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Simultaneous determination of illegal galactagogue adulterants in supplement diets by LC-MS/MS

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Abstract Recently, for successful lactation, many breastfeeding mothers seek various products, including herbal medicine, dietary supplements, and prescribed medicines, to improve milk production. As demand for galactogogues grows, it is highly possible that pharmaceutical galactogogues may be adulterated with illegal products to maximize their efficacy. For continuous control and supervision of illegal products, we developed and validated a simple and sensitive LC-MS/MS method capable of simultaneously determining five galactogogues. Chromatographic separation was conducted using an Agilent Poroshell 120 SB-C_{18} column with a mobile phase consisting of 20 mM ammonium formate (pH 5.4) and 100 % acetonitrile. The total run time was 13 min per analyte. The proposed method was performed according to the guidelines of the International Conference of Harmonization and it produced reliable results. This method showed high sensitivity and specificity, with a limit of detection (LOD) and limit of quantitation (LOQ) of 0.01-0.82 ng/mL and 0.02-2.45 ng/mL, respectively, for the solid- and liquid-type samples. Specificity was evaluated by analyzing matrix-blank samples spiked with the target compounds at LOQ levels, which provided a good separation of all peaks without interference. Additionally, the repeatability and intermediate precision were typically <15 %, whereas the recovery was 80-120 % of the values obtained using blank samples. Thus, we concluded that this method could be used for the identification and quantification of galactogogues in food or herbal products.

Key words: galactogogues, breastfeeding, lactation, LC-MS/MS, validation, dietary supplement

1. Introduction

Maternal milk is considered an optimal food for infants because it influences the growth, development, and health of a baby during the neonatal period.[1-5] Breastfeeding provides many benefits such as increased immunity, improved retinal function, reduced morbidity, and protection against infections.[5] Additionally, breastfeeding is recommended by the World Health Organization and American Academy of Pediatrics

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for 6 months after birth.[1,4] The most common reason for breastfeeding failure is insufficient milk production. Thus, most mothers make an effort to promote breastfeeding and many are interested in herbs and dietary supplements categorized as galactogogues to improve milk production.[1, 6-10]

Galactogogues are substances that maintain, enhance, or increase breast milk production and are categorized as pharmaceutical and herbal.[1-2,6,9] The most common pharmaceutical galactogogues are metoclopramide hydrochloride (HCl), chlorpromazine HCl, sulpiride, and domperidone.[1-2,4,7,11] Unfortunately, these medications have serious side effects such as gastroenteric effects, hyperhidrosis cardiac arrhythmia, depression, and even sudden death in mothers.[1-2,6] Thus, many breastfeeding mothers preferred to use herbs and foods with galactogogue properties.[1,6,12] As preferences about natural galactogogue increase, sales of natural products to stimulate lactation in on-line shop show an increasing trend. As demand for these products grows worldwide, it is very possible that the illegal products may be adulterated with pharmaceutical galactogogue to maximize efficacy of a product. We have studied the various adulterated products, as analysis of phosphodiestrase-5 inhibitors into illicit erectile dysfunction products,[13] monitoring study on weight loss compounds in dietary supplements[14] and screening of steroid adulterants in food and dietary supplements,[15] in a few years. Likewise, some issues on natural products or dietary supplements require firm control and supervision. So, we developed the new method for analysis galactogogue in herbal products or dietary supplements.

Few studies have described methods for analysing each galactogogue compound. There are several published analytical methods for determination of galactogogue compounds in biological samples as follows: liquid chromatography with UV[16-18] or fluorescence,[19-20] gas chromatography-mass spectrometry (GC-MS).[21] Recently, liquid chromatography-mass spectrometry (LC-MS) has been frequently used because above methods have poor sensitivity and specificity. Chlorpromazine and sulpiride as antipsychotics in human blood were analysed by LC-MS/MS.[22-23] A LC-MS method was developed and validated for the determination of metoclopramide and domperidone in human plasma.[24-26] SPE equipped with LC-MS for analysing sulpiride in river water was suggested by Kubo *et al*.[27]

Most analysis methods for galactogogue compounds are used to evaluate human plasma. There are no simultaneous analysis methods for the evaluation of galactogogues. Therefore, the aim of this study was to develop and validate a new method for evaluating galactogogues in dietary supplements by LC/MS/MS.

2. Experimental

2.1. Chemical and reagents

Metoclopramide hydrochloride (HCl), domperidone, and medroxyprogesterone acetate were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). Sulpiride and chlorpromazine HCl were obtained from European Directorate for the Quality of Medicines (EDQM) (Strasbourg, France). HPLC-grade methanol (MeOH) and acetonitrile (ACN) were obtained from Merck (Darmstadt, Germany). Formic acid and ammonium formate were purchased from Sigma-Aldrich. High-purity deionised water (DW) was obtained by purification with a Milli-Q purification system (Millipore, Billerica, MA, USA). Standard stock solutions (1000 ng/mL) of the galactogogues were prepared in MeOH and stored at 4 °C. Aliquots of the calibration standard mixture consisting of each standard stock solution (0.1-100 ng/mL) were prepared daily.

2.2. LC-MS/MS apparatus and chromatographic conditions

Chromatographic separation were performed using a Shiseido SP HPLC (Shiseido Co., Ltd, Tokyo, Japan) with a Agilent Poroshell 120 SB-C_{18} (2.1×75 mm, 2.7 μm; Agilent Technologies Inc., Santa Clara, CA, USA) maintained at 40 °C. Gradient elution with 20 mM ammonium formate in DW adjusted to pH 5.4 with formic acid (mobile phase A) and 100 % ACN (mobile phase B) were used. The gradient elution flow rate was set to 0.3 mL/min and an initial gradient

composition was maintained 10 % B for 1 min. Gradient conditions were linearly changed to 95 % B over 5 min and maintained for 4 min. Next, the gradient composition was changed to the initial condition over 0.1 min and maintained for 2.9 min. The injection volume was 1 μL and total run time was 13 min. Mass spectrometric analysis was conducted on an API 5500 triple quadrupole mass spectrometer (AB Sciex, Concord, Ontario, Canada). The ionization performed in positive ESI mode and ionspray source temperature was optimized at 500 °C and ion voltage was set to 5500 V. The curtain, collision, and ion source gas pressures were 30, 8, and 50 psi, respectively. The structures of galactogogues are presented in *Fig.* 1. The MRM parameters such as parent and quantification ion of each compound, collision energy, and cone voltage are shown in *Table* 1.

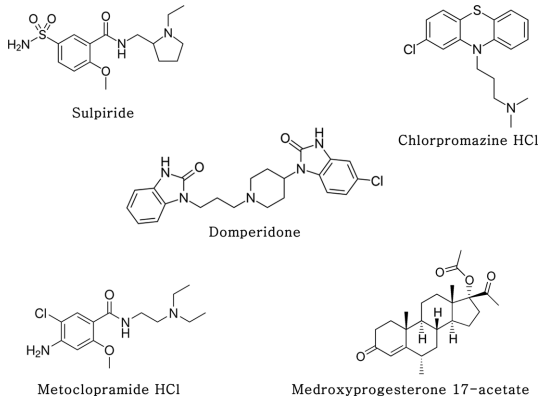

Fig. 1. Structures of the five galactogogues.

Table 1. Summary of diagnostic ions and the MRM transition parameters for the five galactogogues

Compound	Q1	Q3*	DP (volts)	CE (volts)	CXP (volts)
Sulpiride	342.0	**112.2**	100	40	13
		84.1	100	40	20
Chlorpromazine HCl	319.1	**86.1**	100	31	14
		58.2	100	55	14
Domperidone	426.2	**175.0**	100	32	15
		147.0	100	45	20
Metoclopramide HCl	300.1	**227.1**	100	20	13
		184.0	100	45	25
Medroxyprogesterone acetate	387.0	**327.2**	100	21	14
		123.1	100	33	12

*Quantitative ion is marked **in bold font**.

2.3. Samples and sample preparation

Dietary supplements advertised as effective for inducing milk production were purchased from a website. The 11 samples purchased consisted of liquid (2), powders (2), capsule (1), and leached teas (6). The samples (1 g) were extracted with 50 mL of 70 % MeOH and followed by sonication for 30 min. The extracts were filtered using a 0.22 μm polytetrafluoroethylene syringe filter (Whatman International Ltd., Maidstone, Kent, UK) and injected into the LC-MS/MS systems for analysis.

2.4. Method Validation

This method validation was carried out using parameters such as selectivity, specificity, limit of detection (LOD), limit of quantification (LOQ), linearity, precision (intermediate precision and repeatability), accuracy, recovery, and stability. The quantification ion was used for the calculation of the validation parameter. Selectivity and specificity were confirmed by analysing the LC-MS/MS chromatogram profiles of two matrix-blank samples (solid and liquid type). The LOD and LOQ were defined from spiked matrix-blank samples as the lowest concentration at a signal-to-noise (S/N) ratio of 3 and 10, respectively. Linearity was assayed by triplicate injection of external calibration standards curves with 7 points. Based on the LOQ, the ranges of calibration standard curves were 0.33-20.86 ng/mL for sulpiride, 0.17-10.80 ng/mL for chloropromazine HCl, 0.66-42.32 ng/mL for domperidone, 0.16-10.54 ng/mL for metoclopramide HCl, and 1.63-104.40 ng/mL for medroxyprogesterone acetate. The precision (intermediate precision and repeatability), accuracy, recovery, and stability were evaluated at three different levels (n=3 at each level). The low, medium, and high concentration levels of standards were 0.65, 2.61, and 10.43 ng/mL for sulpiride, 0.34, 1.35, and 5.40 ng/mL for chloropromazine HCl, 1.32, 5.29, and 21.16 ng/mL for domperidone, 0.33, 1.32, and 5.27 ng/mL for metoclopramide HCl, and 3.26, 13.05, and 52.20 ng/mL

for medroxyprogesterone acetate. The intermediate precisions and repeatability were evaluated in triplicate over three consecutive days and on the same day, respectively. Precision was expressed as the RSD (%). The accuracy was determined by comparing nominal and measured concentrations. The recovery assay was performed by spiking standard mixtures of three levels into the matrix-blank samples. Stability was tested using standard solutions at three different levels after 6 h at room temperature (21-23 °C), after 24 h of storage in the autosampler (4 °C), and after 48 h of storage in the autosampler (4 °C). The stored solutions were compared with freshly prepared solution.

3. Results and Discussion

3.1. Optimization of instrument conditions

The MS/MS experimental conditions for confirmation of each compound were developed by infusion of each standard solution diluted in 50 % MeOH at 1 ng/mL. All compounds exhibited $[M+H]^+$ ions and the ions were selected as Q1. In positive mode, two fragment ions with high intensity were selected as Q3. The quantification ions were obtained using the MRM transition at m/z 342.0 → 112.2 for sulpiride, at m/z 319.1 → 86.1 for chlorpromazine HCl, at m/z 426.2 → 175.0 for domperidone, at m/z 300.1 → 227.1 for metoclopramide HCl, and at m/z 387.0 → 327.2 for medroxyprogesterone acetate (*Table* 1). Chromatographic separation was performed by HPLC. The chromatographic conditions such as column, mobile phases, and gradient were investigated to optimize specificity and selectivity. In an initial experiment, a Shiseido capcell pak C_{18} column MGII (2.0×50 mm, 3.0 μm) was selected for separations. The flow rate was set to 0.25 mL/min and the column oven was maintained at 40 °C. Buffer consisting of 20 mM ammonium formate in DW at pH 4.3 adjusted with formic acid and 100 % ACN was used as the mobile phase. While all target compounds were suitably separated, all peaks showed appreciable tailing in the initial experiment. The initial conditions showed that the target analytes were significantly affected by the pH of the mobile phase. In order to improve the peak tailing, the pH of the 20 mM ammonium formate in DW was varied from 3.5 to 5.8. After several attempts, a gradient of mobile phases composed of 20 mM ammonium formate in DW (pH 5.4) and 100 % ACN was used. However, a few compounds such as sulpiride, metoclopramide HCl, and chloropramazine HCl showed peak tailing. Thus, we used an Agilent poroshell 120 SB-C_{18} column

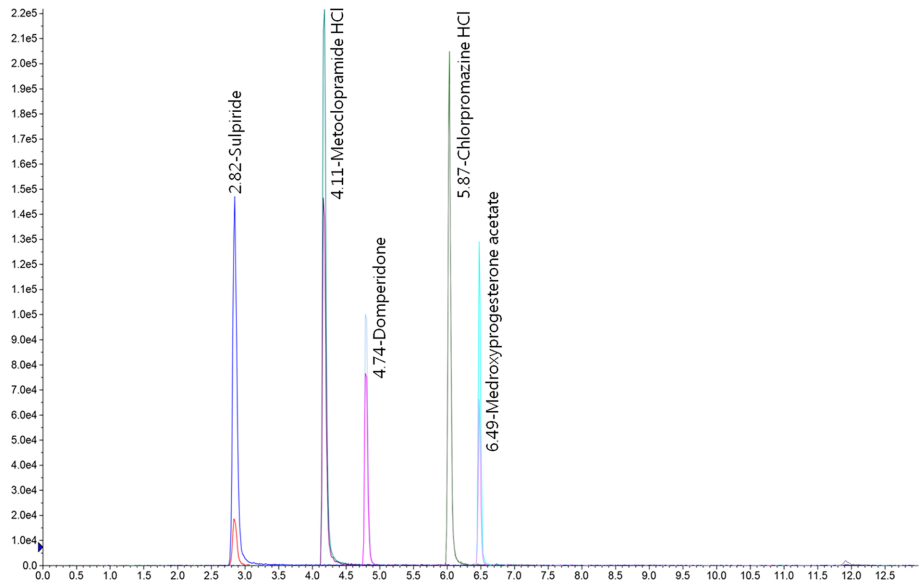

Fig. 2. Typical chromatogram of the five galactogogues obtained using the developed method.

(2.1×75 mm, 2.7 μm) and the flow rate was changed to 0.3 mL/min. Using the Agilent column, excellent peaks without appreciable tailing were observed for all the compounds.

3.2. Method validation and application

The typical chromatogram obtained using the developed LC-MS/MS method is shown in *Fig.* 2. The specificity and selectivity of the developed method was evaluated by analysing matrix-blank samples spiked with LOQ levels of the compounds. *Fig.* 3 shows a chromatogram of matrix-blank sample (A) and galatogogue compounds spiked in blank sample (B). The chromatogram showed good separation of all peaks without interference and the selectivity was confirmed by MS/MS. The low LOD (S/N ≥ 3) demonstrated the high sensitivity of this method. The LOD and LOQ of target compounds indicated appropriate precision of 0.01-2.45 ng/mL (*Table* 2). The calibration graphs were obtained from three consecutive injections over seven different levels of each compound. All calibration graphs showed excellent linearity with $r^2 = 1.00$ (*Table* 2). The precision (intermediate precision and repeatability) and accuracy are presented in *Table* 3. The intra- and inter-day precision of all compounds were less than 12 % and

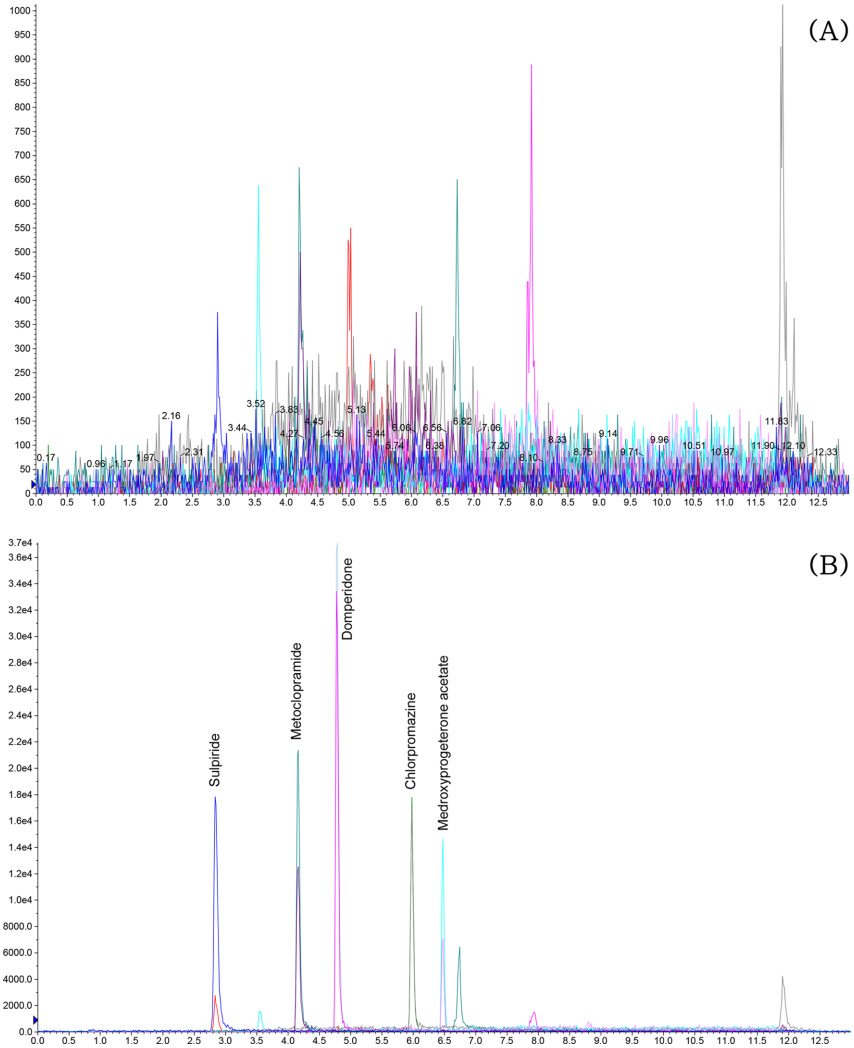

Fig. 3. Chromatograms of the matrix-blank sample (A) and five galactogogues spiked in blank sample (B).

Table 2. Retention time, calibration parameters, LOD, and LOQ of the five galactogogues

Compound	Retention time (min)	R^2	Solid		Liquid	
			LOD (ng/mL)	LOQ (ng/mL)	LOD (ng/mL)	LOQ (ng/mL)
Sulpiride	2.82	1.00	0.08	0.24	0.04	0.12
Chlorpromazine HCl	5.87	1.00	0.08	0.25	0.08	0.25
Domperidone	4.74	1.00	0.33	0.99	0.08	0.25
Metoclopramide HCl	4.11	1.00	0.03	0.08	0.01	0.02
Medroxyprogesterone acetate	6.49	1.00	0.82	2.45	0.27	0.82

Table 3. Repeatability (Intra), intermediate precision (Inter), and accuracy of the five galactogogues (n=3)

Compound	Standard concentration	Intra		Inter	
		Precision (%RSD)	Accuracy (%)	Precision (%RSD)	Accuracy (%)
Sulpiride	Low	3.43	89.58	7.86	86.58
	Medium	2.40	102.37	6.57	97.39
	High	2.78	100.73	7.45	96.96
Chlorpromazine HCl	Low	1.32	94.58	4.10	89.96
	Medium	3.52	100.11	6.45	94.10
	High	2.17	101.45	6.37	93.09
Domperidone	Low	2.23	90.63	3.96	80.03
	Medium	4.94	101.11	9.37	99.69
	High	2.04	103.23	7.77	99.69
Metoclopramide HCl	Low	6.24	91.56	4.57	88.29
	Medium	1.21	101.49	5.08	97.38
	High	1.71	102.67	4.33	97.49
Medroxyprogesterone acetate	Low	1.90	98.72	3.43	83.20
	Medium	9.83	105.25	10.90	91.95
	High	4.53	99.88	11.56	87.47

Table 4. Recoveries (%) of the five galactogogues in the blank samples (n=3)

Compound	Standard concentration	Solid		Liquid	
		Recovery	Precision	Recovery	Precision
Sulpiride	Low	105.75	0.31	82.51	0.20
	Medium	102.36	0.87	83.19	3.67
	High	95.35	1.71	81.17	1.18
Chlorpromazine HCl	Low	107.95	1.67	116.37	2.45
	Medium	103.72	0.37	112.30	2.23
	High	96.57	0.91	108.79	0.82
Domperidone	Low	80.86	0.27	105.52	0.68
	Medium	80.69	0.15	90.63	2.88
	High	83.41	1.28	85.46	3.36
Metoclopramide HCl	Low	104.78	1.76	108.44	1.73
	Medium	100.54	1.38	100.60	2.40
	High	93.53	1.69	95.64	1.90
Medroxyprogesterone acetate	Low	94.07	0.47	107.14	1.73
	Medium	87.48	2.09	95.82	0.70
	High	87.13	2.43	95.53	0.45

Table 5. RSD (%) between the peak areas obtained with freshly and stored standard solutions (n=3)

Compound	Standard concentration	Storage conditions		
		6 h at room temperature	24 h stored in autosampler	24 h stored in autosampler
Sulpiride	Low	2.58	9.62	8.93
	Medium	2.64	8.56	11.13
	High	5.91	8.75	7.70
Chlorpromazine HCl	Low	0.65	7.03	8.34
	Medium	3.47	7.68	7.68
	High	7.03	7.30	5.44
Domperidone	Low	0.34	8.18	7.67
	Medium	0.89	10.79	9.41
	High	4.40	9.47	9.14
Metoclopramide HCl	Low	1.15	6.86	5.78
	Medium	3.37	4.88	7.25
	High	3.62	7.24	6.67
Medroxyprogesterone acetate	Low	5.92	2.04	5.83
	Medium	4.94	6.24	1.08
	High	5.83	2.87	2.34

the accuracies were 80.03-105.25 %. These results indicate considerable precision and accuracy. The average recovery (%) was 81.17-105.75 % for sulpiride, 96.57-116.37 % for chlorpromazine HCl, 80.69-105.52 % for domperidone, 93.53-108.44 % for metoclopramide HCl, and 87.13-107.14 % for medroxyprogesterone acetate in two types of matrix-blank samples (*Table* 4). *Table* 5 shows that the %RSD of the stability was within 12 %. The standard solutions of each compound were considered to be stable after each storage period. As a result, the new method was suitable for the analysis of the galactogogue compounds. The developed method was applied to 11 dietary supplements to stimulate lactation of various dosage forms such as liquid (2), powder (2), capsule (1), and leached tea (6).

4. Conclusions

The interest in products to improve lactation has increased in women who have difficulty breastfeeding, such as a lack of milk production. Recently, many dietary supplements to help lactation have been introduced in online shops. Based on our previous studies (13-15) on phosphodiestrase-5 inhibitors, weight loss compounds, or steroid as adulterants in food, herbal products and dietary supplements, the continuous control and supervision about popular dietary supplements are required. So far, methods for analysing galactogogue compounds have not been developed. In this study, a new LC-MS/MS method was developed for simultaneous determination of galactogogue compounds. The method was validated and showed high-quality results (high selectivity, linearity, good precision, accuracy, recovery, and stability). In the future, our method can be used to monitor and identify galactogogue compounds in various adulterated products and will be helpful for analysing galactogogues to obtain preliminary data.

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