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Matrix effects caused by phospholipids in the multi-residue analysis for beta-agonists with liquid chromatography-electrospray tandem mass spectrometry

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It was firstly confirmed that 5 glycerophospholipids (GPs, (15.6 mg L^{-1})) including lysophosphatidylcholine (LPC C18:0), lysophosphatidylcholine, LPC (C16:0), phatidylcholine, PC (C16:0/C18:2), phatidylcholine, PC (C16:0/C18:1) and phatidylethanolamine, PE (C18:0/C20:4) could significantly suppress ionization of 9 beta-agonists (2 μg L^{-1}) using post-column infusion during liquid chromatography-electrospray tandem mass spectrometry analyses. Under the optimal experimental conditions, most beta-agonists such as fenoterol, clorprenaline, tulobuterol, clenbuterol and especially penbuterol were co-eluted with the GPs, and a positive linear correlation (R^2>0.85) between the amount of GPs and the extent of ionization suppression was observed for the five analytes, respectively. And the linear correlation was of statistical significance (P<0.001). GPs including LPC C18:0 (*m/z* 496.2), PC C16:0/C18:2 (*m/z* 758.5), PC C16:0/C18:1 (*m/z* 760.5), PE C18:0/C20:4 (*m/z* 768.6) and plasmalogen phosphatidylcholine, PLPC C18 (Plasm)/C18:1 (*m/z* 772.6) were detected in the final extracts of porcine liver sample. These endogenous GPs may be main effectors that cause matrix effects in beta-agonists residues detection in porcine liver.

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1. Introduction

Beta-agonists, a group of synthetic phenethanolamine compounds such as clenbuterol, salbutamol, ractopamine, terbutaline, cimaterol, etc., can improve the carcass characteristics and promote growth of animals. However, illicit use of these compounds in food-producing animals may cause chemical food-poisoning if the meat products obtained from the treated animals are eaten by consumers.[1] That's why they have been banned as growth promoter of farm animals in many countries.[2] Nevertheless, their illegal use in animal husbandry still happens from time to time.[3] Therefore, the monitoring of beta-agonists residues in animal-derived food is indeed necessary for consumer health.

Due to its high selectivity and sensitivity, liquid chromatography-tandem mass spectrometry (LC-MS/MS) has become a primary analytical technology for multi-residue analysis of veterinary drugs and banned substances in animal-derived food.[4,5] However, with the rapid development of this technique and its wide application, a few problems such as matrix effects (ME) and unavailable qualifier ion for few compounds have gradually caused concerns. The ME is a phenomenon in which analyte ionization is suppressed or enhanced due to interferences co-eluting with the analyte.[6] ME can drastically affect the precision, accuracy and linearity of the method and lead to poor and unreliable data in a quantitative assay.[7] Both electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI) suffer from ME, but ESI has been proved to be more prone to ME than APCI.[8] The mechanism of ME is not fully understood, but the ionization competition between the nonvolatile materials and the analytes in ion source is considered as the most probable cause.[9] For these reasons, one should pay much attention to assessment of ME during the early development of LC-MS/MS methods.[10]

It has been reported that the ME might result from either endogenous components such as lipids (especially phospholipids), proteins, salts, drugs and metabolites,[10] which were originally present in samples and retrieved in the final extracts, or exogenous materials such as plastic and polymer residues,[11] ion-pairing reagents[12] and organic acids,[13] which were introduced during the process of sample preparation and analysis. Although both origins of ME have been widely investigated, the endogenous interferences have received more attention in the development of bioanalytical methods. [14] Phospholipids have been identified as main matrix effectors and used as a marker to evaluate ME in biological samples, especially plasma[15] in the LC-MS/MS analysis. Bennett et al.[16] proposed that the existence of phospholipids in the extracts could lead to retention time shifts, elevated baselines, and divergent curves even if they did not co-elute with target analytes. Thus, phospholipids could affect assay performance. Considerable literatures have been published on detection, removal and separation of phospholipids in plasma.[17] Chambers[18] et al systematically investigated a comprehensive strategy for reducing ME of drugs in plasma in LC-MS/MS analyses. The combination of polymeric mixed-mode SPE, the appropriate mobile phase pH and UPLC technology provides significant advantages for reducing matrix effects resulting from plasma matrix components and in improving the ruggedness and sensitivity of bioanalytical methods. However, compared to the extensive studies in plasma samples, little attention has been devoted to investigate the influence of phospholipids in edible animal tissues on matrix effects. Moragues et al.[19] evaluated matrix effects during the determination of beta-agonists in animal liver and urine using liquid chromatography-ion trap mass spectrometry (more suitable for qualitative analysis). It was suggested that the hexane washing step in solid phase extraction (SPE) can remove phospholipids, fatty acids, etc. responsible, to a great extent, for ion suppression phenomenon.

The main purpose of this work is further to determine what glycerophospholipids (GPs) are involved in causing matrix effects during the residue analysis of beta-agonists in porcine liver by LC-MS/MS in positive ESI mode. The study focuses on the specific relationship between GP's amount and extent of matrix effect of analytes. It is helpful to reduce or compensate matrix effects caused by endogenous phospholipids during the determination of drug residue in porcine liver.

2. Experimental

2.1. Reagents and standards

Standards of salbutamol, terbutaline, cimaterol, fenoterol, ractopamine, clorprenaline, tulobuterol, clenbuterol and penbuterol were provided by Dr. Ehrenstorfer (Augsburg, Germany). Standards of lysophosphatidylcholine, LPC (C18:0) and lysophosphatidylcholine, LPC (C16:0) were purchased from Sigma (St Louis, MO, USA), and the standard solutions of phatidylcholine, PC (C16:0/C18:2), phatidylcholine, PC (C16:0/C18:1) and phatidylethanolamine, PE (C18:0/C20:4) in chloroform were provided by Supelco (Bellefonte, PA, USA). Acetonitrile, methanol and formic acid purchased from Fisher Scientific Co. (Pittsburgh, PA, USA) were of HPLC grade. Other reagents were of analytical grade or higher quality.

Stock solutions of nine beta-agonists were prepared in methanol at concentration of 1 mg mL^{-1} and stored at -20 ^{0}C. Working solutions were diluted from the stock solutions with 10% methanol in water containing 0.1% formic acid before use for all experiments except for the co-elution study. Standards of LPC C18:0 and LPC C16:0 were first dissolved in proper amount of chloroform and then diluted to 1 mg mL^{-1} with methanol (as stock solutions). Working solutions of five GPs were diluted from the stock solutions with 10% methanol in water containing 0.1% formic acid before use.

2.2. Instruments and apparatus

An Agilent 1200 HPLC system (Palo Alto, CA, USA) coupled to an Applied Biosystems API 4000 triple-quadrupole mass spectrometer (Foster City, CA, USA) equipped with an ESI source was employed. A syringe pump (Harvard Apparatus Inc., Holliston, MA, USA) with a 1 mL or 10 mL Hamilton microliter syringe was employed to introduce the solutions into ESI interface. Universal 32R Centrifuge was purchased from Hettich Inc. (Tuttlingen, Germany).

2.3. Sample preparation

Porcine liver samples were collected from local markets in China. The liver samples were homogenized and then stored at -20 ^{0}C before analysis. A previous analysis was conducted to ensure the absence of target analytes in the porcine liver samples. Samples were treated and analyzed by LC-MS/MS according to the developed method.[20] Briefly, 2 g blank liver sample was extracted with 10 mL of acetonitrile and 1 mL of 10% sodium carbonate solution. The sample was ultrasonicated about 20 min and centrifuged at 8 000 rpm for 10 min at 4 ^{0}C. The supernatant was transferred into another tube and the sediment was extracted with 5 mL acetonitrile once again. All the supernatants were combined and evaporated in a rotary evaporator until near dryness (about 0.5 mL). Then the residues were reconstituted in 5 mL of 0.02 mol L^{-1} ammonium acetate solution (pH=5.2). The SPE cartridge (a synthesized polymer in acetone) as described elsewhere[20] was initially conditioned with 5 mL of methanol followed by 5 mL of water and 5 mL of 0.02 mol L^{-1} ammonium acetate solution (pH=5.2), and then the reconstituted extracts were loaded onto the cartridge that was sequentially washed with 5 mL of water and 5 mL of methanol. Finally, the cartridge was eluted with 5 mL of 4% ammonia in methanol. The eluates were collected, evaporated to dryness under a gentle stream of nitrogen at 50 ^{0}C, and the residues were reconstituted in 2 mL of

10% methanol in water containing 0.1% formic acid for the subsequent study.

2.4. **LC-MS/MS analytical conditions**

Chromatographic separation was performed using a Luna C_{18} column (150 mm × 2.0 mm, 5 µm) purchased from Phenomenex (Torrance, CA, USA). Solvent A and B were 0.1% formic acid in water and acetonitrile, respectively. A gradient elution was used for all experiments to analyze the nine beta-agonists except for post-column infusion experiment where isocratic elution (80% B) was adopted. The linear gradient profile consisted of 0-2.0 min: 0%-45% B; 2.0-6.0 min: 45% B; 6.0-7.0 min: 45%-0% B; 7.0-15 min: 0% B. Flow rate was at 0.25 mL min^{-1} and the column temperature was 35 ^{0}C. Injection volume was 5 µL.

The mass analyses were performed using an ESI source in positive ionization mode. Selected reaction monitoring (SRM) experiments were carried out. The operation conditions were as follows: ionspray voltage, 5.0 kV; source temperature, 650 ^{0}C; curtain gas, 20 psi; ion source gas 1 and gas 2 were at 60 and 55 psi, respectively. Dwell time was 150 ms for all nine beta-agonists. The other optimized parameter values and SRM transitions of the analytes are given in Table 1 or published elsewhere.[6]

The positive ESI mode was employed to monitor phospholipids. The mass spectrometer acquisition parameters and SRM transitions for five phospholipids are listed in Table 2. On the basis of literature,[17,21] the following characteristic fragment ions were selected as product ions. Specifically, a protonated phosphocholine moiety (product ion, m/z 184) was used to detect PCs, and the positive ion (neutral loss of 141 Da) was used to monitor PE. The other instrument conditions were the same as those used for nine beta-agonists analysis.

2.5. Experimental design of influence of GPs on analyte ionization

In order to investigate the influence of phospholipids on analytes ionization, post-column infusion experiment was carried out. The solution containing nine beta-agonists (each at 2 μg L^{-1}) was constantly infused into the MS system with the syringe pump at the flow rate of 10 μL min^{-1}, while the solution containing five GPs (each at 15.6 mg L^{-1}) was injected in triplicates from the autosampler under the isocratic elution (80% B) conditions as described above. The schematic of the post-column infusion system is shown in Scheme 1. As a comparison, a solution (10% methanol in water containing 0.1% formic acid) was injected from the autosampler as well. Any variation of the signal of analyte indicated the effect caused by the elution of phospholipids.

2.6. **Determination of the extent of ion suppression of beta-agonists**

Firstly, nine beta-agonists (2 μg L^{-1}) in the neat solution (10% methanol in water containing 0.1% formic acid) were analyzed by LC-MS/MS in SRM mode, and peak areas of all the analytes were recorded. Secondly, the beta-agonists (2 μg L^{-1}) in the solution containing the five GPs at 2.0, 3.9, 7.8, 15.6, 31.3, 62.5 and 125 mg L^{-1} were prepared and analyzed by LC-MS/MS in SRM mode, respectively. The corresponding peak area of the analyte was recorded. The extent of ion suppression (IS, %) is defined as (100 - B/A × 100),[22] where A and B represent the average peak area of the neat standard solution and the standard solution spiked with GPs (n = 5), respectively. Thereafter, the least-squares linear regression line is generated by using IS of each analyte versus the corresponding concentration of GPs and fitted to the equation: $y = b\ x + a$, indicating the relationship between the extent of ion suppression of analyte and the concentration of phospholipids. And linearity test was conducted using statistical analysis by SPSS software package, version 17.0 (SPSS Inc, Chicago, IL, USA). A P-value of <0.01 was considered statistically significant.

2.7. Determination of GPs in final extracts of porcine liver sample

To find out the presence of GPs in real samples after pretreatment, 20 blank porcine liver tissues from five different markets were extracted with basified acetonitrile and purified by SPE as described above. The final extracts were spiked with mixture of nine analytes at the concentration level of 2 μg L^{-1} prior to LC-MS/MS analysis. Besides monitoring the characteristic ion pairs of the phospholipids references, GPs mentioned in the literature,[17] such as plasmalogen phosphatidylcholine (PLPC C18 (Plasm)/C18:1, *m/z* 772.6/184.1) and lysophosphatidylethanolamine (LPE C16:0, *m/z* 454.3/313.3) were also monitored during the LC-MS/MS analysis.

3. Results and discussion

Post extraction addition and post column infusion are the two main approaches used to evaluate ME. In post extraction addition, the value of ME is evaluated as the ratio of analyte response in blank sample extracts to the response of the same concentration in neat solution.[8] It provides information about ME at the time of analyte elution. Post column infusion is carried out by monitoring response of an analyte constantly being infused by an infusion pump and then injection of the blank sample extracts using the LC system under the desired chromatographic conditions.[23] Through this technique, the chromatographic regions where an analyte most likely suffers from ME can be identified. If the main sources that give rise to ME are identified, we can remove them more purposefully. Thus, matrix effects will be overcome or diminished.

3.1. Influence of GPs on analyte ionization

In the absence of any interferences eluting from the analytical column, the post-column infusion of an analyte should obtain a constant background signal in the SRM channel. Any variation in the

constant signal profile of the analyte indicates the presence of matrix effects. To investigate the effects of the phospholipids on the analytes ionization, the neat solvent and the mixed GPs solution of LPC (C18:0), LPC (C16:0), PC (C16:0/C18:2), PC (C16:0/C18:1) and PE (C18:0/C20:4) were injected via the autosampler, respectively, while the analytes were being constantly infused from post-column. The results showed that for the neat solvent (10% methanol in water containing 0.1% formic acid) injected pre-column, steady signal profiles for nine beta-agonists were observed except for a dip at about 1.3 min due to injection (see Figure 1). On the contrary, for the results following injection of the GP solution presented several significant signal dips (see Figure 2). Therefore, it was suggested that the ion suppression of the analyte was caused by the presence of GPs. Representative Chromatograms of five phospholipids are shown in Figure 3. It was observed that one peak was eluted for PE (C18:0/C20:4) (Figure 3a) and PC (C16:0/C18:1) (Figure 3b), that's why more than two signal dips were produced at the elution time windows of two phospholipids (see Figure 2). Furthermore, the peaks of PE (C18:0/C20:4) and PC (C16:0/C18:2) (Figure 3d) were partially overlapping with those of PC (C16:0/C18:1) and LPC (C18:0) (Figure 3e), which led to increase of the signal suppression at the time windows of 5 min (see Figure 2).

3.2. Relationship between the extent of IS and the amount of GPs

Since the post-column infusion experiment has demonstrated that GPs caused significant ion suppression for the nine beta-agonists, the co-elution of GPs and target analytes was further detected. Firstly, the mixed standards solution of nine beta-agonists and five GPs was prepared, and the most intensive SRM transition of each compound was monitored during LC-MS/MS analysis. The results showed that the retention time windows (peak widths) of five GPs covered from 7.0 to 13 min. Most target analytes including fenoterol, ractopamine, clorprenaline,

tulobuterol, clenbuterol and penbuterol were also eluted during these periods, and were not well separated from the GPs interferences. Typical extracted ion mass chromatograms of the co-eluates (nine beta-agonists and five GPs) are shown in Figure 4. Three beta-agonists including salbutamol, terbutaline and cimaterol were eluted at 6.5 min.

Secondly, the extent of ion suppression was determined under different concentrations of GPs (15.6 mg L^{-1}, which is the maximal mixed concentration obtained by diluting the purchased GPs solutions), and IS of analytes versus concentration of GPs was fitted with least-squares linear regression. The results show that a positive linear correlation is found between the GPs and each of six beta-agonists including fenoterol, ractopamine, clorprenaline, tulobuterol, clenbuterol and penbuterol, which were co-eluted with GPs. The regression curves and correlation coefficients are shown in Figure 5. The R^2 values of the six beta-agonists except for ractopamine (0.68) were more than 0.85. In addition, the results of linearity test of the curves showed that *P* values were all <0.001 for R^2 of those six analytes, indicating the linear correlation was of statistical significance. That's to say, for these target analytes, the extent of IS increased linearly with increase of GPs in porcine liver sample. Therefore, we could deduce that GPs played an important role in causing matrix effects of beta-agonists in LC-MS/MS analysis.

3.3. GPs in the final extracts of liver samples

Although it was proven that the GPs had profound effects on ME of the beta-agonists, whether they still existed in the final extracts of real sample had to be identified. After being extracted with basified acetonitrile and then purified by weak cation exchange SPE,[20] the sample extracts were injected into LC-MS/MS system under the optimized conditions for beta-agonists. As shown in Figure 6, significant amounts of endogenous GPs were detected in porcine liver samples. The LPC (*m/z* 496.2), PC (*m/z* 758.5), PC (*m/z* 760.5), PE (*m/z* 768.6) and PLPC (*m/z* 772.6) presented large and wide peaks, and the peak heights of them were ranged from 1.6×10^3 to 7.2×10^4 and the peak

width from 5.4 min to 5.7 min. The results suggested that GPs remained in the extracts of porcine liver tissues had great effects on the MEs of beta-agonists. In our experiments, hexane was also used to remove phospholipids before and after SPE. The results indicate that hexane can remove phospholipids to some extent, but the difference between the steps using hexane or not is not significant ($P>0.05$), and SPE using a weak cation polymer synthesized in our laboratory might significantly remove the phospholipids in porcine liver samples ($P<0.05$). SPE is better than hexane to remove phospholipids, which is consistent with the findings reported by Chambers[18] et al. Completely removing phospholipids from animal tissue samples is difficult. Various efficient strategies have been used to reduce or compensate matrix effects, such as optimizing sample preparation, manipulating chromatographic parameters and using a stable isotope internal standard, and however, in some cases, the above-mentioned means cannot be performed. The strategy of spiking appropriate GPs to standard solution (naming standard addition method) for compensating matrix effects in complex biological samples is considerable in LC-MS/MS analysis. The matrix-matched calibration curve should be adopted for reliable quantification.

4. Conclusions

Glycerophospholipids might cause significant ion suppression effects on signals of nine beta-agonists including salbutamol, terbutaline, cimaterol, fenoterol, ractopamine, clorprenaline, tulobuterol, clenbuterol and penbuterol. A positive linear correlation ($P<0.01$) was found between the extent of ion suppression of five beta-agonists and the amount of phospholipids with the correlation coefficients more than 0.85. Considerable amounts of phospholipids such as LPC (*m/z* 496.2), PC (*m/z* 758.5), PC (*m/z* 760.5), PE (*m/z* 768.6) and PLPC (*m/z* 772.6) were detected in the final extracts of porcine liver samples. Glycerophospholipids were suggested to be used as general substances to compensate matrix effects in complex biological samples with LC-MS/MS analysis.

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Figure captions

Scheme 1. Schematic of the post-column infusion system

Fig. 1 The post-column infusion spectra of beta-agonists (2 μg L^{-1}) after injection of solvent (10% methanol in water containing 0.1% formic acid)**.** (a) salbutamol, (b) terbutaline, (c) cimaterol, (d) fenoterol, (e) clorprenaline, (f) ractopamine, (g) tulobuterol, (h) clenbuterol, (i) penbuterol.

Fig. 2 The post-column infusion spectra of beta-agonists (2 μg L^{-1}) after injection of glycerophospholipids standards mixture (15.6 mg L^{-1}). a, b, c, d, e, f, g, h and i identification as in **Fig. 1**.

Fig. 3 Representative chromatograms of glycerophospholipids standards. (a) PE (C18:0/C20:4), (b) PC (C16:0/C18:1), (c) LPC (C16:0), (d) PC (C16:0/C18:2), (e) LPC (C18:0).

Fig. 4 Co-elution of nine beta-agonists (2 μg L^{-1}) and five glycerophospholipids standards (125 mg L^{-1}). (a) salbutamol, (b) terbutaline, (c) cimaterol, (d) fenoterol, (e) clorprenaline, (f) ractopamine, (g) tulobuterol, (h)clenbuterol, (i) penbuterol. (j) PE (C18:0/C20:4), (k) PC (C16:0/C18:1), (l) LPC (C16:0), (m) PC (C16:0/C18:2) (n) LPC (C18:0).

Fig. 5 Correlation between ion suppression (IS) of beta-agonists and phospholipids concentration (n = 6). (a) fenoterol, (b) clorprenaline, (c) ractopamine, (d) tulobuterol, (e) clenbuterol, (f) penbuterol. IS (%) = 100 - B/A × 100, where A and B represents peak area of analyte in pure solvent and in solution spiked with different amounts of phospholipids, respectively. The regression curves and correlation coefficients were obtained using EXCEL, version 2003, and P-value was obtained by statistical software package SPSS, version 17.0.

Fig. 6 Typical SRM chromatograms of 5 phospholipids. A and B represents final extracts of porcine liver sample and 10% methanol in water containing 0.1% formic acid, respectively; (a) PE ($C_{18:0}/C_{20:4}$), (b) PC ($C_{16:0}/C_{18:1}$), (c) LPC ($C_{16:0}$), (d) PC ($C_{16:0}/C_{18:2}$), (e) $C_{18:0}$ (Plasm)/$C_{18:1}$ PLPC.

TABLES

Table 1. Selected reaction monitoring settings for MS/MS analysis of 9 beta-agonists

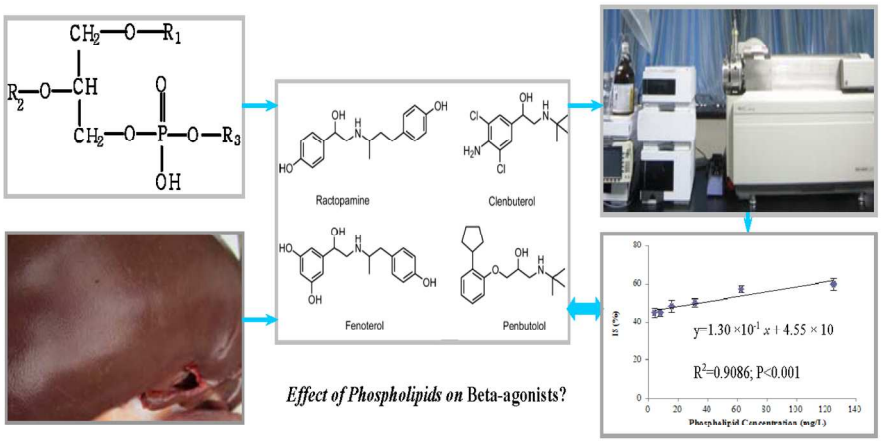
analyte	precursor ions $[M+H]^+$, *m/z*	product ions, *m/z*	DP[a], V	CE[b], eV
ractopamine	302.2	284.2, 164.1[c]	54	20, 23
clenbuterol	277.1	258.9, 203.0[C]	48	19, 23
salbutamol	240.0	222.0, 148.0[C]	60	19, 24
terbutaline	226.1	169.8, 152.1[C]	54	23, 24
cimaterol	220.0	143.2, 202.2[C]	55	21, 16
fenoterol	304.2	134.8, 107.0[C]	54	14, 15
clorprenaline	214.2	196.1, 154.1[C]	56	17, 18
tulobuterol	228.1	171.8, 154.1[C]	54	20, 24
penbuterol	292.2	201.2, 236.2[C]	54	30, 25

[a]DP represents declustering potential. [b]CE represents collision energy. [c]quantification ions.

Table 2. Single selected reaction monitoring settings for MS/MS analysis of 5 phospholipids

Compounds	Precursor ion $[M+H]^+$, (*m/z*)	Product ions (*m/z*)	DP[a] (V)	CE[b] (eV)
Lysophosphatidylcholine (LPC), C18:0	524.4	184.2	180	35
Lysophosphatidylcholine (LPC), C16:0	496.2	184.2	160	35
Phosphatidylcholine (PC), C16:0/C18:2	758.5	184.1	156	43
Phosphatidylcholine (PC), C16:0/C18:1	760.5	184.1	140	45
Phosphatidylethanolamine (PE), C18:0/C20:4	768.6	627.5	138	45

[a] DP, Declustering potential. [b] CE, Collision energy.



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