Journal of Chromatography A, 1456 (2016) 242–248

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Metabolomic investigation of porcine muscle and fatty tissue after Clenbuterol treatment using gas chromatography/mass spectrometry

Guanglei Li, Yuhua Fu, Xiaosong Han, Xinyun Li, Changchun Li*

Key Lab of Agriculture Animal Genetics, Breeding, and Reproduction of Ministry of Education, College of Animal Science and Technology, Huazhong Agricultural University, Wuhan 430070, People's Republic of China

ARTICLE INFO

Article history:
Received 3 October 2015
Received in revised form 16 May 2016
Accepted 5 June 2016
Available online 6 June 2016

Keywords:
Clenbuterol
Muscle tissue
Fatty tissue
Metabolomic
Gas chromatography/mass spectrometry

ABSTRACT

Clenbuterol is a β-adrenergic agonist used as additive to increase the muscle mass of meat-producing animals. Previous studies were limited to evaluations of animal growth performance and determination of the residues. Several studies have focused on urine samples. Little information about the underlying molecular mechanisms that can explain Clenbuterol metabolism and promote energy repartition in animal muscle and fatty tissue is available. Therefore, this research aims to detect the metabolite variations in muscle and fatty tissue acquired from Chinese pigs fed with Clenbuterol using gas chromatography/mass spectrometry (GC/MS). Ten two-month old Enshi black pigs were fed under the same condition; five of which were fed with basic ration containing Clenbuterol for one month, whereas the other five pigs were fed only with basic ration. Muscle and fatty tissue were subjected to metabolomics analysis using GC/MS. Differences in metabolomic profiles between the two groups were characterized by multivariate statistical analysis. The muscle samples showed that 15 metabolites were significantly different in the Clenbuterol-treated group compared with the control group; 13 potential biomarkers were found in the fatty tissue. Most of the metabolites were associated with fatty acid metabolism and amino acid metabolism. Glycerol, phenylalanine, and leucine were the common metabolites between the muscle and fatty tissue. These metabolites may provide a new clue that contributes to the understanding of the energy reassignment induced by Clenbuterol.

1. Introduction

Clenbuterol is a kind of β-adrenergic agonist, which was first used as bronchodilator and tocolytic agent in clinical management, and then widely applied to meat-producing livestock as additive. β-adrenergic agonist increases the muscle mass and fatty tissue decomposition [1]. Previous studies showed that β-agonist increased the expression level of alpha-actin in the skeletal muscle of pigs and decreased protein degradation in sartorius and pectoral muscles of neonatal chicks [1,2]. Clenbuterol can inhibit masseter muscle atrophy caused by the dexamethasone through IGFI, AKT pathway, and mTOR pathway [3]. It can also alleviate muscle atrophy through its effects on the ubiquitin-proteasome pathway in rats [4]. β-agonist could inhibit the transcription of KLF-15 and the enzyme activity of the fatty acid synthase in birds [5]. Although β-agonist has powerful function in producing more skeletal muscles, its residue with long half-life period is harmful to humans [6]. The residues of β-agonist also affected the behavior and physiology in finishing pigs [7]. In general, the residues of β-agonist vary in different tissues, and the eyes have the most residues in all the tested tissues [8,9]. Impaired calcium homeostasis can explain the underlying mechanisms for dysfunction of the fast-switch muscle [10]. Several countries have banned the use of Clenbuterol in livestock [11,12], whereas Ractopamine can still be used in some countries [13]. A number of methods have been developed to detect the residue of β-agonists to supervise the abuse of them [14–18].Although Clenbuterol has been banned for many years in most countries, incidents of Clenbuterol poisoning have been frequently reported. Thus, development of other safe additives that can increase the skeletal muscle mass without toxicity to humans is necessary. We can get information about the underlying mechanism of energy redistribution from the Clenbuterol.

In recent years, omics have developed effective methods in disease diagnosis and biomarker screening [19]. However,genomics, transcriptomics, proteomics and epigenomics cannot explain all things because the compensation of the other genes or pro-

* Corresponding author.
E-mail addresses: liguanglei9@126.com (G. Li), 397701514@qq.com (Y. Fu), hanxiaosong2011@163.com (X. Han), hzaulxy@163.com (X. Li), lichangchun@mail.hzau.edu.cn (C. Li).

http://dx.doi.org/10.1016/j.chroma.2016.06.017
0021-9673/

teins neutralizes the function of the changed gene or proteins. Metabolomics is a method that can detect low-molecular-weight metabolites from tissues or biological fluids. Metabolites are the final products of genes or proteins, so diagnosis of the variation of metabolites may be more accurate for tracking several diseases and drugs [20]. The metabolomics of residue of β-agonist have been reported. Wu et al. detected the metabolites of three β-agonists in swine urine using ultra-HPLC combined with the quadrupole time-of-flight MS [21]. 2-Indolecarboxylic acid and fluorometholone acetate may be considered as diagnostic markers to distinguish the illegal use of β-agonist. Gaud et al. implemented metabolomics plus a statistical model to detect the residue of β-agonist in bovine urine by LC-HRMS [22].

Previous studies focused the metabolites analysis on the body fluids, while muscle and fatty tissue are the target tissue of Clenbuterol. Target tissue might provide higher sensitivity and specificify than serum [23].Thus, it is necessary to identify metabolites from the muscle and fatty tissue of pigs feeding clenbuterol In this study, we conducted the metabolomics profiles of fatty and muscle tissues in Enshi black pigs, a famous Chinese indigenous pig breed, by using gas chromatography/mass spectrometry. We found different metabolites in the fatty and muscle tissues, and these biomarkers may be useful for exploring new additives and contributing the genetic improvement of livestock.

2. Materials and method

2.1. Ethics statement

In this study, all the pigs were raised in Enshi Black pig conservation farm in Xianfeng county of Enshi Tujia and Miao Autonomous Prefecture in Hubei Province of China. Animal care and all experimentations were conducted in accordance with the guidelines pre-approved by Huazhong Agricultural University Institutional Animal Care and Use Committee (HZAUSW2015-0003).

2.2. Animals and sample collection

Ten two-month-old Enshi black boars (Chinese local pig breed) were randomly divided into two groups. All of these pigs were castrated during lactation, and the weight of these pigs was in the same level. Five pigs were fed with basic ration without Clenbutoral (Jinhe Pharmaceutical Co. Wuhan, China), while the other five pigs were fed with basic ration with Clenbuterol (25 mg/Kg). After one month, all pigs were sacrificed in Sile Company, Enshi City, Hubei Province of China. The skeletal muscle (biceps femoris) and fatty tissue (at the fifth lumbar vertebra level) were collected, stored at −80 °C, and then processed for GC/MS analysis.

2.3. Specimen processing

For GC/MS analysis, the samples were stored and freeze-dried at −80 °C refrigerator and then was ground into powder and made homogenization before extraction. 50 mg of each sample was rapidly transferred to a 5 mL glass centrifuge tube. Approximately 2 mL of the homogeneous mixture of chloroform–methanol–water (20:50:20, v/v/v) was then added to each tube and 100 μL ribitol (0.1 mmol/L) was added to each sample as internal standard. The sample was conducted to homogenate using a homogeniser at 4 °C and then was centrifuged at 15,000*g* for 10 min 200 μL of the supernatant was transferred to a GC vial and then evaporated to dryness under a stream of nitrogen gas.80 μL of methoxy pyridine hydrochloride (15 mg/mL) was added to the vial. The sample was incubated at 37 °C for 90 min after vortexed for 20 s. After incubation, 80 μL of BSTFA with 1% TMCS was added to the vial, and the sample was incubated at 70 °C for 30 min.

2.4. GC/MS analysis

The Agilent 7890A GC system (Agilent, Shanghai, China) equipped with a 30.0 m × 0.25 mm i.d. fused-silica capillary column having 0.25 μm HP-5MS stationary phase (Agilent J&W Scientific) was used to conduct the analysis. 1 μL aliquot of derivative sample was injected splitless into the above system. The injector temperature was set at 270 °C. Helium was used as carrier gas at a constant flow rate of 1 mL/min through the column [24]. The column temperature program is described as follows: the initial temperature was maintained at 70 °C for 2 min and then increased to 140 °C at a rate of 10 °C/min. After the above procedure, the temperature increased to 240 °C at a rate of 4 °C/min and then reached to 300 °C at a rate of 10 °C/min and maintained for 8 min. The column effluent was introduced into the ion source of an Agilent 5975C mass selective detector (Agilent). The temperature of the EI ion source was set at 230 °C. The temperature of the quadrupole rod was set at 150 °C. Masses were acquired from *m*/*z* 50–600.

2.5. Data processing and multivariate statistical analysis for GC/MS

We used our programs (R language) to extract the raw data of the GC total ion chromatograms (TICs) and fragmentation patterns, which were then imported into the TagFinder software. In the acquiescent state, the software adjusted the retention time, aligned the peaks, and analyzed the fragment position. The data exported from the TagFinder was edited as two-dimensional matrix using EXCEL from Office software. The peaks intensities were normalized depending on the internal standard, which was adjusted to 1000. The value was set as 0.01 or smaller when the data was missing (zero) in some samples. The resulting data were exported into Simca-P software (Umetrics) for multivariate data analysis, including principal component analysis (PCA), partial least square-discriminant analysis (PLS-DA), and orthogonal partial least square-discriminant analysis (OPLS-DA) [25]. PCA analysis was mainly used to visualize the trend of the samples. PLS-DA and OPLS-DA were used to determine the difference between the two groups. The relevant R^2 and Q^2 were employed to evaluate model stability and ability to explain the raw data. PCA models were regarded as valid only if $R^2 > 0.4$. The PLS-DA models were regarded as valid only if $Q^2 > 0.4$.

2.6. Identification of potential biomarkers

The potential biomarkers to discriminate the Clenbuterol-treated group and the control were found by combining variable importance in projection (VIP > 1) values of the OPLS-DA model and student *t*-test ($P < 0.1$)[26]. The potential biomarkers were identified by comparison with a standard mass spectrum in the National Institute of Standards and Technology (NIST) library using the NIST 2.0 software. Peaks with similarity index of more than 70% were assigned compound names, whereas the others were listed as unknown metabolites [24].

3. Results

3.1. Metabolite profiling of muscle and fatty tissue

The representative TICs of the muscles and fatty tissue from the Clenbuterol-treated group and the control group are displayed in Fig. 1. In this study, we got 327 and 272 peaks in the muscle and fat tissue respectively, and the detail information was supplied