Development of HPLC Methods for the Determination of Cyadox and Its Main Metabolites in Goat Tissues

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Rapid and sensitive high-performance liquid-chromatographic methods were developed for the determination of cyadox, an antimicrobial growth-promoter, and its main metabolites (1,4-bisdesoxycyadox, quinoxaline-2-carboxylic acid) in goat muscle, liver, kidney and fat. Cyadox (CYX) and 1,4-bisdesoxycyadox (BDCYX) in fat were extracted with acetonitrile, and in other tissues with ethyl acetate. Quinoxaline-2-carboxylic acid (QCA) was isolated from tissue hydrolysates by solvent extraction, cleaned up with ion-exchange chromatography, followed by a final liquid-liquid extraction step. UV detections of CYX, BDCYX and QCA were performed at 305, 280 and 320 nm, respectively. The average recoveries of CYX, BDCYX and QCA in spiked tissues at levels of 25, 50, 100 µg/kg were 65 - 92%. The inter-day relative standard deviation for three compounds in different tissues was 5 - 16%. The quantitation limit was 25 µg/kg, and the detection limit was 15 µg/kg for three compounds in various tissues. Incurred goat tissues were analyzed to demonstrate the validity of the described methodologies. The present methods were highly selective and could be used in the metabolism and residue studies of cyadox.

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Fig. 1 Molecular structures of cyadox and its main metabolites.

Introduction
Cyadox (CYX) is an antimicrobial growth-promoting agent, like carbadox and olaquindox, belonging to quinoxaline-1,4-dioxides. The widespread uses of these agents in food-producing animals raise the possibility of residues remaining in edible tissue after slaughter. The presence of drug residues in tissues of food-producing animals is undesirable from a public safety standpoint. It is necessary to have sensitive methods for determining drug residues in animal tissues. Metabolism has shown that cyadox is converted into monoxyocyadox, bisdesoxycyadox (BDCYX), quinoxaline-2-carboxyl glycine and quinoxaline-2-carboxylic acid (QCA) in the urine of pig (Fig. 1). So far, the studies for analyzing cyadox and its bisdesoxycyadox metabolites in animal tissues are rare. QCA is used as a marker compound for the monitoring of carbadox in livestock animals. Analytical methods published for QCA were focused on porcine liver, and for other tissues were rarely reported. The extraction and cleanup procedures for QCA from tissues in this article were based on their reports. We firstly developed methods for the determination of CYX and its main metabolites in goat edible tissues. The methods reported here are sensitive, which can be used in kinetics and metabolism studies and a routine monitoring program.

Experimental

Materials and reagents
Methanol, acetonitrile, hexane, ethyl acetate, formic acid and chloroform were of analytical reagent. AG MP-50 ion-exclusion resin (100 - 200 mesh) was obtained from Bio-Rad Laboratories, CA 94547. CYX (99% 20020604) and BDCYX (98.0% 20010320) were provided by Institute of Veterinary Pharmaceutics (Huazhong Agricultural University, Wuhan, Hubei, China). QCA (97%) was obtained from Sigma-Aldrich Co. Ltd.
Solutions
Various solutions were prepared as follows: Citric acid buffer (0.5 M, pH 6.0) was prepared with 210 g of citric acid (1H2O) dissolved in 1000 mL of water, and the pH was adjusted to 6.0 with 5 mol/L NaOH. 1 mol/L HCl was prepared by adding 83.3 mL of concentrated HCL to 1000 mL of water. 3 mol/L NaOH was prepared using 120 g of sodium hydroxide pellets dissolved in 1000 mL of water. 5 mol/L NaOH was prepared using 200 g of sodium hydroxide pellets dissolved in 1000 mL of water.

Standard solutions
Approximately 10 mg of each reference standard was weighed into a 10 mL brown volumetric flask and diluted with dimethylsulfoxide (with methanol for QCA) to make stock solutions (1 mg/mL). 0.1 mL CYX and BDCYX stock solutions were combined and diluted with methanol to make a working solution I (10 µg/mL). 0.1 mL QCA stock solution was diluted to 10 mL with methanol to make working solution II (10 µg/mL). The standard solutions were stored at 4˚C and protected from light.

Apparatus
HPLC apparatus. HPLC separation was conducted using an Agilent 1100 Series high-performance liquid chromatograph. The column was a Hypersil-Keystone RP-18 column (250 mm i.d., 5 µm particle sizes). The mobile phase containing a 1% formic acid solution and methanol (60:40) was used for QCA, and the detection was at 320 nm. A fine separation for CYX and BDCYX was achieved by water and acetonitrile with a gradient elution condition. The detailed gradient elution program was as follows: time (min), water (%), acetonitrile (%); 0, 85, 15; 10, 75, 25. The detection for CYX and BDCYX was at 305 nm and 280 nm, respectively. The flow rate was 1 mL/min.

Packed ion-exclusion column. About 7 g AG MP-50 resin was dissolved in methanol and transferred to a 25 cm × 10.5 mm i.d. glass column containing a small glass-wool plug to retain the resin.

High-speed refrigerated centrifuge, Model CR 21G (Japan); vortex machine, Model XW-80A (China); centrifuge, Model TDL-5-A (China)

Goat tissues
Control goat muscle, liver, kidney and fat were purchased from a local market. Incurred tissues were from five goats fed CYX for 3 consecutive months at a dose of 750 mg/kg.

Sample preparation
Extraction and clean-up for CYX and BDCYX. Control tissue samples (5 g) were added into 50 mL centrifuge tubes. Working solution I was diluted with methanol to obtain working standard solutions of 1.25, 2.5, and 5 µg/mL, respectively. 100 µL of each standard working solution was spiked into each of the tubes and the tissues were homogenized by a vortex machine at the maximum speed for 2 min with 2 mL of water and 10 mL of ethyl acetate (fat tissue was homogenized with 10 mL acetonitrile). The organic phase was collected after centrifugation for 10 min at 4200 rpm. The residue was evaporated to dryness and dissolved in 0.5 mL of methanol for HPLC analysis. Blank tissues were prepared as described above.

Extraction and clean-up for QCA. Control tissue samples (5 g) were added into 50 mL centrifuge tubes. Working solution II was diluted with methanol to obtain working standard solutions of 1.25, 2.5, and 5 µg/mL, respectively. 100 µL of each standard working solution was spiked into each of the tubes. The samples were allowed to equilibrate for 10 min before processing. 3 mol/L NaOH (10 mL) was added to all tubes, which were then placed into a water bath at 95 - 100˚C for 30 min (note: the level of the water bath should exceed that of the tissue sample). The tubes were removed and allowed to cool to room temperature. Concentrated hydrochloric (4 mL) was added into each tube, and the contents were mixed for 30 s. Ethyl acetate (10 mL) was added and the tube mixed for 1 min and centrifuged (10 min, 4200 rpm). The upper layer (ethyl acetate layer) was transferred into a 50 mL centrifuge tube, and the extraction was repeated twice with ethyl acetate (20 mL). The three extracts were then combined. Citric acid buffer (5 mL) was added, mixed, and centrifuged (4200 rpm, 5 min) to let the lower phase clarify. The aqueous phase was transferred to a 50 mL tube. The ethyl acetate phase was re-extracted with an additional 5 mL of citric acid buffer, and the aqueous extracts were combined. Concentrated hydrochloric (2 mL) was added into each tube and mixed for 30 s. The acidified aqueous extract was applied onto the ion-exclusion column (wash resin in sequence with 50 mL of methanol, 50 mL of water and 50 mL of 1 mol/L HCl before the extract was applied) and washed with 1 mol/L HCl (50 mL). The resin was eluted with 75 mL of methanol-water (10:90 v/v) into a 250 mL separatory funnel. The column was allowed to run dry in this step. Concentrated hydrochloric acid (1 mL) was added to each separatory funnel. Three 25 mL portions of chloroform were used for extraction. The extracts were collected into a 125 mL round-bottom flask and evaporated to dryness on a rotary evaporator at 45˚C. Residues were dissolved with 2 mL of methanol, and centrifuged for 5 min at 10000 rpm to clarify the methanol.
phase. The upper layer was taken for HPLC analysis.

Limit of detection and limit of quantitation
A series of drug solutions with different concentrations diluted from working solution I or II with methanol were added into four kinds of blank tissue samples. Samples were then prepared by the described extraction and clean-up procedure. The level resulting in a peak with a height typically three-times as high as the baseline noise without interference was considered to be the limit of detection (LOD). The lowest level that gave reasonable accuracy and precision was considered to be the limit of quantitation (LOQ).

Recovery and precision
Samples of muscle, liver, kidney and fat tissues were fortified with CYX, BDCYX or QCA at 25, 50, 100 µg/kg. Five replicates of samples at each level were analyzed in one day to evaluate the intra-day recoveries and variations. Also, samples of muscle, liver, kidney and fat tissues fortified with CYX, BDCYX or QCA at 25, 50, 100 µg/kg were analyzed on different days (n = 5) to evaluate the inter-day recoveries and variations of the analytical procedure. The recoveries of three compounds were calculated by means of the standard calibration curves with the peak area.

Standard calibration curves
Working solution I or II was diluted with methanol to prepare seven different levels, including 0.02, 0.04, 0.08, 0.16, 0.32, 0.64 µg/mL. Each level was injected into the HPLC system three times. A certain average peak area was regressed with a certain level to calculate the calibration equation.

Results
Specificity
Each compound was scanned by diode-array-detection (DAD) to find their maximum absorption wavelength before analysis, which indicated that the best sensitivities for CYX, BDCYX and QCA were 305, 280, 320 nm, respectively. Figure 2 shows chromatograms of blank kidney and blank kidney spiked with CYX and BDCYX. Figure 3 shows chromatograms of blank muscle and blank muscle spiked with QCA. Figure 4 shows chromatograms of CYX, BDCYX and QCA standard. The chromatograms indicated that the assay was selective, because no interfering substances appeared under the chromatographic conditions.

Limit of detection and limit of quantitation
The LOD of three compounds in four tissues was 0.015 µg/g, while the LOQ was 0.025 µg/g.

Recovery and precision
Table 1 summarizes the recoveries and inter-day RSD of spiked tissues for CYX, BDCYX and QCA. The recoveries of CYX ranged from 65% to 79% with an inter-day RSD ranging from 6.7% to 11.1%, and the recoveries of BDCYX ranged from 70% to 92% with an inter-day RSD ranging from 4.7% to 15.9%, while the recoveries of QCA ranged from 70% to 82% with an inter-day RSD ranging from 6.6% to 10.0%.

Standard calibration curves
Table 2 gives the data on calibration curves for three compounds, which demonstrated excellent linearity for CYX, BDCYX and QCA at ranges of 0.02 - 0.64 µg/mL with the coefficients of correlation of 0.9999, 0.9997 and 0.9997, respectively.

Incurred tissues
To test the methods, cyadox residues were measured in the tissues (muscle, liver, kidney and fat) of goats fed medicated feed with cyadox after the last administration of medicated feed. The experimental data are reported in Table 3, where all data were derived directly from calibration curves. The concentrations of CYX and BDCYX in all tissues were below the limit of quantitation of the analytical method, while QCA could be detected in liver and kidney tissues in five goats. Since the calibration curves were prepared with pure standard
solutions, and not with extracted samples, a correction factor had to be introduced into the chromatogram integration method. The recoveries of QCA in spiked liver and kidney tissues at 100 µg/kg were introduced as the correction factor, because the concentrations of QCA in incurred animals were most close to the concentration of 100 µg/kg. The concentrations of QCA in liver and kidney tissues ranged from 76 µg/kg to 250 µg/kg.

Figure 5 shows chromatograms of the control tissue and incurred kidney and liver tissues. A small background response was observed near to the retention time of QCA in the kidney control samples. The background response was less than 10% of the response of the lowest fortified concentration level, which would not affect evaluating the true concentration of QCA.

Discussion

Extraction procedure

Because cyadox is similar to carbadox in chemical structure, we initially selected the extraction reagent with reference to carbadox. Several reagents, such as methanol and acetonitrile, were used, but extractions with these solvents were found to be of lower recovery, or to have interference with blank samples. We obtained better recoveries with water-ethyl acetate for CYX and BDCYX. When CYX and BDCYX were prepared from tissues, 5 mL of combined organic extracts was used for future processing. If the total combined extracts were used, more substances in tissues would interfere with the detection of CYX. CYX was sensitive to light, and would decrease in amount when it was exposed to light, and CYX was not stable even when it was put naturally for a long time. Thus, it was necessary to protect the samples from strong light during the sample pretreatment, and standard working solutions had to be made just before the analysis was needed. We used this procedure for the extraction of QCA: alkaline hydrolysis, liquid–liquid extraction and filtration with strong cation-exchange resin (AGMP-50 resin). QCA usually exists in tissues as its conjugated form, and can be liberated by hydrolyzation with NaOH. QCA was quantitatively extracted into ethyl acetate from a strong acidic solution (pH < 1) and easily extracted into aqueous buffered solutions at pH 6 or higher from ethyl acetate, because it is a strong carboxylic acid. The aqueous extract was suitable for purification by ion-exclusion.
chromatography. The ion-exclusion process was necessary, since it provided the means for the trace collection of QCA and separation of it from interference substances. AG MP-50 was selected because of its non-swelling properties and its apparently greater sorptive capacity for non-electrolytes. The drawback of using AG MP-50 is manual loading of the cation-exchange resin, which was of low throughput. We had tried to use SPE cartridge with 0.5 g of non-endcapped benzenesulfonic acid (SCX) to replace AG MP-50; however, the sorbent used did not produce enough cation-exchange sites for protonated analytes under such a highly acidic condition to overwhelm the high concentration of H⁺. This result was consistent with that of Sin et al.6

Chromatographic conditions
A mobile phase system containing methanol and water was firstly selected for three compounds. However, the peak shape of CYX was not good enough. After changing methanol to acetonitrile, a better separation for CYX and BDCYX was achieved with a gradient elution condition. There were interference substances with QCA under the chromatographic condition for CYX and BDCYX. The mobile phase containing 1% formic acid solution and methanol could separate QCA from interference well, and the shape was fine. DAD was used to find their maximum absorption wavelength before detection, which indicated that the best sensitivity for CYX was 305 nm, that for BDCYX was 280 nm and that for QCA was 320 nm.

Recovery and precision
The recovery of CYX was lower than that of BDCYX in tissues. Compared with BDCYX, CYX is a strong polarity compound, which makes its extraction from samples easy. The overall relative standard deviation ranged from 4.7% to 15.9%, it could be concluded from these data that the within laboratory reproducibility was excellent in the tested concentration range. The limit of detection and the limit of quantitation were 15 µg/kg and 25 µg/kg, which could be used for residue detection.

Animal experiment
The parent compound and one of its metabolites, BDCYX, were not detectable in five incurred goat tissues, which indicated that they were eliminated quickly in goat. The levels of QCA in tissues were higher than CYX and BDCYX, which indicated that QCA was more stable than CYX and BDCYX, and was depleted more slowly than them. However, it must be noted that these data reflected the results only from five goats for this drug, and may not generally reflect the rate of depletion of CYX from the goat. The metabolism and depletion for CYX in goat need to be further investigated. Also, these methods are suitable for such investigations.

Conclusion
The methods described here were sensitive and rapid for the determination of the residue of CYX and its main metabolites. The sample preparation and the extraction procedure were simple and easy to operate. Animal experiments proved that these methods meet the requirement for monitoring CYX residues in goat tissues. The described methods can be used in metabolism and residue studies.

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References