Method development and validation of testosterone in muscle by using LC-MS/MS in positive ESI mode

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A simple, accurate and precise LC-MS/MS method has been developed and validated for determination of testosterone in muscle. The method has been validated as per the guidelines of ICH and FDA. The finalized method is revealed with significantly shorter retention time of 2.0 min with simple isocratic programme. The MRM transitions corresponding to m/z 289>109 are used for quantification. The unit of detection of the method is found to be 0.20 ng/kg and unit of quantification has been calculated as 0.40 ng/kg. The method is found to be linear in the range of 0.25 ng/mL to 10 ng/mL. The recoveries of testosterone from the spiked samples of muscles are found between 85-98%. The results of the study show that the proposed method is simple, rapid, precise and accurate, which is useful for the estimation of testosterone in muscle sample and other food products of animal origin with accuracy and reproducibility.

Keywords: Testosterone, LC-MS/MS method, food products, animal origin, method development, validation

In the earlier ages, products of animal origin harvested by hunting were considered as noble food contributing to strength, health, longevity and the well being of man\textsuperscript{1}. However, with advances in science, many xenobiotic and natural compounds (steroids, hormones and \(\beta\)-agonists) have been used and sometime misused to improve the growth of cattle, sheep and other livestock animals\textsuperscript{2}. Testosterone is one such steroid hormone from the androgen group (C-19 steroids) and is found in mammals, reptiles\textsuperscript{3}, birds\textsuperscript{4} and other vertebrates. Androgens stimulate or control the development and maintenance of masculine characteristics and most often used as anabolic steroids\textsuperscript{5}. Steroid hormones are legally used in veterinary medicine under veterinary prescription. However, besides their use under regulated conditions, their use for growth promotion is forbidden. Nevertheless, several synthetic hormones including 17-methyl-testosterone are still offered on the ‘black’ market for animal fattening purposes. Steroid hormones are used in animal fattening because of their capacity to increase weight gain and to reduce the feed conversion ratio, which is the average feed intake in relation to the weight gain. In addition, their synergetic effects and their ability to reduce nitrogen retention and to increase the water retention and fat content have been reported in literature\textsuperscript{6-8}. In 1981, the European Union prohibited the use of testosterone and other such substances having a hormonal action for growth promotion of farm animals. Directive 88/146/EEC was promulgated prohibiting the administration of testosterone for growth promotion or fattening purposes\textsuperscript{9}. Based on the available literature, with emphasis on multi-residue methods for steroids in meat, a number of analytical methods have been developed and are described\textsuperscript{10-12}. Fewer methods are described for kidney fat\textsuperscript{13}, kidney\textsuperscript{10}, liver\textsuperscript{10} and muscle\textsuperscript{10,14}. De Brabander \textit{et al.} have extensively reviewed the possibilities of mass spectrometry in the determination of residues of banned substances (amongst other things EGAs and corticosteroids) in matrices of meat producing animals\textsuperscript{15}. Although, many LC–MS methods have been developed to measure steroid hormones and hormone-like substances in environmental samples\textsuperscript{16-21} or urine\textsuperscript{22,23}, the application to edible matrices of animal origin are rather limited\textsuperscript{10,12,24,25} compared to the number of detection methods based on gas chromatographic separation and mass spectrometric detection. The current method has been tested and validated on the basis of principal criteria, like accuracy, specificity, precision and practicability by which quantitative analytical methods are usually evaluated\textsuperscript{26}. The results of the study show that the proposed method is simple, rapid, precise and accurate, which is useful for the estimation of testosterone in muscle sample with accuracy and reproducibility.

Results and Discussions

Extraction procedure

For the extraction of testosterone from the muscle samples, current extraction procedure has been developed which is better than the reported analytical
method. The reported method has used the acetic acid buffer and SPE techniques for sample cleanup which not only makes the sample preparation cumbersome but the method was also prone to errors resulting in low recoveries and accuracy of the results. Based upon the past experience of the authors, the extraction method was thereby simplified. Since testosterone is soluble in solvents like methanol and acetonitrile, a combination of methanol and acetonitrile was employed for extracting the residues of testosterone from muscle samples. Any fat components which might have been co-extracted along with the testosterone residues were washed off with n-hexane saturated with acetonitrile. The extract was dried under nitrogen and the dried extract was dissolved in 0.5 mL methanol and injected into LC-MS/MS.

Method development

The liquid chromatographic separation and quantitation is achieved using isocratic system of elution with no salts used in the mobile phase. The chromatographic conditions have been used as mentioned in the experimental section. With the use of proposed method, a well resolved peak for testosterone is obtained within 2 min of injection. The composition of mobile phase has been optimized through several trials to achieve good resolution with systematic peak shape. Different volumes of methanol-water and acetonitrile-water were tried as mobile phase, along with additives, like acetic acid and formic acid in varying concentrations. Finally, a mixture comprising of water with 0.1% formic acid and methanol with 0.1% formic acid in the ratio of 20 and 80, respectively, was found as the most suitable mobile phase which eluted the testosterone at 1.37 min. The use of X Terra MS C-18 column (2.1 mm × 100 mm; 5 µm) provided separation of testosterone with no interference from the matrix.

For the purpose of evaluating the parent ion and the product ions, the reference standard solution of testosterone 500 ng/mL was infused using both the positive and negative ESI mode, the intensity of positive mode was much higher due to protonation. No signal for testosterone was observed when infused in the negative ionization mode. The use of 0.1% formic acid in the mobile phase further augmented the response of precursor [M+H]+ ions at m/z 289 in the MS full scan spectrum. The two most abundant product ions at m/z 97 and 109 were distinctly observed in the positive ionization mode. A MRM acquisition method was set up based on the transition of the respective [M+H]+ ions towards either m/z 97 or 109. Figure 1 explains the fragmentation of testosterone parent ion m/z 289 into two product ions at m/z 97 and 109.

The data on MS spectrum of testosterone and MS/MS spectrum of testosterone in ESI positive mode is shown in Table I.

**Table I** — MRM settings for positive ion mode analysis of testosterone

<table>
<thead>
<tr>
<th>Parent ion (Da)</th>
<th>ESI mode</th>
<th>Product ion (Da)</th>
<th>Collision energy</th>
<th>Cone energy (V)</th>
<th>Dwell time (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>289</td>
<td>Positive</td>
<td>97, 109</td>
<td>30</td>
<td>30</td>
<td>50</td>
</tr>
</tbody>
</table>

**Figure 1** — Fragmentation process of testosterone

Although the current extraction procedure was simpler compared to the ones already reported earlier, the extracts obtained through it were free from any interference which may generally occur due to
presence of other components in the matrix and moreover, the current method gives good specificity. It was important to establish criteria that can be used as the basis for rigorous identification of testosterone through the replicated analysis of muscle samples spiked at different concentration levels i.e., 0.5, 2.5 and 10 µg/kg. The ion ratio for the ions at m/z 97 > 109 was found within the acceptable range of ± 20% against the predicted ion ratio of 1.16, considering 97 as the quantifier ion and 109 as the qualifier ion. Combining these ion ratio criteria with the requirement of the analyte to fall within the retention time window of 1.37 ± 0.5% (1.37 ± 0.069 = 1.30 to 1.44) min, provided the basis for identification criteria.

**Method performance characteristics**

The method was validated as per the International Conference on Harmonization (ICH) guidelines

**Linearity**

Six calibration standards run in triplicate were evenly spread over the concentration range of interest. The calibration curve prepared using the pure standards were found to be linear in the range of 0.25 ng/mL to 10 ng/mL with correlation coefficient of 0.9993. Linearity of the matrix-matched calibration standards in the concentration range of 0.040 ng/kg to 1.60 ng/kg and at the same concentration levels as that for the calibration standards was also evaluated in triplicate. The calibration curve (Figure 2) for the matrix-matched standards was also found to be linear with correlation coefficient of 0.9998. Calibration equation was

\[ y = 371.46x + 23.013 \]

\[ R^2 = 0.9998 \]

**Specificity**

The chromatographic interferences from the muscle samples were investigated by comparing the chromatograms of blank and the spiked samples
(Figure 3). For this purpose, samples were prepared using the same procedure as mentioned in preparation of sample and the specificity of the method was measured. It was found that the presence of interferences did not have any effect on the quantitative results of the analyte of interest thus providing reliability of the LC-MS/MS method for determination of testosterone.

**Precision**

The precision evaluated as the repeatability of the method was studied on sample (containing 0.25 ppb, 1 ppb and 2.5 ppb of testosterone) by calculating the percentage relative standard deviation (%RSD) for 6 determinations performed on the same day (intra day) and on three different days (inter day) and by different analysts (Table II). The low %RSD value within the acceptable norms obtained for intra-day and inter-day variations showed that the proposed method is precise and can be adopted for analysis.

**Accuracy**

The recoveries (Table III) of testosterone in spiked samples were calculated to study the effect of matrix on the determination of testosterone. The recovery studies were carried out at three different concentrations. For this, three different portions of pre-analyzed muscle samples were spiked with 0.08 µg/kg, 0.40 µg/kg, and 1.6 µg/kg, respectively, in triplicate on three different days and then extracted and determined by the same method as mentioned in preparation of sample. The recoveries of testosterone from the muscle samples were found in the range of 85% to 98%.

**Robustness**

Robustness of the method was determined by analyzing the same set of spiked samples (i.e. samples spiked at concentration levels of 0.25 µg/kg, 1.0 µg/kg and 2.5 µg/kg) under different parameters such as same column chemistry from different manufacturers, different analysts, and different injection volumes. The method was found to be robust even with small changes in analytical conditions: change in flow rate (±0.05 mL/min), a change in column temperature (±5°C), use of same column from different manufacturer (Waters C-18 and Agilent C-18 columns). Under all of these conditions, the analytical values of the spiked samples were not affected and it was in accordance with the actual values.
LOD and LOQ study

LOD was determined by considering signal to noise (S/N) ratio of 3:1 for the strongest mass transition with respect to the background noise obtained from the blank sample, whereas, LOQ was determined similarly by considering S/N ratio of 10:1. Based upon the mean noise level for the ten injections for each of the matrix blank of two muscle samples, lowest detection limit of the instrument was calculated as 0.20 ng/kg and confirmed using standard solutions with concentration of 0.20 ng/kg. The lowest concentration level that could be quantified with reproducible values was determined as 0.40 ng/kg, which was further confirmed by injecting matrix matched standard solution of testosterone having concentration of 0.40 ng/kg. The level of detection and the level of quantitation of the method were calculated, taking into account the sample weight and the dilution factors; in this case the concentration factor. Since the dried extract contains 1.5 g of muscle sample, the volume was reduced to 0.25 mL.

Materials and Methods

Reference standard of testosterone with purity of 98% was purchased from Sigma Aldrich. Acetonitrile, water and methanol (liquid chromatographic grade) were purchased from Merck Specialties Private Limited. Formic acid and n-hexane (analytical reagent grade) were purchased from S.D. Finechem Limited and anhydrous sodium sulphate was purchased from Rankem Chemicals Limited.

Instrumentation

LC-MS/MS system (Waters 2695 series, USA) with a Triple Quadrupole Mass Spectrometer, Quattro micro API (Micro mass, UK) equipped with an electro spray interface and masslynx 4.1 software (Micro mass) for data acquisition and processing was used. Balance (Afoset 3200, Mettler toddler) with readability of 0.01 mg and capacity of 180 g, Vortex (Model-Spinix (Tarsons Products Pvt Ltd), Syringe filters (Advanced Microdevices Private Limited) of pore size 0.2 mm and 0.45 mm with diameter of 25 mm, disposable 50 mL conical centrifuge tubes with screw caps (Tarsons Products Pvt Ltd), Rapid Vap (Labconco Corporation) nitrogen evaporator and high-speed refrigerated centrifuge (Remi Sales and Engineering Ltd) with rotor head suitable for eight 50 mL sample tubes were used.

Method

Preparation of standard solution

Approximately 5.0 mg ± 0.1 mg of testosterone reference standard was accurately weighed and dissolved in approx. 20.0 mL methanol in a 100 mL volumetric flask. Volume was made upto 100 mL with methanol. This gave a stock solution of 50 µg/mL. From the stock solution 1 mL of aliquot was taken and diluted to 100 mL to give a standard solution of testosterone having a concentration of 0.5 µg/mL. The solutions were stored at 2 to 8°C.

Preparation of calibration standard solutions

From the standard solution having concentration of 0.5 µg/mL, appropriate aliquots were taken and further diluted with methanol so as to give a series of calibration standard solutions having testosterone concentrations of 0.25, 0.5, 1.0, 2.5, 5.0 and 10.0 ng/mL, respectively. All solutions were stored at 2 to 8°C.

Preparation of matrix- matched calibration standard solutions

For the matrix-matched calibration curve, six portions of 1.5 ± 0.1 g sample of muscle extract were taken separately in six individual centrifuge tubes and extracted using the procedure as given under the heading for preparation of sample. The individual extracts obtained were evaporated to dryness and reconstituted using testosterone solutions containing 0.25, 0.5, 1.0, 2.5, 5.0 and 10.0 ng/mL respectively, so as to give matrix matched calibration standards having concentrations 0.040, 0.080, 0.16, 0.40, 0.80, and 1.60 ng/mL.

Preparation of mobile phase

The mobile phase was prepared by mixing two solutions, solution A (0.1% formic acid in water) and solution B (0.1% formic acid in methanol) in the ratio of 20 and 80, respectively, filtered through 0.45-micron filter membrane using a Millipore filtration unit.

Preparation of sample

Approximately 3.5 g of the muscle sample was taken in the centrifuge tube and extracted with 10 mL of 30:70 mixtures of acetonitrile and methanol using vortex mixer. The sample was then centrifuged at ambient temperature for 10 min at 7000 rpm followed by centrifugation again at 4°C and at 7000 rpm for further 15 min. The supernatant layer was collected in a dry separating funnel. The extraction process was repeated twice and the combined organic solvent
collected from all the three extractions. The combined extract was dried over anhydrous sodium sulphate and washed with n-hexane saturated with acetonitrile. This solvent was then evaporated to dryness under a stream of nitrogen and the dried extract was re-dissolved in 0.5 mL methanol before injecting into LC-MS/MS.

**LC-MS/MS conditions**

**Column**

Chromatographic analysis of testosterone was performed using X Terra MS C-18 column (2.1 mm × 100 mm; 5 μm) and mobile phase comprising of solution A (0.1% formic acid in water) and solution B (0.1% formic acid in methanol) in the ratio of 20 and 80, respectively, in the isocratic mode. The LC column was set at 40°C.

**ESI Interface**

Optimal parameters of the ESI interface were optimized by infusing 500 ng/mL standard solution of testosterone in the mobile phase using a Harvard syringe pump. LC-MS/MS determination was performed by operating the mass spectrometer in positive ionisation mode.

**Typical MS settings**

Capillary voltage (kV): 3.5; cone voltage (V): 30; source temperature (°C): 120; desolvation temperature (°C): 450. Mass spectra was acquired over a scan range of m/z from 100 Da to 500 Da for MS/MS mode, product ion scan mass spectra of protonated molecules of testosterone was acquired in MS/MS mode, product ion scan mass spectra of over a scan range of m/z from 100 Da to 500 Da for characteristic fragments in the mass range of 50 Da to 300 Da. Two different characteristic fragments i.e. 289>97 and 289>109 were monitored for testosterone in multiple reaction monitoring mode (MRM) using a dwell time of 50 milli seconds and collision energy of 30 (V) was used.

**Conclusion**

The developed method using positive ESI LC-MS/MS allows the detection, quantitation and confirmation of testosterone in muscle present at trace levels with high precision, accuracy and sensitivity by using a simple extraction procedure. In spite of using a simple extraction procedure, no interferences were observed from the matrix components during the determination of testosterone residues. The method can be used for the routine analysis of testosterone residues in muscle with added advantages of speed and economy. The method can also be tried for testosterone content in other animal products, like meat and poultry.

**References**