Concentration of Steroid Hormones in Chicken Meat Samples from Iranian Markets Using Liquid Chromatography–Tandem Mass Spectrometry

Nabi Shariatifar

Tehran University of Medical Sciences

Samaneh Nabizadeh (✉ snabizadeh@yahoo.com)

Tehran University of Medical Sciences

Seveh Anboyeh

Tehran University of Medical Sciences

Maryam Amirahmadi

Tehran University of Medical Sciences

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Abstract

The aim current study was to analysis of anabolic hormones (methyltestosterone and androstenedione) residue in meat poultry samples collected from Tehran market of Iran by a method based on liquid chromatography–tandem mass spectrometry (LC/MS/MS). In this regard, the extraction method of "quick, easy, cheap, effective, rugged and safe" (QuEChERS) was applied. The method of optimized was validated; obtaining suitable results for all validation parameters in the chicken matrices were assessed. Recovery values ranged from 76.3% to 116.7%. The detection limit (LOD) was established for two compounds as 0.16 µg/kg, and the quantification limit (LOQ) was determined as 0.5. Finally, 72 chicken meat samples were analyzed and two hormones (methyltestosterone and androstenedione) were evaluated, which were not detected in any of the chicken meat samples. Therefore, it can be concluded that the amount of contamination of chicken meat available in the Iranian market with hormones is often similar to that found in other countries, and in accordance with the European Commission (EC) standard. Therefore, due to the lack of hormones in chicken meat, its use cannot be dangerous for humans.

Introduction

Hormones are chemical substances which act as messengers and releases from a cell and reach the targeted cell (near or far away), and cause the response in the cells and target tissues in body. These chemical substances have important role at physiological functioning and maturation of bodies of human and animals. Hormones are steroids or proteins. Steroid hormones have two vital actions: anabolic and androgenic. Some natural or synthetic steroids (Testosterone, progesterone, estradiol, methyltestosterone and androstenedione) are widely used in meat producing animals and poultry to anabolic effect and improve weight ¹.

Of public concerns about the presence of steroid hormones in a wide range of edible matrices (meat, sausage, milk, egg, kidney and liver), the use of hormones had been banned in number of European country for example Denmark, Germany, Italy since 1960s. European Commission (EC) set austere legislation that prohibited the use of all hormones for animal fattening except for therapeutic treatment in 1980. These hormones must be prescribed by a veterinarian and animals and poultry should be slaughtered after withdrawal time. Since 1980s, World Health Organization (WHO), Food and Agriculture Organization (FAO) and Office International des Epizooties (OIE) started to studies the safety of these hormones in meat production ². In later years, number of studies state excessive exposure to the steroids can be toxic to organ (liver and kidney) and systems and their residues may be infusing a variety of diseases (cancer of prostate, ovary and breast) ^{3,4}.

During the last decades, scientific studies for the level of steroid hormones in edible matrices, as muscle ⁵, liver ⁶, kidney ^{6,7}, milk ⁸ and egg ⁴ has been conducted. Several methods were used to determine the quantity of steroid hormones. Among the methods used for the determination of steroid hormones in the food samples, the Liquid chromatography–mass spectrometry (LC-MS), liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) high performance liquid chromatography (HPLC), gas chromatography mass spectrometry (GC/MS) and gas chromatography tandem mass spectrometry (GC–MS/MS) Due to saving the time, specificity and more sensitivity of liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS), was reported as better method⁹. Di Donna et al. ¹⁰, Kaklamanos et al. ¹¹ and Vanhaecke et al. ¹² discussed the use of LC–MS/MS, and concluded that these analytical methods were able to isolate and determine the residual compounds of steroids in food. Precision and accuracy of the method were satisfactory. Presence of a wide range of steroid hormones in edible matrices should be monitored. Considering the high consumption of chicken meat (the average per capita consumption is 23.5kg) in the meat and meat products basket worldwide, it is important to examine the amount of hormones in chicken meat. Therefore, in the current study, a method has been presented to quantitative determine of methyltestosterone and androstenedione in chicken meat based liquid chromatography coupled to electrospray ionization tandem mass spectrometry using multiple reaction monitoring (MRM) scan mode (positive mode). In electrospray ionization, matrix effects may lead to suppression or enhancement of the analyte signal and reduce sensitivity. Therefore, the signal analyzes in the standard solution and the blank samples were compared to determine the decrease or increase of signals in ionization. Also, the presence of different compounds in the matrix can affect the accuracy, precision and linearity. In this study, due to the effects of the matrix, the calibration curve is plotted by method matrix match.

Materials And Methods

2.1. Chemicals and reagents

The standard of methyltestosterone and androstenedione were purchased from company of Sigma (Sigma–Aldrich, USA). Methanol, acetonitrile, acid formic, sodium acetate (CH₃COONa), magnesium sulfate (MgSO₄), Primary secondary amine (PSA) and C18 (HPLC reagent grade) were obtained from company of Merck (Darmstadt, Germany).

The stock standard solutions (1mg/mL) and Mixed standard solutions (mix1: 10 µg/mL, mix2: 1 µg/mL and mix3: 0.1 µg/mL) were prepared in methanol and stored at –20 °C.

2.2 Samples collection

The blank samples collected from untreated chicken meat samples and for the presence of the steroid hormones, 72 chicken meat samples from famous brands were collected from markets of Tehran, Iran. The samples were placed at –20 °C until analysis.

2.3. Sample preparation

Initial extraction "quick, easy, cheap, effective, rugged and safe (QuEChERS): About 0.5 g meat poultry was placed into 50 mL centrifuge tube. The mixture of internal standards was added. 3 mL deionized water was added to samples and vortexed for 30 s. After addition of 5 mL acetonitrile and vortexes for 60 s, 0.75g (CH₃COONa) and 3g MgSO₄ added and vortexed for 60 s and were centrifuged for 10 min (8500 rpm). 1 mL aliquot of supernatant was transferred to cleanup tube that contains 0.45g of MgSO₄, 0.075g PSA sorbent and 0.075 g C18 sorbent. Then vortexed for 60 s and were centrifuged for 15 min (5000 rpm). 4 mL aliquot of supernatant was transferred to vial and was evaporated under a nitrogen stream. After the addition of 500µL methanol and 500µL deionized water, vial was vortexed. Finally, 20 µL was injected to LC-MS/MS.

2.4. LC–MS/MS analyses

The mobile phase was composed of solvent A (deionized water + acid formic 0.1%) and solvent B (acetonitrile + acid formic 0.1%).

The gradient program used was as follows: 40% solvent B + 60% solvent A at the start (t = 0 min, held for 5 min), 100% solvent B (t= 5min, held for 5 min), 40% solvent B + 60% solvent A (t = 11 min, held for 1 min) and 60% solvent B + 40% solvent A (t = 22 min). Reversed-phase C18 column (100 mm×2 mm, Blueorchid 175-108, A KNAUER) was used for the analyses. The flow rate was kept at 0.8 mL/min and injection volume was 20µL. In order to determine the concentration of hormones simultaneously, electrospray ionization (ESI) was used in the positive mode in the MRM program.

2.5. Statistical analysis

One sample t-test was used to statistically analyze the data, and to compare the mean concentration of sample hormones with the allowable value of FDA. The data analyzed using SPSS version21.0 (SPSS Inc., Chicago, IL, USA). The significance level was <0.05.

Results And Discussion

In the present study, levels of two steroid hormones were detected by LC/MS/MS. Our results showed that all samples collected from all grocery stores and main food supply locations no had contamination in the allowable level of EC. Based on this study, it has been shown that all important stages of meat production in Iran are studied thoughtfully.

The steroid hormones in chicken meat may be due to two reasons: first, prescription by a veterinarian or over-the-counter use by a poultry owner, and second, the presence of natural steroid hormones secreted from the gonads and adrenal glands of

chickens¹³. Excessive consumption of these hormones can lead to various physical and psychological complications. Complications of these substances in short term including gynecomastia, hair loss in men, growth of facial hair especially in women, menstrual problems and a deeper voice in women. Complications of these hormones in the long term can be serious, causing tumor of liver, abnormal cholesterol levels, heart disease, testicular shrinkage in boys, and short stature in adolescents, as well as behavioral, mood, and aggression changes¹⁴. Considering the relatively high proportion of chicken meat in the meat basket and meat products in Iran, it is important to routinely assess the concentration of steroid hormones in food is very important. Because the use of these hormones can have many side effects for humans¹⁵. Therefore, according to the above, it was necessary to use a precision and accuracy measuring device (LC/MS/MS) in order to measure steroid hormones.

So to optimization of mass spectrometer parameters and determine the parent ion, daughter ion and collision energy for the MRM workflow, each steroid standard with concentration of 5 µg/mL was directly infused into the system (Table 1). Fig 1-4 shown chromatograms of identification methyltestosterone and androstenedione parent ions and daughter ions.

Determinations of HPLC mobile phases have a significant influence on separation efficiency, MS/MS responses and ionization of the analytes and increase the sensitivity. Mixture of water and acetonitrile with gradient elution shown higher peaks and shorter retention time, whereas the water and methanol mixture had better separation of the peaks but the peaks were wider with a longer retention time (Fig 5 and 6).^{16,17} Formic acid (1%) used for optimized the mobile phases and ionization of the target analytes.¹⁷

To eliminate the matrix effects, calibration curves was evaluated with matrix-matched, using five concentrations (0.5, 1, 2, 5 and 7 ng/g) of mixed standard spiked to blank check samples. All correlation coefficients R-squared (R²) were > 0.999.

Under optimized high performance liquid chromatography-QuadrupoleTime-of-Flight-MS (HPLC-QqQ-MS) conditions, five spiking levels (0.75, 1.25, 3, 4.5 and 6 ng/g) on blank chicken meat samples were processed and analysed, and recovery was measured for each steroid hormone at both low and high concentration levels. By measuring the inter-day repeatability (5 days) at the spiking level of 0.1 µg/mL, the method precision was evaluated; RSDs of the recoveries were lower than 9.51%. This method in positive ionization mode proved accurate for methyltestosterone and androstenedione with calculated mean recoveries, 76.3% to 116.7% (Table 2).

In the present study, the concentration of two hormones in 72 chicken meat samples were not detectable or lower than LOD (LOD=0.16 ng/g). Bussche et al.¹⁸ in 2014 used QqToF-MS for analysis of growth promoting agents in meat and relative standard deviations for repeatability and reproducibility were below 20%. In this study, we used triple quadrupole (QqQ-MS); RSDs of the recoveries were lower than 9.51% and have good sensitivity and confirmatory performance in multiple reaction monitoring. Triple quadrupole system was equipped with electrospray ionization (ESI) source. López-García et al. (2018)¹⁹ state electrospray ionization provided higher sensitivity than atmospheric pressure chemical ionization and reduced mass error of all the target analytes in the analysis of steroid hormones. Conventionally, extraction and purification of solid samples are conducted with organic solvents using liquid-liquid extraction (LLE) and/or liquid solid extraction (LSE). Fuh et al. (2004)²⁰ and Blasco et al. (2007)¹⁶ used acetonitrile for extraction of steroid hormones and Shao et al. (2005)⁶, Impens et al. (2002)⁷, Marchand et al. (2000)²¹ and Seo et al. (2005)²² performed extraction with methanol. López-García et al. (2018)¹⁹ applied acetonitrile based on the QuEChERS method for meat samples of chicken, beef and pork and clean-up step was conducted adding aluminum oxide and florisil. They compared QuEChERS and "dilute and shoot" extraction method for the meat samples; results were better in QuEChERS approach. Guedes-Alonso et al. (2017)²³ applied methanol and acetonitrile as extractants for extraction of steroid hormones from fish tissue; recoveries were below 15% when used acetonitrile. For the mixture of acetonitrile: water (50:50, v/v), the signal suppressions were high (over 30%). Recoveries for pure methanol and mixture of methanol: water 50:50 (v/v) were over 50%, whereas signal suppressions of methanol: water 50:50 (v/v) was 36%. For these reasons, the chosen organic solvent for microwave-assisted extraction was pure methanol. They selected Phenomenex Phree cartridges to remove phospholipids and proteins from fish sample. Regarding this method, recovery obtained from muscle extracts was approximately 56%.

We used acetonitrile to extract samples and separation of lipids from chicken matrices. C18 and PSA sorbent used for clean-up and purification and mean of recovery obtained was 96.5%.

Conclusion

Considering the high consumption of chicken meat (the average per capita consumption is 23.5kg) in the meat and meat products basket worldwide, it is important to examine the amount of hormones in chicken meat. For this reason, there is always a concern about the presence of hormones in this food. The results indicated that LC-MS/MS with QuEChERS extraction method can monitor the trace residues of steroid hormones (methyltestosterone and androstenedione) in chicken meat samples with high sensitivity and selectivity. In addition, our data showed that no hormones (methyltestosterone and androstenedione) was observed in all samples. Therefore, it can be concluded that the amount of contamination of chicken meat available in the Iranian market with hormones is often similar to that found in other countries, and in accordance with the European Commission (EC) standard. Therefore, due to the lack of hormones in chicken meat, its use cannot be dangerous for humans.

Declarations

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Author statement

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Authorship contributions

Nabi Shariatifar: Conceptualization, Supervision, Design of study, Writing- Reviewing and Editing,. Samaneh Nabizadeh.: Data curation, Writing- Original draft preparation, Visualization, Investigation. Serveh Anboyeh: Software, Methodology. Maryam Amirahmadi : Software, Validation:

Best regards

Corresponding author

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Tables

Table 1. Precursor and product ions of hormones

Compound	Parent ion [M+H] ⁺	daughter ions (m/z)	CE(eV)	DP(eV)	EP(eV)
methyltestosterone	303.4	97.2	29	36	9
		109.2			
androstenedione	287.3	97.1	29	36	9
		109.1			

CE: Collision Energy

DP: Declustering Potential

EP: Entrance Potential

Table 2. Results obtained from the determination of hormone in chickenmeat samples

Samples	Number	amount	Hormone	LOD (ng/g)	LOQ (ng/g)	Spiking Level (ng/g)	Recovery (mean) %	RSD %
Chicken meat	72	*ND	methyl testosterone	0.16	0.5	0.75	76.3692	5.21
						1.25	102.2715	7.23
						3	111.1720	9.51
						4.5	93.2249	6.54
						6	105.1773	6.33
			androstenedione	0.16	0.5	0.75	116.7001	6.21
						1.25	80.8810	8.76
						3	102.4505	8.32
						4.5	92.5432	7.56
						6	107.4718	8.31

* non detected

Figures

Figure 1

Chromatogram of identification methylestosterone parent ion

Figure 2

Chromatogram of identification methylestosterone daughter ions

Figure 3

Chromatogram of identification andoseterone Parent ion

Figure 4

Chromatogram of identification andoseterone daughter ions

Figure 5

Chromatogram of separation of two hormones with mobile phase, water and methanol

Figure 6

Chromatogram of separation of two hormones with mobile phase, water and acetonitrile