Journal of Pharmaceutical and Biomedical Analysis 50 (2009) 1044–1049

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Short communication

Development and validation of LC–MS/MS method for the determination of cyproheptadine in several pharmaceutical syrup formulations

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ARTICLE INFO

Article history:
Received 24 December 2008
Received in revised form 1 June 2009
Accepted 3 June 2009
Available online 12 June 2009

Keywords:
Cyproheptadine
Diphenylpyraline
LC–MS/MS
Pharmaceuticals
Food residues
Method validation

ABSTRACT

A rapid and sensitive liquid chromatographic–tandem mass spectrometric (LC–MS/MS) method was developed and validated for the qualitative and quantitative assay of cyproheptadine (CP) in pharmaceutical samples. Diphenylpyraline hydrochloride (DPP) was used as an internal standard (IS). Two multiple reaction-monitoring (MRM) transitions for each analyte were observed: 288.1/96.1 and 288.1/191.2 for CP and 282.1/167.2 and 282.1/116.3 for DPP. The retention time of the drug was 7.29 min. The analytical method was successfully validated for linearity (1–100 ng/ml), intra-day precision, inter-day precision, and accuracy. The limit of detection (LOD) and limit of quantification (LOQ) were 0.86 and 0.98 ng/ml, respectively. The proposed method was applied to analyse the cyproheptadine content from seven different syrup formulations.

1. Introduction

Cyproheptadine hydrochloride (CP, Fig. 1) (4-(5H-dibenzo[a,d]-cyclohepten-5-ylidene)-1-methylpiperidine hydrochloride) is an antihistaminic, antiserotonergic agent, known to have inhibitory activities for L-type calcium channels [1].

Previously, CP was evaluated in patients for the treatment of anorexia, migraines, and atopic dermatitis [2–4]. Investigations with animals suggested that CP has obvious therapeutic effects on traumatic brain oedema [5], can protect spinal cord ischemic injury [6], increase cerebral blood flow causing cerebral vasodilation [7], and can induce anti-shock effects [8]. More recently, CP was identified as the lead novel therapeutic agent for the treatment of cancer, as an inhibitor of D-cyclin expression, leading to induced cell death and delayed tumour growth in mouse models [9].

In veterinary medicine, CP is indicated to treat anorexia, weight loss, lack of growth, convalescence, and muscular weakness; it is additionally used as a non-specific tonic. In all cases, CP use is specifically not allowed for animals destined for food production. However, we are suspicious about the illegal use of CP in meat production, because the existence of a black market for chemical cocktails used illegally for growth promotion in food-producing animals is well known. Furthermore, CP is included on the list of prohibited ingredients in cosmetics by the Japanese Pharmaceutical Affairs Act [10].

On other hand, special attention has recently been paid to CP in the literature, because significant structural similarities between CP and tricyclic antidepressants (TCAs) have induced false positive results in TCAs analysis. Cyproheptadine has a 3-ringed molecular structure resembling the TCAs, and can affect the assays to detect the presence of these antidepressants, as reported in a previous work [11].

This has been of interest not only in the fields of clinical toxicology and pharmacology, but also in forensics, because TCAs are often involved in intoxications [12–14].

In consulting the scientific literature, several analytical methods have been reported for the detection of CP, but in all of the cases, attention focused on human samples or laboratory animals. In these reports, gas–liquid chromatography [15], high-performance liquid chromatography with photodiode-array detection [16,17] and only a few MS detection [18–20] methods were employed. Methods for the determination of CP in pharmaceutical preparations were reported previously, based on their reaction with ammonium molybdate [21], using a stability-indicating high-performance liquid chromatographic assay [22], electrochemistry

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doi:10.1016/j.jpba.2009.06.006

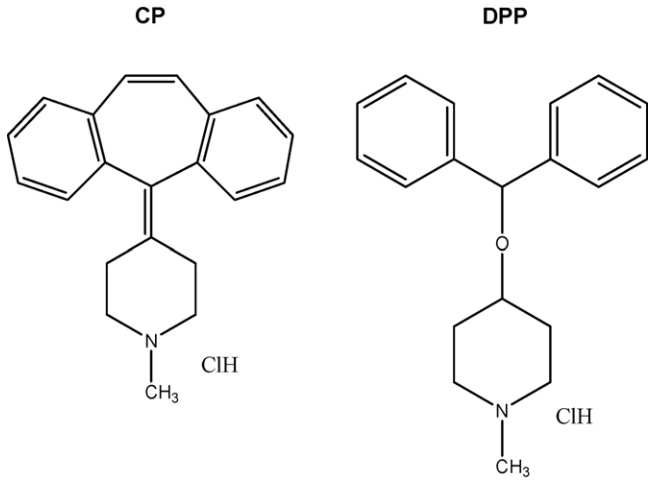

Fig. 1. Chemical structures of cyproheptadine hydrochloride (CP) and diphenylpyraline hydrochloride (DPP).

[23] or by high-performance liquid chromatography (HPLC) and chemometric methods [24].

The work presented here describes a simple method for the detection and quantification of CP in several pharmaceutical syrups, using a sensitive and specific LC–MS/MS method that is suitable for detection in the parts per billion (ppb) range.

2. Experimental

2.1. Chemicals

Cyproheptadine hydrochloride, the internal standard (IS) diphenylpyraline hydrochloride (DPP) (Fig. 1), nandrolone, boldenone, trenbolone, dexamethasone, betamethasone, flumethasone, prednisolone, triancinolone, topazole, mercaptobenzimidazole, salbutamol, clenbuterol and zilpaterol were supplied by the Sigma Company (St. Louis, MO, USA). Acetonitrile was purchased from Scharlau Chemie (Sentmenat, Barcelona, Spain). Formic acid was purchased from Acros Organics (Geel, Belgium). Anhydrous (100%) methanol and acetic acid (glacial) 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, USA) was used.

2.2. Solutions

2.2.1. Preparation of standard solutions

Cyproheptadine hydrochloride and DPP reference standard (100 mg) were transferred to a 100-ml volumetric flask and dissolved by sonication in methanol (final concentration of 1 mg/ml). These solutions were kept at 4 °C, in the dark for no longer than 1 month. From this solution, CP standard working solutions of lower concentrations (1, 5, 10, 25, 50, 75 and 100 ng/ml) were freshly prepared by an appropriate dilution in with 10 ml water. The IS solution was similarly prepared to a final concentration of 10 ng/ml.

Table 1
Operating conditions for the chromatography separation.

Step	Time (min)	Flow rate (μl/min)	Solvent A[a] (water)	Solvent B[a] (acetonitrile)
1	1	300	90	10
2	3	300	45	55
3	6	300	55	45
4	8	300	90	10
5	10	300	90	10
6 (equilibration)	10	300	90	10

[a] Expressed in %, both with 0.1% formic acid.

2.2.2. Preparation of quality control samples

A synthetic pharmaceutical sample free of CP was prepared by dissolving 100 mg of carnitine, lysine chlorhydrate, vitamin B_{12}, sucrose, anabolic steroids (nandrolone, boldenone and trenbolone), corticosteroids (dexamethasone, betamethasone, flumethasone, prednisolone and triancinolone), thyreostats (topazole and mercaptobenzimidazole) and β-agonist (salbutamol, clenbuterol and zilpaterol) into a 100-ml volumetric flask. The solution was sonicated for 10 min with 10 ml of methanol, and the volume was made up to 100 ml with water.

Quality control (QC) samples were prepared daily by spiking synthetic pharmaceutical samples with the required volume of one of the working solutions mentioned above, to produce final concentrations equivalent to 1 ng/ml (low level), 25 ng/ml (middle level) and 100 ng/ml (high level) of CP.

2.2.3. Preparation of samples

Pharmaceutical syrup samples (Table 4) were shaken vigorously for 1 min and sonicated for 5 min. Next, a sample equivalent to 1 ml of the pharmaceutical dosage form was accurately measured, transferred to a 100-ml volumetric flask and sonicated for 10 min with 50 ml of methanol. Finally, the volume was made up to 100 ml with water. A portion of the solution was used to prepare the other diluted solutions in water, spiked with IS at a concentration of 10 ng/ml before analysis.

2.3. Liquid chromatography tandem mass spectrometric analysis

Separations were performed on an 1100 series LC system consisting of a quaternary pump, degasser and autosampler from Agilent Technologies (Minnesota, USA). A hybrid triple quadrupole linear trap Q-Trap 2000 mass spectrometer with the 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. The unit mass resolution was set in both the mass-resolving quadrupoles Q1 and Q3. The mobile phase was water mixed in gradient mode with acetonitrile, each with 0.1% formic acid (Table 1).

The flow rate of the mobile phase was 300 μl/min. A Synergi Fusion-RP (150 mm × 2 mm) 4-μm column and a guard column both from Phenomenex (Torrance, CA, USA) were used, and the injected volume in the column was 10 μl.

Table 2
Mass spectrometer parameters and monitorised transitions (RT, retention time; DP, declustering potential; EP, entrance potential; CEP, collision cell entrance potential; CE, collision energy) for cyproheptadine (CP) and diphenylpyraline hydrochloride (DPP).

Compound	RT	Q1 mass (amu)	Q3 mass (amu)	Dwell (ms)	DP	EP	CEP	CE
CP	7.29	288.10 288.10	96.10 191.20	150	46	4.5	14	41
DPP	7.13	282.11 282.11	167.20 116.30	150	46	9.5	26	75

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 optimised 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).

2.4. Validation study

The method validation was performed in terms of specificity, linearity, precision (repeatability), accuracy, limit of detection (LOD) and limit of quantification (LOQ) using the program ResVal version 2.2 obtained from the Community Reference Laboratory (CRL) (RIVM, Bilthoven, The Netherlands). The characterization of the method involved repeated daily plotting of calibration curves for CP for 3 days. Three repeats of each level were made every day. The data were generated by three different analysts, using different solutions on each day. All results were referred to as ratios between CP and DDP, which was used as the internal standard. The concentrations of the analyte in pharmaceutical samples were interpolated from calibration curves constructed each day by calculating the area ratio of the CP peak area/IS peak area, versus the analyte concentration with QC samples of CP. The IS was added to a concentration of 10 ng/ml.

For specificity, 10 QC samples were analysed for any interference in the region of the chromatogram where CP and the IS are expected to elute. The calibration graph was constructed by injecting QC samples containing variable concentrations of the drug over the range of 1–100 ng/ml into the LC–MS/MS. The LOD and LOQ were determined as the lowest concentration giving a response 3 and 10 times, respectively, the average of the baseline noise defined from six synthetic pharmaceutical samples free of CP.

2.5. Stability assay

Stability tests of the analyte were performed on six replicates of three QC sample concentrations (at 1, 25 and 100 ng/ml) after (a) 12 h at room temperature, (b) six freeze (−20 °C)-and-thaw cycles, (c) at 4 °C for 24 h and (d) storage at −20 °C for a month. The concentrations of CP in the QC stored samples were compared with freshly prepared standard solutions at the same nominal concentrations.

2.6. Extraction recovery

The recovery of CP through the extraction procedures was determined at three different concentrations (1, 25 and 100 ng/ml). A known amount of the CP was added to the QC samples prior to the analysis as described above. The extraction recovery was calculated by comparing the peak area ratio of CP/DDP of the extracted QC samples to the peak area ratio of the CP/DDP standards.

2.7. Sample dilution

To test whether the method can be applied to samples where the CP concentration is higher than the upper limit of quantitation, CP-spiked QC samples at 1, 0.25 and 0.1 mg/ml were prepared and diluted with water, in order to ensure the concentration fell within the range of the calibration curve.

3. Results and discussion

3.1. Method development

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

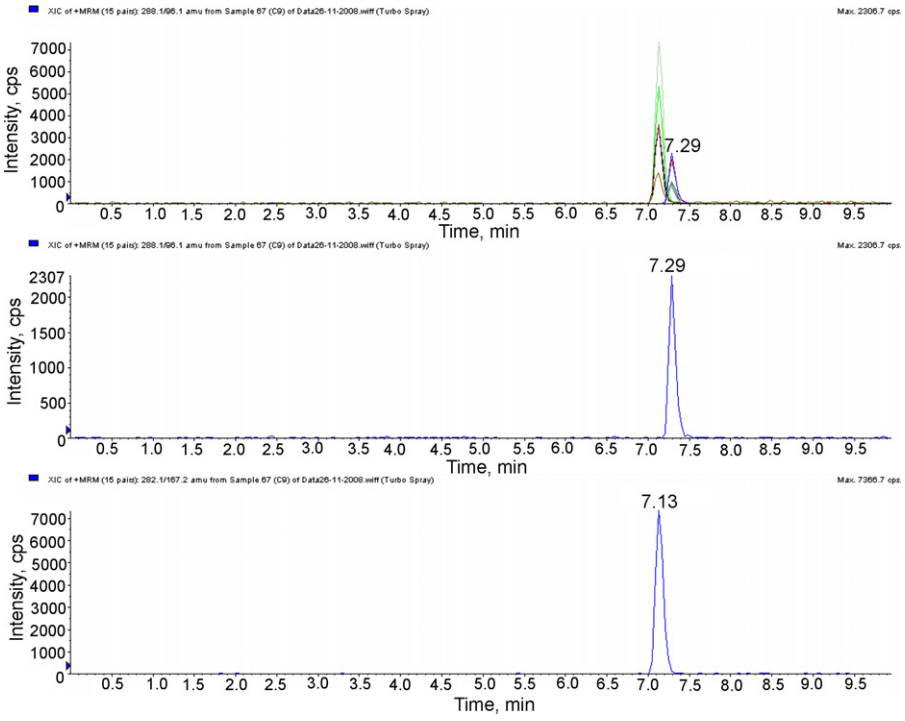

Fig. 2. Chromatogram of the acquisition window for the two MRM transitions for CP and DPP (IS): 288.1 > 96.1 and 288.1 > 191.2 (CP), 282.1 > 167.2 and 282.1 > 116.3 (DPP) corresponding to the QC sample with a CP concentration of 10 ng/ml.

Table 3
Summary table of the calibration parameters and stability of cyproheptadine under tested conditions.

Calibration			
Equation[a] (r)	Range (LOD/LOQ)[b] (ng/ml)	Reproducibility[c]	Repeatability[d]
$y = 1243x + 3567$ (0.996)	1–100 (0.86/0.96)	1.1	0.9
	Stability test[e]		
	1[f]	25[f]	100[f]
At room temperature for 12 h	1.01 ± 0.02[g]	25.05 ± 0.10[g]	100.07 ± 0.09[g]
Six freeze at −20 °C-and-thaw cycles	0.99 ± 0.05[g]	24.94 ± 0.13[g]	99.92 ± 0.14[g]
At 4 °C for 24 h	1.07 ± 0.04[g]	24.98 ± 0.05[g]	100.02 ± 0.05[g]
At −20 °C for a month	0.98 ± 0.13[g]	24.09 ± 0.10[g]	98.90 ± 0.11[g]

[a] The linear regression was obtained by fitting peak area ratios (y) of cyproheptadine to the IS against the spiking concentrations (x).
[b] Limit of Detection (LOD): signal-to-noise ≥3; limit of quantification (LOQ): signal-to-noise ≥10.
[c] Reproducibility of the method, defined as the highest R.S.D. (%) for all of the contamination levels recorded for the 3 days.
[d] For repeatability a QC sample spiked with 10 ng/ml of cyproheptadine was analysed six times.
[e] Stability tests of the analyte were performed in six replicates of three QC sample concentrations.
[f] Concentration added.
[g] Concentration found in ng/ml (mean ± S.D.).

molecules in a 50:50 mobile phase. The analyte was quantified in multiple reaction-monitoring (MRM) mode. To obtain quantitative results, the analyte peak area was divided by the IS peak area. Two MRM transitions (one precursor, two product ions, four identification points) were monitored (150 ms dwell time/transition).

The chromatographic separation was achieved using a Synergi Fusion-RP column filled with a hybrid polymer. Under the chromatographic conditions described in Section 2, a complete separation of the CP in the analytes and the internal standard DPP was possible. Good efficiency and peak shape were obtained in a 10 min analysis time. Fig. 2 shows the chromatogram of the acquisition window for the two MRM transitions for CP and DPP (IS): 288.1 > 96.1 and 288.1 > 191.2 (CP), 282.1 > 167.2 and 282.1 > 116.3 (DPP) corresponding to a QC sample with a CP concentration of 10 ng/ml.

3.2. Method validation

The linearity of the method was determined over 3 days by the addition of 1, 5, 10, 25, 50, 75 and 100 ng of CP, and 10 ng of DPP, as an internal standard, to 1 ml of QC sample (CP-free). Each sample assay was repeated three times. The ratio of the peak areas (CP/DDP) was used for the calculation of the calibration functions by a least-squares linear regression. The calibration curve, $y = 1243x + 3567$, gave a good linear correlation, with $r \geq 0.996$. The reproducibility of the method, defined as the highest relative standard deviation for all of the contamination levels recorded for the 3 days, was 1.1% for CP. For repeatability, a QC sample spiked with 10 ng/ml of CP was analysed six times, and the R.S.D. obtained was 0.9%. The detection limit (signal-to-noise ≥3) was estimated to be 0.86 ng/ml for CP, and the quantification limit (signal-to-noise ≥10) was determined to be 0.98 ng/ml for CP. Table 3 summarises the results of the calibration.

Because CP is not included in Annex I, II or III of the EU Council Regulation 2377/90 [25], this substance cannot be authorised as a veterinary medication for use in food-producing animals. However, the potential use of this compound is evident, based on a set of interviews with some farmers, because it is a cheap and widely available piperidine antihistamine that increases the appetite, through its antiserotonergic effect on 5-HT2 receptors in the brain, and also has sedative effects. The development of a black market for chemical cocktails that illegally promote growth in food-producing animals via feed additives or via the drinking water at trace levels, is well known [26,27].

Therefore, we investigated the selectivity and specificity for CP and DPP of the method when analysing medicated feed water or an unknown drug powder. Ten QC samples were prepared using a cocktail of a combination of growth promoters (anabolic steroids, corticosteroids, thyreostats and β-agonist) derived from illegal practice. In each run, a blank QC sample (processed without the IS) was analysed to confirm the absence of interferences but was not used to construct the calibration function. The analysis of a synthetic pharmaceutical sample, without analyte but with IS, showed no endogenous interference at the retention positions of CP. No interfering peaks were observed at the retention time for the transitions monitored (Fig. 2).

3.3. Stability, extraction recovery and dilution controls

The stability of CP was investigated at three levels, as described in Section 2.5. Table 3 summarises the results of the difference between the tested samples and the freshly prepared standard solutions at the same CP concentrations. The results showed reliably stabile behavior of CP under the conditions tested.

The data of extraction recovery measured for CP in the QC samples was consistent, precise and reproducible. The mean absolute

Table 4
Results of the analysis of cyproheptadine in pharmaceuticals.

Pharmaceutical preparations[a]		Cyproheptadine content[b]		
Brand name	Company name	Labeled	Found[c]	S.D.
Anti-Anorex Triple (a)	Laboratorios Lesvi	0.8	0.83	0.01
Tónico Juventus (b)	Laboratorios Juventus	1	0.99	0.01
Pantobamín (c)	Laboratorios Medix	0.5	0.48	0.02
Pranzo (d)	Laboratorios Viñas	0.5	0.51	0.01
Desarrol (e)	Iquinosa Farma Faes Grupo	0.2	0.23	0.02
Medenorex (f)	Laboratorios Medea	0.15	0.17	0.01
Ciproheptadine-G Chinfield (g)	Chinfield	1	0.83	0.03

[a] Per 1 ml of solution: (a) 0.1 g of dimethylaminoethanol acetylglutamate and 1 mg of metoclopramide HCl; (b) 250 mcg of cobamamide; (c) 0.0625 g of L-lysine, 75 mg of L-carnosine, 0.025 g of L-threonine, 5 mg of thiamine nitrate, 2.5 mg of pyridoxine HCl, 2.5 mg of cianocobalamine, 1.25 mg of dexpanthenol, 1.25 mg of riboflavin sodium phosphate and 1.250 mcg of cobamamide; (d) 0.0625 g of carnitine HCl, 1.25 mg of lysine HCl and 0.0489 g of ethanol; (e) 0.05 g of carnitine HCl, 0.025 g of arginine aspartate, 0.4 g of sacarose and 0.250 g of sorbitol 70%; (f) 0.02 g of triethanolamine aspartate, 0.02 g of lysine orotate, 0.02 g of carnitine chloride, 1 mg of Vit. B_1, 2 mg of Vit. B_2, 2.5 mg of Vit. B_6, 20 mg of Vit. B_{12}, 2 mg of Vit. B_3 and 1 mg of pantenol and (g) 10 g of lysine HCl, 33 g of bethaine HCl and 20 μg of Vit. B_{12}.
[b] mg of CP per ml of pharmaceutical preparation.
[c] Average, $n = 3$.

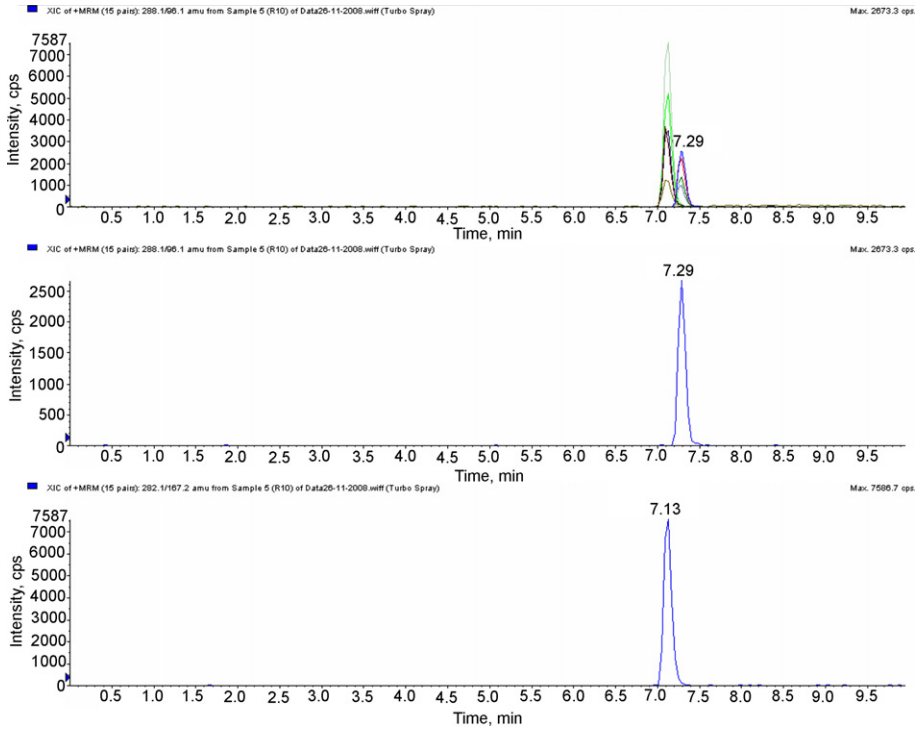

Fig. 3. Chromatogram of the acquisition window for the two MRM transitions for CP and DPP (IS): 288.1 > 96.1 and 288.1 > 191.2 (CP), 282.1 > 167.2 and 282.1 > 116.3 (DPP) corresponding to the analysed pharmaceutical form.

extraction recovery of CP at each QC level (1, 25 and 100 ng/ml) was 98.8 ± 0.9, 99.7 ± 0.7 and 99.9 ± 0.5%, respectively.

Dilution controls were applied to test if the calibration curve was suitable for measuring samples whose concentration is higher than the upper limit of quantitation. QC samples were spiked to a nominal concentration, which was diluted 1–100 prior to analysis. The sample was measured, which allowed for the calculation of the reported concentrations for the diluted QC samples. The difference between the nominal concentration of the dilution controls and the reported concentration was less than 1.1%.

3.4. Application to commercial pharmaceutical dosage forms

The developed method was applied to the determination of CP in seven commercially available pharmaceutical syrup, which are used in both human and veterinary medicine, and contain CP as the active substance. The complete pharmaceutical formulation revealed that they are authentic "anabolic cocktails." This asseveration was included by the manufacturer on some of the labels of the analysed pharmaceutical samples. The results, expressed as mg of CP per ml of pharmaceutical dosage forms, are presented in Table 4. Fig. 3 shows the chromatogram of the acquisition window for the two MRM transitions corresponding to an analysed sample.

4. Conclusions

In summary, a method was described for the quantification of cyproheptadine in pharmaceutical presentations using LC–MS/MS in positive ionization mode with multiple reaction-monitoring. This developed method was shown to be reproducible, reliable and sensitive, and was used to characterize cyproheptadine in several syrup pharmaceutical presentations. In addition, this method is free of interferences due to of the presence of common drugs employed in illegal growth cocktails. The method has a potential use in the detection and control of unlawful cyproheptadine in food-producing animals by simple analysis of animal feed water or suspected medicated powder.

Acknowledgements

This investigation was funded by grant PGIDT05TAL20801PR from the Xunta de Galiza (Consellaría de Agricultura e Política Agroalimentaria) and CICYT AGL2005-08073 from the Spanish Ministerio de Ciencia y Tecnología. Xesús Feás would also like to thank the Spanish Ministerio de Ciencia e Innovación (José Castillejo program for young researchers, grant no.: JC2008-00118), Patricia Regal for technical assistance and Carmen Ortiz de Galisteo for providing the pharmaceutical dosage forms.

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