



Determination of Atropine Residues in Beef by High Performance Liquid Chromatography

Shuhuan Li^{1*}, Yongheng Bo², Youzhi Li³ and Xiuzhen Yang²

¹Department of Food Science and Engineering, Shandong Agricultural and Engineering University, Jinan 250100, China

²Shandong Livestock Product Quality and Safety Center, Jinan 250022, China

³Institute of Veterinary Drug Quality Inspection of Shandong Province, Jinan 250022, China

Shuhuan Li and Yongheng Bo contributed equally to this article.

ABSTRACT

Atropine is an anticholinergic drug from natural plants, and has been widely used in the clinical applications of animals and humans. However, in livestock production, excessive or improper use of atropine will lead to atropine residues in meat. When people eat animal meat from these sources, it will pose a potential threat to human health. Thus, in production practice, atropine residues in meat are usually determined quantitatively. In this study, high performance liquid chromatography (HPLC), a simple but potent method was used to quantify atropine residues in beef. The results indicated that the method could measure atropine residues accurately, with the average recoveries between 82.37 and 88.31 % when the additional concentration ranged from 0.5 to 5.0 mg/kg, and a detection limit of 0.25 mg/kg, providing an effective and reliable method for the detection of atropine residues in beef.

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Authors' Contribution

YB and YL conceived and designed the experiments. SL and YB performed experimental work and analyzed the data. YB wrote the article. SL revised the manuscript. XY provided the experimental basis for the research.

Key words

Atropine, Drug Residues, HPLC, Atropine Level in Beef

INTRODUCTION

With the rapid economic development and the improvement of people's living standard, the demand of animal-derived foods is also increasing. Under such circumstances, it is urgent to establish effective and low-cost method for their drug residues detection.

Atropine is a kind of alkaloid extracted from *Solanaceous* plants, which can be synthesized artificially (Cirlini *et al.*, 2019; Sramska *et al.*, 2017; Ciechomska *et al.*, 2016). Atropine has important applications in clinical medicine as a drug (Wang *et al.*, 2017; Perera *et al.*, 2017; Bratcher *et al.*, 2016), and has been used as the first choice drug for the treatment of organophosphorus poisoning (Samprathi *et al.*, 2020; Jiang *et al.*, 2019; Liu *et al.*, 2015). In veterinary practice, it can also be used as a neuromuscular blocker, anesthetist and cardiac sympathetic balance agent (Lagarde *et al.*, 2014; Poletto *et al.*, 2011; Clutton and Glasby, 2008). It must be noted

that, atropine residues remained in human and animals will cause serious impact and potential harm to health (Daoud *et al.*, 2019; Samsamshariat *et al.*, 2019; Akemi *et al.*, 2018; Moudgil *et al.*, 2018; Adamse *et al.*, 2014).

In addition, it is reported that as atropine can reduce gland secretion and induce animals to be thirsty to drink a lot of water, some illegal traders inject atropine into animal bodies in the slaughter process (Wang *et al.*, 2019). Once atropine residues from animals are ingested, they will make people vehement and agitated, delirious and blacked and may cause death, so it is urgent to strengthen the supermarket monitoring of atropine, and meanwhile it is necessary to establish effective and rapid determination method for atropine residues from animal meat.

Many methods such as liquid chromatography-mass spectrometer (LC-MS) (Wang *et al.*, 2019), gas chromatography-mass spectrometer (GC-MS) (Papoutsis *et al.*, 2012), liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Baslé *et al.*, 2020), ultra high performance liquid chromatography (UPLC) (Chen *et al.*, 2019) and ultra performance liquid chromatography/tandem mass spectrometry (UPLC-MS/MS) (Castilla-Fernandez *et al.*, 2021; Arvadiya and Dahivelker, 2013) have been reported for atropine determination in pharmaceuticals, plants, blood, serum and plasma, and the detection limit of the above methods were all lower than the HPLC method.

HPLC has been widely used in synthetic chemistry,

* Corresponding author: lishuhuan418@126.com
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food inspection, environmental monitoring, and determination of many kinds of veterinary drug residues in meat (Yashin and Yashin, 2020; Chitescu *et al.*, 2011; LeBoulaire *et al.*, 1997), eggs (Canton *et al.*, 2019), milk (Legrae *et al.*, 2020; Prado *et al.*, 2015) and other foods.

In this study, a HPLC method which has the advantages of low cost, high popularity and simple operation has been used to determine the level of atropine in beef.

MATERIALS AND METHODS

Reagents and materials

Standard atropine (99.9 % purity) was obtained from Dr. Ehrenstrofer GmbH (Germany). Methanol, acetonitrile and n-hexane (Chromatographic purity grade) were supplied by Fisher Company (America). The other reagents used in this study were all AR grade chemicals, and ultrapure water was used in the experiment. The beef as blank control without atropine was from Hualian supermarket in Jinan and it had an official animal quarantine certificate. The atropine positive beef sample was from the hind leg muscles of a dead cattle on a farm in Tai'an of Shandong Province. The cattle was poisoned after eating feed containing organophosphorus pesticides and died after being treated with the antidote atropine. The drug of atropine was firstly given at a dose of 60 mg/kg, followed by a dose of 400 mg/kg in 5 times 5 h later.

Preparation of atropine standard solution

A standard stock solution of 100 µg/mL was prepared in methanol and concentrations ranging from 0.5 to 20.0 µg/mL were used for preparation of standard curve using HPLC for quantification of atropine in samples.

Experimental conditions of HPLC

HPLC measurements were conducted on a Waters Alliance E2695-C18 (4.6 mm × 25 mm, 5 µm) system with a PDA detector, the eluent i.e. the mobile phase was phosphoric acid solution (0.025 mol/L, pH = 3) and acetonitrile (volume ratio being 87:13). UV detection was at 210 nm, flow rate was 1.0 mL/min, and the injection was 20 µL for all samples and standard solutions at 30 °C.

Pretreatment and purification of samples

For pretreatment and purification of samples, the uniformly ground beef sample (2.00 g/10.00 mL acetonitrile) were shaken on vortex mixer for 1 min, and then on multi-speed oscillator for 15 min. After centrifugation, the supernatant was separated. After repeated extractions procedures, the combined supernatant was blow dried with nitrogen. The obtained residues were dissolved in NaCl solution (4%, 5 mL) and n-hexane (5 mL). The mixture was then stirred and layered. The n-hexane layer was discarded and the water layer was preserved for purification by HLB

solid phase extraction (SPE) column.

Firstly, the column was activated with 3 mL methanol and 3 mL water, then all the sample was poured into the column, washed with 3 mL methanol (5%) and 3 mL water respectively. Next, the column was dried and eluted with 3 mL acetonitrile, and the eluate was dried with nitrogen at 50°C. Lastly, atropine residues in the sample would be determined by HPLC after the sample was dissolved with 1.00 mL mobile phase in fixed volume and filtered. Figure 1 shows the absorption curve, chromatographic elution curve and standard curve of the extraction and purification of atropine.

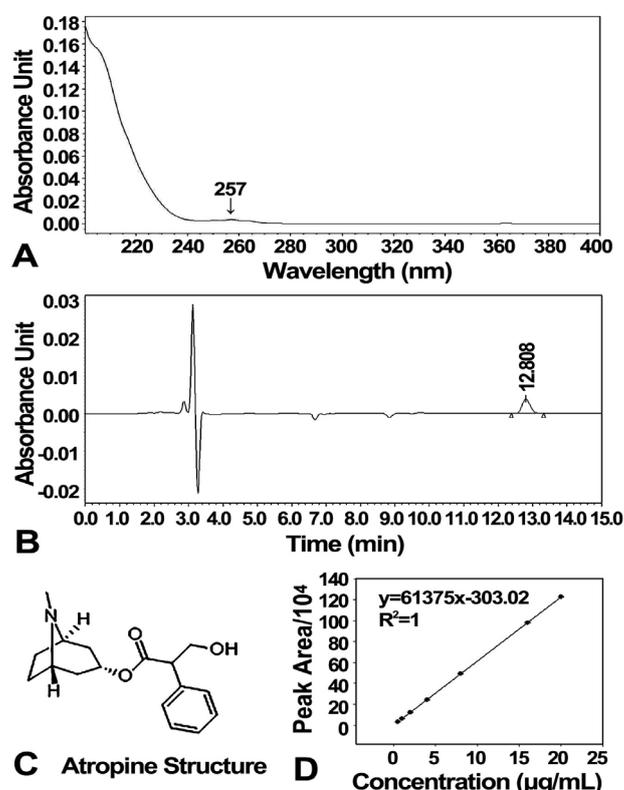


Fig. 1. A, absorption curve of atropine in UV spectrum; B, chromatographic elution curve of atropine (1 µg/mL); C, chemical structure of atropine; D, Standard curve of atropine.

Sensitivity test and accuracy test by HPLC

To perform the sensitivity test, standard atropine with four different concentrations (0.25, 0.5, 1.0 and 5.0 mg/kg) were added to the four different groups of blank beef sample respectively, and then determined by HPLC. The spiking blanks experiments were used to evaluate the accuracy and precision of the method. Standard atropine with three different concentrations (0.5, 1.0 and 5.0 mg/kg) were separately added to blank beef sample and were determined by HPLC. Each concentration was tested in 5

parallel samples and was repeated 3 times.

The experiments were performed in triplicate, and result was expressed as mean value \pm standard deviation.

RESULTS

In the process of purification, it is necessary to avoid some operations that are not conducive to sample purification. For example, it is not recommended to conduct the ultrasonic treatment on the sample after it is extracted with acetonitrile, because it will lead to the increase of cell fragments, which is not conducive to the adverse for the precipitation of impurities during centrifugation, and the column was also more likely to be blocked under such circumstances. In addition, avoid to dry the sample thoroughly with nitrogen gas, otherwise the recovery rate will decrease. In a word, after extraction, fat removal, and SPE purification, the impurities in the sample were effectively removed, providing a reliable condition for the subsequent HPLC separation and determination.

Figure 2 shows that the maximum absorption wavelength of atropine was between 202 and 210 nm. To increase the accuracy of the experiment and further reduce the absorption of acetonitrile, 210 nm was chosen as the detection wavelength and to improve the separation effect, 0.025 mol/L phosphoric acid solution (pH = 3.0) and acetonitrile was chose as the mobile phase (v/v=87/13). Under this condition, the retention time of standard atropine was found at 12.8 min (Fig. 2C).

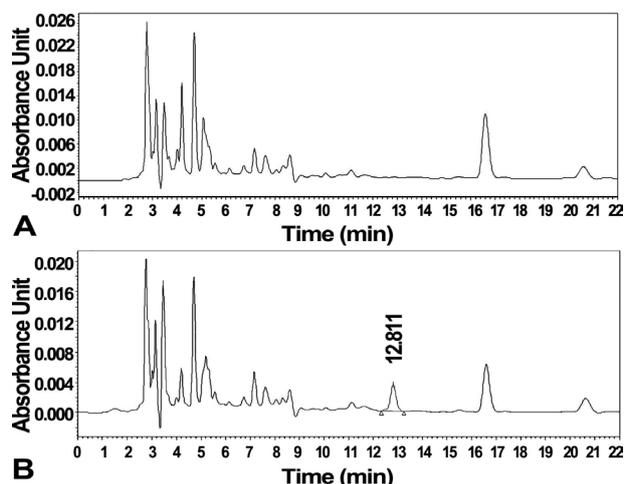


Fig. 2. The chromatographic elution curves of blank beef (A) and beef added with 0.5 mg/kg standard atropine (B).

For the sample of beef, no atropine was found as no peak was found at or near 12.8 min (Fig. 2A). When the atropine standard solution was added to the sample of blank beef group, it can be seen that, a peak appeared

at 12.8 min which can be identified as atropine. It was obvious that the peak of atropine separated well (Fig. 2B) from other substances, with a resolution higher than 1.5, and this was considered as a standard for complete separation of two substances. In fact, under the same experimental conditions, atropine in pork and lamb can also be effectively separated according to our previous study (Bo *et al.*, 2020). Therefore, the optimized HPLC conditions here can not only be used for the determination of atropine in beef, but also for the determination of atropine in pork and mutton.

Sensitivity and detection limit analysis of HPLC

Four different concentrations of standard atropine solutions (0.25, 0.5, 1.0 and 5.0 mg/kg) were added to the sample of blank beef separately to evaluate the sensitivity and detection limit of the method. The detection limit can be found when the signal-to-noise ratio (S/N) is more than 3. In the result, when the additional concentration of atropine was 0.25 mg/kg, the value of S/N was 9.101651, so the detection limit of the method was determined as 0.25 mg/kg. Similarly, 0.5 mg/kg can be determined as the quantitative limit of the method when the value of S/N is greater than 10.

Accuracy and precision analysis of HPLC

The standard curves of atropine was first obtained by plotting of the peak area versus the concentrations of standard atropine solutions. From Figure 1D, it can be seen that the correlation coefficient R^2 of the standard curve is 1 (> 0.999) in the concentration range between 0.5 and 20.0 $\mu\text{g/mL}$, indicating that the linearity of the standard curve is very good, thus can meet the requirements for determination.

Subsequently, the recoveries were obtained through the spiking blanks experiments to evaluate the accuracy of the method. Three different concentrations of standard atropine solutions (0.5, 1.0 and 5.0 mg/kg) were added to the sample of blank beef separately, 5 parallel tests were performed for each concentration and repeated for 3 times. The average recoveries and relative standard deviations were listed in Table I. It can be seen that, under the experimental conditions established in this study, the recoveries of atropine were between 82.37 and 88.31%, and RSD in batch and between batches were all controlled within 10%.

Determination of Positive Samples

Based on the HPLC method established in this study, the positive beef sample was pretreated and determined. Results showed that the detection result was 245.1 ± 5.1 mg/kg, indicating that the method was effective for the measurement of atropine residues in beef samples.

Table I. Average recovery and RSD in batch, and RSD between batches of atropine in beef.

| Additional concentration of atropine / (mg·kg ⁻¹) | Average recovery in batch (%) | | | RSD in batch (%) | | | RSD between batches (%) |
|--|-------------------------------|-------|-------|------------------|-----|-----|----------------------------|
| | 1 | 2 | 3 | 1 | 2 | 3 | |
| 0.5 | 87.01 | 83.81 | 84.02 | 5.9 | 2.7 | 2.7 | 4.2 |
| 1.0 | 86.09 | 86.55 | 88.31 | 4.2 | 5.1 | 4.1 | 4.3 |
| 5.0 | 82.58 | 82.37 | 82.39 | 2.0 | 1.5 | 2.2 | 1.8 |

DISCUSSION

According to the literatures, the detection limits of LC-MS, GC-MS, LC-MS/MS, UPLC-MS/MS for atropine can reach ppb level (10^{-9}), but the detection limit of HPLC method in this study is of ppm level (10^{-6}). However, the structure of chromatography coupled with mass spectrometry is complex, the operation is time-costing and troublesome, and the maintenance cost is much higher than that of HPLC. So the HPLC established here can meet the requirements for food safety testing, and the detection of atropine in beef. In another experiment, the New Zealand white rabbits were killed 5 h later after unocular instillation of 0.05 mL of 1% atropine, with LC-MS and matrix-assisted laser desorption ionization-imaging mass spectrometry, atropine was detected with a concentration of 19.05 ± 5.57 mg/kg (Wang *et al.*, 2019), which is much higher than the quantitative limit of 0.5 mg/kg of the HPLC method in this paper.

In another respect, atropine has been mainly used as a drug for treatment currently, but its negative effects needs further investigation, especially the detection of atropine in food both domestically and abroad, is still very scarce, which should be paid more attention to (Chen *et al.*, 2021).

CONCLUSIONS

In this paper, a HPLC method was established after optimization for the determination of atropine residues in beef. Before the experiment, the beef was purified thoroughly to ensure the separation and determination of atropine. Under the established HPLC conditions, the detection limit and quantitative limit of the method was determined as 0.25 mg/kg and 0.5 mg/kg, respectively. Recoveries of the method was between 82.37%-88.31%. This method has been proved to be effective and sensitive in the detection of atropine residues in positive beef sample, provided a strong technical support for the effective monitoring of atropine residual risk in meat products.

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Statement of conflict of interest

The authors have declared no conflict of interest.

Ethical compliance

There are no researches conducted on animals or humans.

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