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Development of high performance liquid chromatographic methods for the determination of cyadox and its metabolites in plasma and tissues of chicken

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ABSTRACT

Cyadox (CYX) is an antimicrobial growth-promoter of the quinoxalines. It is highly effective on improving growth and feed conversion of chicken with little toxicity. For food safety concerns, HPLC-UV methods were developed for the sequential determination of CYX and its major metabolites, 1,4-bisdesoxycyadox (BDCYX) and quinoxaline-2-carboxylic acid (QCA), in plasma, muscle, liver, kidney and fat of chicken. For CYX and BDCYX, samples were subjected to a deproteinisation, a degrease and a liquid–liquid extraction. For QCA, samples were subjected to an alkali hydrolysis, a liquid–liquid extraction and a cation exchange column (AG MP-50 resin) clean-up. Analysis was performed on a RP-C18 column by UV detection with a gradient program of wavelength. Gradient elution was performed at a flow of 1 mL/min. The limits of quantification for CYX, BDCYX and QCA in plasma and tissues were 0.025 μg/g, and 0.002 μg/g for QCA in muscle. The recoveries of three compounds in plasma and tissues were 70–87% with inter-day relative standard deviation (R.S.D.) less than 10%. An animal experiment was performed to show the applicability of the present methods in real biological samples, which demonstrated a satisfactory applicability since all compounds could be detected nearly in all tissues. The present methods were highly sensitive and accurate, and could therefore be useful in pharmacokinetic and residue studies for cyadox in chicken. The developed methods will be further applied in the residue screening of cyadox and its metabolites in chicken.

1. Introduction

Cyadox (CYX), a derivative of quinoxaline-*N*-dioxide, is a promising antimicrobial growth-promoting agent. It is more safer to animals than carbadox (CBX) and olaquindox (OLA), well-known members of the quinoxalines, which were banned or strictly limited for the use in food-producing animals for their genetic or potential toxicities [1–5]. In the last years, research has performed to evaluate the efficiency and toxicity of cyadox in poultry. The results demonstrated that cyadox was active to pathogenic bacteria in poultry [6], and could promote the growth and feed conversion of poultry with little toxicity [7]. This indicated that CYX could be a promising safe and effective candidate of quinoxalines to poultry compared with its congeners. For practice use and food safety concerns, further pharmacokinetic and residue studies should be performed. Consequently, it is necessary to develop sensitive and accurate bio-analytical methods for the quantification of CYX and its metabolites.

CYX has great structural similarities with CBX and OLA, especially with CBX (see Fig. 1), which indicates that CYX could be metabolized in the same way as CBX. Early studies demonstrated that CBX is rapidly transformed into its desoxy and carboxylic acid derivatives which can be further metabolized into quinoxaline-2-carboxylic acid (QCA) (see Fig. 2) [8]. Since desoxy-metabolites were potentially toxic to animals, and QCA was set as the marker residue of CBX [8], the parent drug, desoxy metabolites and QCA were usually the major compounds to be monitored for quinoxalines residues. Moreover, a metabolization study in pigs performed in our laboratory demonstrated that CYX, 1,4-bisdesoxycyadox (BDCYX) and QCA could be detected in edible tissues of pigs. Therefore, it is mostly possible that the same metabolites could be produced in chicken.

Up to now, only two papers reported the quantification of CYX in porcine feed, plasma and contents of the gastro-intestinal tract. One paper described an HPLC method with fluorimetric detection to quantitate CYX in porcine feed and contents of the gastro-intestinal tract [9]. The other paper described a pulse polarography method to detect the CYX and quinoxaline-2-formylglycine in plasma [10]. For the quantification of CYX in tissues, only one paper reported an HPLC-UV method to detect CYX and its metabolites in goat tissues [11]. No methods had been published for the sequential

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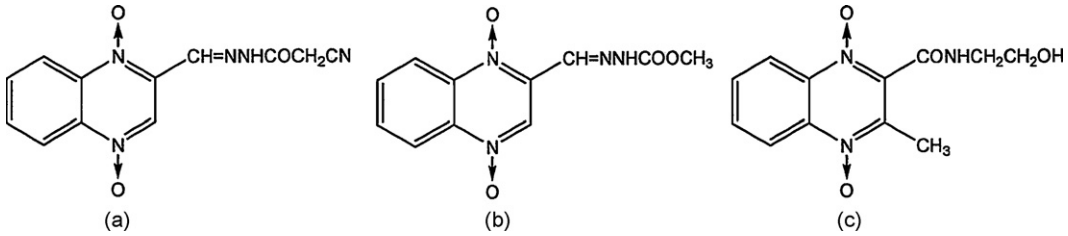

Fig. 1. Structures of CYX (a), CBX (b) and OLA (c).

determination of CYA and its metabolites in plasma and tissues of chicken.

Based on the requirement of the FDA Guidance for Industry Bioanalytical Method Validation [12], the present study described sensitive and accurate methods for the sequential determination of CYX and its metabolites in chicken plasma and tissues by HPLC with UV detection. Liquid–liquid extraction and ion-exclusion column clean-up techniques were used to improve the sample treatment procedure. The present methods would facilitate the further pharmacokinetic and residue studies of CYX in chicken, and contribute to the residue monitoring of CYX.

2. Experimental

2.1. Chemicals and reagents

CYX (99.5%) and BDCYX (99.8%) were obtained from Institute of Veterinary Pharmaceuticals (Huazhong Agricultural University, Wuhan, PR China). QCA (97%) was purchased from Sigma–Aldrich (Milwaukee, WI, USA). Stock solutions were prepared by dissolving BDCYX and QCA in methanol (MeOH) and CYX in dimethylsulfoxide (DMSO). Working solution I (10 μg/mL) was prepared weekly by combining CYX and BDCYX stock solutions and diluting with acetonitrile (ACN). Working solution II (10 μg/mL) was prepared weekly by diluting QCA stock solution with MeOH. All solutions were kept in brown container and stored at <10 °C.

AG MP-50 ion-exclusion resin was purchased from Bio-Rad library (Richmond, USA). DMSO, ethyl acetate, chloroform ($CHCL_3$), MeOH, ACN, and hydrochloric (HCL) were of analytical reagent grade. De-ionized water (Milli-Q, Millipore Corp.) was used throughout the study.

2.2. Equipments

A Varian Prostar ISO9001 HPLC system comprising Prostar 5.5 workstation, a 230 solvent delivery module, 400 autosampler and 310 UV–vis detector was used (CA, USA). A Hypersil® ODS2, 5 μm, 250 mm × 4.6 mm (Thermo Electron Corporation, 2005) HPLC column protected with 4 mm × 3.0 mm C_{18} guard columns (Phenomenex, USA) was used. High-speed refrigerated centrifuge (HIMAC CR 21 G, Japan) and vortex shaker (XW-80A, China) were used through the sample preparation.

Ion-exclusion column was prepared as followed: 7 g AG MP-50 resin was dissolved in MeOH and transferred to a 25 cm × 10.5 mm i.d. glass column containing a small glass wool plug to retain resin. The resin was washed in sequence with 30 mL MeOH, 30 mL water and 30 mL 1N HCL, and then packed to a height of 10–11 cm. 1N HCL was maintained slightly above resin bed.

2.3. Sample preparation

Controlled muscle, kidney, fat and liver were randomly collected from grocery outlets. Controlled plasma was obtained from chickens with no antimicrobial addition. Incurred samples were obtained from six chickens fed with CYX for ten consecutive days at a dose of 100 mg/kg. All tissues were frozen at −20 °C untill analysis. After thawing, the samples were homogenized in a high-speed food blender.

2.3.1. Extraction and clean-up for CYX and BDCYX

Plasma (0.3 mL) was added into 5 mL polypropylene centrifuge tube. Deproteinisation was performed by vortex mixing each of the samples with MeOH (0.3 mL) for at least 2 min. The samples were then centrifuged (2000 × *g*, 4 °C, 10 min). The supernatant was collected for HPLC analysis.

Tissues (2 g) were added into a 10 mL polypropylene centrifuge tube followed by vortex mixing each of the samples with water (1 mL) for at least 1 min. Ethyl acetate (MeOH/ACN = 1:1 (v/v) for fat) (4 mL for the first time, then 2 × 2 mL) was piped into the mixtures, followed by a vortex mixing and ultrasonic step. The mixtures were centrifuged (2000 × *g*, 4 °C, 10 min), and the supernatants were combined in a 10 mL glass tube followed by evaporating to

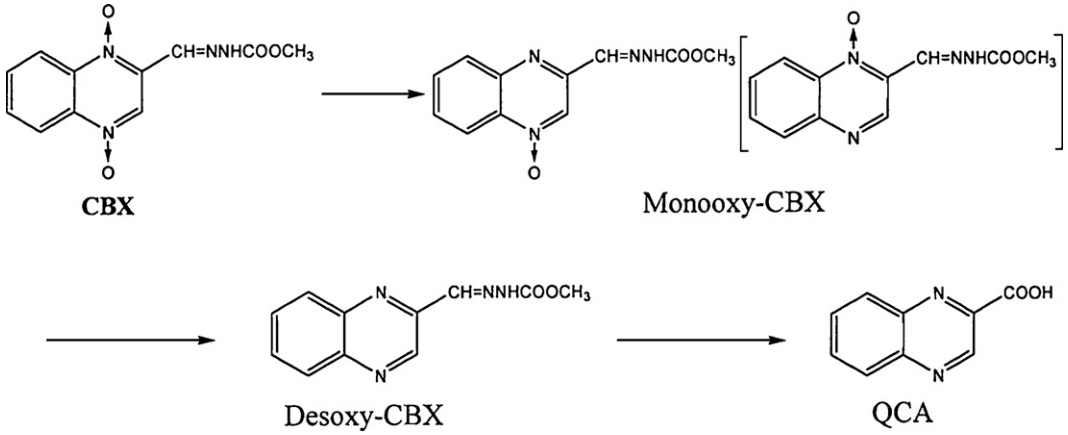

Fig. 2. The main metabolic pathway of CBX in pig.

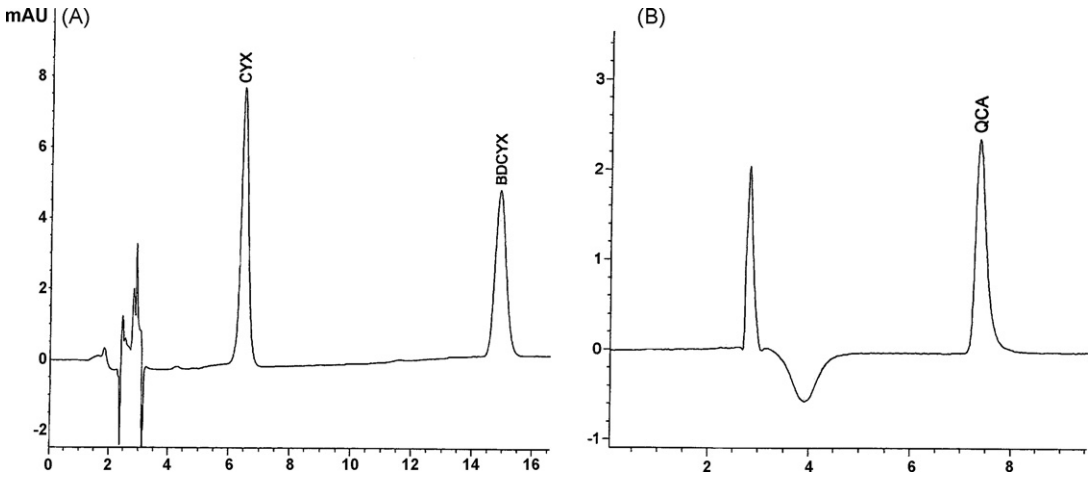

Fig. 3. Chromatograms of standard solutions of CYX, BDCYX (A) and QCA (B) at the level of 0.1 μg/mL.

dryness under a gentle nitrogen stream in a warm water bath at 45–50 °C. The residue was dissolved in CAN (2 mL) and degreased with *n*-hexane (2 × 3 mL). The *n*-hexane layer was removed, and the lower ACN phase was evaporated to dryness under a gentle stream of nitrogen at about 50 °C water-baths. The residue was dissolved in ACN (1 mL) for HPLC analysis.

2.3.2. Extraction and clean-up for QCA

Samples (5.0 g for tissues, 0.5 mL for plasma) were transferred into polypropylene centrifuge tube followed by vortex mixing with 3N NaOH (7 mL for tissues, 1 mL for plasma). The mixtures were hydrolyzed in a water-bath at 95–100 °C for 30–40 min, and then cooled at room temperature. Concentrated HCL (2 mL for tissues, 0.5 mL for plasma) was added into the alkaline hydrolysate followed by a vortex mixing with ethyl acetate (2 × 10 mL for tissues, 2 × 3 mL for plasma) for 1–2 min. The mixture was centrifuged (2000 × g, 5 min), the ethyl acetate phase was transferred into a glass tube and the extracts were washed with water (2 × 5 mL for tissues, omitted for plasma). The organic extracts were back extracted with 0.5 M citric acid buffer (2 × 5 mL for tissues, 1 × 0.5 mL for plasma) by vigorously vortex mixing for 1–2 min. The mixture was centrifuged (800 × *g*, 5 min), and the lower aqueous phase was collected. For plasma, the collected solution was analyzed directly by HPLC. For tissues, further clean-up of the buffer extracts was performed by ion-exclusion column. Concentrated HCL (2 mL) was added into the buffer extracts and mixed for about 30 s. The acidified aqueous extract was transferred gently onto the ion-exclusion column (Section 2.2) followed by an elution with 1N HCL (30 mL) and MeOH/water (10/90, v/v) (40 mL). The HCL effluents were discarded, and the elute of MeOH and water was collected into a separatory funnel. Concentrated HCL (2 mL) was added into the elute, and the elute was extracted with $CHCL_3$ (2 × 15 mL). The extract was evaporated to dryness on a rotary vapor in a WWB at 45–50 °C. The residue was dissolved in MeOH (2 mL, 2 × 2 mL for muscle), and vortex mixed for 1–2 min followed by centrifuging (2000 × g, 5 min) to clarify the MeOH phase for analysis. For muscle, the MeOH layer was further evaporated to dryness in a stream of nitrogen at 45–50 °C water-baths. The residue was dissolved in MeOH (0.2 mL) for analysis.

2.4. HPLC analysis

For the analysis of CYX and BDCYX, the mobile phase consisted of CAN/water (15:85) was used at 0–8 min. The wavelength was set at 305 nm. Over the next 7 min the mobile phase was altered to ACN/water (25:75) using a linear gradient with a wavelength of 280 nm. For the analysis of QCA, the mobile phase containing MeOH/water/formic acid (40:60:10) was used until the completion of the run at 15 min with a wavelength of 320 nm. A 20 μL aliquot was injected onto the HPLC column. The flow rate was 1 mL/min, and the column temperature was 30 °C.

2.5. Method validation

2.5.1. Specificity

Specificity was performed by analyzing ten of blank samples from different sources to evaluate possible endogenous interferences in samples. The sample preparation and chromatographic condition were optimized to guarantee that no interferences incurred at the retention time of the tested compounds.

2.5.2. Limit of detection (LOD) and limit of quantitation (LOQ)

Five sets, each of five, of blank samples were spiked with a series level of working solutions I or II. When analyzed as the methods described above, the amount that results in a peak with an area typically three times the baseline noise level was considered to be LOD. It was performed by comparing measured signals from samples with known low concentrations of CYX, BDCYX or QCA with those of blank samples. The lowest level that gave reasonable accuracy and precision was designated to be LOQ.

2.5.3. Accuracy

Working solutions I or II were diluted with MeOH to make the concentration of CYX, BDCYX and QCA to be 0.5, 1.0 and 2.0 μg/mL. Three sets, each of five, of blank samples were fortified with 0.1 mL (0.015 mL for plasma) of the diluted standard solutions to obtain the concentrations of 0.025, 0.05 and 0.1 μg/g in plasma, kidney and fat tissues. Muscle was fortified with 0.02 mL of the diluted standard solutions to obtain the level of 0.002, 0.004 and 0.008 μg/g for QCA. The samples were prepared and analyzed as described above. The accuracy is defined by the mean absolute recovery which was calculated by comparing the peak area of extracted samples with those of standard working solution at the corresponding levels.

2.5.4. Precision

Three sets, each of five, of blank samples were fortified with suitable working solutions I or II as described in the accuracy

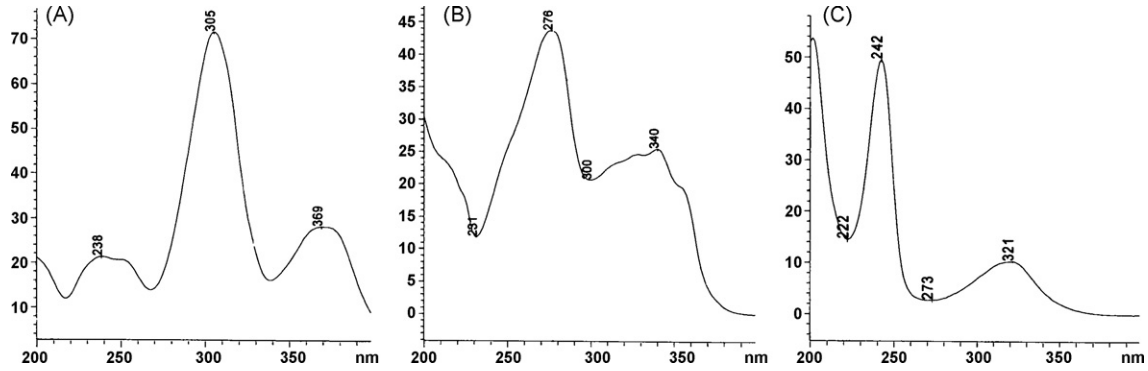

Fig. 4. UV-apctra of CYX (A), BDCYX (B) and QCA (C).

evaluation, and analyzed. The precision was defined by the relative standard deviation (R.S.D.) and estimated from the fiveplicate analysis of three samples during five days.

2.5.5. Calibration curve

Blank tissues were spiked with working solutions I or II to make the spiked concentrations to be 0.025, 0.05, 0.1, 0.2, 0.4 and 0.8 $\mu g\,kg^{-1}$ in plasma and tissues, and 0.002, 0.005, 0.01, 0.05, 0.25 and 0.5 $\mu g\,kg^{-1}$ for QCA in muscle. All samples were prepared and analyzed as described above. The certain average area regressed with the certain level, and the slope, intercept and coefficient correlation were calculated from the fiveplicate analysis of six samples during five days.

2.5.6. Incurred samples validation

Six chickens were fed with CYX for consecutive ten days at a dose of 100 mg/kg. Blood, muscle, fat, liver and kidney were collected at the time of 0.5 h after administration. The incurred samples were prepared and analyzed as described above to check the applicability of the methods in real life samples. The residue amount of three

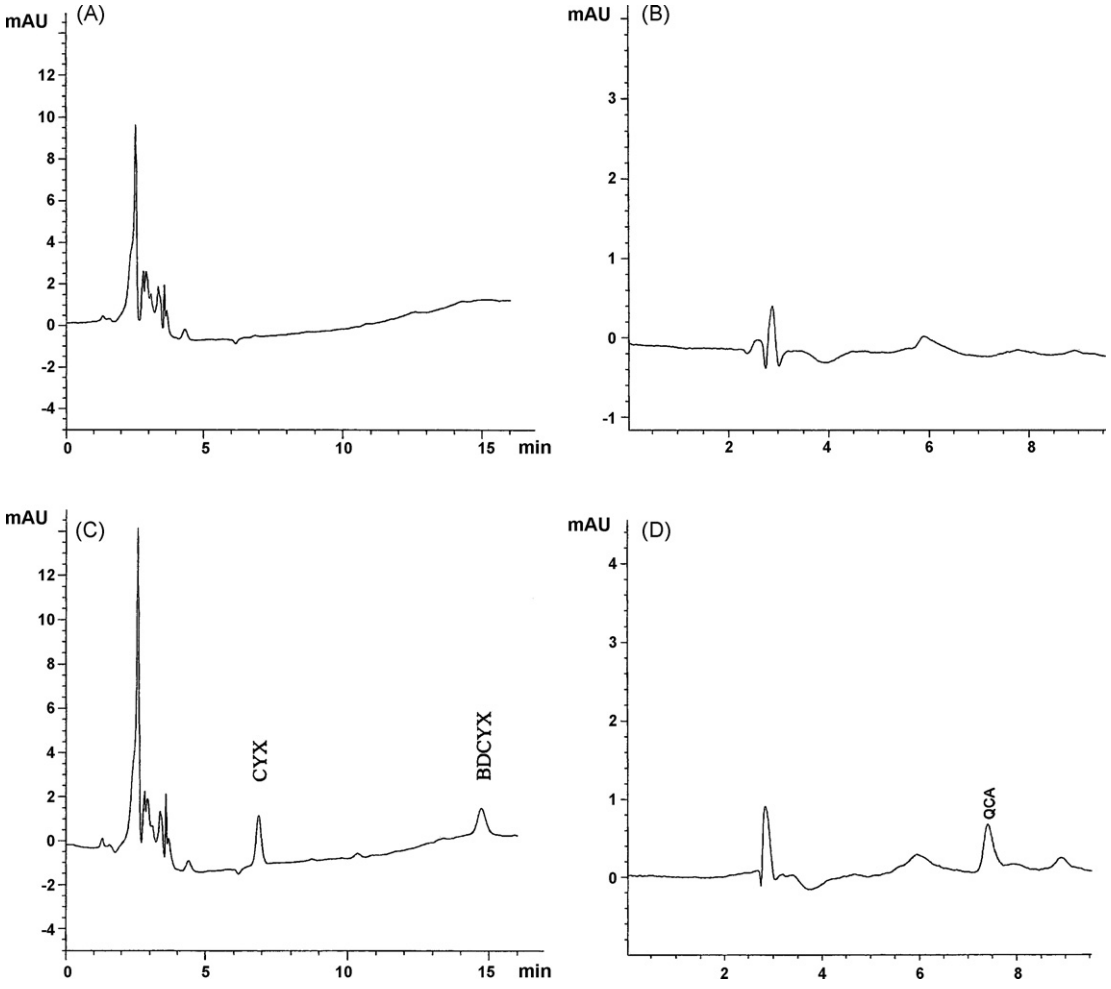

Fig. 5. Chromatograms of blank plasma (A and B) and fortified plasma with CYX and BDCYX (C) and QCA (D) at the level of 0.05 μg/mL.

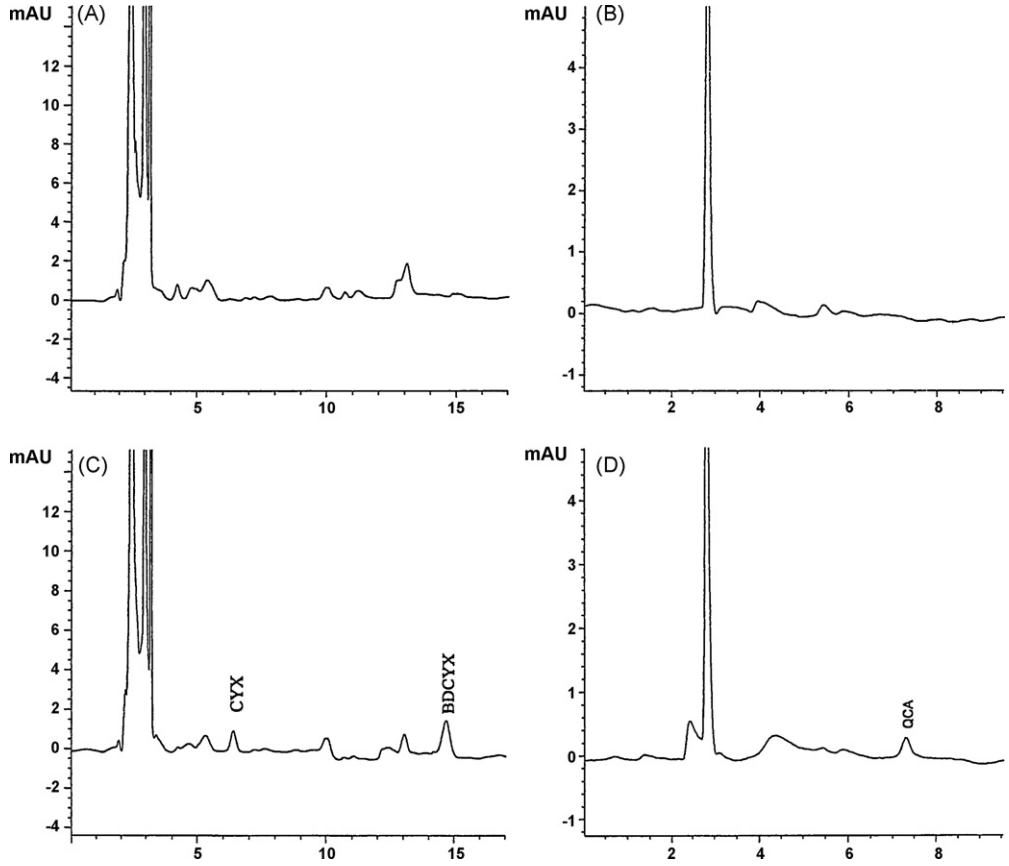

Fig. 6. Chromatograms of blank muscle (A and B) and fortified muscle with CYX and BDCYX (C) (0.05 μg/g) and QCA (D) (0.004 μg/g).

compounds in incurred samples was calculated by the calibration curve.

3. Results and discussion

3.1. Chromatographic separation

Fig. 3A and B showed chromatograms of standard solution for CYX, BDCYX and QCA, respectively, demonstrating the good separation had been achieved in the present chromatographic condition.

During our study, the wavelength, mobile phase and flow rate were investigated. Each compound was scanned by a DAD set at maximum wavelength to find their most significant UV-wavelength for quantitative purposes. The results showed the best sensitivity was 305 nm for CYX and 280 nm for BDCYX (see Fig. 4A and B). There were two significant strong absorptions for QCA at 245 and 320 nm (see Fig. 4C). Since too much interferences were found and so difficult to be cleaned up at the wavelength of 245 nm, a wavelength of 320 nm was selected for the detection of QCA.

Several mobile phases were investigated during our study. When a mobile phase consisted of MeOH/water was used, the peak

Table 1
Data on calibration curves for CYX, BDCYX and QCA in different matrix ($n = 15$)

Matrix	Compounds	Concentration ranges (μg/g)	Slope ($\bar{X}$ + S.D.)	Intercept ($\bar{X}$ + S.D.)	Equations
Plasma	CYX		142053 ± 9865	452 ± 35	$Y_A = 142053X_c + 452$ ($r = 0.9991$)
	BDCYX	0.025–0.8	60976 ± 4786	368 ± 32	$Y_A = 60976X_c + 368$ ($r = 0.9999$)
	QCA		31542 ± 2897	2048 ± 175	$Y_A = 31542X_c + 2048$ ($r = 0.9999$)
Muscle	CYX	0.025–0.80	110000 ± 9876	1099 ± 114	$Y_A = 110000X_c - 1099$ ($r = 0.998$)
	BDCYX		68166 ± 654	0.96 ± 0.12	$Y_A = 68166X_c - 0.96$ ($r = 0.999$)
	QCA	0.002–0.5	26413 ± 985	826 ± 72	$Y_A = 26413X_c + 826$ ($r = 0.998$)
Fat	CYX	0.025–0.80	110662 ± 8749	274 ± 25	$Y_A = 110662X_c + 274$ ($r = 0.999$)
	BDCYX		84241 ± 734	952 ± 76	$Y_A = 84241X_c + 952$ ($r = 0.998$)
	QCA		42224 ± 2576	711 ± 58	$Y_A = 42224X_c + 711$ ($r = 0.999$)
Liver	CYX	0.02–0.80	84667 ± 7586	741 ± 65	$Y_A = 84667X_c + 741$ ($r = 0.997$)
	BDCYX		34006 ± 2547	863 ± 72	$Y_A = 34006X_c + 863$ ($r = 0.997$)
	QCA		88859 ± 6842	1130 ± 94	$Y_A = 88859X_c - 1130$ ($r = 0.999$)
Kidney	CYX	0.025–0.80	92487 ± 7328	574 ± 47	$Y_A = 92487X_c + 574$ ($r = 0.998$)
	BDCYX		64834 ± 4356	759 ± 57	$Y_A = 64834X_c + 759$ ($r = 0.997$)
	QCA		73582 ± 6354	1025 ± 89	$Y_A = 73582X_c + 1025$ ($r = 0.998$)

Table 2
Recoveries and inter-day R.S.D. of cyadox and its metabolites in plasma ($n = 15$)

Compounds	Fortified concentrations (μg/mL)	Rec. (%)	Inter-day R.S.D. (%)
CYX	0.025	84 ± 6	7.14
	0.05	85 ± 6	7.06
	0.1	87 ± 4	6.02
BDCYX	0.025	83 ± 5	6.02
	0.05	83 ± 4	4.82
	0.1	86 ± 6	6.98
QCA	0.025	75 ± 4	5.33
	0.05	77 ± 6	7.79
	0.1	79 ± 5	6.33

shapes of CYX and BDCYX were not sharp and symmetrical, the peak shapes can be improved significantly with a mobile phase of ACN/water. Therefore, ACN/water with a linear gradient was chosen as the mobile phase for the separation of CYX and BDCYX. For the separation of QCA, a 1% formic acid solution was prepared in the mobile phase of MeOH/water. QCA exhibited weak acidity properties and therefore, peak tailing was seen in reversed phase chromatography. Moreover, the retention time was too early to be separated from the solvent front. To avoid the tailed peak and early retention time, 1% formic acid as the modificatory solvent was added into the mobile phase. Good peak shape and reasonable retention time were obtained (see Fig. 3B).

It had been recognized that quinoxalines were sensitive to light [13]. Macintosh et al. had reported that desoxy compounds were more sensitive to light than the parent compounds [14]. During our study, we also found that BDCYX would decrease in amount when exposed to light or put naturally for a long time. Therefore, it was necessary to protect the samples from strong light during the sample preparation, and the standard solutions should be made just before the analysis was needed.

3.2. Extraction and clean-up procedure

Several organic reagents, such as MeOH, ACN, acetone (ACT), $CHCL_3$ and ethyl acetate, had been investigated for the extraction of CYX and BDCYX. A simple deproteinisation step with MeOH was enough for the separation of CYX and BDCY. For muscle, liver and kidney, only 100% ethyl acetate with the addition of 1 mL water into the tissues gave the best recovery and cleanest separation. Since ethyl acetate was aliphatic and not suitable for the extraction of CYX and BDCYX for fat, MeOH/ACN (1:1) was used instead and gave satisfactory recovery and separation.

QCA, the residue marker of CBX, was possibly the final metabolite of CYX in chicken. Earlier studies showed that QCA usually existed in the tissues in its conjugated form, and it can be liberated by hydrolyzing tissues with NaOH [9,15]. The extraction and clean-up procedures described here were similar with the previously published GC/MS method for confirmatory identification of QCA [15], in which QCA was quantitatively extracted into ethyl acetate from a strong acidic solution (pH < 1) followed by a back extraction with buffered solutions at pH 6.0. The aqueous extract was suitable for purification by ion-exclusion chromatography. Several improvements had been made based on the GC/MS method. For plasma, aqueous buffered solutions was injected into HPLC for analysis directly to simplify the clean-up procedure, because QCA had been extracted into the buffer solution completely with little interferences and good recovery. For tissues, before the ethyl acetate extract was back extracted into the aqueous buffered solutions, a step of washing the extract with water was added. If not, a strong interference would occur at the retention time of QCA. The ion-exclusion process was useful because it provided the means for trace collection of QCA and its separation from non-electrolytes. Like other aromatic and aliphatic carboxylic acids, QCA in its non-ionized form ($pH \leq 1$) was sorbed by the matrix of the resin polymer and was excluded as the dissociated species. Among various strong cation exchange resins, the macroporous resin AG MP-50 was selected because of its non-swelling properties and its apparently greater sorptive capacity for non-electrolytes. Since the extraction and clean-up of QCA involved several processes, to ensure good efficiency during the extraction and purification, it was necessary to prepare the ion-exchange column in a suitable density and height with the flow rate less than 1.2 mL/min. This resin should be discarded after each assay, but may be regenerated by washing in sequence with MeOH, water, and 1N HCL.

3.3. Method performance characteristics

The method performance was validated according to the FDA recommendations proposed in 2001 [12]. Several parameters were evaluated, such as specificity, recovery, repeatability, limit of detection, limit of quantification and linearity.

The specificity of the method was checked by analyzing different blank samples. The chromatograms of blank samples of plasma, muscle and liver were shown in Figs. 5–7A and B, those of spiked tissues were shown in Figs. 5–7C and D, respectively. No interfering peaks were observed at the designated retention time (see Figs. 5–7) in the chromatograms of blank samples, which indicated the methods were highly selective.

Table 3
Recoveries and inter-day R.S.D. of cyadox and its metabolites in edible tissue of chickens (n = 15)

Samples	Fortified con. (μg/g)	Cyadox		Desoxycyadox		QCA	
		Rec. (%)	Inter-day R.S.D. (%)	Rec. (%)	Inter-day R.S.D. (%)	Rec. (%)	Inter-day R.S.D. (%)
Muscle	0.025/0.002[a]	74 ± 5	6.76	74 ± 5	6.76	70 ± 8	11.43
	0.05/0.004[a]	77 ± 5	6.49	77 ± 5	6.49	72 ± 7	9.72
	0.10/0.008[a]	81 ± 4	4.94	80 ± 4	5.00	75 ± 6	8.00
Fat	0.025	74 ± 5	6.76	74 ± 5	6.76	73 ± 5	6.85
	0.05	74 ± 4	5.41	76 ± 5	6.58	76 ± 7	9.21
	0.10	78 ± 4	5.13	79 ± 5	6.33	78 ± 7	8.97
Liver	0.025	71 ± 7	9.86	75 ± 5	6.77	74 ± 6	8.11
	0.05	73 ± 4	5.48	76 ± 6	7.89	77 ± 8	10.39
	0.10	75 ± 7	9.33	78 ± 5	6.41	78 ± 7	8.97
Kidney	0.025	75 ± 6	8.00	73 ± 4	5.48	72 ± 5	6.94
	0.05	76 ± 6	7.89	79 ± 6	7.59	74 ± 7	9.46
	0.10	80 ± 4	5.00	81 ± 6	7.41	78 ± 6	7.69

[a] The fortified concentration of QCA in muscle.

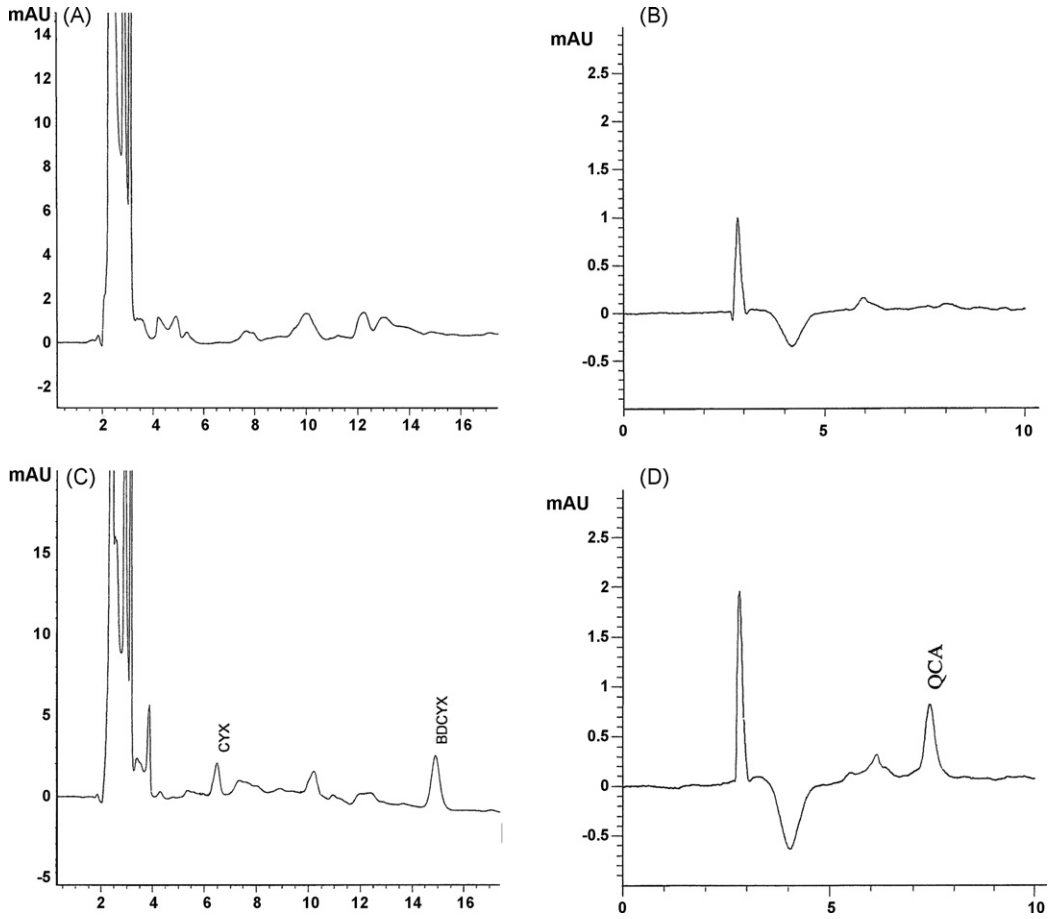

Fig. 7. Chromatograms of blank liver (A and B) and fortified liver with CYX and BDCYX (C) and QCA (D) at the level of 0.05 μg/g.

The LOD has been described as the lowest concentration that an analyte can be reliable detected. It can be defined as a ratio of the analyte signal to the background signal (S/N), and a ratio of 3:1 (S/N) was usually set as LOD. In the analysis of drug residues in food, the LOQ is practically defined as the lowest drug concentration in food samples that can be measured with a desired level of accuracy and precision. In our study, the LOD and LOQ for CYX, BDCYA and QCA in plasma and tissues was 0.025 μg/mL(g) except that the LOQ for QCA in muscle was 0.002 μg/g.

The linearity of the methods was checked by the matrix-matched calibration curves which were performed by applying the extraction procedure to analysis of spiked animal tissues at six different concentrations ranging from LOQ to much higher levels. The working ranges, slopes and intercepts and regression equations were summarized in Table 1. The matrix-matched calibration curves for three compounds showed good linearity with a high coefficient correlation more than 0.999 within the tested range (see Table 1). All these results gave evidences to demonstrate that the detection conditions were feasible and unbiased, and could be applied to quantitative analysis for CYX and its metabolites.

The accuracy and precision were the most important criteria for judging the performance of an analytical method. The accuracy of an analytical method is estimated as the percentage difference between the mean values generated by the method and the true or known concentrations. The precision describes the closeness of individual measures of an analyte when the procedure is applied repeatedly to multiple aliquots of a single homogeneous volume of biological matrix. In our study, the accuracy is assessed by analyzing a sample of known concentration and compare the measured value with the true value as supplied with the material, and the precision is expressed as the percentage relative standard deviation (R.S.D.%). Extraction recovery and repeatability (inter-day and intra-day) were used to evaluate the accuracy and precision, respectively. Tables 2 and 3 gave the overview of the recoveries and inter-day R.S.D. of the methods in plasma and edible tissues. The recoveries of CYX, BDCYX and QCA in plasma and tissues were from 71% to 87%. And the inter-day R.S.D. was less than 12%. All the recoveries and R.S.D. of the present methods were in accordance with

Table 4
Concentrations (μg/g) of CYX, BDCYX and QCA in incurred plasma and tissues of chickens

	Plasma	Muscle	Fat	Liver	Kidney
CYX	0.029 ± 0.003	0.034 ± 0.013	0.030 ± 0.018	0.066 ± 0.020	0.050 ± 0.011
BDCYX	ND	0.027 ± 0.005	0.027 ± 0.011	0.098 ± 0.039	0.107 ± 0.039
QCA	ND	0.004 ± 0.001	ND	0.104 ± 0.024	0.174 ± 0.064

ND: not detectable.

the requirement of veterinary residues analysis for food-producing animal [16].

The applicability of the methods was tested in a real residue study. Six chickens were administrated CYX for consecutive ten days at a dose of 100 mg/kg (feed) which was the clinical recommended dose. Since it is described that the parent drug eliminated rapidly from the tissues [13], the chickens were sacrificed at 0.5 h after cessation of medication. The residue amounts of CYX, BDCYX and QCA in incurred tissues were calculated by the matrix-matched calibration curves, and the results were showed in Table 4. Only a little parent drug was detected in plasma, while no metabolites were detected. In most edible tissues, CYX and its metabolites were detected. The results indicated that CYX could be metabolized into BDCYX and QCA. The methods established in our study were applicable for their quantitative determination. A small amount of CYX and BDCYX was detected in fat, while no QCA was detected. Further investigations should be carried out to illustrate the overall characteristics of CYX in chickens, while the methods presented in this study would facilitate the investigations.

4. Conclusions

Methods were developed for the sequential quantification of CYX and its metabolites, BDCYX and QCA, in the plasma and edible tissues of chicken. Good linearity was demonstrated over the concentrations ranged from 0.025 to 0.8 μg/g for three compounds in most tissues, and 0.002 to 0.5 μg/g for QCA in muscle. The methods were sensitive enough with considerable low LOQ of 0.025 μg/g (mL) for three compounds in plasma and tissues, and 0.002 μg/g for QCA in muscle. Accuracy and precision at the spiking levels for the method were in accordance with the requirement of veterinary residues analysis for food-producing animal. An animal experiment was performed to show the applicability of the presented methods in real life samples, which demonstrated that the sensitive and accurate methods presented in this study could be applied to the further pharmacokinetic and residue study of CYX in chickens. In the near future, the methods will be applied for the residue screening of CYX and its metabolites in plasma and tissues of chickens.

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