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Determination of ractopamine in monkey plasma and swine serum by high-performance liquid chromatography with electrochemical detection

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Abstract

A high-performance liquid chromatographic (HPLC) method is described for the determination of ractopamine (LY031537) in monkey plasma and swine serum. Plasma or serum (0.5 ml) was diluted with phosphate buffer pH 7.0. Ractopamine was isolated from the plasma matrix using ion exchange on a polymeric carboxylic acid solid-phase extraction cartridge followed by partitioning with ethyl acetate. An isocratic HPLC method using electrochemical detection at +700 mV was used to separate and measure ractopamine in the purified extract in 6.5 min of run time. Standard area response was linear with respect to concentration of ractopamine over the range of 0.5 to 40 ng/ml. Validation data were collected using rhesus monkey plasma and swine serum. The method precision and accuracy were evaluated in the range 1.0 to 20 ng/ml using fortified samples of monkey plasma. The method limit of quantitation was estimated at 2 ng/ml as determined in monkey plasma.

Keywords: Ractopamine

1. Introduction

Ractopamine hydrochloride (Fig. 1) is a phenethanolamine repartitioning agent belonging to the general class of β-adrenergic agonists [1,2]. It is currently under development as a feed additive for swine, cattle, and turkeys. Studies have shown that ractopamine reduces fat and increases muscle mass while improving growth and carcass performance, and feed utilization efficiency when fed to swine [3,4], cattle [5], and turkeys [6].

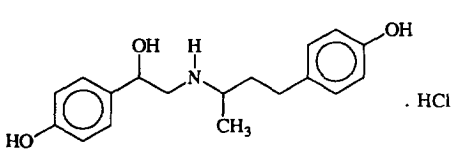

Fig. 1. The structure of ractopamine HCl.

The development of a sensitive residue method for ractopamine was necessary to obtain data on plasma and serum levels of the drug in various species as a function of dose. These data may be used to calculate pharmacokinetic parameters or to examine bioavailability of the drug using various formulations. It was desired that the method conform to the residue guidelines established by the Center for Veterinary Medicine of the Food and Drug Administration (CVM/FDA) and the European Community (EC) for compounds with hormonal or thyrostatic effects [7,8].

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The most commonly employed techniques for quantitative trace analysis of β-agonists in biological fluids have been gas chromatography–mass spectrometry, HPLC, and immunoassay [9–11]. The β-agonists that have been most frequently examined with these techniques are clenbuterol, salbutamol, and cimaterol due to their illicit use in equine and meat-producing animals [12,13]. Sensitive methods for the assay of ritodrine (Yutopar), a related phenethanolamine, in human plasma and sheep serum have been developed using HPLC–electrochemical detection (EC) and GC–MS [14–16].

The method described in this paper was developed based on extensive experience with isolating ractopamine from animal tissues and feeds [17]. The plasma assay described used weak ion-exchange solid-phase extraction (SPE) to isolate ractopamine directly from diluted plasma or serum. Further purification was achieved using liquid–liquid partitioning with ethyl acetate. Electrochemical detection was chosen based on the demand for high sensitivity and selectivity.

2. Experimental

2.1. Materials

HPLC-grade acetonitrile, methanol, and dichloromethane and reagent-grade ammonium hydroxide (28%) were purchased from Mallinckrodt (Paris, KY, USA). Ethyl acetate was HPLC grade from Burdick and Jackson (Muskegon, MI, USA). Reagent-grade phosphoric acid (85%) was from CMS (Houston, TX, USA). Sodium hydroxide (0.2 *M*), sodium phosphate monobasic, and sodium phosphate dibasic were from Fisher (Fair Lawn, NJ, USA). Ammonium phosphate monobasic and sodium borate decahydrate were reagent grade from EM Science (Gibbstown, NJ, USA). Ractopamine HCl reference standard was obtained from the corporate repository (Eli Lilly, Indianapolis, IN, USA). The purity of the reference standard was 98.0%. Water was obtained from a Milli-Q system (Millipore, Milford, MA, USA). A 0.1 *M* phosphate buffer pH 7.0 was prepared with sodium phosphate monobasic and sodium phosphate dibasic, and a 0.25 *M* borate buffer pH 10.3 was prepared with sodium borate decahydrate and 0.2 *M* sodium hydroxide. Ultra-pure helium was from Air Products (Shelbyville, IN, USA). The Clean-Up II carboxylic acid SPE cartridges (200 mg, Cat. No. CUCCX223), were obtained from World Wide Monitoring (Horsham, PA, USA). Control rhesus monkey plasma was obtained from an internal source. Swine serum was obtained from Pel-Freez Biologicals (Rogers, AK, USA).

2.2. Instrumentation

A Beckman System Gold HPLC consisting of a Model 126 pump and Model 507 autosampler (Fullerton, CA, USA) was used for all analyses. The HPLC was controlled via System Gold software (Version 7.12) running on a Compaq 20e/386 computer. The pump was equipped with two tandem pulse dampers from Scientific Systems (State College, PA, USA) and Varian (Palo Alto, CA, USA). The autosampler used a Rheodyne Model 7010 injection valve (Cotati, CA, USA). The detector was an ESA Model 5200 Coulochem equipped with a Model 5020 guard cell and a Model 5010 analytical cell (Bedford, MA, USA). The detector output signal was sent simultaneously to the Compaq computer via a Beckman Model 406 A/D interface, and to a HP1000 data system (Hewlett-Packard, Palo Alto, CA, USA) via a PE Nelson Model 900 A/D interface (Norwalk, CT, USA). The C_{18} analytical column (LC-18-DB, 250×4.6 mm, I.D., 5 μm) was obtained from Supelco (Bellefonte, PA, USA).

2.3. Chromatographic conditions

The mobile phase was 79:21.5 (v/v) 0.05 *M* ammonium phosphate buffer–acetonitrile (pH 4.5). The mobile phase was filtered through an 0.2 μm membrane (Gelman Sciences, Ann Arbor, MI, USA) and sparged with helium before use. The pump was operated in an isocratic mode with a flow-rate of 1.0 ml/min. The column was maintained at ambient temperature (23–25°C). All samples were injected using a full loop injection volume of 50 μl. The 5020 guard cell was set at a potential of +900 mV. The first electrode of the 5010 analytical cell was used as a screening electrode and set at a potential of +400 mV. The second electrode was used as the analytical electrode, from which the signal was

recorded, and set at +700 mV. The response time for electrode no. 2 was set at 2 s and the detector range was either 100 or 200 nA fullscale.

2.4. Standard and fortification solutions

A stock standard solution was prepared by adding 100 mg of ractopamine HCl to a 100-ml volumetric flask and diluting to the mark with methanol. The stock solution was serially diluted with methanol to give a 1 μg/ml standard. The 1 μg/ml standard was diluted to prepare fortification solutions or analytical standards. The fortification solutions used to spike control plasma or serum were prepared fresh daily using water as the diluent. The analytical standards were prepared using mobile phase as the diluent. The standards were stable for at least three months at ambient temperature. Normally, fortification and analytical standards were prepared by serial dilution to give concentrations of 20, 10, 5.0 and 1.0 ng/ml.

2.5. Sample preparation

Plasma or serum samples (0.5 ml) were transferred to a 16×100 mm Pyrex screw-cap tube. Five ml of phosphate buffer pH 7.0 were added to the tube followed by brief vortexing. A Clean Up II carboxylic acid SPE cartridge was installed on an SPS-24 sample processing station (Jones Chromatography, Lakewood, CO, USA) and washed with 2 ml of methanol (≤10 ml/min) followed by 4 ml of phosphate buffer pH 7.0 (1–2 ml/min). The sample was then poured into the reservoir. The empty tube was vortex-mixed briefly with 1 ml of buffer pH 7.0 which was added to the reservoir. The sample was allowed to drain through the cartridge at a flow-rate of 1–2 drops per second. The cartridge was washed with 4 ml of phosphate buffer pH 7.0, and then dried under house vacuum (≥0.05 kPa) for 5 min. The cartridges were washed with 2 ml of dichloromethane (1–1.5 ml/min) and then dried again for 2 min under house vacuum (≥0.05 kPa). Ractopamine was eluted from the cartridge using two 2-ml portions of 2% (v/v) ammonium hydroxide in ethyl acetate with a flow-rate of 1–2 drops per second. The eluent was collected into a 16×100 mm Pyrex screw-cap tube. The elution solution was prepared fresh daily. Ractopamine was stable in this solution for at least 2 h.

Two ml of borate buffer pH 10.3 were added to the tube. The tube was capped and hand shaken vigorously for 30 s. Ten minutes were allowed for phase separation. (As a faster alternative, samples could be centrifuged for 5 min with a relative centrifugal force of 1500 *g*.) The upper ethyl acetate phase was transferred to a clean 16×100 mm Pyrex tube using a 5-ml disposable serological pipette. The aqueous phase was reextracted with 4 ml of ethyl acetate as before, combining the second aliquot with the first. The extract was evaporated to dryness at 40±5°C using a TurboVap (Zymark, Hopkinton, MA, USA). Dried extracts were reconstituted by vortexing for 30 s with 500 μl of mobile phase. A portion of the sample was then transferred to a 300-μl limited volume insert HPLC vial using a disposable Pasteur pipette.

3. Results

3.1. Linearity

Standard solutions of ractopamine were prepared at 1.0, 5.0, 10 and 20 ng/ml. The standards were injected in duplicate during validation runs on four separate days. A linear response was obtained over the entire concentration range for all standard curves. The correlation coefficients ranged from 0.9986 to 0.9999 with the coefficients of determination ranging from 0.9993 to 0.9999. The coefficients of variation (%C.V.) ranged from 1.0 to 4.9. A standard curve was prepared covering two orders of magnitude in concentration using standards at 0.5, 1.0, 5.0, 10, 20 and 40 ng/ml. The correlation coefficient and coefficient of determination were 0.9956 and 0.9978 respectively, and the %C.V. was 5.3.

The effect of the monkey plasma matrix on the linearity of the standard responses was examined. Control plasma samples (0.5 ml) were processed per the method. The dried extracts were diluted with 0.5 ml of 1.0, 5.0, 10, and 20 ng/ml standard solutions. The spiked extracts and matrix free standards were analyzed per the method. The standard curves exhibited parallelism and there was no significant matrix effect on the linearity of the standard curve (*no matrix*: slope=15850, intercept=4460, r=

1.0000, %C.V.=0.32; *matrix*: slope=16100, intercept=3041, r=0.9997, %C.V.=2.55).

3.2. Precision

Method precision was determined by assaying fortified samples of rhesus monkey plasma and swine serum. Samples were prepared according to the method by spiking control plasma with an appropriate volume of a fortification solution. Duplicate samples were prepared at 1, 5, 10 and 20 ng/ml each day for four days. Additional 5 and 20 ng/ml recoveries were assayed on a single day as part of an analyst reproducibility study. The precision data are summarized in Table 1. The method demonstrated acceptable precision over the concentration range examined, except for the 1 ng/ml recoveries which were below the limit of quantitation of 2 ng/ml. Linear regression of the entire data set using SAS JMP software (SAS Institute, Cary, NC, USA) yielded a correlation coefficient of 0.9946, coefficient of determination of 0.9973, and a %C.V. of 7.9. Limited precision data were also generated for swine serum on a single day at levels of 5 and 20 ng/ml (Table 2).

3.3. Accuracy

Method accuracy was determined as percent recovery obtained from spiked samples. Recovery data were obtained from the same samples reported in Table 1 and Table 2 for method precision. The acceptable recovery range for the method is 60 to 110% based on CVM/FDA guidelines [7]. Method recoveries were within the acceptable range at all levels tested.

Table 2
Intra-day precision and accuracy of the assay for samples of swine serum fortified with ractopamine HCl

Day	n	Concentration (ng/ml)	Accuracy (%)[a]	C.V. (%)
1	4	0.0	<LOD[b]	–
	4	5.0	86.0	2.9
	4	20.0	87.0	1.8

[a]Calculated as (mean observed concentration/nominal concentration) multiplied by 100.
[b]LOD =0.5 ng/ml.

3.4. Specificity

Fig. 2 shows example chromatograms of a standard, control plasma, plasma fortified with ractopamine HCl at 10 ng/ml, and a plasma extract obtained from plasma collected 0.5 h post dose from a monkey dosed at 0.5 mg/kg with ractopamine HCl. No interfering peaks were present due to the endogenous matrix.

3.5. Sensitivity

The limit of detection (LOD) of the method was estimated by spiking control plasma with ractopamine HCl over the range 0.125 to 20 ng/ml. Samples were processed according to the method. The LOD was estimated on a single day at 0.500 ng/ml at a signal-to-noise ratio (S/N)=3 compared to the background response of the control matrix. The LOD corresponds to 25 pg injected onto the column (50 μl loop). Fig. 3 displays the chromatograms of recovery samples used in estimating the method LOD. The estimated limit of quantitation (LOQ) of the method is 2 ng/ml (S/N=10). The LOD can be affected by many variables, including the age and condition of the HPLC column and

Table 1
Inter-day precision and accuracy of the assay for samples of monkey plasma fortified with ractopamine HCl

Day	n	Concentration (ng/ml)	Accuracy (%)[a]	C.V. (%)
Overall	6	1.0	84.2	23.8
	19	5.0	86.9	15.3
	8	10	82.9	2.6
	19	20	84.6	5.3

LOD=0.5 ng/ml.
[a]Calculated as (mean observed concentration/nominal concentration) multiplied by 100.

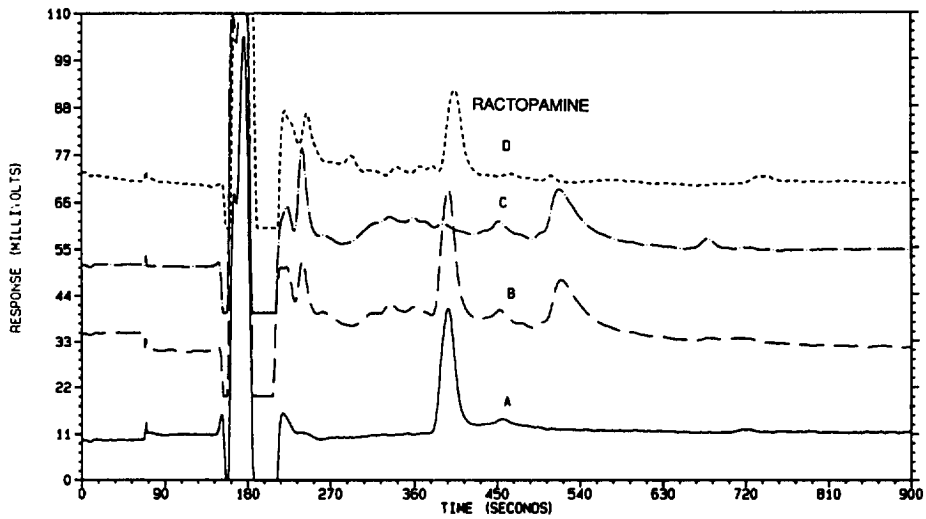

Fig. 2. Representative chromatograms of monkey plasma spiked with ractopamine HCl. (A) 10 ng/ml ractopamine HCl reference standard; (B) control plasma spiked at 10 ng/ml; (C) control plasma; (D) plasma extract collected 0.5 h post dose from a monkey dosed at 0.5 mg/kg.

electrochemical cell and the variability associated with sample preparation. (These estimates are based on a 0.5 ml plasma sample and a final dilution volume of 500 μl. Lower detection limits may be obtainable using a final dilution volume of 250 μl.)

3.6. Stability

Ractopamine HCl was stable in methanol and the ammonium phosphate–acetonitrile buffer solution for at least three months when kept at ambient

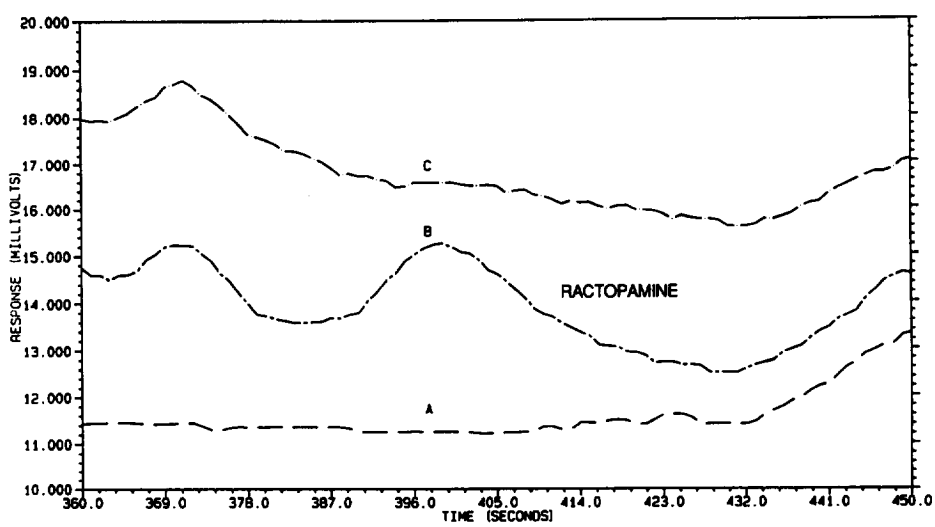

Fig. 3. Representative chromatograms demonstrating the limit of detectionof the method. (A) mobile phase blank; (B) control monkey plasma spiked at 0.5 ng/ml; (C) control monkey plasma.

temperature in subdued light. Standards were stored at 4°C in subdued light. The plasma extracts were stable in the mobile phase for at least 7 days at ambient temperature. The plasma extracts were stable for at least 10 days at 4°C. Studies have shown that ractopamine is stable in frozen plasma (−20°C) for at least one year.

3.7. Ruggedness

The pH of the borate buffer used for the liquid–liquid extraction was not critical as long as the buffer pH was in the range 10.0±0.5. The pH of the aqueous phase during extraction was found to be stable for at least 1 h if left in contact with ethyl acetate. Two Supelco LC-18-DB columns were tested with the method and found to meet system suitability requirements.

Six replicate 0.5-ml plasma samples were spiked with 50 ng each of ractopamine HCl (100 ng/ml) and processed by the method. The mean recovery was 73% with a %C.V. of 9.7, indicating that the method is suitable for plasma concentrations up to 100 ng/ml without dilution of the sample. A 500-mg Clean-UP II cartridge was also available for high level work or when additional column capacity was needed.

4. Discussion

Ractopamine has been isolated from feeds and tissues using ethyl acetate partitioning followed by SPE using an acid-washed silica cartridge [17]. The extraction of ractopamine from feeds and tissues required an initial partition with ethyl acetate to provide an extract suitable for final purification by SPE. For plasma, initial development efforts involved the direct partitioning of buffered plasma with ethyl acetate. Interferences prevented accurate quantification below 10 ng/ml. Efforts then focused on isolating ractopamine directly from plasma using SPE. Initial experiments with C_{18} cartridges did not provide adequate discrimination from the matrix. Bond Elut and World Wide Monitoring CleanScreen C_8/strong ion-exchange cartridges gave erratic recoveries. Weak cation exchange provided adequate retention of ractopamine and reproducible recoveries.

The choice of SPE conditions was driven by the physicochemical properties of the drug. The free base (pK_a in water=9.42) is soluble in moderately polar solvents such as ethyl acetate. The pK of the carboxylic acid ion-exchange sorbent is 4.8. Optimal retention in weak cation exchange is usually found 2 pK_a units above the pK of the sorbent, justifying the use of the pH 7.0 buffer for application of the sample to the sorbent [18]. Low level interferences were still present in some plasma extracts necessitating the need to further purify the extract. As the SPE elution solvent was ammoniated ethyl acetate, further purification was most conveniently accomplished by partitioning with borate buffer.

Quantification of ractopamine was performed using an external calibration curve. The use of an internal standard was unnecessary. The matrix did not have any significant effect on standard curve detector response or linearity. The precision of the assay was satisfactory using external standardization and autoinjection. Furthermore, the proper use of an internal standard would have required extensive validation data for the internal standard, the collection of which, would not have been justified based on current method performance [19].

Ractopamine is a mixture of four stereoisomers in approximately equal proportions (*RS*, *SR*, *RR*, *SS*). This HPLC method does not distinguish between these stereoisomers, and thus results in a single peak for all four stereoisomers present in a particular sample. It was possible to partially separate the diastereoisomeric pairs. Partial resolution of the diastereoisomeric pairs occurred when using mobile phases containing <20% acetonitrile and high efficiency C_{18} columns such as the LC-18-DB.

The LC-18-DB HPLC column was chosen because it provided adequate resolution of ractopamine from the endogenous matrix and did not split the diastereomers of ractopamine, thereby simplifying the analysis. The LC-18-DB has been used extensively for other ractopamine assays, and has proved to have a high degree of column to column reproducibility. A Vydac HS-54 column was also examined in early work. This column gave excellent peak shape, but the retention time of ca. 4 min brought ractopamine too close to the early eluting matrix causing interference.

HPLC with electrochemical detection was favored because ractopamine is a phenethanolamine molecule containing two phenolic groups. Phenols can be reversibly oxidized by an electrochemical detector. The analytical electrode was set at an applied potential for specific oxidation of the phenolic groups of ractopamine. This type of electrochemical detector has been shown in the literature to be extremely sensitive and selective with a high degree of accuracy and reproducibility for a variety of electroactive compounds [20]. Furthermore, the detector does not suffer from short-term sensitivity decline, due to electrode fouling, as has been reported for the analysis of other β-agonists in urine using glassy carbon disk electrode detectors [11].

The analytical cell was electrochemically cleaned at least weekly during extended use. This was accomplished by switching the analytical electrode to −500 mV for 2 min with the mobile phase flowing, and then returning to +700 mV. This procedure was not performed just before starting a run as it took up to 30 min to restabilize the baseline.

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