In Situ Single-Pass Perfused Rabbit Intestinal study sample analysis of Ractopamine by UPLC

ABSTRACT

Researchers have struggled in recent years to anticipate how oral drugs would be absorbed by people, and numerous permeability study approaches have been created for a variety of uses, including biowaiver procedures. The Single-Pass Intestinal Perfusion (SPIP) technique, used on rats, can produce permeability results that are most similar to in vivo circumstances. Since SPIP in rats is the closest approximation to in vivo settings, calculations for absorption prediction in humans and SPIP in rats may show the transport pathways and/or pre-systemic metabolism implicated on permeation processes of pharmaceuticals. In order to increase feed efficiency and promote growth in cattle, ractopamine hydrochloride, a commercial beta-adrenergic agonist, is frequently used as a dietary supplement. Currently, muscle and liver are the only regulatory target tissues (as authorised in the New Animal Drug Application with the Food and Drug Administration) for ractopamine residue testing. The study's goal is to create and validate a UPLC assay method to assess whether or not perfused ractopamine is detectable and quantifiable in cow small intestine. Acquity UPLC BEH C_{18} (150 mm x 2.1 mm, 1.7 µm) reversed phase column is used for the separation. Acetonitrile and 0.01M ammonium formate are used as the mobile phase in gradient mode with a flow rate of 0.5 mL/min. The guiding principles are satisfied by the precision, accuracy, extraction recovery, matrix effect, and stability. The created approach was successfully applied to a perfusion research in the small intestine of a rabbit, and the samples were analysed by Empower software, in order to verify the sensitivity and selectivity of the method in a real-time context. Ractopamine has a P_{eff} value of 0.022 0.003 x 10^{-4}, indicating that the jejunum region has minimal permeability for it.

KEYWORDS: UPLC, Ractopamine, Perfusion, Small Intestine, Validation

1. INTRODUCTION

A phenol-based TAAR1 agonist and adrenoreceptor agonist, ractopamine hydrochloride (Figure-1) activates β1 and β2 adrenergic receptors[1]. Ractopamine, when added to food as a food additive, can be transported by the blood to the muscles, where it acts as a complete agonist to the murine (mouse or rat) TAAR1 receptor protein (though not necessarily in humans). The USFDA approved the use of ractopamine in swine in 2000 in order to improve feed efficiency, weight increase, and carcass leanness[2]. Since then, it has been utilised in the production of cattle in more than 20 nations, although questions about potential hazards to human health persist. Ractopamine increases protein synthesis while reducing protein breakdown and fat synthesis[3]. In turkey, pork, and cattle meat, ractopamine, an animal feed ingredient, is known to generate lean muscle and reduced fat. If used in accordance with the label and accepted norms in a number of nations like the USA, South Korea, and Japan, it is a safe addition for human consumption. When used too much, it harms animals and results in broken limbs, hyperactivity, and trembling. The FDA advises doing numerous studies on humans and animals to observe how

they respond to the drug before deciding if it is safe to administer any prescription or feed supplement to animals. Ractopamine is safe and effective when used at 4.5-9 grammes per tonne of feed in the final three to four weeks before slaughter, according to numerous research conducted over the years. Ractopamine analysis in tissues of slaughtered animals falls below the established limits of 50 ppb in the liver and 15 ppb in meat when this medication is rigorously utilised in accordance with FDA standards. Calculating the allowable daily intake (ADI) of meat is made easier by taking into account the permitted concentration and daily meat consumption. ADI is the total amount of drugs that a person can take in a single day (24 hours) without experiencing side effects or putting their health at danger. All four potential stereoisomers of ractopamine can be found in commercial ractopamine. Additionally, it is a positional isomer of the related substance dobutamine. The beta2-adrenoceptor is thought to be fully agonistic by the RR-isomer of ractopamine, while the beta-adrenoceptor is thought to be just somewhat agonistic. High performance liquid chromatography (HPLC) with fluorescence detection was discovered to be a method for the study of ractopamine residues in liver or muscle in bovine and swine tissue in 2007[4]. Utilising immunosorbent assays[5] and LC-MS techniques[6–10], very few ractopamine methods have been published to date. As of right now, no approach for the UPLC perfusion research of ractopamine has been disclosed. As of right now, there is no method documented for the UPLC perfusion research of ractopamine. The goal of the current study is to develop an ultra-performance liquid chromatography method for the quick, accurate, sensitive, and straightforward quantification of ractopamine in samples from rabbit jejunal perfusion studies. The method will also be validated in accordance with the USFDA guidelines and the Veterinary International Conference on Harmonisation (VICH) guideline for analytical methods to be used in residue depletion studies. Following thorough validation, the technique was used to analyse rabbit sample analyses for the SPIP investigation.

2. EXPERIMENTAL

2.1 Instrumentation and Chromatographic Conditions

UPLC–UV Analysis

A Waters Acquity UPLC with Empower software and a photodiode array detector made up the LC system. The temperature was kept at 20°C while a Waters Acquity UPLC BEH RP C18 column (2.1 mmx150 mm, 1.7µm particle size) served as the stationary phase. Acetonitrile (A) and 0.01M ammonium formate (B) were the components of the mobile phase, which was pumped in gradient mode at a flow rate of 1.0 mL/min. 95% ammonium formate was initially used in the gradient programme. 1.1–2 min: 50% A, 50% B; 2.1-3 min: 95%A, 5% B; 3.1-4 min: 95%A, 5% B; 4.1–4.5 min: 5% A, 95% B; 4.6–5 min: 5% A, 95% B. The analysis took place for 5 minutes using a 10 µL injection volume and a 220 nm detection wavelength. The autosampler maintained at 4°C.

2.2 Chemicals

Sigma-Aldrich Trading Co., Ltd. (Shanghai, China) is where one can obtain ractopamine and the internal standard (imipramine). All additional chemicals, including HPLC-grade acetonitrile and methanol, were purchased from Merck in Mumbai, India. All other chemicals (GR grade) and ammonium formate were bought from Merck Chemicals Ltd. in Mumbai. Millipore (Milford, MA, USA)'s Milli-Q water purification equipment was utilised to prepare the water for the entire analysis.

2.3 Preparation of Perfusion buffer

In 1000 mL of filtered water, 0.35 g of KCl, 1.37 g of NaHCO3, 7.8 g of NaCl, 0.22 g of NaH2PO4, 0.02 g of MgCl2, and 1.48 g of glucose were dissolved. The pH of this solution was determined to be 6.5.

2.4 Preparation of Calibrators and QC Samples

The preparation of a standard stock solution of ractopamine involved dissolving standard 50 mg of ractopamine into a 50 ml volumetric flask, to which 30 ml of methanol was added, and sonicating the mixture for 10 minutes at a temperature not exceeding 20°C. waited for the solution to reach room temperature before adding methanol to dilute it until it reached a 1000 g/mL concentration. In order to create calibration standard and quality control (QC) samples, relevant working solutions were diluted with a 50:50 mixture of methanol and water. 200 µL of perfusion buffer was spiked with 10 mL of the appropriate diluted stock solution at various concentrations and 10 mL of IS at a fixed concentration to produce calibration sample final concentrations of 50, 100, 200, 300, 400, 500, 800, and 1000 ng/mL. IS was at a final concentration of 100 ng/mL. Similar to LLOQ samples, QC samples were made at four different concentration levels: LQC (200 ng/mL), MQC (500 ng/mL), and HQC (800 ng/mL).

2.5 Sample preparation

Perfusion samples were taken at specific intervals and centrifuged for 10 minutes at 5000 rpm. 10 µL of the solution was added to the separated supernatant layer after it had been filtered using 0.45 µm syringe filters and separated for UPLC analysis.

2.6 Analytical Validation

The method is going to be validated interms of specificity, linearity, accuracy and precision. The intra and inter-day precision and accuracy, stability and extraction recovery of analyte need to be determined. The proposed method has resulted no matrix effect with respect to intestinal fluids. The developed bioanalytical method has been validated according to Guidance for Industry[11,12], ICH[13] and VICH guidelines[14]. In order to verify the sensitivity and selectivity of the method in a real-time situation, the developed method is successfully applied to a perfusion study in rabbit small intestine.

Assay Specificity and Selectivity

By confirming that there was no significant interference in the biological control medium with relation to the retention duration of the compound(s) to be tested, specificity was evaluated. By contrasting the chromatograms of the blank matrix and the spiking matrix with the analyte at LOQ concentration, the specificity of the approach was validated. There were no interfering endogenous peaks seen near the retention time.

Linearity

For each run, a calibration curve between 50 and 1000 ng/mL of ractopamine was created. The calibration samples were split in half and examined at the start and the end of the run. The most straightforward calibration model and weighting method were employed. The ratio of the peak Ractopamine/IS regions to the Ractopamine concentration was used to calculate the curve's parameters. The linear regression equation for the curve, which was produced, was used to compute the ractopamine concentrations in the samples.

Except at the lowest concentration level, where the accuracy should be within ±20%, the accuracy of back-calculated calibration samples should be within ±15% of the corresponding nominal concentration. Except for the LLOQ and upper limit of quantification (ULOQ, 800 ng/mL), a maximum of 33% of the calibration samples per calibration curve may deviate from these requirements. Each curve reflected at least six different concentration levels.

Matrix Effect, Extraction Recovery, and Process Efficiency

The instrument response for the low, medium, and high QCs (n = 4 per level) injected directly in the mobile phase (neat solutions), the addition of the same amount of analyte to extracted blank samples (post extraction spiked

samples), and the addition of the same amount of analyte to the biological matrix prior to extraction (pre extraction spiked samples) were compared. This allowed researchers to track the impact of the matrix on the quantification of ractopamine. From the ratio of mean Ractopamine peak regions in extracted validation samples compared to pristine unextracted samples, total process efficiency was estimated. This phrase takes into consideration any signal loss resulting from the extraction procedure or the matrix effect. Ractopamine mean peak areas in extracted validation samples compared to blank samples spiked after extraction were used to calculate extraction recovery. The ratio of mean Ractopamine peak areas in blank samples spiked after extraction vs neat unextracted samples was used to calculate the absolute matrix effect. It was assumed that there would be an external matrix influence if the ratio was 85% or 115%.

Matrix Variability

The selectivity of the developed method was examined using 6 different lots of blank buffer samples spiked with IS at the LLOQ level (n = 3 per lot) and blank buffer samples without IS (n = 3 per lot) against a calibration curve to ensure that the biological matrix would not interfere with the assay. If the accuracy from each matrix lot was between 80% and 120% and the precision from each lot was less than ±20%, the results for the LLOQ samples were deemed acceptable. The raw peak areas discovered at the retention periods of Ractopamine and IS used as the acceptance criterion for the analysis of the blank samples from the six different lots. The peak areas of the blank samples cannot exceed 20% of the average peak area of ractopamine in the LLOQ QCs in more than 10% of the cases.

Stability studies

Both aqueous and matrix-based samples were used to evaluate stability. Utilising freshly made quality control samples (comparison samples), stability assessments of the matrix were carried out against recently spiked calibration standards. Benchtop stability, long-term stability, short-term stability, and freeze-thaw stability tests were used to gauge the stability of ractopamine in perfusion buffer. In an auto sampler set to 10°C, the processed samples were examined for stability. By comparing the mean response ratio of the stability samples to the comparison samples, stability is evaluated at both low and high QC levels.

Animals Selection

Rabbits (1200±50 g) were kept in a clean environment with a relative humidity of 50±5%, a temperature range of 22±2°C, and 12-hour light/dark cycles. Rabbuts were kept in cages with access to free water and standard laboratory food. In every study, the animals (n=6) were given free access to water but were denied food 12 hours before to treatment.

3. RESULTS AND DISCUSSION

3.1 Chromatographic and detection parameters

A reversed-phase C18 column was used to run various mobile phases under ideal chromatographic conditions. Symmetry C18, Luna C18, and Zorbax C18 were the three distinct columns that were tried. The Acquity UPLC BEH C18 column (2.1 mm x 150 mm, 1.7 µm particle size) produced the best results when used in gradient mode with acetonitrile (A) and 0.01M ammonium formate (B) as the mobile phases. The resolution did not significantly alter when the column temperature varied between 20 and 30°C, however differences in retention time were seen. The flow rate through the column was 1.0 mL/min at 20°C. The procedure allows for analyte and IS separation in 5 minutes (Figure 2 runtime).

3.2 Specificity, Linearity, Accuracy and Precision

By contrasting the chromatograms of the blank matrix and the spiking matrix with the analyte at LOQ concentration, the specificity of the approach was validated. Around their retention times, there were no interfering endogenous peaks found. The analyte's eight-point calibration curve revealed a linear relationship between peak area and concentration. According to calibration data (Table 1), the detector response for all standard solutions between 50 and 1000 ng/mL was linear ($r^2 > 0.99$; Figure 3). The LOQ was discovered to be 50 ng/mL, and the limit of detection by UPLC was determined to be 20 ng/mL. Each benchmark and sample was administered in three different ways. Numerous injections demonstrated low standard error and great reproducibility of the results. A recovery experiment was conducted to validate the method's precision. Low QC, Mid QC, and High QC quantities of the standard stock solution were spiked into blank intestinal fluid (perfusion buffer), which was then collected and subjected to an optimised analysis. All samples from perfusion had extraction recoveries ranging from 92.7 to 110.7% with relative standard deviations smaller than 10.0%, proving the sample preparation method is effective for extracting. Table 2 presents the recovery results. By analysing QC samples on two consecutive days, the method's intra- and inter-day accuracy was assessed. The results showed that the method had intra-day precisions that ranged from 92.0 to 107.1% and inter-day precisions that ranged from 95.7 to 109.8%. Table 3 and Table 4 present the results. Each validation run using a calibration curve contained a blank sample that was analysed immediately following the sample at the ULOQ calibration level in order to look into carry-over from one sample to the next in the autosampler. In the blank sample, the response of the interfering peak(s) shouldn't be more than 20% of the response of the component peak at the concentration of the LLOQ calibration sample. The dilution integrity was evaluated with validation samples spiked with the test chemical at 2-, 4-, and 10-fold the concentration of the high QC to show that the procedure is appropriate for perfusion samples with test compound concentration higher than the ULOQ. By raising the IS concentration by the proper dilution factor, the dilution test was carried out. Following extraction, a volume of injection solvent that was likewise multiplied by the same factor was added to the dried extract. It would appear that samples containing ractopamine at a concentration greater than the ULOQ can be diluted using the previously tried dilution approach because the predicted concentrations were accurate to within a range of 85%-115% of the nominal values.

Both aqueous and matrix-based samples were used to evaluate stability. The stock solutions were stable for 24 hours at room temperature and for 90 days at 1 to 10 degrees Celsius. Utilising freshly made quality control samples (comparison samples), stability assessments of the matrix were carried out against recently spiked calibration standards. The processed samples remained stable in the auto sampler at 10°C for up to 36 hours. Over a 90-day period, the long-term matrix stability was assessed at 20°C. Over the stability time and circumstances, no appreciable analyte degradation was seen. The results for long-term stability shown in Table 5 ranged from 85 to 115%. By contrasting the mean response ratio of the stability samples to the comparison samples, stability in intestinal fluid was assessed at both low and high QC levels. The analyte's short-term stability at room temperature ranged from 85 to 115% for up to 24 hours. The stability findings are shown in Tables 6 and 7. Ractopamine was stable over 4 freeze-thaw cycles and up to 10 hours on a bench top at room temperature. The freeze-thaw experiment was done on mouse blood, and the findings are shown in Table 8. The unpredictability of the matrix effect in intestinal fluid has led to extremely slight changes in the calibration curve's recovery of middle concentration. The outcomes of the Matrix effect are shown in Table 9.

3.3 Application of the method to pharmacokinetic study in Mice

A 3 cm midline abdominal incision was done after the rabbits (1200g ± 0.50) were placed on a heated surface kept at 37°C (Harvard Apparatus Inc., Holliston, MA) and given an intramuscular injection of 1 mL/kg of ketamine-

xylazine solution (9:1 respectively) to induce anaesthesia. Starting 2 cm below the Treitz ligament, a 10 cm length of jejuna was cannulated on both ends and washed with a blank perfusion buffer. A 37°C water bath was used to incubate all of the solutions. A perfusion solution with the test substance, 10 mM perfusion buffer, pH 6.5, 135 mM NaCl, 5 mM KCl, and 0.01 mg/mL phenol red was injected into the intestinal segment at the beginning of each experiment at a flow rate of 0.2 mL/min. In order to establish steady state conditions, the perfusion buffer was perfused for 1h without sampling. Following this, samples were obtained every 10 min for a further 1h of perfusion (Figure 4). To ensure that there had not been any pH changes during perfusion, the collected samples' pH was checked at the outflow (pH 6.5). The samples were evaluated right away by UPLC. At the conclusion of the study, the perfused intestinal segment's length was assessed. The following equation was used to calculate the effective permeability (P_{eff}, cm/sec) through the rabbit gutwall.

$$P_{eff} = -Q \ln(C_{out}/C_{in})\ 2\pi RL$$

Where Q is the perfusion buffer flowrate (0.2 mL/min), C_{out}/C_{in} is the ratio of the outlet and the inlet concentration of drug that has been adjusted for water transport via the non-absorbable marker phenol red, R is the radius of the intestinal segment (set to 0.2 cm), and L is the length of the perfused intestinal segment. All the samples were analyzed by UPLC in the proposed method.

As with the reference chemical (metoprolol), it was discovered that the intestinal permeability in rabbits was segmental-dependent, with higher P_{eff} at distal intestinal sections (with higher average pH) than in proximal segments. According to the findings of the current investigation, a medicine is said to be very permeable if its permeability in any part of the intestine is more than metoprolol's permeability in the jejunum. Metoprolol was found to have a P_{eff} of $0.31 \pm 0.07 \times 10^{-4}$, whereas Ractopamine has a P_{eff} of $0.022 \pm 0.003 \times 10^{-4}$ (Table 10). The molecule's low P_{eff} implies that the jejunum region has low permeability for it (Figure 5). A reliable estimate of the effective permeability was provided by in situ single-pass intestinal perfusion in rabbits.

3.4 Greenness of the method using AGREE

AGREE software produces a clockwise circular diagram in which numbers 1 to 12 are arranged in the edge, indicates 12 ideologies of green analytical chemistry. Each of the 12 principles' result is based on an aggregate scale of 0 to 1 based on inputs and weights provided.[15,16] The score is the net result of all the 12 principles Figure 5 shows the AGREE diagram of the proposed method. The representative AGREE score of the proposed UPLC was estimated as 0.73 (Figure-6) which suggested the significant greenness for the estimation of Ractopamine.

4. CONCLUSIONS

The current UPLC approach has a number of advantages over the pricey, previously described LC-MS methods. Any preclinical and clinical study involving various biological species might assess the concentration of ractopamine with the stated linear dynamic range. The current approach can be utilised to estimate the low doses employed in perfusion and diffusion tests, however it may not accurately identify the medicines for permeability. The outcomes also point to the significance of figuring out a drug's regional permeability in predicting whether or not it will work with ER (extended release) formulations.

Statements and Declarations

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Conflict of Interest

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

All authors contributed to the study conception and design. All authors read and approved the final manuscript.

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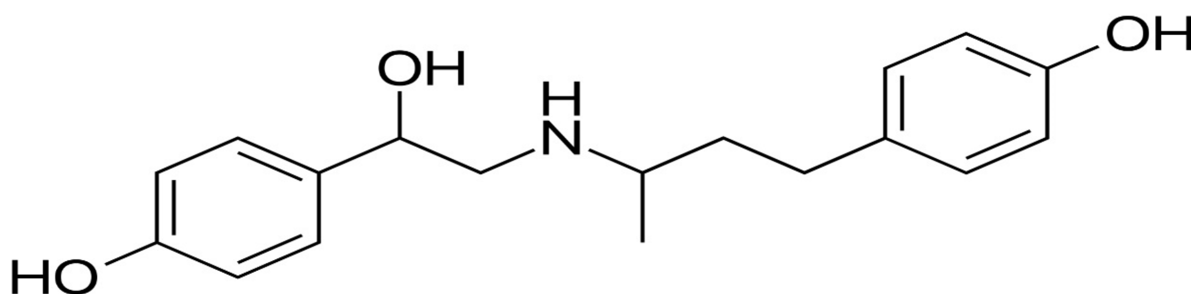

Figure 1: Chemical structure of Ractopamine

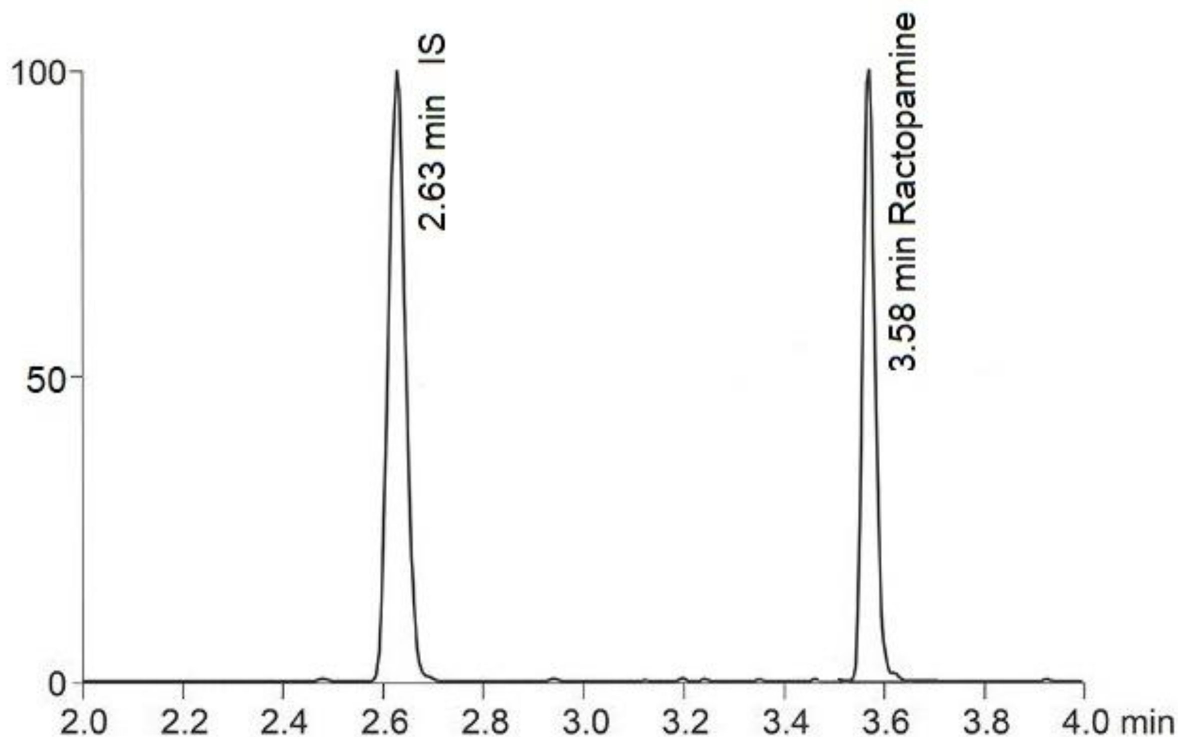

Figure 2: LOQ chromatogram showing the separation of the analyte from IS

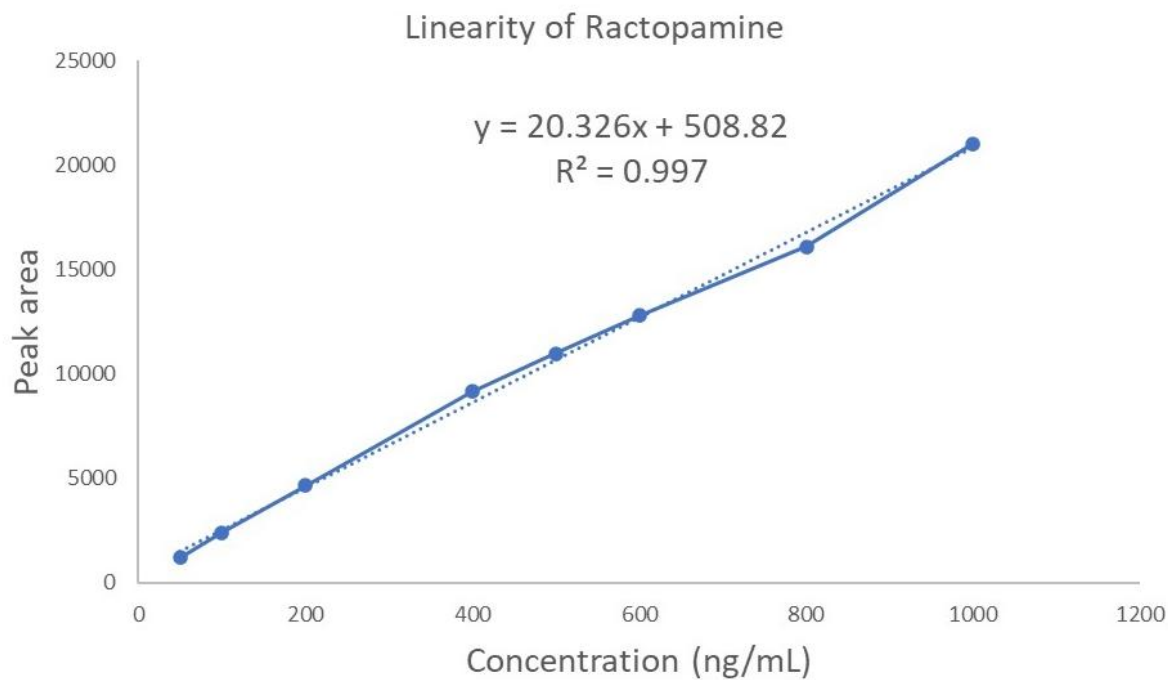

Figure 3: Linearity curve of Ractopamine for Calibration standards

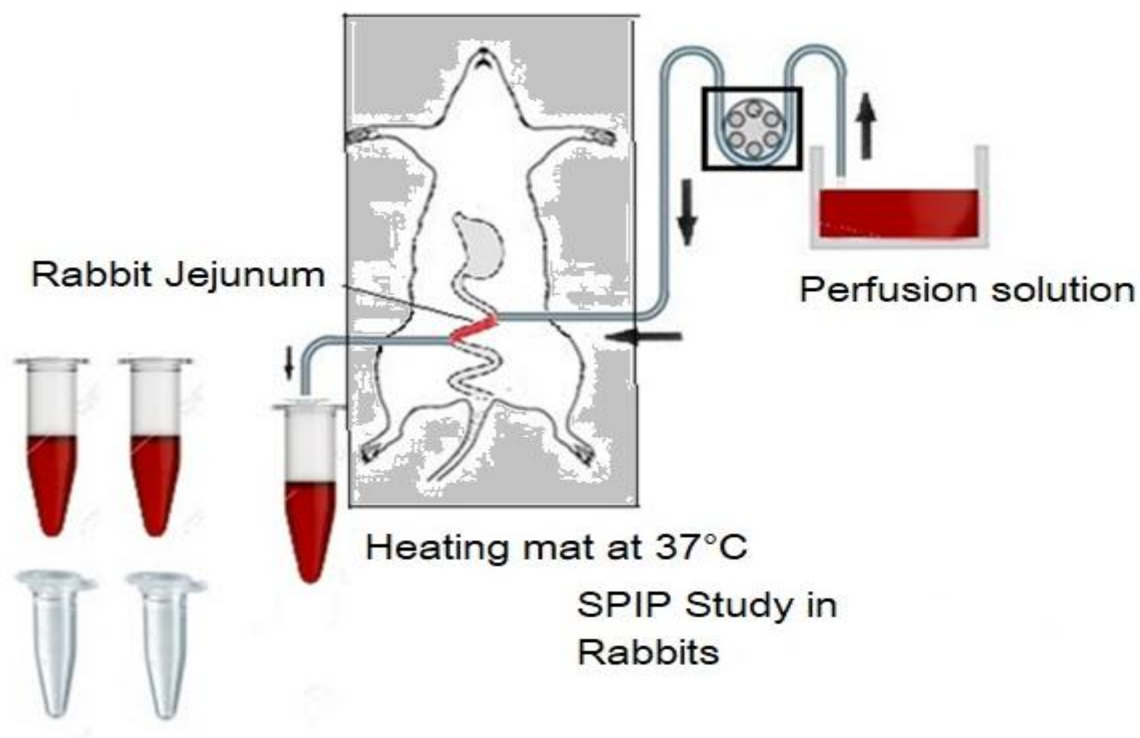

Figure 4: Perfusion study and sample collection of Ractopamine in rabbit

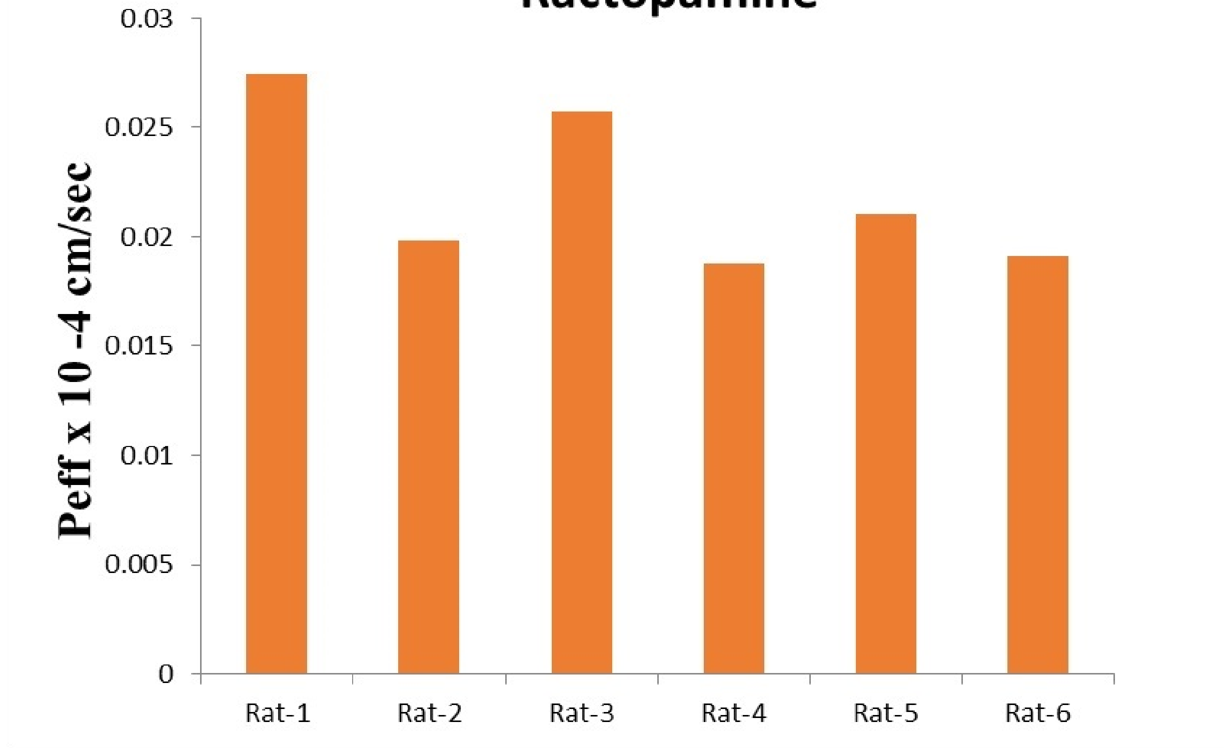

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Table 1: Linearity data of Ractopamine

Concn (ng/mL)	Peak Area
50	1205
100	2425
200	4652
400	9142
500	10977
600	12785
800	16069
1000	21005

Table 2: Recovery Results of Ractopamine

	LLOQ QC-50 ng/mL		LOW QC-200 ng/mL		MID QC-400 ng/mL		HIGH QC-800 ng/mL	
	Concn found	% Recovery	Concn found	% Recovery	Concn found	% Recovery	Concn found	% Recovery
Recovery	55.496	110.659	203.965	101.938	512.305	102.340	814.627	101.462
	55.473	110.615	188.379	94.148	514.237	102.726	800.305	99.679
	55.771	92.719	206.166	103.038	510.745	102.028	808.002	100.637
	55.193	110.055	197.514	98.714	514.368	102.752	826.329	102.920
	55.020	109.710	214.016	106.961	500.450	99.971	790.015	98.397
	49.231	98.167	193.846	96.880	513.470	102.572	833.311	103.790
N	6	6	6	6	6	6	6	6
Mean	**54.364**	**105.321**	**200.648**	**100.280**	**510.929**	**102.065**	**812.098**	**101.148**
SD	**2.528**		**9.239**		**5.310**		**16.140**	
CV(%)	**4.650**		**4.604**		**1.039**		**1.987**	

Table 3: Intra-day Precision & Accuracy Results

Ractopamine								
Batch ID	**LLOQ QC 50 ng/mL**		**LOW QC 200 ng/mL**		**MID QC 500 ng/mL**		**HIGH QC 800 ng/mL**	
	Concn found	**% Recovery**	**Concn found**	**% Recovery**	**Concn found**	**% Recovery**	**Concn found**	**% Recovery**
Intra-day	51.739	103.478	208.290	103.916	519.490	103.775	800.305	100.038
	52.229	104.458	202.519	101.037	515.513	102.980	809.002	101.125
	46.000	92.000	206.242	102.894	512.271	102.333	783.556	97.945
	49.057	98.114	186.433	93.012	533.801	106.634	807.985	100.998
	51.744	103.488	209.008	104.274	536.056	107.084	760.929	95.116
	53.060	106.120	198.224	98.894	523.831	104.642	767.894	95.987
N	6	6	6	6	6	6	6	6
Mean	**50.638**	**101.276**	**201.786**	**100.671**	**523.494**	**104.575**	**788.278**	**98.535**
SD	**2.640**		**8.524**		**9.694**		**20.728**	
CV(%)	**5.213**		**4.224**		**1.852**		**2.630**	

Table 4: Inter-day Precision & Accuracy Results

Ractopamine								
Batch ID	**LLOQ QC 50 ng/mL**		**LOW QC 200 ng/mL**		**MID QC 500 ng/mL**		**HIGH QC 800 ng/mL**	
	Concn found	**% Recovery**	**Concn found**	**% Recovery**	**Concn found**	**% Recovery**	**Concn found**	**% Recovery**
Inter-day	55.088	109.846	198.807	99.381	511.919	102.263	784.562	98.070
	52.829	105.342	199.263	99.610	513.445	102.567	767.334	95.917
	53.646	106.971	187.562	93.760	497.055	99.293	858.067	107.258
	48.010	95.733	209.392	104.673	527.146	105.304	785.684	98.211
	51.069	101.833	208.955	104.455	503.486	100.578	804.812	100.602
	53.720	107.119	189.763	94.861	493.993	98.681	790.307	98.788
N	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**
Mean	**52.394**	**104.474**	**198.957**	**99.457**	**507.841**	**101.448**	**798.461**	**99.808**
SD	**2.722**		**8.971**		**11.376**		**35.019**	
CV(%)	**5.195**		**4.509**		**2.240**		**4.386**	

Table 5: Long term stability study Results (n-6) after 90 days

	Ractopamine			
	0 Hr-Low QC	**0 Hr-HQC**	**Day-90-LQC**	**Day-90-HQC**
Long term stability after 90 days	189.568	808.998	194.984	815.501
	211.278	804.206	201.102	807.602
	208.045	808.019	208.896	815.116
	192.526	775.501	217.845	785.216
	194.040	807.602	200.151	800.000
	188.698	882.994	198.880	785.213
N	**6**	**6**	**6**	**6**
Mean	**197.359**	**814.553**	**203.643**	**801.441**
SD	**9.778**	**35.884**	**8.314**	**13.794**
CV(%)	**4.954**	**4.405**	**4.083**	**1.721**
% Change	**n/a**	**n/a**	**3.184**	**-1.610**

Table 6: Short term stability study Results (n-6)

	Ractopamine					
	0 Hour		4 Hour		24 Hour	
	Low QC	HQC	Low QC	HQC	Low QC	HQC
Short term stability	202.918	785.624	222.019	799.600	199.989	801.252
	210.980	788.147	207.501	800.000	194.699	816.186
	194.206	812.521	197.602	802.531	208.018	795.414
	220.145	800.852	199.967	784.120	192.244	803.732
	196.069	798.632	195.936	792.510	179.495	870.610
	192.971	805.258	211.976	797.241	192.296	889.362
N	**6**	**6**	**6**	**6**	**6**	**6**
Mean	**202.881**	**798.506**	**205.834**	**796.000**	**194.457**	**829.426**
SD	**10.803**	**10.206**	**10.026**	**6.730**	**9.466**	**40.186**
CV(%)	**5.325**	**1.278**	**4.871**	**0.845**	**4.868**	**4.845**
% Change	**n/a**		**-1.913**		**1.299**	

Table 7: Freeze thaw stability (after IV cycle) study Results (n-6) conducted below -20°C

	LOW QC		HIGH QC	
	200 ng/mL		800 ng/mL	
	Conc found	% Recovery	Conc found	% Recovery
Freeze Thaw Cycle-IV below -20°C	207.441	103.721	785.241	98.155
	209.630	104.815	809.854	101.232
	192.511	96.256	791.214	98.902
	189.581	94.791	790.521	98.815
	209.589	104.794	777.524	97.191
	208.586	104.293	786.213	98.277
N	**6**	**6**	**6**	**6**
Mean	**202.890**	**101.445**	**790.095**	**98.762**
SD	**9.255**		**10.849**	
CV(%)	**4.562**		**1.373**	

Table 8: Freeze thaw stability (after IV cycle) study Results (n-6) conducted below -50°C

	LOW QC		HIGH QC	
	200 ng/mL		800 ng/mL	
	Conc found	% Recovery	Conc found	% Recovery
Freeze Thaw Cycle-IV below -50°C	191.522	95.761	788.521	98.565
	208.587	104.294	802.147	100.268
	191.696	95.848	814.214	101.777
	192.214	96.107	799.654	99.957
	190.253	95.126	787.123	98.390
	210.584	105.292	778.214	97.277
N	**6**	**6**	**6**	**6**
Mean	**197.476**	**98.738**	**794.979**	**99.372**
SD	**9.423**		**12.866**	
CV(%)	**4.772**		**1.618**	

Table 9: Matrix effect Results

Intestinal fluid No.	Ractopamine 500 ng/mL	
	Neat standard sample Concentration	**Extracted blank plus spiked sample peak concentration**
Unit No.: 1	11817	11048
Unit No.: 2	10814	10450
Unit No.:3	11891	10650
Unit No.: 4	10908	9862
Unit No.: 5	10779	9997
Unit No.: 6	10832	9627
N	**6**	**6**
Mean	**11173.500**	**10272.333**
SD	**529.317**	**536.002**
CV(%)	**4.737**	**5.218**
Matrix effect (%)	**0.919**	

Table. 10: Concentration of Ractopamine found in rabbit intestine (n=6)

S.No.	Concentration (ng/ml)
Rabbit-1	0.027427859
Rabbit -2	0.0198284
Rabbit -3	0.025704077
Rabbit -4	0.018782709
Rabbit -5	0.021004077
Rabbit -6	0.019123292
Avg	**0.021978402**
S.D	**0.003674593**
CV	**16.71910849**