Determination of eight kinds of glucocorticoids residues in chicken muscle with on-line clean up combined HPLC-MS/MS

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ABSTRACT

A sensitive and rapid method using HPLC-MS/MS was developed for the determination of eight glucocorticoids residues in chicken muscle simultaneously by Turbo Flow. The eight glucocorticoids were prednisone, prednisolone, hydrocortisone, methylprednisolone, dexamethasone, betamethasone, beclomethasone and fludrocortisones. Samples were extracted with ethyl acetate and on-line cleaned up through a Turbo Flow solid-phase extraction column without time-consuming pretreatment before HPLC-MS/MS analysis. Sample pretreatment conditions, Turbo Flow conditions and mass spectral parameters were optimized and obtained eight glucocorticoids calibration curves. These curves showed good linearity over the concentration from 0.2 µg/kg to 50 µg/kg with an average recovery from 71.63% to 117.36%. This method could be applied on real samples and provided simple, rapid, sensitive and highly selective analysis, which made it feasible to be adopted in food inspection organizations or carry out quantitative analysis for other banned substance.

KEYWORDS

glucocorticoid, chicken muscle, Turbo Flow, HPLC-MS/MS, on-line cleanup

INTRODUCTION

Glucocorticoids, the main substance of cortisol are a class of steroid hormones secreted by adrenal cortex zona. It not only functions as adjusting sugar, fat and protein biosynthesis and metabolism, but also has anti-inflammatory, antitoxic, antiallergic, antishock, nonspecific immunosuppressive and antipyretic effects. Glucocorticoid was divided into endogenous glucocorticoids and synthetic glucocorticoids [1]. Synthetic glucocorticoids are commonly used in livestock production. It is frequently used to treat livestock inflammation, autoimmune diseases and bovine ketosis because of the function of antipyretic, anti-inflammatory and anti-allergic. Meanwhile, it can improve the feed conversion rate, and promote the growth of livestock and aquatic animals. Therefore, illegal purposes were often used to increase the intake of animal feed to reach the goal weight gain [2].

Long-term intake of glucocorticoids could cause a variety of human adverse reactions, such as obesity, salt and water metabolism disorders, digestive and cardiovascular complications, osteoporosis and vertebral compression fractures, and other neuropsychiatric disorders disease [3]. Therefore, maximum residue levels in different countries in foods of animal origin glucocorticoids have been stipulated. For example, the EU has banned growth-promoting effects of these drugs, and provided that the maximum residue limit of...
dexamethasone and betamethasone in bovine, porcine muscle and kidney was 0.75 μg/kg and MRLs in the liver was 2 μg/kg. Currently, many sensitive methods were developed to analyze the glucocorticoid for its low limit. ELISA kits had been developed specifically for screening samples glucocorticoid for its sensitivity and rapidity; unfortunately, this method was limited to use in the qualitative detection [4]. The commonly used method of quantitative analysis include gas chromatography [5–6], high performance liquid chromatography [7–8], gas chromatography-mass spectrometry [9, 10], liquid chromatography-mass spectrometry [11, 12]. Gas chromatography and high performance liquid chromatography cannot be applied to the detection of trace, because of low sensitivity, poor selectivity and the lack of specificity. Gas chromatography - mass spectrometry can meet the requirement but it needs derivatization of sample, which was not only cumbersome but also increases the uncertainty of the data [13, 14]. Liquid chromatography - mass spectrometry is high sensitivity, good selectivity and specificity, but the significant matrix effects in the testing process can affect limit of quantitation (LOQ), limit of detection (LOD), linearity, accuracy and precision. Turbo Flow technology which based on the principle of chromatographic provides a new choice for it is a kind of on-line automatic processing technology which combines diffusion, chemical and volume exclusion principle. This technology could provide a sensitive, accurate and rapid determination without using isotope situation [15].

In order to develop a simple pretreatment, selective sensitivity of practical on-line cleanup method to analyze eight glucocorticoids drugs residues in Chicken Muscle, a more effective pretreatment way was proposed to get more concentrated purer samples as well as to improve signal and reduce noise through the optimization of sample pretreatment conditions, Turbo Flow conditions and mass spectral parameters, as well as the examination of LOD, LOQ, linearity, range and recovery of this development method.

**MATERIAL AND METHODS**

**Instruments and reagents**

Instruments included Turbo Flow on-line cleanup liquid chromatography tandem mass spectrometry (thermo electric TSQ Quantum ultra, USA), on-line cleanup column Cyclone-P (0.8 × 50 mm), HPLC Columns, ACQUITY UPLC BEH C18 (2.1 mm × 100 mm, 1.7 μm), homogenizer (ULTRA-TURRAX Tube Drive, Germany), high-speed refrigerated centrifuge (Germany SIGMA 4K-18), nitrogen analyzer (USA Zymark TurboVap), whirlpool oscillator (USA vortex-Genie2), and 0.22 μm organic membrane.

Reagents included acetonitrile (HPLC grade), methanol (HPLC grade), acetone (HPLC grade), ethyl acetate (HPLC grade), hexane (HPLC grade), analytical grade (AR) and ultra-pure water.

Standard substances included prednisone, prednisolone, dexamethasone, betamethasone, fludrocortisone, methylprednisolone, beclomethasone, hydrocortisone and deuterated hydrogenated prednisolone. The standard substances were purchased from Germany Dr Ehrenstorfer GmbH Company. Reagent purity is greater than or equal to 98%.

**Preparation of standard substance**

Acetonitrile saturated n-hexane: it was made by 100 mL acetonitrile mixing with 300 mL n-hexane, and then kept the upper layer of saturated n-hexane after shaking and standing:

Standard stock solution: nine kinds of standard substances were dissolved in methanol prepared as 100 μg/mL respectively for standard solution and stored at −20°C or lower.

Working solution: standard solutions in concentrations of 0.2 ng/mL, 0.5 ng/mL, 1 ng/mL, 2 ng/mL, 5 ng/mL, 10 ng/mL and 50 ng/mL were prepared by above mentioned standard stock solution and acetonitrile-water (2:8) respectively.

**Sample preparation**

Crushed, then were divided into two parts into sealed clean plastic bags respectively and stored at dark and low temperature of −18°C. Five gram sample was taken into 50 mL stopped centrifuge tubes, vortex mixing with 50 μL of 100 ng/mL internal standard mixture and then adding 5 g anhydrous sodium sulfate and 25 mL of ethyl acetate. After homogenized extracting 1 min, samples were centrifuged at 5,000 r/min for 10 min and 5 mL extract were placed in water bath at 45°C and dried with nitrogen. The dry residues were added with 1 mL acetonitrile-water (2:8) and 3 mL acetonitrile saturated n-hexane and vortex mixed for 1 min, and then was centrifuged at 3,000 r/min for 5 min. The lower layer was filtered by 0.22 μm membrane for on-line cleanup and HPLC-MS/MS analysis.

**Instrument parameters and measurement conditions**

On-line cleanup procedure involved two processes for purification and separation. The purification process that the sample pump drives purify mobile phase was completed in the Turbo Flow column. The separation process that elution pump drives analysis mobile phase was done in HPLC analysis column. This process was done by two six-port valves that they were switched to achieve change in the flow path. Flow diagram of on-line cleanup modes was shown in Fig. 1.

**On-line cleanup conditions.** Turbo Flow On-line cleanup column: Cyclone-P (0.8 × 50 mm); injection volume: 50 μL; the composition of mobile phase and conditions of gradient elution were shown in Table 1.

**Conditions of liquid chromatography.** Chromatographic column: ACQUITY UPLC BEH C18, 2.1 mm × 50 mm, 1.7 μm; column temperature: 30°C; the composition of...
mobile phase and conditions of gradient elution were shown in Table 2.

**Conditions of mass spectrometry.** Sheath gas pressure: 30 psi; auxiliary gas pressure: 45 psi; electrospray voltage: ESI-, 4,000V; capillary temperature: 350°C; induced dissociation of endogenous voltage: 10V; collision gas: high purity argon; collision gas pressure: 1.5 mTorr; other MS parameters are shown in Table 3.

### RESULTS AND DISCUSSION

**Optimization of extraction conditions**

Glucocorticoids are a class of fat-soluble hormones with more hydrogen cyclopentane derivatives in structure and low polarity. The extraction agents mainly included methanol, dichloromethane, acetonitrile, ethyl acetate, and mixed extraction solvent because of their high extraction ability [16, 17]. However, considering the cost and toxic of solvents, methanol, acetonitrile and ethyl acetate as the extraction solvents were tested. Results showed that more impurities were found from the methanol extract than other two solvents extracts, which may relate with strong polarity of methanol [16]. More impurities could decline accuracy of the subsequent processes of purification and online analysis. Especially, drying time in this extraction process was too long to obtain satisfied recoveries.

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**Table 1. The composition and flow rate of TurboFlow on-line cleanup**

| Step | Time (min) | Waters (%) | ACN (%) | ACN:IPA:Acetone (1:1:1) | Flow rate (µL/min) | Purpose |
|------|------------|------------|---------|-------------------------|--------------------|---------|
| 1    | 0.0        | 100        | 0       | 0                       | 2,000              | Loading |
| 2    | 1.0        | 0          | 100     | 0                       | 100                | Transfer |
| 3    | 3.0        | 0          | 0       | 100                     | 1,000              | Cleaning |
| 4    | 3.5        | 0          | 0       | 100                     | 1,000              | Cleaning |
| 5    | 6.5        | 0          | 0       | 100                     | 1,000              | Cleaning |
| 6    | 9.5        | 0          | 100     | 0                       | 1,000              | Cleaning |
| 7    | 10.5       | 20         | 80      | 0                       | 1,000              | Loop filling |
| 8    | 12.0       | 100        | 0       | 0                       | 1,000              | Conditioning |

**Table 2. The composition and flow rate of LC mobile phase gradient**

| Step | Time (min) | Waters (%) | Acetonitrile (%) | Flow rate (µL/min) |
|------|------------|------------|------------------|--------------------|
| 1    | 0.0        | 90         | 10               | 500                |
| 2    | 1.0        | 90         | 90               | 500                |
| 3    | 3.0        | 75         | 25               | 500                |
| 4    | 3.5        | 75         | 25               | 500                |
| 5    | 6.5        | 72         | 28               | 500                |
| 6    | 9.5        | 10         | 90               | 500                |
| 7    | 10.5       | 10         | 90               | 500                |
| 8    | 12.0       | 90         | 10               | 500                |

**Fig. 1.** Flow diagram of on-line cleanup modes. The gradient elution and flow rate in each step were shown in Table 1.
When acetonitrile was used as the extractant, the chemical recovery efficiency was poor. This may due to the different polarity from acetonitrile and extract. Relatively, c was a suitable extraction agent compared with above two agents because of its weak polarity as well as its high recoveries. Chen et al. extracted glucocorticoids from pork using ethyl acetate for the determination of glucocorticoids residues with UPLC-MS/MS method. However, same problem was found that ethyl acetate can dissolve part of meat fat leading to high fat content in the final extract [19]. To resolve this problem, acetonitrile saturated n-hexane and anhydrous sodium sulfate were added through the method of liquid-liquid extraction. In addition, anhydrous sodium sulfate added during the extraction process served for absorbing water and assisting to extract analytes.

Optimization of MS conditions

Selection of ion scan mode. The mass spectrometry conditions of glucocorticoids included the ESI ionization source model, positive mode, negative mode, the parent ion and the daughter ion and the selection of the mobile phase was also different [1, 19]. The positive mode and negative mode were compared in four different aqueous phases (5 mM ammonium acetate solution, 0.2% formic acid, ultra-pure water and 0.2% ammonia solution) respectively and organic phase (acetonitrile) with the ratio of 1:1 in the flow rate 200 μL/min. Jin-Zhong et al. checked steroid hormone residues in chicken, and found all chemicals can be assayed in 10 min by ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) using electrospray ionization in positive ion mode and multiple reaction monitoring mode (MRM) [1]. However, our results showed that dexamethasone could obtain higher ionization efficiency in negative mode in 0.2% formic acid and ultra-pure water, although ionization efficiency of eight glucocorticoids was extremely low in ammonium acetate solution and 0.2% ammonia solution. Thus, negative mode was chosen in the following study.

Selection of mobile phase and parent ion. Deprotonation molecule [M—H], formate anion molecule [M+HCOO]− and formate anionization molecule lost CH2OH [M+HCOO−CH2OH] were observed in negative mode. This result was consistent with previous report that many adduct forms in the negative model were formed when glucocorticoid drugs were ionized in electrospray ionization mass spectrometry [18]. The [M+HCOO]− was selected as a monitoring parent ion for its high abundance. Then, quantitative and qualitative secondary ion fragments were obtained based on the optimization of MRM mode. The optimized MRM parameters including capillary voltage, cone voltage, and collision energy were shown in Table 3. The daughter ion scan spectra of Glucocorticoids were shown in Fig. 2.

Optimization of Turbo Flow on-line cleanup conditions

Turbo Flow on-line cleanup column (TFC) was filled by macroporous filler, which had size exclusion effects and chemical factors, leading to small molecules dispersing and macromolecules and other impurities were eluted rapidly [15]. Glucocorticoids compounds commonly were purified with reversed-phase solid phase extraction (RP-SPE) columns, such as SPE HLB, C18, and C8. Because of relatively weak polar, polarity of the modified Cyclone-P (0.8 × 50 mm) was selected as the on-line cleanuping column.

On-line cleanup process contained loading, elution transferring and rinsing. Pure water and 0.1% ammonia solution were selected as loading mobile phase in order to improve the response of detection signal in the negative ion mass spectrometry mode, and both the water and 0.1 ammonia solutions could effectively retain the glucocorticoids compounds in Cyclone-P column.
Water was chosen as mobile phase for the convenience of laboratory operations. Meanwhile, 20% aqueous acetonitrile, 50% aqueous acetonitrile and 80% aqueous acetonitrile solution were investigated in eluting transfer step; results showed that 80% aqueous acetonitrile could completely elute the sample. The proportion of acetonitrile was more than 80%, some unwanted impurities would be eluted, which affected the sensitivity of subsequent analysis. 80% aqueous acetonitrile was chosen as the solvent of elution metastasis.

TFC column was rinsed with strong elution ability solvent using purification pump, and then eluting ring was filled with elution solvent to prepare for the next analysis of samples. The mixture of acetonitrile + acetone + isopropyl alcohol (1:1:1) was choose as strong eluting solvent to fully rinse TFC column in on-line cleanup Methods, and then the TFC column was initialized with pure water. MS schematic of the optimized result of on-line cleanup process was shown in Fig. 3.

Optimization of chromatographic conditions

Selection of mobile phase. A variety of substances was in form of [M + HCOO]⁻ in negative ion mode under water and acid water system. Compared the acid water and pure water as aqueous phase in mobile phase, mass response of
each substance was not changed obviously. To meet low limit of detection and convenience preparation of mobile phase, the pure water was selected as aqueous phase; Chromatogram peaks of betamethasone and dexamethasone were not separated in the methanol mobile phase. Thus, the acetonitrile was selected as mobile phase.

Selection of chromatogram column. There were polar differences for eight kinds of glucocorticoid compounds, especially for isomers of betamethasone and dexamethasone (437.2 > 361.2, 437.2 > 391.2) [19]. So it was necessary to achieve chromatographic separation for quantitative and qualitative analysis. A conventional C18 column (Therom Hypersil Gold C18, 150 mm × 2.1 mm) and an ultra-high pressure liquid phase C18 column (Waters ACQUITY UPLC BEH C18, 2.1 mm × 50 mm, 1.7 μm) were compared in the gradient elution method under acetonitrile/water mobile phase system. The ultra-high pressure liquid phase C18 column was selected considering the separation efficiency. Eight kinds of glucocorticoid compounds chromatogram were shown in Fig. 4.

Method validation

Eight kinds of glucocorticoids standard curves were obtained, and the matrix calibration curve for each glucocorticoids compound was linear ($r > 0.99$) for their concentration ranged from 0.2 μg/kg to 50 μg/kg (Table 4). A chicken sample without glucocorticoid was chosen as negative control, and a quantity of standard mixture was added to test the limits of detection (LOD). The LOD of this method for most glucocorticoids except for hydrocortisone was 0.2 μg/kg (Table 4). The detection limit of this method was lower than that standards of the maximum residue limit of the hormone in China, the U.S. and the EU [19, 20].

This method also showed high recoveries and good repeatability (Table 4). Overall recoveries for this method were between 71.63% and 117.36%, and the relative standard deviations (RSD) were between 1.43% and 17.7%. Blank matrix samples were shown in Fig. 5; matrix spiked in Fig. 6.
Fig. 4. LC ESI-MS/MS chromatograms of 8 kinds of glucocorticoid hormones

Table 4. Linear ranges, precision and Recovery of the method (n=6)

| name           | Linear equation | Coefficient of association | Detection limit (μg/kg) | Spiked level (μg/kg) | Average recovery (%) | RSD (%) |
|----------------|-----------------|----------------------------|-------------------------|----------------------|----------------------|---------|
| prednisone     | $Y = -12,123.9 + 143,541^*X$ | 0.9998                     | 0.2                     | 0.2                  | 92.00                | 5.58    |
|                |                 |                            | 0.5                     | 84.52                | 6.93                 |
|                |                 |                            | 1.0                     | 108.15               | 12.57                |
|                |                 |                            | 5                       | 98.33                | 3.20                 |
| prednisolone   | $Y = 90.897 + 163,769^*X$ | 0.9999                     | 0.2                     | 0.2                  | 74.92                | 15.73   |
|                |                 |                            | 0.5                     | 82.09                | 7.02                 |
|                |                 |                            | 1.0                     | 103.85               | 9.27                 |
|                |                 |                            | 5                       | 95.04                | 4.97                 |
| hydrocortisone | $Y = 12,910.5 + 147,191^*X$ | 0.9998                     | 0.5                     | 0.2                  | 55.43                | 17.07   |
|                |                 |                            | 0.5                     | 71.63                | 21.07                |
|                |                 |                            | 1.0                     | 100.03               | 5.40                 |
|                |                 |                            | 5.0                     | 95.07                | 5.49                 |
| methylprednisolone | $Y = -3,489.47 + 259,020^*X$ | 0.9998                     | 0.2                     | 0.2                  | 75.46                | 1.61    |
|                |                 |                            | 0.5                     | 75.61                | 4.33                 |
|                |                 |                            | 1.0                     | 100.71               | 8.95                 |
|                |                 |                            | 5.0                     | 92.81                | 2.25                 |
| dexamethasone  | $Y = 6,949.58 + 608,452^*X$ | 0.9999                     | 0.2                     | 0.2                  | 75.54                | 6.86    |
|                |                 |                            | 0.5                     | 77.68                | 17.70                |
|                |                 |                            | 1.0                     | 102.27               | 8.35                 |
|                |                 |                            | 5.0                     | 95.14                | 2.00                 |
| betamethasone  | $Y = -13,354.8 + 563,257^*X$ | 0.9998                     | 0.2                     | 0.2                  | 82.57                | 6.93    |
|                |                 |                            | 0.5                     | 84.86                | 11.15                |
|                |                 |                            | 1.0                     | 104.81               | 6.93                 |
|                |                 |                            | 5.0                     | 94.80                | 1.43                 |
| beclomethasone | $Y = -21,129.7 + 175,910^*X$ | 0.9997                     | 0.2                     | 0.2                  | 117.36               | 3.73    |
|                |                 |                            | 0.5                     | 89.57                | 2.41                 |
|                |                 |                            | 1.0                     | 104.88               | 8.78                 |
|                |                 |                            | 5.0                     | 89.40                | 5.94                 |

(continued)
### Table 4. Continued

| name                | Linear equation | Coefficient of association | Detection limit (µg/kg) | Spiked level (µg/kg) | Average recovery (%) | RSD (%) |
|---------------------|------------------|----------------------------|------------------------|----------------------|----------------------|---------|
| fludrocortisones    | $Y = -26,001 + 235,250 \times X$ | 0.9998                     | 0.2                    | 0.2                  | 118.34               | 11.58   |
|                     |                  |                            | 0.5                    | 100.34               | 9.67                 |         |
|                     |                  |                            | 1.0                    | 109.16               | 7.05                 |         |
|                     |                  |                            | 5.0                    | 97.58                | 0.99                 |         |

**Fig. 5.** LC ESI-MS/MS blank chromatograms of 8 kinds of glucocorticoid hormones

**Fig. 6.** LC ESI-MS/MS matrix spiked chromatograms of 8 kinds of glucocorticoid hormones (0.2 ng/mL)
Some researchers also developed determination methods for hormone residues in animal tissues. e.g. Jin-Zhong et al. checked eleven steroid hormone residues in chicken by UPLC-MS/MS and LODs were from 0.3 μg/kg to 0.4 μg/kg, recoveries were 62.3%–105%, and RSD were 0.5%–15% [1]; Rocha et al. examined hormonally growth promoting agent residues in bovine muscle by HPLC-MS/MS method, and showed that LODs were from 0.29 μg/kg to 0.79 μg/kg, recoveries were 86.9%–114.1%, and RSD were 3.5%–32.2% [16]. Compared with these studies, our method showed a good detection limit and acceptable recoveries. In addition, the sample in the analytical column was more pure with the on-line cleanup system leading to the reducing of noise and artifact peaks in some extent.

CONCLUSION

A more sensitive and rapid method using on-line cleaned up combined HPLC-MS/MS was developed for the determination of eight glucocorticoids residues in Chicken Muscle simultaneously. Chicken samples were extracted by ethyl acetate with auxiliary addition of acetonitrile saturated n-hexane and anhydrous sodium sulfate, and then purified by Turbo Flow column and assayed by HPLC-MS system. Calibration curves of eight glucocorticoids were obtained after the optimization of extraction conditions, MS conditions, Turbo Flow on-line cleanup conditions and chromatographic conditions. The linearity of calibration curves was greater than 0.99 over the concentration range from 0.2 μg/kg to 50 μg/kg. Overall recoveries were from 71.63% to 117.36%, and the relative standard deviations were between 1.43% and 17.7%. The limits of detection for all analytes were from 0.2 μg/kg to 0.5 μg/kg. This method saved the time-consuming pretreatment before HPLC-MS/MS analysis and provided accurate, rapid, sensitive and specified determination for glucocorticoids residues in chicken muscles. This method could be also the reference for applying other banned substance quantitative analysis.

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1In the original published version, the project was identified by the non-standard English translation ‘Hebei Province Key R & D projects’. In this version we replaced it – upon authors’ request – by the formal English project name ‘S & T Program of Hebei’. The project number remained unchanged.