Determination Method for Ractopamine in Swine and Cattle Tissues Using LC/MS

(Received February 21, 2007)

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Simple and reliable methods using LC/MS have been developed for the determination of the β-agonist ractopamine in swine and cattle tissues. Ractopamine was extracted with ethyl acetate from muscle and liver, and the ethyl acetate layer was evaporated to dryness. The residue was purified by partition with acetonitrile/n-hexane. In the case of fat, ractopamine was extracted and purified by partition with acetonitrile/n-hexane. The resulting acetonitrile solutions were evaporated to dryness. The residue was dissolved in methanol, and subjected to LC/MS. The LC separation was performed on a Wakosil-II 3C18HG column (150 mm x 3 mm i.d.) in isocratic mode with 0.05% trifluoroacetic acid/acetonitrile (80 : 20) as a mobile phase at a flow rate of 0.4 mL/min. The MS detection was performed in the selected ion recording (SIR) mode, with detection of the M+H+ ion of ractopamine (m/z 302) produced by electrospray ionization (ESI). The mean recoveries of the drug from swine muscle (0.01 µg/g fortified), fat (0.01 µg/g fortified) and liver (0.04 µg/g fortified) were 99.7%, 99.5% and 100.8%, and those from cattle samples were 108.3%, 97.0% and 109.4%, respectively. The relative standard deviations (RSDs) ranged from 0.1% to 9.5%. The limit of quantification (LOQ) of the drug was 1 ng/g.

Key words: ractopamine; β-agonist; swine; cattle; LC/MS

Introduction

β-Adrenergic agonists (β-agonists) are widely used as bronchodilators, tocolytics and heart tonics in clinical and veterinary medicine1). Ractopamine (Fig. 1) is a β-agonist, and the impact of this drug on growth performance and carcass quality has been clearly demonstrated in pig, mainly in terms of increased weight gain and lean tissue accretion, as well as improved feed conversion ratio2–4). The advantages of feeding pigs with this drug have been reported as increased daily weight gain, improved feed efficiency, saving on feed, increased nitrogen retention and shortened breeding period5, 6).

Recently, veterinary drug residues have become a matter of public concern because of possible adverse effects on human health owing to carryover from drug-treated animals to the human diet. Ractopamine has been approved as a feed supplement in the U.S.A. and Australia. On the other hand, the EU has officially banned the use of such adrenergic drugs as growth-promoting agents and the import of ractopamine-treated meat. In Japan, the use of this drug has also been banned and maximum residue limits (MRLs) have been set for the drug as shown in Table 1.

Several screening methods for ractopamine have been reported based on enzyme immunoassay (EIA) or enzyme-linked immunosorbent assay (ELISA)7–9). The detection limits of these methods were in the 1–50 ng/mL (ppb) range, and the cross-reactivities for other β-agonists were generally less than 0.5%. These methods are able to detect ractopamine without complicated purification, due to the specificity of antibody used, though matrix effects often occur. Other screening methods using HPLC with electrochemical detection10, 11), UV detection12, and fluorescence

Table 1. Maximum residue limits (MRLs) for ractopamine in Japan

<table>
<thead>
<tr>
<th>Animals</th>
<th>Tissues</th>
<th>MRL (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cattle and Swine</td>
<td>Muscle</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>Fat</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td>Other edible parts</td>
<td>0.04</td>
</tr>
</tbody>
</table>
Detection have also been reported. These methods are sensitive, but generally require complicated pretreatments and are also time-consuming for detection of ppb levels of ractopamine (Table 1). The methods using HPLC with tandem mass spectrometry (LC/MS/MS) are highly sensitive, but these instruments are expensive.

On the other hand, LC/MS systems are widely available, and are sensitive enough to detect ppb levels of ractopamine as listed in Table 1. So, if a simple and reliable method using LC/MS is developed, more inspection agencies will be able to perform residue analysis of ractopamine.

The aim of this study was to develop a simple and reliable method for the quantification and confirmation of trace amounts of ractopamine in swine and cattle tissues using LC/MS. The procedure was developed for use in routine monitoring of ractopamine residues, as mandated by the inspection agencies.

Materials and Methods

Samples, chemicals and materials

Swine and cattle tissues (muscle, fat and liver) were purchased from a market in Tokyo, Japan. These samples were stored at −40°C until analysis. Standard ractopamine hydrochloride was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Ractopamine standard solutions were prepared as described below. A standard stock solution containing 100 µg/mL of ractopamine was prepared in methanol, and the solution was diluted successively with methanol. These standard solutions were stored at −20°C until analysis. All the solvents used were of LC grade and other chemicals used were of analytical grade unless otherwise stated.

Liquid chromatograph and mass spectrometer

Alliance 2695 separation module liquid chromatograph (Waters, Co., Milford, MA, USA) coupled with a micromass ZQ 2000 mass spectrometer (Waters).

LC/MS conditions

Column: Wakosil-II 3C18HG (150 × 3 mm i.d., 3 µm, Wako Pure Chemical Industries, Ltd.)
Column oven temperature: 40°C
Mobile phase composition: 0.05% trifluoroacetic acid-acetonitrile (80:20)
Flow rate: 0.4 mL/min
Source temperature: 100°C
Desolvation temperature: 350°C
Gas: nitrogen, flow rate: ca. 360 L/hr
Capillary voltage: 3.5 kV
Ionization mode: ESI, positive ion mode
Measuring mode: SIR
Monitoring ion: m/z 302
Cone voltage: 20 V

Sample preparation

For meat and liver, a finely chopped sample (5.0 g) was weighed into a 50 mL centrifuge tube. Then 20 mL of ethyl acetate and 1 mL of 4 mol/L potassium carbonate solution were added to the tube, and the sample was homogenized for 2 min and centrifuged for 10 min at 3000 rpm. The ethyl acetate layer was transferred to a recovery flask, and 20 mL of ethyl acetate was added to the pellet in the tube. The mixture was homogenized and centrifuged. The resulting ethyl acetate solutions were combined and evaporated to dryness below 40°C. The residue was dissolved in 30 mL of acetonitrile, and the mixture was homogenized for 2 min and centrifuged for 10 min at 3,000 rpm. The acetonitrile layer was transferred to a recovery flask, and 30 mL of acetonitrile was added to the glass tube. The mixture was homogenized and centrifuged. The two acetonitrile layers were combined and defatted with 30 mL of n-hexane saturated with acetonitrile. The acetonitrile layer was evaporated to dryness, and the residue was redissolved in 1.0 mL of methanol.

For fat samples, preparation of the test solution was performed as described below. A sample (5.0 g) was weighed into a 250 mL glass tube. Then 30 mL of acetonitrile and an equivalent volume of n-hexane saturated with acetonitrile were added, and the sample was homogenized for 2 min and centrifuged for 10 min at 40°C. The residue was dissolved in 30 mL of acetonitrile, and the mixture was homogenized and centrifuged. The two acetonitrile layers were combined and defatted with 30 mL of n-hexane saturated with acetonitrile. The acetonitrile layer was evaporated to dryness, and the residue was redissolved in 1.0 mL of methanol.

Determination of ractopamine

Determination of ractopamine was performed by LC/MS. Aliquots of 2 µL of the ractopamine standard solutions and the test solutions were injected into the LC/MS system. The quantification was performed by calculating the peak areas. Detection was performed by SIR at m/z 302.

Results and Discussion

MS conditions

Ractopamine is a basic compound possessing an amino group (Fig. 1). Therefore, the positive ESI mode was applied for the analysis of this drug. In the MS spectrum of ractopamine, the proton adduct of the ractopamine molecule (M+H)+ (m/z 302) was observed as a base peak. Therefore, the m/z 302 ion was selected as the monitor ion for SIR. The cone voltage was set to 20 V, since the monitoring ion (m/z 302) was observed most strongly.

Fig. 1. Structure of the β-agonist ractopamine

The asterisks indicate asymmetric carbon atoms.
Almost all added ractopamine was examined based on the one peak.

Extraction and clean-up

A standard C18 column, which is generally used for the analysis of veterinary drugs, was selected as the analytical HPLC column.

Acetonitrile and 0.05% trifluoroacetic acid were selected as the mobile phase and the flow rate was set to 0.4 mL/min, based on the Multiresidue Method I for Veterinary Drugs, etc. by HPLC (Ministry of Health, Labour and Welfare, Japan).

The standard solution of ractopamine was subjected to LC/MS using 2 different mobile phases (0.05% trifluoroacetic acid–acetonitrile (80:20) and 85:15). The chromatograms obtained are shown in Fig. 2. The peak obtained was very sharp with 0.05% trifluoroacetic acid–acetonitrile (80:20), whereas it was slightly broadened with 0.05% trifluoroacetic acid–acetonitrile (85:15). It was considered that the isomers of ractopamine were partially separated in the latter mobile phase.

The MRLs for ractopamine are set as the total concentration of ractopamine. Therefore, we used ethyl acetate as a solvent for extraction. The results of recovery tests are shown in Table 2. The mean recoveries of ractopamine from swine samples were 99.5±1.9% and from cattle samples 97.0±0.4%. These results meet the standards for residual analysis of veterinary drugs in foods established by the Codex Alimentarius Commission (CAC). Therefore the method proposed in this study offers good performance for the analysis of ractopamine residues in foods.

Next, clean-up procedures were investigated using several cartridge columns (polymer, C18 reverse phase and cation-exchange type). The retention and elution of ractopamine were good in the case of the standard solution, but less good in the case of food matrices, and the recoveries varied widely. So, the chosen clean-up procedure consisted of only partition with acetonitrile/n-hexane, without using the cartridge columns.

With such a simple method, the extraction of ractopamine was achieved with high recovery and high reproducibility, without interfering peaks on the chromatograms.

HPLC conditions

A standard C18 column, which is generally used for the analysis of veterinary drugs, was selected as the analytical HPLC column.

Acetonitrile and 0.05% trifluoroacetic acid were selected as the mobile phase and the flow rate was set to 0.4 mL/min, based on the Multiresidue Method I for Veterinary Drugs, etc. by HPLC (Ministry of Health, Labour and Welfare, Japan).

The standard solution of ractopamine was subjected to LC/MS using 2 different mobile phases (0.05% trifluoroacetic acid–acetonitrile (80:20) and 85:15). The chromatograms obtained are shown in Fig. 2. The peak obtained was very sharp with 0.05% trifluoroacetic acid–acetonitrile (80:20), whereas it was slightly broadened with 0.05% trifluoroacetic acid–acetonitrile (85:15). It was considered that the isomers of ractopamine were partially separated in the latter mobile phase.

The MRLs for ractopamine are set as the total concentration of ractopamine. Therefore the mobile phase of 0.05% trifluoroacetic acid–acetonitrile (80:20) was selected in this study and the ractopamine was determined based on the one peak.

Extraction and clean-up

First, solvents for the extraction of ractopamine were investigated. Almost all added ractopamine was extracted by using methanol/acetic acid, methanol or acetonitrile, but materials which interfered with the determination of ractopamine were also extracted. Therefore, we used ethyl acetate as a solvent for extraction, and transferred un-ionized ractopamine to the ethyl acetate layer by adding 4 mol/L potassium carbonate. This method could extract ractopamine almost entirely, without extracting any interfering material. In the case of fat, ractopamine was extracted and purified by partition with acetonitrile/n-hexane, because no interfering material was extracted.

<table>
<thead>
<tr>
<th>Table 2. Results of recovery tests for ractopamine</th>
</tr>
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<tbody>
<tr>
<td>**Fortified ( ppm)</td>
</tr>
<tr>
<td>-------------------</td>
</tr>
<tr>
<td>Muscle</td>
</tr>
<tr>
<td>Fat</td>
</tr>
<tr>
<td>Liver</td>
</tr>
</tbody>
</table>

\( n=3 \)

Conclusion

A simple and reliable method using LC/MS has been developed for the determination of ractopamine in swine and cattle tissues. The method consists of extraction with ethyl acetate for muscle and liver and with acetonitrile/n-hexane for fat, clean-up by extraction with acetonitrile/n-hexane saturated with acetonitrile, and determination using LC/MS (SIR mode).

The mean recoveries of ractopamine from swine tissues were 99.5±100.8%, and those from cattle tissues were 97.0–109.4%. The RSDs \( (n=3) \) ranged from 5.7% to 9.5% for swine samples, and from 0.1% to 1.9% for cattle samples. The LOQ was 1 ng/g \( (S/N=10) \). These results satisfied the standards for residual analysis of veterinary drugs in foods established by the Codex Alimentarius Commission (CAC). Therefore the method proposed in this study offers good performance for the analysis of ractopamine residues in foods.

References

1) Antignac, J. P., Marchand, P., Bizec, B. L., Andre, F. Identification of ractopamine residues in tissue and urine

![Fig. 2. Chromatograms of ractopamine standard solutions with two different mobile phases](image-url)


