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Development and Validation of an LC-MS/MS Confirmatory Method for Residue Analysis of Cyproheptadine in Urine of Food-Producing Animals

CRISTINA A. FENTE,* PATRICIA REGAL, BEATRIZ I. VÁZQUEZ, XEXÚS FEÁS, CARLOS M. FRANCO, AND ALBERTO CEPEDA

Analytical Chemistry, Nutrition and Bromatology, Veterinary Faculty, University of Santiago de Compostela, 27002 Lugo, Spain

The possible off-label and illegal use of cyproheptadine (CYP) as an appetite stimulant for food-producing animals creates the need for methods capable of detecting it. A high-performance liquid chromatography tandem mass spectrometry method (LC-MS/MS) was developed to identify CYP in bovine urine, according to Commission Decision 2002/657/EC. Two multiple reaction monitoring (MRM) transitions for each analyte were monitored: 288.1/96.1 and 288.1/191.2 for CYP and 282.1/167.2 and 282.1/116.3 for diphenylpyraline hydrochloride (DPP), which was used as an internal standard. The solid phase extraction technique without a liquid−liquid step gives good results in urine samples from treated animals. The analytical method was successfully validated for linearity (0.15−10 ng/mL), with intraday precision of 9.4%, interday precision of 20.4%, and accuracy of 96.7%. The decision limit (CCα) and detection capability (CCβ) were 0.48 and 0.82 ng/mL, respectively.

KEYWORDS: Cyproheptadine; LC-MS/MS; bovine urine

INTRODUCTION

Veterinary drugs used in animal husbandry to treat diseases or prevent sickness are very frequently the same as used in human medicine. Cyproheptadine hydrochloride (CYP) (**Figure 1**) is often used to treat allergic disorders (*1*) but has also been employed as a human appetite stimulant to assist weight gain (*2*).

In veterinary medicine, cyproheptadine is also recommended because of its antihistamine effects and has been prescribed as an appetite stimulant for cats, dogs, or horses in several cases. In all cases, cyproheptadine use is specifically not allowed for animals destined for food production (including horses). Nevertheless, there are quite a few countries (i.e., Argentina, Mexico) where cyproheptadine can be administered in calves during the weight gain period.

Cyproheptadine is not included in Annex I, II, or III of EU Council Regulation 2377/90 (*3*) and, according to EU Directive 2001/82 (*4*), this substance cannot be authorized for a veterinary medication in animals intended for food production. However, it was not difficult for us to recognize the possible use of this compound after interviews with farmers. The possible off-label use of this drug for the illegal purpose of inducing weight gain in calves could result in the presence of CYP and its derivatives in meat and/or milk destined for human consumption. This may pose a health hazard to consumers, such as allergic or other reactions. This has created a need for methods capable of detecting CYP and its derivatives in food.

In the scientific literature, several analytical methods have been reported for the detection of cyproheptadine hydrochloride, but in all cases attention was focused on human samples or laboratory animals. In these papers, gas−liquid chromatography (*5*), high-performance liquid chromatography with photodiode array detection (*6*, *7*), and only a few MS detection (*8*−*10*) methods have been reported.

This work describes a simple method for the detection of cyproheptadine hydrochloride in urine of meat-producer calves. A solid-phase extraction (SPE) technique is used coupled with a sensitive and specific LC-MS/MS method that is suitable for detection in the parts per billion (ppb) range. This is the first time that CYP has been analyzed as an incurred residue in bovine urine samples by a confirmatory LC-MS/MS method that allows for the

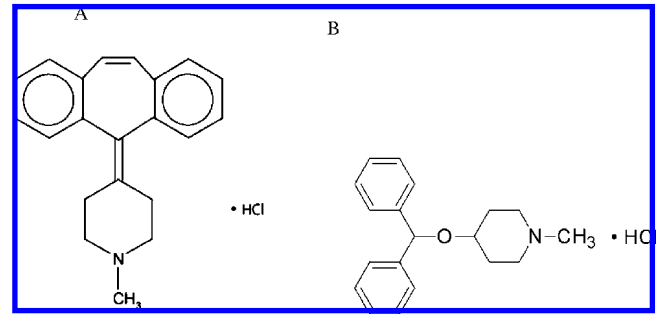

Figure 1. (**A**) Cyproheptadine hydrochloride structure; (**B**) diphenylpyraline hydrochloride structure.

* Author to whom correspondence should be addressed (e-mail cristina.fente@usc.es).

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Table 1. Gradient Elution for Chromatographic Separation[a]

step	total time (min)	flow rate (μL/min)	solvent A (%)	solvent B (%)
0	0.00	300	10	90
1	2.00	300	10	90
2	5.00	300	40	60
3	10.00	300	50	50
4	11.00	300	60	40
5	15.00	300	10	90
6	20.00	300	10	90

[a] Solvent A, acetonitrile; solvent B, water, each with 0.1% formic acid.

Table 2. Mass Spectrometer Parameters[a]

compd	Q1 mass (amu)	Q3 mass (amu)	DP	EP	CEP	CE
CYP	288.10	96.10	46	4.5	14	41
	288.10	191.20	46	4.5	14	41
DPP	282.11	167.20	46	9.5	26	47
	282.11	116.30	46	9.5	26	47

[a] DP, declustering potential; EP, entrance potential; CEP, collision cell entrance potential; CE, collision energy.

definitive identification required for a substance that is forbidden according to European Community legislation.

MATERIALS AND METHODS

Reagents and Chemicals. Cyproheptadine hydrochloride, the internal standard (*8*) diphenylpyraline hydrochloride (DPP) (**Figure 1**), and β-glucuronidase from *Helix pomatia* were supplied by Sigma Chemical Co. (St. Louis, MO). Acetonitrile was purchased from Scharlau Chemie (Sentmenat, Barcelona, Spain). Formic acid was purchased from Acros Organics (Geel, Belgium). Methanol, sodium hydroxide solution (2 mol/L), and acetic acid (glacial) 100% anhydrous were supplied by Merck (Darmstadt, Germany). All chemicals and solutions were of analytical reagent grade. A Milli-Q Gradient A10 water purification system from Millipore (Bedford, MA) was used.

Standard solutions (1 μg/mL) were prepared by dissolving the appropriate amounts of CYP and DPP in methanol. Solutions were stored at 4 °C in the dark for no longer than 2 months. Standard working solutions in 50:50 mobile phase (10 ng/mL) were prepared freshly each day.

Samples. Twenty-four Holstein calves (approximately 3 months old) were used for this study. Animals were fed a diet usually employed in the animal husbandry practice and had ad libitum access to water. Twenty animals were used to collect urine blank samples. Four calves were treated orally with 10 mg per day of CYP in aqueous solution for 6 consecutive days. Samples were collected at the end of the treatment.

Preparation of Samples. Urine samples (50 mL) were homogenized by manual shaking and were then centrifuged at 2500 rpm for 15 min. An aliquot of 2 mL from the supernatant was used for the analysis. Urine pH was adjusted to 5 with acetate buffer (2 M, pH 5.2). Then, 100 μL of internal standard working solution and 30 μL of β-glucuronidase from *H. pomatia* were added. Hydrolysis was performed over 2 h at 52 °C. Prior to SPE the pH of the hydrolysate was adjusted to 9 (pK_a of CYP = 9.3) with sodium hydroxide solution.

The OASIS HLB 3 cm^3 (60 mg) extraction cartridges from Waters (Milford, MA) were conditioned with 5 mL of methanol followed by

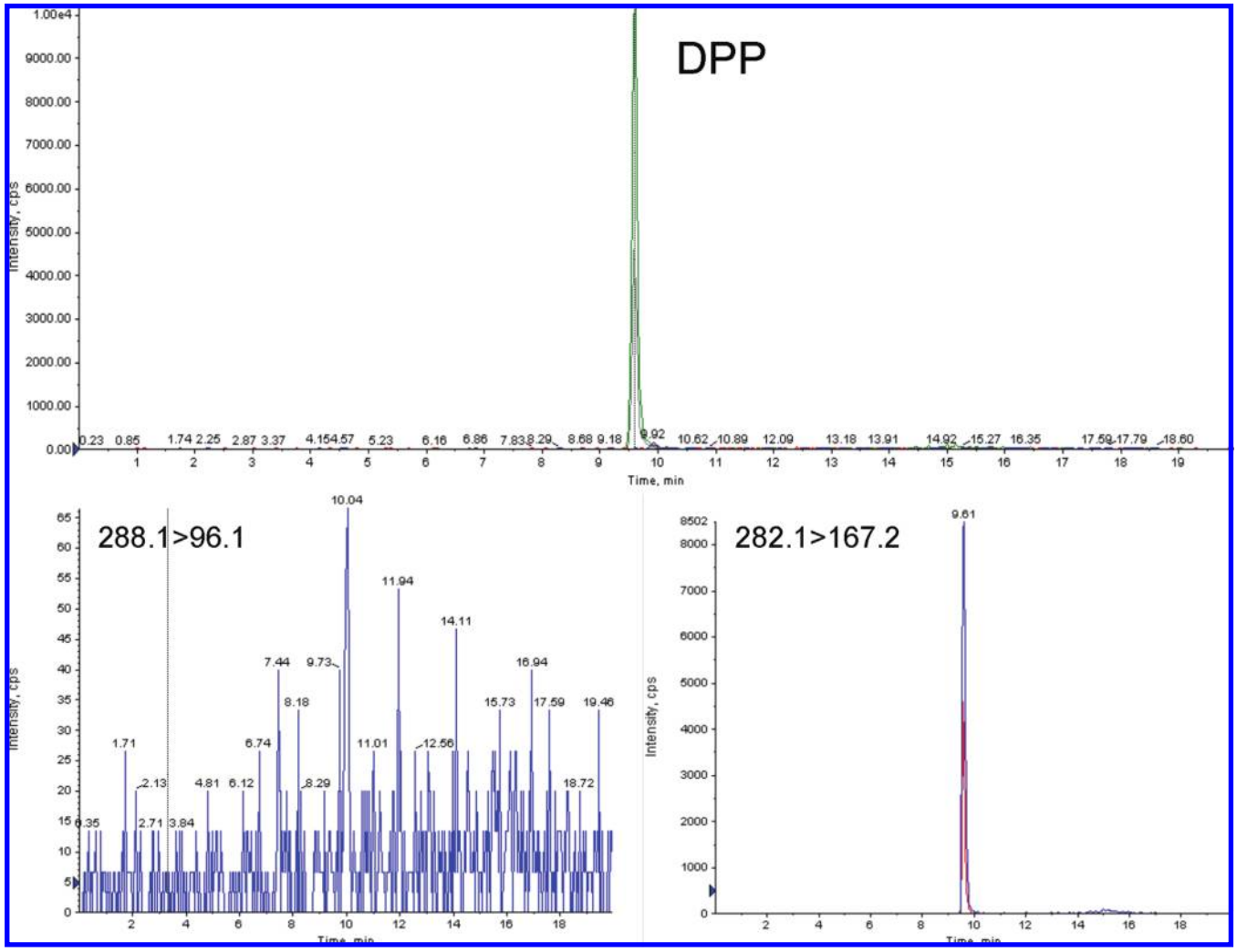

Figure 2. Chromatogram corresponding to a bovine urine blank sample processed as indicated under Materials and Methods.

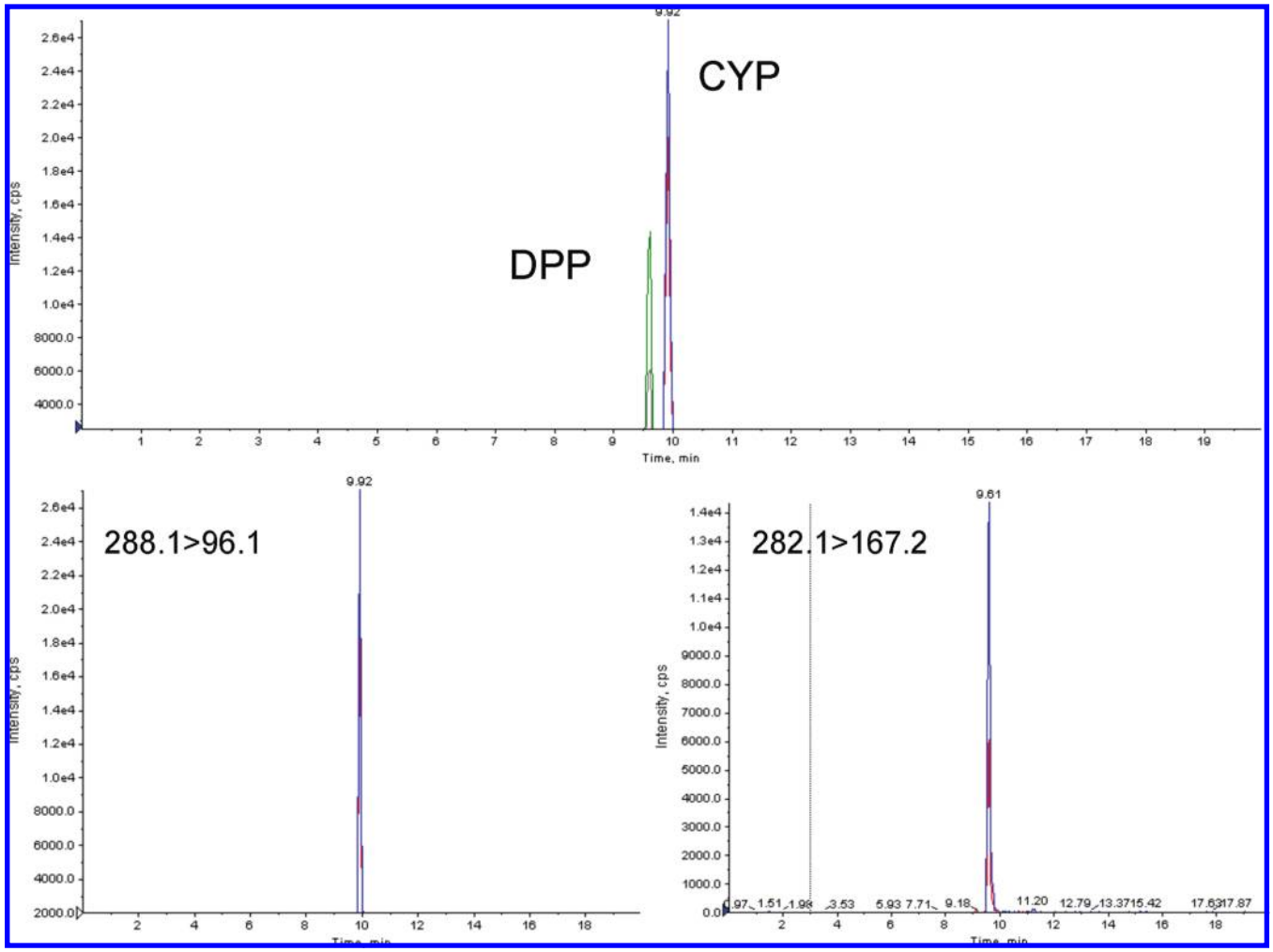

Figure 3. Chromatogram corresponding to a real sample from a treated animal (121 ng/g of CYP).

5 mL of water. The cartridges were washed with 5 mL of water and 5 mL of methanol/water (50:50, v/v). The analytes were eluted from the cartridges with 5 mL of methanol. Eluate was evaporated to dryness under a nitrogen stream at 40 °C in a nitrogen evaporation system, with a thermostat-regulated heating plate from New Brunswick Scientific. The extracts were redissolved with 100 μL of 50:50 mobile phase, and 10 μL was injected into the chromatographic system.

LC-MS/MS Method. Separations were performed on an 1100 series HPLC system consisting of a quaternary pump, degasser, and autosampler from Agilent Technologies (Waldbronn, Germany). A hybrid triple-quadrupole linear trap Q-Trap 2000 mass spectrometer with an Ion Source Turbo Spray from Applied Biosystems MSD Sciex (Toronto, Canada) was used. Nitrogen produced by a high-purity nitrogen generator (PEAK Scientific Instruments Ltd., Chicago, IL) was used as the curtain, nebulizer, and collision gas. Unit mass resolution was set in both mass-resolving quadrupole Q1 and Q3. A Synergi Fusion-RP (150 × 2 mm) 4 μm column and a guard column, both from Phenomenex (Torrance, CA), were used. The mobile phase was water mixed in gradient mode with acetonitrile, each with 0.1% formic acid, at a flow rate of 300 μL/min (**Table 1**). The ion source was operated at 350 °C in the positive ion mode. Multiple reaction monitoring (MRM) mode was used, with two transitions for each molecule. The optimized parameters for mass detection are shown in **Table 2**. Data were collected using a Dell Optiplex GX400 workstation and processed by the Analyst 1.4.1 software package (MDS SCIEX).

Validation Study. The method was fully validated following the criteria specified by European Commission Decision 2002/657/EC (*11*). Method validation was performed in terms of specificity, linearity, precision, accuracy, decision limit (CCα), and detection capability (CCβ) using the program ResVal version 2.2 obtained from the Community Reference Laboratory CRL for hormones (RIVM, Bilthoven, The Netherlands). A homogeneous mixture of blank urine was divided into 63 subsamples. Each day (over the course of 3 days) 21 fortified samples were analyzed: 1 unspiked sample, 6 samples spiked at 0.5 ng/mL, 6 samples spiked at 1 ng/mL, 6 samples spiked at 1.5 ng/mL, 1 sample spiked at 2 ng/mL, and 1 sample spiked at 5 ng/mL. Additionally, representative blank samples ($n = 20$) were analyzed for any interference in the region of the chromatogram where cyproheptadine and the internal standard were expected to elute. The concentration of the analyte in the validation and real samples was interpolated from calibration curves constructed each day by calculating the area ratios of analyte peak area/IS peak area versus analyte concentration with standard solutions of cyproheptadine at concentrations of 0.15−10 ng/mL. The internal standard was added at the concentration of 10 ng/mL.

RESULTS AND DISCUSSION

The mass spectrometric conditions were optimized to obtain the maximum signal intensity for CYP and DPP using direct infusion of 1 μg/mL of each analyte in 50:50 mobile phase mixture. These molecules were easily ionizable in positive mode by using an electrospray ionization source (ESI) and gave a strong protonated molecule $[M + H]^+$. The ion spray voltage was 5500 V. The optimized parameters to achieve better detection as a declustering potential, entrance potential, collision cell entrance potential, and collision energy are shown in **Table 2**. Collision cell exit potential was 4 V for the two molecules. The electrospray source parameter setting was optimized for intensity under LC conditions using FIA of the mixture of both molecules in 50:50 mobile phase. The analyte was quantified

in MRM mode. To obtain quantitative results, the analyte peak area was divided by the IS peak area. Two MRM transitions (one precursor and two product ions, four identification points) were monitored (150 ms dwell time/transition), according to the 2002/657/CE decision (*11*).

Chromatographic separation is achieved using a Synergi Fusion-RP column filled with a hybrid polymer. Good efficiency and peak shape were obtained in a 20 min analysis time. **Figure 2** shows the chromatogram corresponding to a blank urine sample. Similarly, **Figure 3** shows the chromatogram corresponding to a real sample (CYP concentration = 121 ng/mL) from treated animals. Bovine urine was processed as indicated under Materials and Methods.

Several authors reported methodology to detect CYP, but in multidrug systems and human matrices (*6*−*8*). Average recoveries obtained turned out to be lower than that obtained by the liquid−liquid method, but the authors concluded that, in contrast, more reproducible results could be achieved with SPE systems. The method developed in this work for bovine urine analysis combines the simplicity of an SPE technique without a liquid−liquid step with good results in the parts per billion range, as we demonstrate with the validation study.

Method Validation. Selectivity and specificity of the method were established for CYP and DPP with urine samples fortified and unfortified. No interfering peaks were observed at the retention time for the transitions monitored (**Figures 2** and **3**).

The linearity of the chromatographic response was studied in standard solutions covering the entire working range of 0.15−10 ng/mL. In bovine urine samples, linearity was also examined in the range of 0.5−5 ng/mL. Calibration curves [$y = (0.8228 \pm 0.0455)x + (0.0805 \pm 0.060)$] gave a good linear correlation, with $R = 0.9923 \pm 0.0049$. The intraday precision and interday precision were 9.4 and 20.4%, respectively, and the accuracy (recovery) was determined as 96.7%. There is no limit set for CYP, so working ranges were chosen by taking into account the response of the analyte in the linearity study. The decision limit (CCα) is defined as the limit at and above which it can be concluded (with an error probability of 1%) that a sample is noncompliant. The corresponding concentration at the *y*-intercept plus 2.33 times the standard deviation of the intercept equals the decision limit. The decision limit (CCα), the concentration level for a confirmatory method in accordance with the European Decision (*11*), was 0.48 ng/mL. The detection capability CCβ is the smallest content of the analyte that may be detected, identified, and/or quantified in a sample with an error probability of 5%. The corresponding concentration at the decision limit plus 1.64 times the standard deviation of the within-laboratory reproducibility of the mean measured content at the decision limit equals the detection capability. The detection capability CCβ was 0.82 ng/mL.

In accordance with Commission Decision 2002/657/EC, the criteria taken into account to consider a sample as positive were as follows: the relative retention time of the analyte (RRT) should correspond to that of the CYP, from a spiked sample, with a tolerance of ± 2.5%; the relative intensities of the peak from two monitored transitions must correspond to those of the CYP either from calibration standards or from incurred samples, at comparative concentrations, within the tolerance of ±20%. All real samples collected after treatment resulted in noncompliance with the CYP media concentrations of 168 ± 60 ng/mL.

We have developed the first confirmatory LC-MS/MS method to detect cyproheptadine in bovine urine that is validated according to Commission Decision 2002/657/EC and gives results applicable for monitoring programs.

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