Determination of Clenbuterol in Various Edible Parts of Livestock Products by LC-MS/MS and LC-MS/MS/MS Methods

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Abstract
The residual status of clenbuterol in various edible parts of livestock products was investigated using the LC-MS/MS and LC-MS/MS/MS methods. The target food products were the edible tissues of pig and cattle, and clenbuterol was extracted using acetonitrile containing anhydrous sodium sulfate and sodium chloride. Following the dispersive solid-phase extraction using C18 particles and purification with an anion-exchange solid-phase extraction cartridge, the resultant test sample solutions were subjected to the LC analysis. A C18 column was used as the analytical column, and the peak of clenbuterol was eluted at 4.8 min with the gradient elution from 5% acetonitrile to 99% acetonitrile in 10 min. As a result of the LC-MS/MS analysis, interfering peaks were detected around the retention time of clenbuterol. By using the LC-MS/MS/MS analysis, which was developed as a quantitative analysis method that reduces the influence of contaminants, most of the unknown peaks were removed, and it was confirmed that the food samples obtained in Kanagawa prefecture did not contain clenbuterol.

Keywords: β2-Agonist; Clenbuterol; LC-MS/MS; LC-MS/MS/MS; Food safety

1. Introduction
4-Amino-3,5-dichloro-α-[[(1,1-dimethylethyl)amino]methyl]benzenemethanol (clenbuterol) is a β2-adrenoceptor agonist, which is a drug that selectively acts on β2-adrenoceptors in smooth muscle and exhibits a relaxing action [1]. Clenbuterol hydrochloride is used as a human drug for bronchodilation and urinary incontinence prevention. The β2-agonist has the effect of increasing the ratio of muscle to fat, therefore, it is sometimes used for human muscle enhancement purposes [2]. For this reason, β2-agonists are prohibited drugs for doping. Poisoning cases have also been reported when humans intentionally ingested clenbuterol for muscle enhancement purposes [3-6].

For animals, clenbuterol is used as a veterinary drug as a bronchodilator for horses and cattle and a uterine contraction suppressant for cattle [1,7]. The action of the β2 agonist, which increases the ratio of muscle to fat, enhances feed conversion efficiency, and overseas, clenbuterol is sometimes illegally given to livestock for growth promotion purposes. Since clenbuterol is given to edible animals, poisoning occurred in humans who ingested the meat and organs obtained from livestock that clenbuterol has been illegally administered [8-13]. The main symptoms of intoxication are tachycardia, palpitations, headache, dizziness, vomiting, hypokalemia and leukocytosis [13,14]. The onset was observed 10 min after ingestion, and the duration of symptoms varies from 1.5 hours to 6 days. Since clenbuterol is heat-stable, it is difficult to prevent the poisoning by heating. Therefore, the investigation of residual status of clenbuterol in the edible tissues of livestock is greatly expected for food safety.

In Japan, the clenbuterol residual status in foods is regulated to be undetectable in pig, chicken, egg, seafood and honey. For cattle and other animals belonging to terrestrial mammals, the maximum residue limits (MRLs) are set at 0.2 ng/g for muscle and fat, 0.05 ng/g in milk, 0.6 ng/g for liver, kidney and other edible parts [15]. For the analysis of clenbuterol in food samples, the Ministry of
Health, Labour and Welfare (MHLW) Japan has qualified an analytical method using liquid chromatography tandem mass spectrometry (LC-MS/MS) with the limit of quantification (LOQ) of 0.05 ng/g [15,16]. Besides the approved official method, several methods have been reported for the clenbuterol analysis such as enzyme-linked immunosorbent assay (ELISA), gas chromatography mass spectrometry (GC-MS), high-performance liquid chromatography (LC) and LC-MS (or MS/MS) [1,8-12,17-21]. In cases of overseas poisoning, GC-MS, ELISA and LC methods were mainly used to determine clenbuterol [8-13]. However, the selectivity is sometimes insufficient, and an analytical method with a higher selectivity is practically essential. Considering these requirements, we have developed an original LC-MS/MS analytical method (LOQ: 0.05 ng/g), equivalent to the official clenbuterol analysis method qualified by the MHLW Japan, and applied to the clenbuterol residual analysis in pig muscles [22]. However, in the real poisoning cases of clenbuterol, the poisoning was caused by the ingestion of various parts of livestock products such as muscle and liver (theoretically, also possible by the ingestion of other edible parts). Therefore, a higher selective analysis method applicable to various food matrices is required, and we have developed an LC-MS/MS/MS method in the previous study [23]. The LC-MS/MS/MS method was successfully applied to the pig liver samples, and wider applications to various tissues obtained from different edible animals are expected. In the present study, therefore, the residual status of clenbuterol in various edible parts of livestock products has been investigated using the LC-MS/MS/MS method.

2. Experimental

2.1. Chemicals and reagents

Clenbuterol hydrochloride (99.5% purity) and clenbuterol-d₉ (98.5% purity) as the internal standard (IS) were purchased from the FUJIFILM Wako Pure Chemical (Osaka, Japan). Clenbuterol hydrochloride was dissolved in methanol to prepare the standard stock solution (100 μg/mL) and the solution was stored at -30°C. Methanol, acetonitrile and formic acid of LC/MS grade were obtained from FUJIFILM Wako. Anhydrous sodium sulfate, sodium chloride, acetonitrile, acetone and n-hexane of pesticide residue analysis grade and 1-propanol of guaranteed grade were also the products of FUJIFILM Wako. Water was purified by a Milli-Q Integral 5 system (Merck, Darmstadt, Germany).

2.2. Sample preparation

Various livestock edible parts of pig (liver, kidney and small intestine) and those of cattle (liver, kidney, small intestine and stomach) were purchased at a retail store in Fujisawa city in Kanagawa Prefecture (Japan). The samples were shredded by a food processor and stored at -20°C until use. The samples were basically processed according to a previous report [22] as follows. The shredded sample (5.00 g) was transferred to a 50-mL polypropylene centrifuge tube and spiked with 1 ng of clenbuterol-d₉ as the surrogate. To the sample, 20 mL of acetonitrile, 5 g of anhydrous sodium sulfate and 1 g of sodium chloride were added. The mixture was homogenized by a homogenizer (BM-2 Biomixer, Nippon Seiki, Tokyo, Japan) for 1 min, then centrifuged at 1,460 g for 10 min at 4°C. The supernatant was transferred to another 50-mL tube. To the precipitate, 10 mL of acetonitrile was added and vigorously mixed for 5 min, then centrifuged again at 1,460 g for 10 min at 4°C. The supernatant was combined and 0.4 g of C18 particles (InertSep C18, GL Sciences, Tokyo, Japan) was added. The tube was shaken for 1 min, then centrifuged at 1,460 g for 10 min at 4°C. To the supernatant, 10 mL of 1-propanol was added, concentrated under reduced pressure at 40°C, then the concentrated solvent was evaporated under a stream of nitrogen. The residue was dissolved in 2 mL of a mixed solution of acetone and n-hexane (3:7), the solution was loaded onto an anion-exchange solid-phase extraction cartridge (InertSep PSA, 500 mg/6 mL, GL Sciences), and then 15 mL of the same solution was added. The entire effluent was collected and concentrated under reduced pressure at 40°C, then the concentrated solvent was evaporated under a stream of nitrogen. The residue was dissolved in 1 mL of methanol and passed through a membrane filter (Millex-LG, 0.2 μm, Merck). The filtrate (5 μL) was subjected to the LC-MS/MS (or LC-MS/MS/MS) system described in Section 2.3.

2.3. LC-MS/MS and LC-MS/MS/MS determination of clenbuterol

Clenbuterol was basically determined by the previously reported LC-MS/MS and LC-MS/MS/MS methods [22,23] using a tandem mass spectrometer with multi-stage fragmentation (QTRAP 4500, Sciex, Framingham, MA, USA). The target clenbuterol was separated from other compounds by a reversed-phase C18 column (Mightysil RP-18 GP, 2.0 mm i.d. x 100 mm, particle size 3 μm, Kanto Chemical, Tokyo, Japan) at 40°C using a Nexera XR LC system (Shimadzu, Kyoto, Japan). Mobile phase A was comprised of 0.1% formic acid in water and mobile phase B was comprised of 0.1% formic acid in acetonitrile. The mobile phases were delivered by a combination of gradient and stepwise elution modes. The gradient conditions were as follows: 0-10 min from 5 to 99% mobile phase B, and 10-15 min at 99% mobile phase B. The flow rate was 0.2 mL/min.

The eluted clenbuterol was detected by a tandem mass
spectrometer. As for the MS/MS conditions (positive-ion mode), the precursor and product ion pairs for clenbuterol and clenbuterol-d₉ were 277/203 and 286/204, respectively. The ion spray voltage, turbo gas temperature and entrance potential were 4000 V, 500°C and 10 V, respectively. The curtain gas pressure, ion source gas 1 and ion source gas 2 were 40 psi (276 kPa), 50 psi (345 kPa) and 80 psi (552 kPa), respectively. The collision energy was 23 V. The declustering potential for clenbuterol and clenbuterol-d₉ was 11 V and 61 V, respectively. The collision cell exit potential was 10 V.

As for the MS/MS/MS conditions (positive-ion mode), the precursor, primary product and secondary product ion pairs for clenbuterol and clenbuterol-d₉ were 277/203/132 and 286/204/133, respectively. The curtain gas pressure was 50 psi (345 kPa). The declustering potential was 65 V. The excitation energy in the linear ion trap (LIT) and LIT fill time were 0.07 V and 25 msec, respectively. The other conditions were the same as those of the MS/MS.

3. Results and discussion

3.1. Determination of clenbuterol in various livestock edible parts using LC-MS/MS

Using our previously reported LC-MS/MS method [22], the clenbuterol residual status in the various edible parts of the livestock products distributed in Kanagawa Prefecture, Japan, was evaluated. The target matrices were the edible parts of pig (liver, kidney and small intestine) and cattle (liver, kidney, small intestine and stomach) sold in a retail store in Fujisawa city in Kanagawa Prefecture (Japan). Figure 1 shows the LC-MS/MS chromatograms obtained using the edible parts of the pig and cattle samples, and also those obtained using the samples to which clenbuterol was spiked. In the pig liver (Fig. 1A), a peak was detected around the retention time of clenbuterol (4.8 min) and the peak area was equivalent to be 0.237 ng/mL clenbuterol (Table 1). However, using the pig liver sample spiked with the clenbuterol standard solution, two peaks were observed and the peak of clenbuterol was difficult to be separated from the unknown peak in the liver sample. In the pig kidney (Fig. 1B), a trace peak was observed around the retention time of clenbuterol; the peak area was below the calibration range (0.1-1.5 ng/mL [23]). In the pig small intestine, no peak was observed derived from the tissue matrix (Fig. 1C).

As the cattle edible parts, the liver, kidney, small intestine

![Fig. 1](image-url)  
Fig. 1. Application of the LC-MS/MS method to various edible parts of pig ((A) liver, (B) kidney and (C) small intestine) and cattle ((D) liver, (E) kidney, (F) small intestine and (G) stomach). Arrows indicate the retention time of clenbuterol. To evaluate the accuracy, 0.25 ng (per injection) of clenbuterol was spiked in the food samples.
and stomach were selected and these matrices were analyzed by the same LC-MS/MS system as that used for the pig samples. The obtained chromatograms are shown in Fig. 1, together with the results obtained using the samples spiked with clenbuterol. In the cattle liver, kidney and small intestine (Figs. 1D-F), small peaks were detected around the retention time of clenbuterol. Using the cattle liver and small intestine samples spiked with the clenbuterol standard solution, two peaks were observed, and the clenbuterol peak was difficult to be separated from the unknown peaks. In the cattle kidney, a peak was detected at the same retention time as that of clenbuterol, and the peak area was equivalent to 0.154 ng/mL clenbuterol (Table 1). No peak was observed from the tissue matrix of the cattle stomach (Fig. 1G).

Table 1 shows the amounts of clenbuterol determined by the LC-MS/MS method for the edible parts of the pig and cattle. As already described, the unknown peaks derived from the matrices were detected at around the same retention time as that of clenbuterol, and the amounts are equivalent to 0.237 ng/mL clenbuterol in the pig liver and 0.154 ng/mL clenbuterol in the cattle kidney. Trace peaks below the calibration range were also observed in the pig kidney, cattle liver and cattle small intestine. For the samples to which clenbuterol was spiked (0.25 ng clenbuterol), the obtained quantitative values gave large variations due to the presence of the unknown peaks. The recovery rate corrected by the surrogate (accuracy) for the pig small intestine and the cattle stomach, in which no unknown peak was detected, was 105.2% and 100.0%, respectively. On the other hand, for the samples in which the unknown peaks were detected, the accuracy values were 67.6-185.6%.

Several papers have been published reporting the analysis

Table 1. Determination of clenbuterol in various edible parts of pig and cattle by LC-MS/MS.

| Sample          | Pig Liver | Pig Kidney | Pig Small intestine | Cattle Liver | Cattle Kidney | Cattle Small intestine | Cattle Stomach |
|-----------------|-----------|------------|---------------------|-------------|--------------|------------------------|---------------|
| Spiked sample   | 0.237*    | Trace*     | ND                  | Trace*      | 0.154*       | Trace*                 | ND            |
| (ng/mL)         | 0.537*    | 0.258*     | 0.263               | 0.464*      | 0.323*       | 0.355*                 | 0.250         |
| Accuracy (%)    | 120.0     | 103.2      | 105.2               | 185.6       | 67.6         | 142.0                  | 100.0         |

*: peak areas were measured with unknown interfering substances. ND: not detected. To evaluate the accuracy, 0.25 ng (per injection) of clenbuterol was spiked in the food samples.

Fig. 2. Application of the LC-MS/MS/MS method to various edible parts of pig ((A) liver, (B) kidney and (C) small intestine) and cattle ((D) liver, (E) kidney, (F) small intestine and (G) stomach). Arrows indicate the retention time of clenbuterol. To evaluate the accuracy, 0.25 ng (per injection) of clenbuterol was spiked in the food samples.
of clenbuterol in various livestock tissues [8-12,17-21]. These analytical methods have been developed by using GC-MS, LC-ultraviolet and LC-MS/MS. The MHLW Japan has notified that a method using LC-MS/MS with the LOQ of 0.05 ng/g is applicable for the analysis of clenbuterol in food samples [15,16]. In our previous research, the original analytical method for clenbuterol by LC-MS/MS using dispersive solid phase extraction and anion-exchange cartridge (LOQ: 0.05 ng/g) was established [22]. The analytical method has been applied to pig muscle and processed pork food. In the present study, the same LC-MS/MS method was conducted for checking the edible parts of pig (liver, kidney and small intestine) and cattle (liver, kidney, small intestine and stomach) distributed in retail stores. As a result, in the liver (pig and cattle), kidney (pig and cattle) and small intestine (cattle), unknown interference peaks were detected around the retention time of clenbuterol. In these matrices, the accuracy values were not sufficient (67.6-185.6%) due to the influence of the interfering peaks, and the utilization of a more selective method like LC-MS/MS/MS was required to exclude the influence of the livestock tissue matrices.

### 3.2. Determination of clenbuterol in various livestock edible parts using LC-MS/MS/MS

Considering the results of the clenbuterol analyses using the LC-MS/MS instrument for the edible parts of the pig and cattle (Section 3.1.), the unknown peaks derived from the sample matrices were observed close to the retention time of clenbuterol. Also, based on the results analyzing the samples spiked with clenbuterol, an accurate determination could not be carried out. Therefore, the LC-MS/MS with the multistage fragmentation mode (LC-MS/MS/MS) analysis was performed as a quantitative method with reduced effects from the matrices. The MS conditions were determined by direct infusion of the analyte. The precursor ion was 277, which is the protonated molecule [M+H]⁺ of clenbuterol, and m/z=203 was selected for the 1st product ion and m/z=132 was selected for the 2nd product ion. The chromatograms are shown in Fig. 2. Using the LC-MS/MS/MS method, interfering peaks derived from the pig liver (Fig. 1A), pig kidney (Fig. 1B), cattle liver (Fig. 1D), cattle kidney (Fig. 1E) and cattle small intestine (Fig. 1F) detected by the LC-MS/MS analysis were all removed. In the clenbuterol-spiked samples, the peaks derived from the matrices were not detected and the accurate analysis of clenbuterol could be performed. Table 2 shows the results analyzing clenbuterol by the LC-MS/MS/MS system. In all of the livestock tissue samples tested in the present study, clenbuterol was not detected. In the samples spiked with clenbuterol (0.25 ng clenbuterol), the observed clenbuterol amounts were 0.230-0.265 ng/mL, and the accuracy values of clenbuterol were 92.0-106.0%.

Several cases of human poisoning due to the ingestion of muscle and organs of livestock illegally administered clenbuterol have been reported abroad. In these cases of poisoning, 160-291 ng/g of clenbuterol was detected from the cattle liver, 800-7400 ng/g from the beef meat and 375-500 ng/g was detected from the veal liver [8,11,12]. In our previous research, the original LC-MS/MS and LC-MS/MS/MS analytical methods for clenbuterol (LOQ: 0.05 ng/g), which is equivalent to the analysis method certified by the MHLW Japan were developed. Especially, the developed LC-MS/MS/MS method is highly selective and interfering peaks derived from the food sample matrices were drastically reduced [23]. As shown in the above case reports, clenbuterol poisoning was caused by the ingestion of various livestock edible parts such as the muscle and liver. Therefore, in the present study, the clenbuterol residual status was investigated by the LC-MS/MS/MS method in various edible parts of livestock products commercially available in retail stores in Kanagawa Prefecture (Japan). Using the LC-MS/MS/MS method, the accuracy was evaluated with the LOQ of 0.05 ng/g as the spiked concentration, and the obtained accuracy values were 92-106%. All the peaks detected from the livestock tissues by the LC-MS/MS analysis (trace-0.237 ng/mL) were removed by the LC-MS/MS/MS analysis, and it was confirmed that the livestock products tested in the present study did not contain clenbuterol. The present LC-MS/MS/MS method is useful as a highly specific quantitative analytical method for clenbuterol in complicated livestock tissues containing uncountable intrinsic components, and further applications to various foodstuffs are expected.

### 4. Conclusion

In the present investigation, the residual status of clenbuterol in various edible parts of livestock products was evaluated using the LC-MS/MS and LC-MS/MS/MS methods. As a result of the LC-MS/MS analysis, interfering peaks were detected around the retention time of clenbuterol, however, most of the unknown peaks were removed by the LC-MS/MS/MS analysis, and clenbuterol

#### Table 2. Determination of clenbuterol in various edible parts of pig and cattle by LC-MS/MS/MS.

| Sample (ng/mL) | Pig Liver | Pig Kidney | Pig Small intestine | Cattle Liver | Cattle Kidney | Cattle Small intestine | Stomach |
|----------------|----------|-----------|--------------------|-------------|---------------|------------------------|---------|
| Spiked sample (ng/mL) | 0.265 | 0.244 | 0.251 | 0.248 | 0.235 | 0.230 | 0.231 |
| Accuracy (%) | 100.6 | 97.6 | 100.4 | 99.2 | 94.0 | 92.0 | 92.4 |
| ND: not detected. To evaluate the accuracy, 0.25 ng (per injection) of clenbuterol was spiked in the food samples. |

Note: The accuracy values were 92.0-106.0%.
was not detected in any of the test samples. The present LC-MS/MS/MS method is highly selective and is considered to be useful for food safety control.

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