0.0005, 0.001, 0.005, 0.01, 0.05, 0.1, and 0.2 μg/mL of mosapride citrate and M-1 for calibration curves.

2.3. Sample preparation

Equine muscle, liver, kidney, adipose tissue, and intestine from healthy adult Thoroughbreds stored below −18 °C were used under the approval of the Research Institute for Animal Science in Biochemistry and Toxicology. The ethics approval was given by the Law for the Humane Treatment and Management of Animals in Japan (Law No. 105, 1973; the amendment on December 1, 2000). To preserve these samples, tissue samples collected from a euthanized horse was immediately frozen in a

Table 1
Analyte-specific MS/MS parameters

	Mosapride	M-1	I.S.
Q1 mass (*m/z*)	422.3	314.3	438.3
Q3 mass (*m/z*)	198.1	198.3	198.2
Dwell time (ms)	300	300	300
DP[a] (V)	56	56	51
FP (V)	370	350	360
CE[b] (eV)	29	25	29
EP (V)	10	10	10
CXP (V)	12	12	12

Abbreviations used are: DP, declustering potential; FP, focusing potential; CE, collision energy; EP, entrance potential; CXP, collision cell exit potential.
[a] DP is the difference between voltages at the orifice plate and skimmer.
[b] CE is the force of the current of fragmentation.

Table 2
Calibration curves of mosapride citrate and M-1

Analyte	Tissues	Regression equation ($y^a = ax + b$)		*r*
		a	*b*	
Mosapride citrate	Muscle	7.272	0.00626	0.999
	Liver	7.961	0.00516	0.999
	Kidney	7.727	0.00358	0.998
	Adipose tissue	7.640	0.00610	0.999
	Intestine	7.979	0.00632	0.998
M-1	Muscle	6.602	0.00384	0.999
	Liver	5.784	0.00886	0.999
	Kidney	5.212	−0.00086	0.999
	Adipose tissue	5.241	0.00364	0.999
	Intestine	5.449	0.00489	0.999

[a] *y* is the ratio of peak area (Q3 mass) of the analyte vs. I.S. *x* is concentration of the analyte (0.0005–0.2 μg/mL).

sealed plastic bag below −18 °C and used within 6 months. No additional treatment was provided to the samples between their collection and preservation. A piece of frozen samples (5.0 g) was weighed, 40 μL of the internal standard solution (10 μg/mL) and an appropriate amount of mosapride citrate and M-1 were added, and it was allowed to stand at 25 °C for 5 min. To this mixture, 20 mL of acetonitrile and 0.5 g of NaCl were added and homogenized by a Physcotron NS-50W (Microtec Co., Ltd., Funabashi, Japan) for 1 min. The homogenate was centrifuged at 1300 × *g* for 5 min at 5 °C (CR 5DL, Hitachi), and the supernatant was collected as an extraction solution. The extraction solution was mixed well with an equal volume of 0.05% (v/v) formic acid containing 5 mmol/L nonafluoropentanoic acid, and the resulting mixture was injected into LC/MS/MS. To determine recovery of mosapride citrate or M-1, an appropriate amount of mosapride citrate or M-1 in 100 μL of methanol was added to 5.0 g of frozen tissue samples. The samples were treated as described above and its recovery was determined.

2.4. LC/MS/MS

The LC/MS/MS system consisted of an HP 1100 HPLC system (Agilent Technologies, Inc., Palo Alto, CA, USA), an HTC-PAL autosampler (CTC Analytics, Zwingen, Switzerland), an API 3000 triple-quadrupole mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with a Turboionspray ionization source, and a working station installed with an Analyst® ver. 1.4 software (Applied Biosystems). The analytical column was TSK-GEL SUPER ODS (20 mm i.d. × 10 cm) (Tosoh Co., Tokyo, Japan) and its temperature was maintained at 40 °C. The injection volume was 10 μL. The mobile phase consisted of acetonitrile–0.05% (v/v) formic acid containing 5 mmol/L nonafluoropentanoic acid (2:3, v/v). The flow rate was 0.2 mL/min. The temperature of the autosampler for sample collection was set at 10 °C.

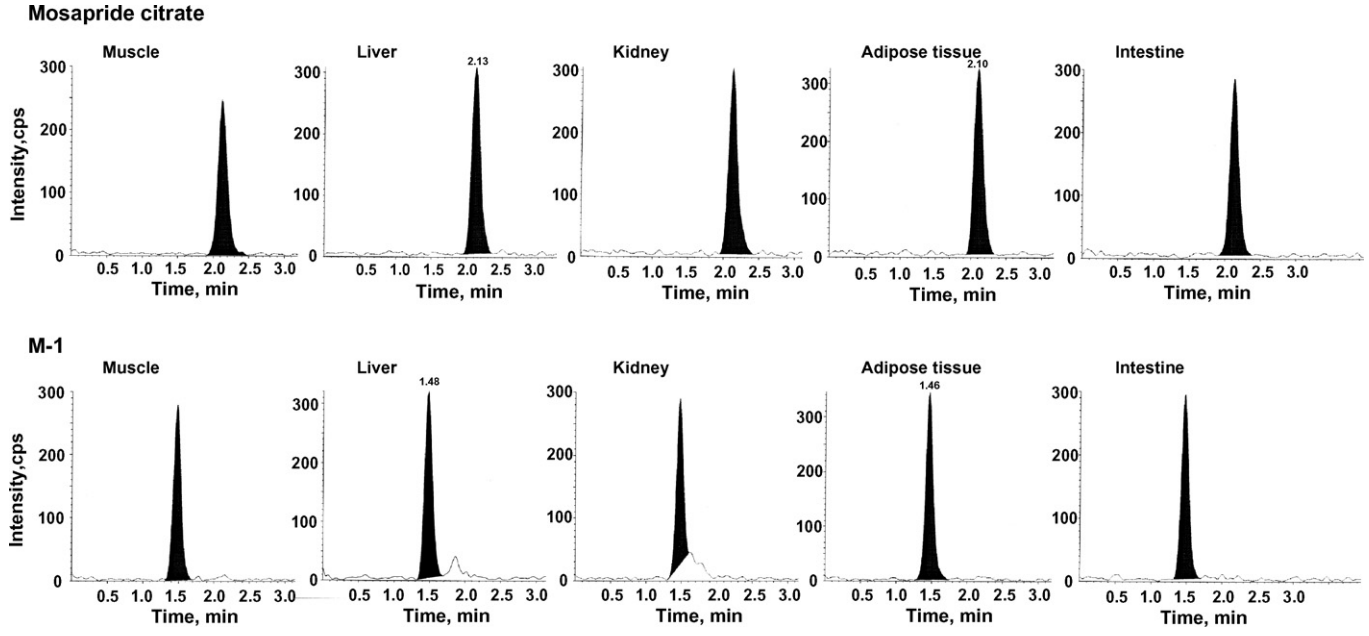

Fig. 2. LC/MS/MS chromatograms of standard mosapride citrate and M-1 at the concentration of 0.0005 μg/mL. The LC/MS/MS conditions were same as described in Section 2.

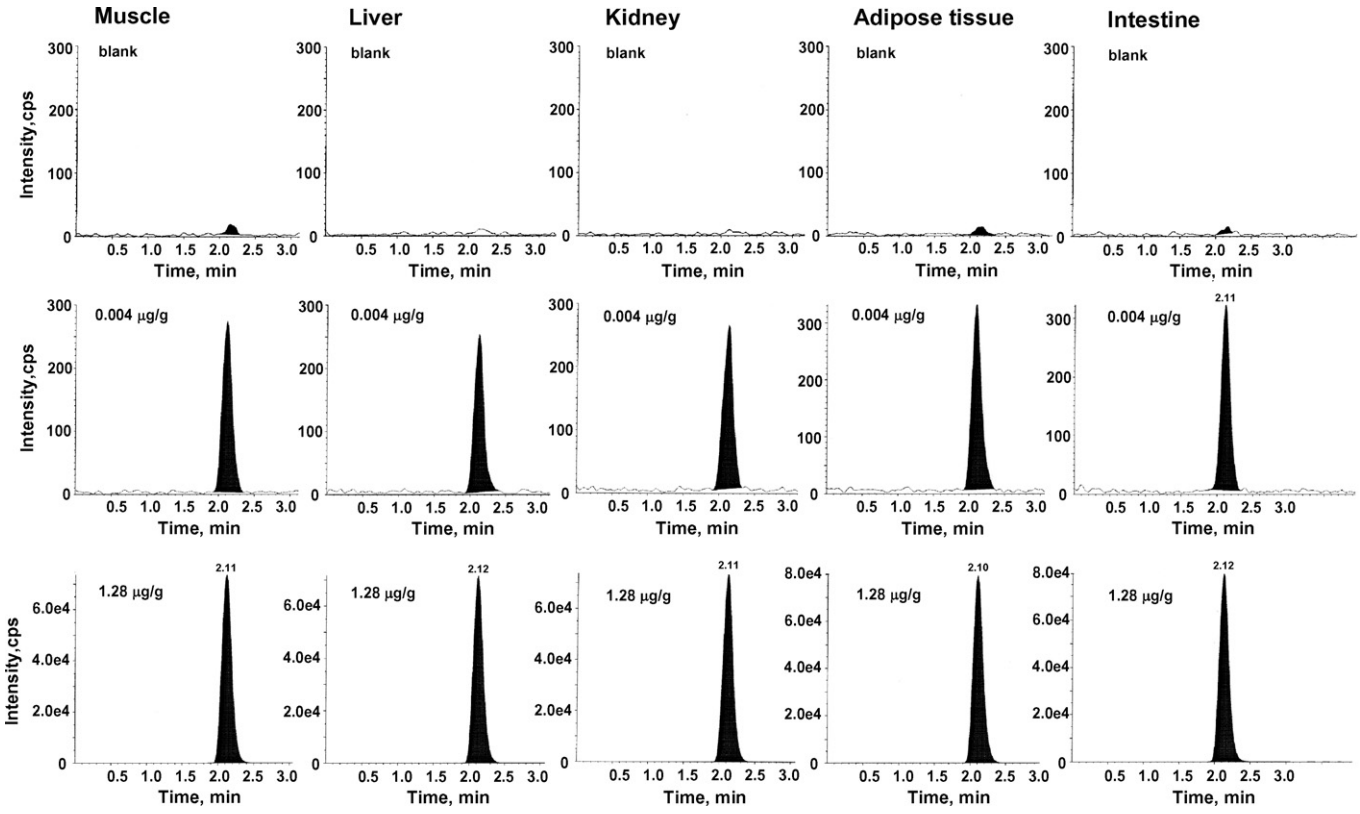

Fig. 3. LC/MS/MS chromatograms of mosapride citrate spiked at 0 (blank), 0.004, and 1.28 μg/g into equine muscle, liver, kidney, adipose tissue, and intestine. The LC/MS/MS conditions were same as described in Section 2.

3. Results and discussion

3.1. Method development

MS data were acquired in the positive ion electrospray ionization (ESI) mode by multiple reaction monitoring (MRM). Source parameters including curtain gas pressure, temperature, and ion spray voltage were optimized by flow injection analysis. This gave following conditions: curtain gas pressure, 12 psi; source temperature, 450 °C; ion spray voltage, 4500 V; collisionally activated dissociation (CAD) gas pressure, 10 psi; Turboionspray gas flow rate, 7.0 L/min. Compound parameters including declustering potential (DP), focusing potential (FP), entrance potential (EP), collision energy (CE), and collision cell exit potential (CXP) were also optimized. The individual compound parameters and analytical conditions are listed in Table 1.

The mass transitions for individual analytes from a precursor ion in the first quadrupole (Q1 mass) to a product ion in the third quadrupole (Q3 mass) were selected (Table 1) based on the positive ESI MS/MS spectrum, and they were used for determination. The mass transition m/z 422 → 198 for mosapride confirmed several previous reports [15,22,23]. The fragmentation pathways have been described previously in several papers [15,22,26].

To select the internal standard, blank tissue samples were analyzed by the present method. The peak area in blank samples at the elution position of I.S. ((±)-4-Amino-5-chloro-2-ethoxy-*N*-[[4-(2-chlorobenzyl)morpholinyl]methyl]benzamide) was less than 1% of 0.01 μg/mL of I.S. in all tissues, suggesting that endogenous compounds do not interfere with the determination. Thus, we selected I.S. as the internal standard for determination of mosapride citrate and M-1.

3.2. Method validation

3.2.1. Calibration curves

To test the correlation of concentrations and signals for calibration purposes, seven-point calibration curves were created. The ratio of the peak area of mosapride and M-1 vs. I.S. was found to be linear over the large working concentration range of 0.0005–0.2 μg/mL with correlation coefficient values ranging from 0.998 to 0.999 in all tissues examined (Table 2). Fig. 2 shows representative LC/MS/MS chromatograms (Q3 mass) of the standard mosapride and M-1 at the lower limit of quantification (LLOQ).

3.2.2. Specificity

Blank tissue samples ($n = 5$) were analyzed to test specificity. The upper panels of Fig. 3 (mosapride) and 4 (M-1) represent LC/MS/MS chromatograms in blank tissue samples. Although there were some interfering peaks of endogenous compounds at the elution position of mosapride and M-1 in some tissues, the peak area did not exceed 20% of the peak area of the spiked mosapride citrate (0.004 μg/g, middle panel, Fig. 3) and M-1 (0.004 μg/g, middle panel, Fig. 4). As mentioned below, the LLOQ of the present method was 0.004 μg/g for mosapride cit-

Table 3
Intra-day recovery[a], inaccuracy[b], and imprecision[c] of mosapride citrate and M-1

Tissues	Spiked concentration (μg/g)	Mosapride citrate			M-1		
		Recovery (%)	Recovery −100 (%)	RSD (%)	Recovery (%)	Recovery −100 (%)	RSD (%)
Muscle	0.004	100	0	6.0	73	−27	4.8
	0.08	105	5	1.2	82	−18	4.3
	1.28	107	7	2.5	92	−8	1.6
Liver	0.004	104	4	7.8	73	−27	4.7
	0.08	97	−3	1.9	74	−26	2.6
	1.28	97	−3	2.2	80	−20	2.6
Kidney	0.004	85	−15	4.0	70	−30	7.1
	0.08	102	2	2.5	90	−10	5.3
	1.28	107	7	2.4	101	1	2.6
Adipose tissue	0.004	98	−2	3.9	91	−9	5.1
	0.08	99	−1	1.7	100	0	5.3
	1.28	101	1	1.1	106	6	3.8
Intestine	0.004	82	−18	4.5	75	−25	7.2
	0.08	100	0	1.9	96	−4	2.3
	1.28	105	5	1.4	102	2	1.9

[a] Mean recovery was determined with five replicates for each concentration in one day.
[b] Inaccuracy was expressed as recovery −100 (%).
[c] Imprecision was expressed as RSD (%).

rate as well as M-1. Therefore, the results satisfy one of the FDA criteria of the LLOQ [27] where the analyte response at the LLOQ should be at least five times the response compared to blank response. All these results clearly show that the present method is highly specific.

3.2.3. Accuracy and precision

Accuracy and precision were assessed by the calculation of intra-day and inter-day variability of recovery of mosapride citrate and M-1 (spiked and extracted) from tissue samples at known concentrations corresponding to low (0.004 μg/g),

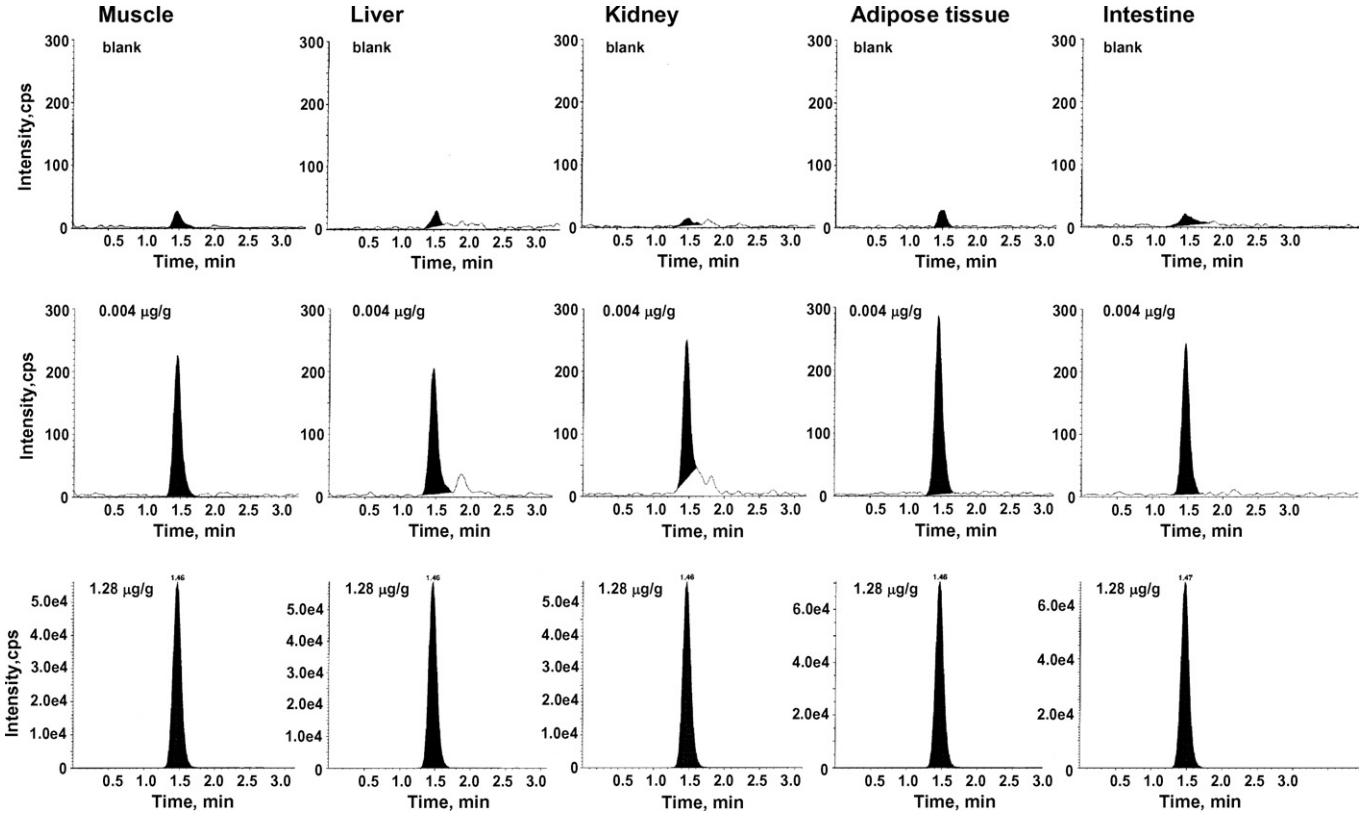

Fig. 4. LC/MS/MS chromatograms of M-1 spiked at 0 (blank), 0.004, and 1.28 μg/g into equine muscle, liver, kidney, adipose tissue, and intestine. The LC/MS/MS conditions were same as described in Section 2.

Table 4
Inter-day recovery[a], inaccuracy[b], and imprecision[c] of mosapride citrate and M-1

Tissues	Spiked concentration (μg/g)	Mosapride citrate			M-1		
		Recovery (%)	Recovery −100 (%)	RSD (%)	Recovery (%)	Recovery −100 (%)	RSD (%)
Muscle	0.004	104	4	4.4	77	−23	4.5
	0.08	106	6	3.0	77	−23	5.5
	1.28	107	7	2.3	83	−17	9.2
Liver	0.004	102	2	3.4	70	−30	7.3
	0.08	100	0	2.3	73	−27	2.9
	1.28	98	−2	1.0	79	−21	2.9
Kidney	0.004	88	−12	4.5	74	−26	9.1
	0.08	102	2	3.9	87	−13	6.8
	1.28	104	4	4.4	90	−10	11
Adipose tissue	0.004	109	9	9.2	94	−6	3.3
	0.08	105	5	5.7	99	−1	5.1
	1.28	105	5	3.4	106	6	5.0
Intestine	0.004	98	−2	13	73	−27	0.8
	0.08	102	2	5.2	89	−11	7.2
	1.28	106	6	0.9	94	−6	8.0

[a] Mean recovery was determined with three replicates ($n = 1$ for 3 days of assay) for each concentration.
[b] Inaccuracy was expressed as recovery −100 (%).
[c] Imprecision was expressed as RSD (%).

medium (0.08 μg/g) and high (1.28 μg/g). Intra-day data were obtained by analyzing five sets of samples in 1 day (Table 3). The intra-day inaccuracy, expressed as "recovery −100 (%)," ranged −18 to 7% for mosapride citrate and −30 to 6% for M-1. The intra-day imprecision, expressed as relative standard deviation (RSD), ranged 1.1–7.8% for mosapride citrate and 1.6–7.2% for M-1. Inter-day data were obtained by analyzing one set of samples on three different days of assay (Table 4). The inter-day inaccuracy ranged −12 to 9% for mosapride citrate and −30 to 6% for M-1. The inter-day imprecision ranged 1.0–13% for mosapride citrate and 0.8–11% for M-1. From the results shown in Tables 3 and 4, we consider that the present method is sufficiently accurate and precise.

3.2.4. Sensitivity

Sensitivity was evaluated by LLOQ of mosapride citrate and M-1. The LLOQ of the present method was 0.004 μg/g for mosapride citrate as well as M-1. At the concentration, the signal-to-noise (S/N) ratio was more than 10 in all tissues. The intra-day inaccuracy ranged −18 to 4% for mosapride citrate and −30 to 9% for M-1 (Table 3). The intra-day imprecision ranged 3.9–7.8% for mosapride citrate and 4.7–7.2% for M-1 (Table 3). As described above, there were no interfering peaks of endogenous compounds that exceed 20% of the peak area of the spiked mosapride citrate and M-1 (0.004 μg/g) at the elution position of these compounds in all tissues. The level of LLOQ seemed to be enough to determine the amount of mosapride residues in tissues after oral administration, based on the results of the tissue distribution analyses of ^{14}C-labeled mosapride in rats, dogs, and monkeys [23,24]. These results show that the present method is sensitive enough for the tissue distribution analyses of mosapride.

3.2.5. Matrix effects

Real samples may have varying amounts of matrix effects [28,29]. To test this possibility, the matrix from five different tissues (equine muscle, liver, kidney, adipose tissue, and intestine) was added to the standard solution of mosapride citrate and M-1 to create matrix standard curves. The tissue matrix (the blank extraction solution) was prepared seven times in a different day, offering seven standard curves in each tissue. Table 2 shows a representative set of the seven sets of the matrix standard curves. To evaluate the matrix effects, statistical differences in the slope and the y-intercept of the seven sets of the matrix standard curves among five tissues were examined. Bartlett's test was used to show that the slope and the y-intercept had equal variances before testing analysis of variance (ANOVA). Next, One-way ANOVA was used to test for differences of the slope and the y-intercept among tissues. The results showed that for both mosapride citrate and M-1 there were no significant differences in the slope and the y-intercept among five tissues ($p < 0.05$). These results indicated that different tissue matrices did not affect the standard curves of mosapride citrate and M-1, concluding that matrix effects can be negligible in the current method.

3.2.6. Stability

Three stability studies (post-preparative stability, stock solution stability, and stability in frozen tissues) of mosapride citrate and M-1 were performed. First, post-preparative stability was examined. The standard solutions for calibration curves that contain the blank extraction solution were stored at 5 °C in the dark for 7 days. We selected 7 days, because 7 days seemed to be enough for practical purposes. Calibration curves were drawn and compared with those of freshly prepared solutions.

Table 5
Stability[a] of mosapride citrate and M-1 in frozen tissues

Tissues		Mosapride citrate			M-1		
		Period of storage at −20 °C (month)			Period of storage at −20 °C (month)		
		0	1	2	0	1	2
Muscle	Recovery (%)	102	102	101	82	78	77
	RSD (%)	1.7	3.4	3.2	1.8	6.0	9.1
Liver	Recovery (%)	98	92	95	75	70	67
	RSD (%)	3.9	1.1	2.2	3.5	2.4	5.7
Kidney	Recovery (%)	97	106	92	76	86	77
	RSD (%)	2.1	2.5	0.63	6.4	0.67	3.4
Adipose tissue	Recovery (%)	98	92	93	83	85	86
	RSD (%)	2.1	2.2	2.2	1.4	3.6	3.0
Intestine	Recovery (%)	90	105	95	80	93	89
	RSD (%)	6.8	2.0	2.6	4.8	4.3	2.6

[a] Stability was determined with three replicates for each time point, and it was expressed as %recovery of spiked mosapride citrate and M-1 (0.08 μg/g).

The differences between stored and freshly prepared solutions were −7 to 15% for mosapride citrate and −11 to 15% for M-1 except for 0.001 and 0.005 μg/mL of M-1 solutions that contain the blank extraction solution from the intestine, suggesting that the standard solutions for calibration curves were mostly stable for 7 days. Second, stock solution stability was tested. The stock standard solution mixture of mosapride citrate and M-1 (100 μg/mL each) was stored at 5 °C in the dark for 18 days. The solution was diluted to 0.1 and 1 μg/mL and compared with solutions prepared from a freshly prepared stock standard solution. The results showed that the difference between stored and freshly prepared solutions was less than 2% in both concentrations, indicating that the stock standard solution mixture was stable at least for 18 days. For the I.S. solution (100 μg/mL), it was stable for 49 days at 5 °C in the dark. Third, to assess the stability in frozen tissues, mosapride citrate and M-1 were spiked into blank tissues (0.08 μg/g), stored at −20 °C, and %recovery was determined. The results summarized in Table 5 show that mosapride citrate and M-1 were stable for 2 months in frozen equine tissues. We conclude that three stability studies show that the analytes in the current study are practically stable.

4. Conclusions

A quantitative LC/MS/MS method has been developed for the determination of mosapride citrate in equine muscle, liver, kidney, adipose tissue, and intestine samples. The method was validated for specificity, accuracy, precision, recovery, sensitivity, and stability. Because, it is expected that mosapride citrate is used in a variety of fields of animal science and veterinary medicine, this method will be a useful tool for food safety as well as *in vivo* research on pharmacological effects of mosapride and its tissue-specific metabolism.

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