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High Performance Liquid Chromatography to Determine Animal Drug Clenbuterol in Pork, Beef and Hog Liver

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ABSTRACT

A semi-micro high performance liquid chromatography (HPLC) was developed to determine clenbuterol in pork, beef and hog liver. The procedure included extraction with 0.4 N perchloric acid, partitionally separation with diethyl ether, and then back-extraction with 0.2 M sulphuric acid. Analytical procedure was performed by HPLC with a Waters Cosmosil column 5C18-MS (2.0 × 150 mm), a mobile phase of 0.05 M NaH_2PO_4 (pH3.0)/acetonitrile solution (80/20, v/v) and absorbance at 212 nm. The recoveries of clenbuterol in pork, beef and hog liver spiked with standards of 0.0005~0.01 ppm were 80.9~90.6 %. The detection limit of clenbuterol in tested samples was 0.0001 ppm, which is lower than the officially allowed level. The equation was linear for detecting 0.0002~0.001 ppm clenbuterol. The method was validated to be usable for market samples. Three out of 150 samples were detected to contain clenbuterol (0.0001~0.00015 ppm), which were less than both JECFA and Department of Health regulation levels (hog liver 0.0006 ppm and beef 0.0002 ppm).

Key words: clenbuterol, semi-micro HPLC, pork, beef, hog liver

INTRODUCTION

Clenbuterol (4-amino-3,5-dichloro-α-tert-butyl amino methyl-benzyl alcohol hydrochloride) is an anabolic hormone and has been used widely as veterinary drug for fattening animals around the world (Figure 1). Clenbuterol has been used as a solid implant to promote growth and improve productivity[(1)]. Clenbuterol is a synthetic β-adrenergic agonist and has high affinity to the adrenergic receptor in swine adipocytes[(2,3)] which bind with limit fat accretion when fed to growing pigs[(1)]. Clenbuterol was used illegally to gain competitive advantage in some animals such as calves, lambs and swine. The abuse of clenbuterol could be a public health concern if animals were to be slaughtered for human food with clenbuterol residues in the liver or in the muscle. In 1995, FSIS (Food Safety and Inspection Service, United States Department of Agriculture, Washington, DC, USA) and FDA (Food and Drug Administration, USA) have taken enforcement actions against the abuse of clenbuterol in animals. Furthermore, a FDA regulatory action (detected limit <0.0002 ppm in meat) against persons was involved in the use or distribution of clenbuterol, and FSIS monitors the residue of clenbuterol in the meat and hog liver to keep contaminated meat away from consumer channels[(4)].

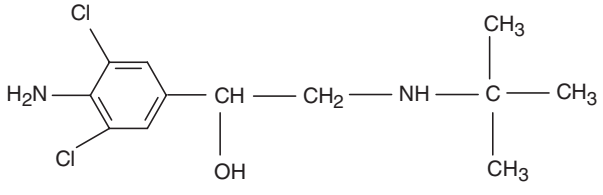

Figure 1. Structure of clenbuterol.

For safety and environmental impact assessment, analytical methods for determining clenbuterol residue are indispensable. There have been several methods to determine clenbuterol, including gas chromatography–mass spectrometry (GC-MS)[(5)], high performance liquid chromatography (HPLC)[(6,7,8)], capillary electrophoresis (CE)[(9)] and capillary electrophoresis-mass spectrometer (CE-MS)[(10)]. Among these methods, all detected limits all were higher than 0.05 ppm, except for GC/MS (0.0001 ppm), while the official detected limit is 0.0002 ppm in beef and 0.006 ppm in cattle liver and kidney[(4)]. Due to the complicated procedure, high expense of facilities, and low recovery (about 60%) for GC/MS method, it is urgent to develop a new simple, rapid and popular method to detect the residue of clenbuterol. Semi-micro HPLC has been reported to have better accuracy, precision and sensitivity than conventional HPLC. There are several types of

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HPLC: micro LC (0.3-1 mm, i.d.), semi-micro LC (1-3 mm, i.d.), conventional LC (4-8 mm, i.d.), semi-preparative LC (10-20 mm, i.d.), preparative LC (20-50 mm, i.d.) and process LC (> 50 mm, i.d.). The tube caliber and the particle size of these HPLC types are less than those of general HPLC. The liquid phase chromatographic instrument tube rate reduces along with the increase of padding granule. It may reduce the eddy diffusion and the nature passes on the resistance. Using the miniature analysis tube may save the motion solution expense and simultaneously reduces the waste liquid pollution in the environment. Compared with traditional HPLC, the semi-micro HPLC has the following merits: 1. less organic solvent amount used, 2. better sensitivity, 3. using small amount (1-2 uL) for injection[11].

Therefore, a simple, rapid, sensitive and semi-micro HPLC method to detect low residue of clenbuterol was developed in this study.

MATERIALS AND METHODS

I. *Reagents and Samples*

Standard clenbuterol was obtained from Sigma Chemical Company (MO, USA). Ten milligrams of clenbuterol was taken and dissolved into 100 mL of distilled water as stock solutions. Then, the stock solution was diluted with 0.01 N hydrochloric acid into a series of standard solutions (0.0001~1 μg/mL). Acetonitrile, ether, triethylamine, hydrochloric acid, perchloric acid, sulphuric acid, sodium dihydrogen phosphate and sodium hydroxide were purchased from Merck (Darmstadt, Germany).

The numbers of tested samples were as follows: pork, 50; beef, 50; and hog liver, 50. The samples were collected from different markets in Taipei during June to October, 2002.

Each sample (5 g) was ground in a blender for 3 min, transferred to 50-mL centrifuge tube, and then homogenized with 20 mL of 0.4 N perchloric acid for 3 min. The homogenate was centrifuged (3500 rpm, 10 min) and the supernatant was transferred to another 50-mL centrifuge tube. The above procedures were performed in duplicate with 20 mL of 0.4 N perchloric acid. The supernatant was mixed with 0.6 mL of 10 N sodium hydroxide, and then adjusted to pH 12.

The above supernatant was added with 10 mL ether and shaken for 5 min. The upper ether phase was collected and the aqueous phase was again added with 10 mL of ether and shaken for 5 min. Ether extracts were combined, transferred to a volumetric flask, evaporated under vacuum in a 45°C water bath, and then dried by nitrogen. The residue was dissolved in 0.2 mL of 0.2 M sulphuric acid, filtered through a 0.45 μm nylon membrane, and then analyzed by semi-micro HPLC.

Five concentrations (0.0002, 0.0004, 0.0006, 0.0008 and 0.001 μg/mL) of standard clenbuterol were prepared as described above and 3.0 μL of each was injected. Standard curves were plotted according to peak areas and concentrations.

Samples and standard solutions, 3.0 μL of each, were individually injected to the HPLC instrument. Peak identification was made by comparing the retention times and spectra of samples with those of standard solutions. The following formula was used to calculate the amounts of clenbuterol in test samples: Amount of clenbuterol (ppm) = C × V/W, where C is the clenbuterol concentration (μg/mL) calculated by standard curve, V is the volume of sample solution (mL), and W is the weight of sample (g).

Recovery test was performed in quadruplicate by spiking standards at 5 levels (0.0005, 0.001, 0.002, 0.005 ,and 0.01 μg /mL) into the respective homogenates of pork, beef, and hog liver. The spiked and blank samples were then analyzed by HPLC. Recovery was calculated by comparing the analyzed concentrations with spiked concentrations. The detection limit was evaluated according to the ratio of sample peak area to noise peak area, which is required to be more than 3.

II. *Apparatus*

Clenbuterol was determined by using a Shimadzu semi-micro liquid chromatograph (Shimadzu, Tokyo, Japan) consisting of a Shimadzu Model LC-10AT pump, a Shimadzu SPD-10A UV-Vis detector, set at 212 nm, and a Shimadzu Model C-R4A Chromato-integrator. A Cosmosil 5C18-MS reversed-phase column (5 μm, 150 mm × 2.0 mm i.d., Nacalir Tesque Inc., Japan) was used for the separation. The mixed solvent of 0.05 M NaH_2PO_4 (pH 3.0)/acetonitrile (80/20, v/v) was used as mobile phase. The flow rate was 0.2 mL/min.

RESULTS AND DISCUSSION

Before analysis, UV spectra scanning at the range of 200~350 nm for clenbuterol were tested using 0.05 M sodium dihydrogen phosphate solution/acetonitrile (80/20) as shown in Figure 2. The maximum UV absorption

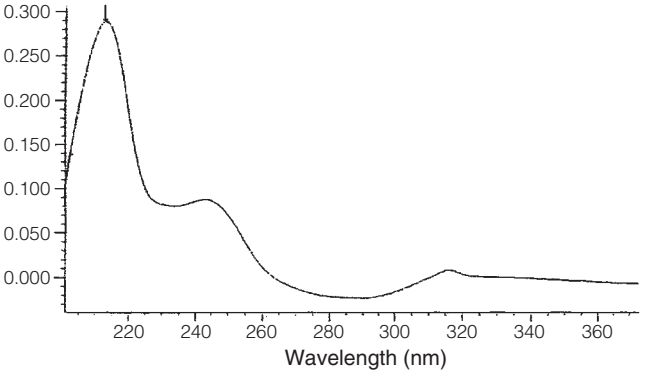

Figure 2. UV-absorption spectrum of clenbuterol dissolved in 0.05M NaH_2PO_4 (pH 3.0)/acetonitrile (80/20, v/v).

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presented at 212 nm was selected for clenbuterol detection in this study.

Botterblom *et al.*[6] and Miyazaki *et al.*[8] pointed out that the peak of clenbuterol appeared after 10 min and was not sharp when using HPLC with C18 column and different ratio of 0.05 M sodium dihydrogen phosphate solution/acetonitrile (65/35, or 70/30). Similarly, Botterblom *et al.*[6] also reported that when the mobile phase in HPLC was acetonitrile (pH 3.0)/sodium dihydrogen phosphate/triethylamine (80/20/1%), the retention time of clenbuterol was tardy and the peak had tailing.

To avoid the tardy and tailing peak, the effect of pH 3-7 on the absorbance of mobile phase (acetonitrile/0.05 M sodium dihydrogen phosphate solution=80/20, v/v) was investigated. The result indicated that increasing pH caused steady increase in absorption, which made the detected peak difficult to be identified, so that pH 3 was the optimal condition. In this study, the mobile phase of 0.05 M sodium dihydrogen phosphate (pH3.0)/acetonitrile (80/20, v/v) solvent was used to analyze clenbuterol by semi-micro HPLC and UV absorption at 212 mm. The semi-micro HPLC profile of standard clenbuterol is shown in Figure

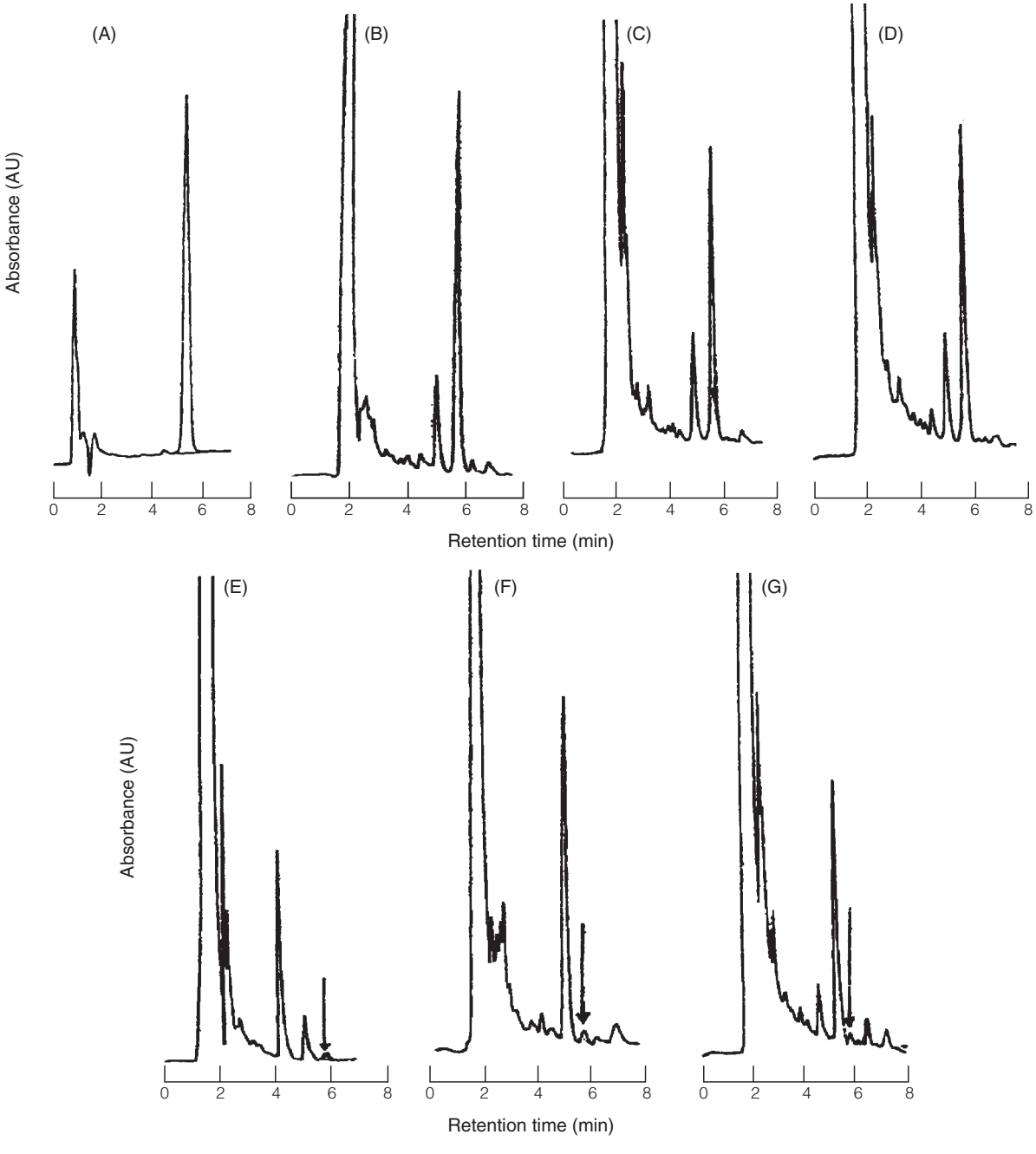

Figure 3. Analysis by semi-micro HPLC chromatography for (A) standard solution of 0.01 μg/mL clenbuterol, (B) pork spiked with 0.002 ppm clenbuterol, (C) hog liver spiked with 0.002 ppm clenbuterol, (D) beef spiked with 0.002 ppm clenbuterol, (E) pork sample containing 0.0001 ppm clenbuterol, (F) hog liver sample containing 0.00015 ppm clenbuterol, and (G) beef sample containing 0.00015 ppm clenbuterol.

3(A), in which the peak of clenbuterol was sharp and appeared at 5.6 min. The retention time of clenbuterol was less than 6 min and the peak was not interfered with other noise peaks. The semi-micro HPLC method possesses the advantages of analytic sensitivity and reducing the volume of mobile phase used.

The standard curve for the clenbuterol in the range of 0.0002, 0.0004, 0.0006, 0.0008 and 0.001 μg/mL was shown to be linear (Figure 4) with the regression equation is $Y = 1.0663 \times 10^7$ X-2.0667 (r = 0.9999), where Y is the peak area and X is the concentration of clenbuterol.

The recovery test was performed in quadruplicate by spiking 0 (as blank), 0.0005, 0.001, 0.002, 0.005 and 0.01 μg/mL of clenbuterol to 5 g samples of pork, beef and hog liver, respectively. Results are shown in Table 1 and their semi-micro HPLC chromatograms are shown in Figure 3. The average recoveries of clenbuterol in pork, beef and hog liver were in the range of 80.9~88.8%, 82.6~90.6% and 80.3~86.9% with coefficients of variation ranging at 0.7~6.3%, respectively. These data were better than that of other reports[5,6,8-14]. The detection limit for clenbuterol in pork, hog liver and beef was 0.0001 ppm. This value is lower than the allowable residual clenbuterol levels of both JECFA and our Department of Health regulations (hog liver 0.0006 ppm and beef 0.0002 ppm).

One hundred and fifty samples purchased at local markets were analyzed by this method. Three samples were found to contain clenbuterol (one was 0.0001 ppm and the other two were 0.00015 ppm), but none of them was over the regulation levels. Hence, the use of semi-micro HPLC method to determine clenbuterol in pork, beef and hog liver was validated.

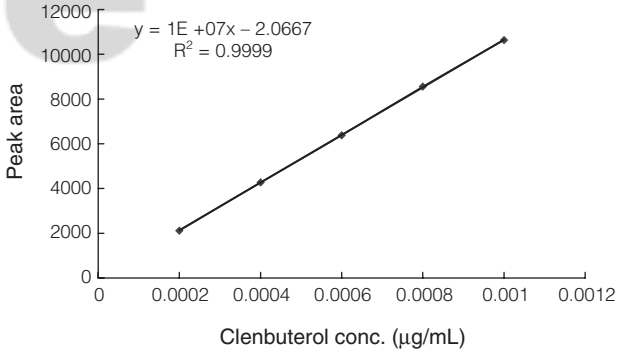

Figure 4. Standard curve of clenbuterol by semi-micro HPLC.

Table 1. Recoveries of clenbuterol spiked in pork, beef and hog liver

Spike level	Recovery[a](%)		
(ppm)	pork	hog liver	beef
0.0005	80.9 (4.59)[b]	80.5 (4.95)	90.6 (6.24)
0.001	88.8 (4.65)	83.3 (4.03)	85.3 (4.57)
0.002	84.1 (3.57)	86.9 (4.50)	87.1 (4.34)
0.005	86.2 (2.22)	80.3 (1.41)	82.6 (1.58)
0.01	88.3 (0.73)	83.2 (1.09)	80.6 (1.10)
Average	85.7 (3.15)	82.8 (3.20)	85.2 (3.57)

[a]Average of quadruplicate analysis.

[b]Number in parentheses represents coefficient of variation (CV, %).

CONCLUSIONS

A method using semi-micro HPLC equipment to analyze clenbuterol in pork, beef and hog liver was developed in this study. This method is easy to operate and its detection limit can be as low as 0.0001 ppm. The average recovery was higher than 80%. Applying this method, 150 samples purchased from local markets were analyzed and three of them were found to contain clenbuterol, but the levels were all lower than those allowed by governmental regulations. This method was proved to be usable.

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