Original Research Article

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

ABSTRACT

In recent years, the prediction of oral drug absorption in humans has been a challenge for researchers and many techniques for permeability studies have been developed for several purposes, including biowaiver processes. The Single-Pass Intestinal Perfusion (SPIP) method performed in rats can provide permeability results closest to in vivo condition. he ~~use~~uses of SPIP in rats and calculation~~s~~ for absorption prediction in humans may indicate the transport mechanisms and/or pre-systemic metabolism involved on permeation processes of drugs, since this model is the closest to in vivo conditions. Ractopamine hydrochloride is a commercial beta-adrenergic agonist commonly used as a dietary supplement in cattle production for improved feed efficiency and growth promotion. Currently, regulatory target tissues (as approved in the New Animal Drug Application with Food and Drug Administration) for ractopamine residue testing are muscle and liver. The aim of the study is to develop and validate an UPLC assay method to determine whether detectable and quantifiable levels of perfused ractopamine in small intestine of cattle or not. The separation is carried on Acquity UPLC BEH C_{18} (150 mm x 2.1 mm, 1.7 µm) reversed phase column using acetonitrile and 0.01M ammonium ~~formate~~format as the mobile phase in gradient mode at flow rate of 0.5 mL/min. The precision, accuracy, extraction recovery, matrix effect, and stability meet the requirements of the guiding principles. 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 small intestine of rabbit and the samples were analyzed by Empower software.

Abstract including: background, material and methods, results and conclusion. But, their material and methods and result not clear.

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

1. INTRODUCTION

Ractopamine hydrochloride (Figure-1) is a phenol-based TAAR1 agonist and β adrenoreceptor agonist that stimulates β_1 and β_2 adrenergic receptors[1]. When used as a food additive, ractopamine added to feed can be distributed by the blood to the muscle tissues, where it serves as a full agonist to murine (mouse or rat) TAAR1, a receptor protein (not necessarily in humans). In 2000, use of ractopamine for the purpose of increasing weight gain, carcass leanness, and promoting better feed efficiency in swine was approved by the USFDA[2]. Since then, it has been used in livestock production in over 20 countries, but concerns remain regarding potential human health risks. ractopamine increase protein synthesis while decreasing degradation of protein and production of fat[3]. Ractopamine is an animal feed additive that is known to produce lean muscle and less fat in turkey, pork and cattle meat. It is a safe additive for human consumption if used according to the label and approved guidelines in a number of countries like the USA, South Korea and Japan. When used excessively, it causes adverse effects to animals like broken limbs, hyperactivity and trembling. Before any medication or feed supplement that is given to animals is determined safe, the FDA recommends several tests on humans and animals to see how they react to

the drug. Several studies have been done over the years and found that ractopamine is safe and effective when administered at 4.5-9 grams per ton of feed in the last three to four weeks to slaughter. When this drug is strictly used according to FDA's guidelines, ractopamine analysis in tissues of slaughtered animals falls below the set limits of 50 ppb in the liver and 15 ppb in meat. The acceptable concentration, together with the amount of meat consumed in a day, is useful in calculating the acceptable daily intake (ADI). ADI is the total drug amount a person can take in a single day (24 hours) without side effects and health risks. Commercial ractopamine is a mixture of all four possible stereoisomers. It is also a positional isomer of dobutamine, a related drug. The RR-isomer of ractopamine is considered to be a full agonist at the beta2-adrenoceptor and a partial agonist at the beta-adrenoceptor. In cattle and swine tissue, it was found in 2007 that a procedure for the analysis of ractopamine residues in liver or muscle can be performed by high performance liquid chromatography (HPLC) with fluorescence detection[4]. Till date very few methods are published for ractopamine using immunosorbent assays[5] and LC-MS methods[6-10]. As of today, no method has been reported for the perfusion study of Ractopamine by UPLC. The objective of the current research is to develop a rapid, reliable, sensitive and simple ultra-performance liquid chromatography method for the quantification of Ractopamine in perfusion study samples conducted in jejunum of rabbits and also to validate the method as per the Veterinary International Conference on Harmonization (VICH) guideline for analytical methods to be used in residue depletion studies and USFDA Guidelines. After complete validation, the method was applied to analyze sample analysis in rabbits by SPIP study.

2. EXPERIMENTAL

2.1 Instrumentation and Chromatographic Conditions

UPLC–UV Analysis

The LC system consisted of a Waters Acquity UPLC with Empower software equipped with a photodiode array detector. A Acquity UPLC BEH RP C18 column (2.1 mm × 150 mm, 1.7 µm particle size) from Waters was used as stationary phase and temperature maintained at 20°C. The mobile phase consisted of Acetonitrile (A) and 0.01M ammonium formate (B) in gradient mode pumped at a flow rate of 1.0 mL/min. Gradient program started initially with 95% ammonium formate. 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. Analysis was performed for 5 min at the detection wavelength of 220 nm and the injection volume was 10 µL. The autosampler maintained at 4°C.

2.2 Chemicals

Ractopamine and internal standard (Imipramine) are purchased from Sigma–Aldrich Trading Co., Ltd. (Shanghai, China). Acetonitrile and methanol of HPLC grade and all other chemicals were obtained from Merck (Mumbai, India). Ammonium formate and all other chemicals (GR grade) were purchased from Merck Chemicals Ltd., Mumbai. Water used in the entire analysis was prepared from Milli-Q water purification system from Millipore (Milford, MA, USA).

2.3 Preparation of Perfusion buffer

0.35 g KCl, 1.37 g $NaHCO_3$, 7.8 g NaCl, 0.22 g NaH_2PO_4, 0.02 g $MgCl_2$, and glucose 1.48 g dissolved in 1000 mL purified water, pH of this solution obtained as 6.5.

2.4 Preparation of Calibrators and QC Samples

A standard stock solution of Ractopamine was prepared by dissolving standard 50 mg of Ractopamine into 50 ml volumetric flask, to this added 30 ml of methanol and sonicated for 10 minutes at a temperature not exceeding

20°C. Allowed the solution to attain room temperature and then diluted to the volume with methanol to have a solution with a concentration of 1000 µg/mL. Calibration standard and quality control (QC) samples were prepared by diluting corresponding working solutions with ~~methanol:water~~methanol: water (50:50). A volume of 10 mL of appropriate diluted stock solution at different concentrations and 10 mL of IS at a fixed concentration were spiked into 200 µL of perfusion buffer to yield final concentrations of calibration samples 50, 100, 200, 300, 400, 500, 800 and 1000 ng/mL. The final concentration of IS was 100 ng/mL. Similarly, QC samples were prepared at four concentration levels LLOQ (50 ng/mL), LQC (200 ng/mL) MQC (500 ng/mL) and HQC (800 ng/mL).

2.5 Sample preparation

Perfusion samples were collected at certain time points, centrifuged at 5000 rpm for 10 minutes. The supernatant layer was separated and filtered through 0.45 µm syringe filters and 10 µL of the solution was injected 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

Specificity was assessed by verifying the absence of significant interference in the biological control medium with regard to the retention time of the compound (s) to be assayed. The specificity of the method was confirmed by comparing chromatograms of blank matrix, spiked matrix with analyte at LOQ concentration. No interfering endogenous peaks were observed around the retention time.

Linearity

A calibration curve was prepared within the range of 50 to 1000 ng/mL Ractopamine in each run. Half of the calibration samples were analyzed at the beginning of the run and half at the end. The simplest calibration model and weighting procedure were used. The calculations of the curve's parameters were based on the ratio of the peak areas of Ractopamine/IS versus the concentration of Ractopamine. Ractopamine concentrations for samples were calculated from the curve's equation obtained by means of linear regression.

Accuracy of back-calculated calibration samples should be within ±15% of the corresponding nominal concentration, except at the lowest concentration level, where the accuracy should be within ±20%. Per calibration curve, a maximum of 33% of the calibration samples, except the LLOQ and upper limit of quantification (ULOQ, 800 ng/mL), may differ from these specifications. At least 6 concentration levels were represented in each curve.

Matrix Effect, Extraction Recovery, and Process Efficiency

The influence of the matrix on the quantification of Ractopamine was monitored using a comparison of: (1) the instrument response for the low, medium, and high QCs (n = 4 per level) injected directly in mobile phase (neat solutions), (2) the same amount of analyte added to extracted blank samples (post extraction spiked samples), and

(3) the same amount of analyte added to the biological matrix before extraction (pre extraction spiked samples). Total process efficiency was calculated from the ratio of mean peak areas of Ractopamine in extracted validation samples versus neat unextracted samples. This term accounts for any loss in signal attributable to the extraction process or matrix effect. Extraction recovery was calculated from the ratio of mean peak areas of Ractopamine in extracted validation samples versus blank samples spiked after extraction. The absolute matrix effect was calculated from the ratio of mean peak areas of Ractopamine in blank samples spiked after extraction versus neat unextracted samples. If the ratio was 85% or 115%, an exogenous matrix effect was inferred.

Matrix Variability

To confirm that the biological matrix would not interfere with the assay, the selectivity of the developed method was tested by analyzing 6 different lots of blank buffer samples spiked with IS at the LLOQ level (n = 3 per lot), and blank buffer samples with no IS (n = 3 per lot) against a calibration curve. The results for the LLOQ samples were considered acceptable if the precision from each matrix lot was ±20% and the accuracy was within the range of 80%–120%. The acceptance criterion for the analysis of the blank samples from the 6 individual lots was based on the raw peak areas found at the retention times of Ractopamine and IS. No more than 10% of the blank samples could have peak areas greater than 20% of the average peak area of Ractopamine in the LLOQ QCs.

Stability studies

Stability evaluations were performed in both aqueous and matrix-based samples. Stability evaluations in matrix were performed against freshly spiked calibration standards using freshly prepared quality control samples (comparison samples). Ractopamine stability in perfusion buffer was evaluated by performing bench top stability, long-term stability, short term stability and freeze-thaw stability. The processed samples were studied for stability in auto sampler at 10°C. Stability evaluated at both low and high QC level by comparing the mean response ratio of stability samples against the comparison samples.

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3. RESULTS AND DISCUSSION

3.1 Chromatographic and detection parameters

Optimal chromatographic conditions were obtained after running different mobile phases with a reversed-phase C18 column. The different columns tried were Symmetry C18, Luna C18 and Zorbax C18. The best results were observed with the Acquity UPLC BEH C18 column (2.1 mm × 150 mm, 1.7 µm particle size) using acetonitrile (A) and 0.01M ammonium formate (B) (gradient mode) as mobile phase. Variation of the column temperature between 20 and 30°C did not cause significant change in the resolution, however changes in retention time were observed. The column was used at 20°C at a flow rate of 1.0 mL/min. The method allowed the separation of analyte with IS in 5 min (Figure 2) runtime.

3.2 Specificity, Linearity, Accuracy and Precision

The specificity of method was confirmed by comparing chromatograms of blank matrix, spiked matrix with analyte at LOQ concentration. No interfering endogenous peaks were observed around their retention times. The ~~eight point~~eight-point calibration curve for the analyte showed a linear correlation between concentration and peak area. Calibration data (Table 1) indicated the linearity ($r^2 > 0.99$) of the detector response for all standard solutions from 50 to 1000 ng/mL (Figure-3). The limit of detection by UPLC was found to be 20 ng/mL and LOQ was found to be 50 ng/mL. All standards and samples were injected in triplicate. Multiple injections showed that

the results are highly reproducible and showed low standard error. A recovery experiment was performed to confirm the accuracy of the method. Blank intestinal fluid (perfusion buffer) was spiked with Low QC, Mid QC and High QC levels of the standard stock solution and then extracted and analyzed under optimized conditions. The extraction recoveries of all samples from perfusion were in the range of 92.7-110.7% with relative standard deviations less than 10.0%, which indicates the sample preparation technique is suitable for extracting. The recovery results are displayed in Table 2. Intra- and inter-day precision of the method was determined by analyzing QC samples on two consecutive days and the obtained intra-day accuracies were in the range of 92.0–107.1% and inter-day accuracies were in the range of 95.7–109.8%. The results are displayed in Table 3 and Table 4. To investigate carry-over from one sample to the other in the autosampler, each validation run containing a calibration curve included a blank sample analyzed directly after the sample at the ULOQ calibration level. The response of interfering peak (s) in the blank sample should not exceed 20% of the response of the component peak at the LLOQ calibration sample concentration. To demonstrate that the method is suitable for perfusion sample with test compound concentration higher than the ULOQ, the dilution integrity was assessed using validation samples spiked with the test compound at 2-, 4-, and 10-fold the concentration of the high QC. The dilution test was performed by increasing the concentration of IS by the appropriate dilution factor. After extraction, the dry extract was taken up with a volume of injection solvent also multiplied by the same factor. Accuracy of the calculated concentrations within the range of 85%–115% of the nominal values would suggest that samples containing Ractopamine at a higher concentration than the ULOQ can be diluted using the above tested dilution method.

Stability evaluations were performed in both aqueous and matrix-based samples. The stock solutions were stable for a period of 24 h at room temperature and for 90 days at 1–10°C. Stability evaluations in matrix were performed against freshly spiked calibration standards using freshly prepared quality control samples (comparison samples). The processed samples were stable up to 36 h in auto sampler at 10°C. The long-term matrix stability was evaluated at −20°C over a period of 90 days. No significant degradation of analytes was observed over the stability duration and conditions. The long-term stability results presented in Table 5 were within 85–115%. Stability in intestinal fluid was evaluated at both low and high QC level by comparing the mean response ratio of stability samples against the comparison samples. The short-term stability of analyte at room temperature was within 85–115% upto 24 h. The stability results presented in Table 6 and Table 7. Ractopamine was stable upto 10 h on bench top at room temperature and over 4 freeze–thaw cycles. In mouse ~~blood ,~~blood, the freeze-thaw study was carried out and the results are presented in Table 8. The variability of the matrix effect in intestinal fluid has resulted a very minute changes in the recovery of middle concentration of calibration curve. The results of Matrix effect area presented in Table 9.

3.3 Application of the method to pharmacokinetic study in Mice

Rabbits (1200g ± 0.50) were anesthetized with an intra-muscular injection of 1 mL/kg of ketamine–xylazine solution (9:1 respectively), placed on a heated surface maintained at 37°C (Harvard Apparatus Inc., Holliston, MA), and a 3 cm midline abdominal incision was made. Approximal 10 cm jejuna segment, starting 2 cm below the ligament of Treitz, was cannulated on two ends, and was rinsed with blank perfusion buffer. All solutions were incubated in a 37°C water bath. At the starting point of each experiment, perfusion solution containing the investigated drug, 10mM perfusion buffer, pH 6.5, 135 mM NaCl, 5mM KCl, and 0.01 mg/mL phenol red, with an osmolarity of 290 mOsm/L, was perfused through the intestinal segment at a flowrate of 0.2 mL/min. The

perfusion buffer was perfused for 1h without sampling, to ensure steady state conditions, followed by additional 1h of perfusion with samples taken every 10 min (Figure-4). The pH of the collected samples was measured at the outlet, to verify that there was no pH change throughout the perfusion (pH 6.5). The samples were immediately assayed by UPLC. The length of the perfused intestinal segment was measured at the end point of the experiment. The effective permeability (P_{eff}, cm/sec) through the rabbit gutwall was determined according to the following equation.

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

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.

The intestinal permeability in the rabbit was found to be segmental-dependent similar to reference compound (metoprolol) with higher P_{eff} at distal intestinal regions (with higher average pH) than in proximal segments. The results from the present study suggest that a drug can be classified as highly permeable if it's permeability in any intestinal region is higher than metoprolol's permeability in jejunum. The P_{eff} of metoprolol was found to be 0.31 ± 0.07 x 10^{-4} whereas for Ractopamine, the P_{eff} value is 0.022 ± 0.003 x 10^{-4} (Table-10). The low P_{eff} of the molecule indicates it has low permeability in jejunum region (Figure-5). *In situ* single-pass intestinal perfusion in rabbits gave a good estimation of the effective permeability.

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.

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4. CONCLUSIONS

The present UPLC method offers significant advantages over those previously reported, LC-MS methods. The linear dynamic range established was adequate to measure the concentration of Ractopamine in any preclinical and clinical study involving different biological species. However, the current method may not exactly classify the drugs for permeability, but it can be used to estimate such low concentrations used in perfusion and diffusion studies. The results also suggest the importance of determining regional permeability of drugs for prediction of their suitability for use in extended release (ER) formulations.

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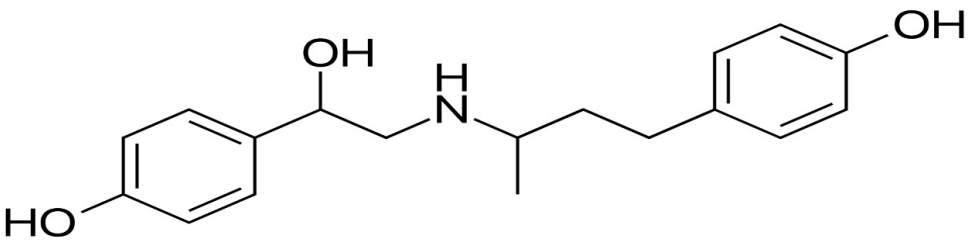

Figure 1: Chemical structure of Ractopamine

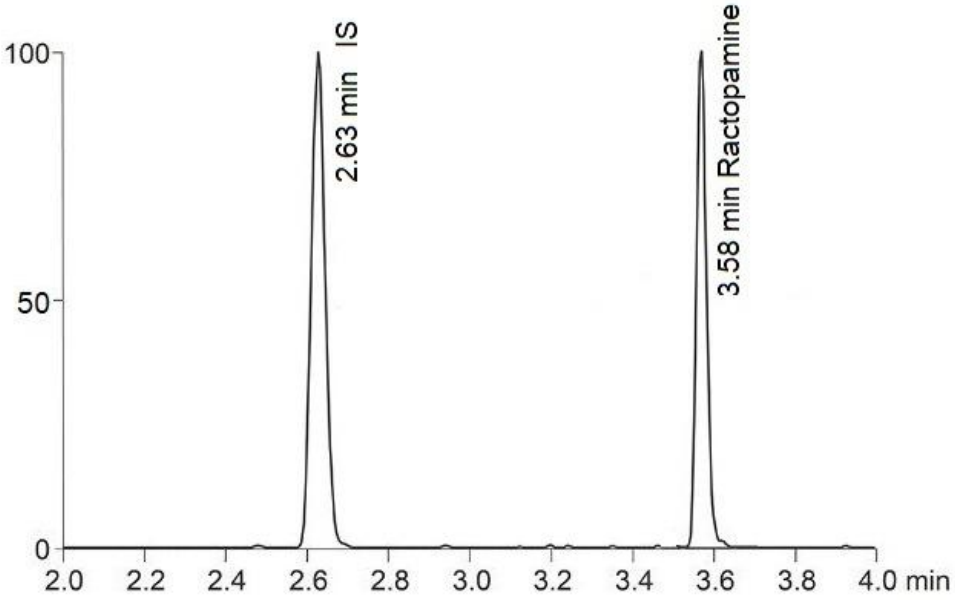

Figure 2: LOQ chromatogram showing the separation of the analyte from IS

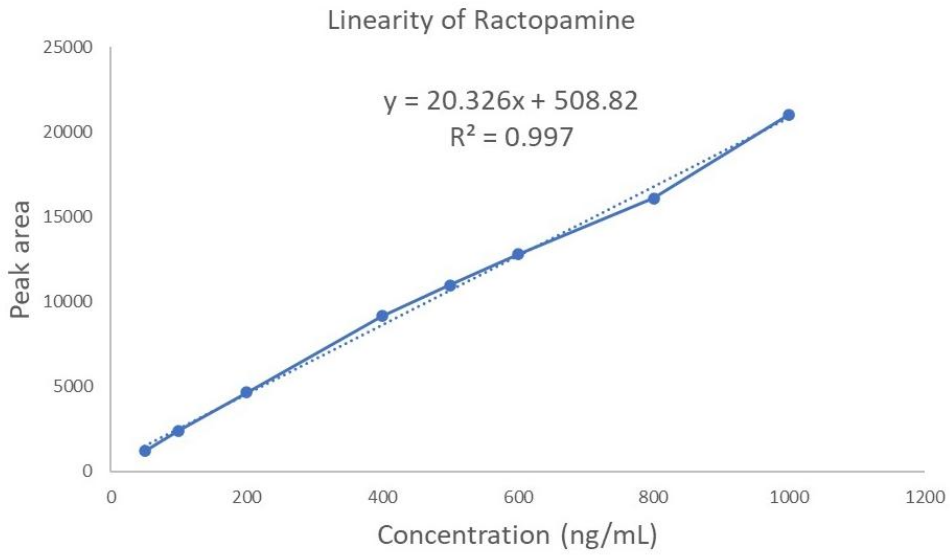

Figure 3: Linearity curve of Ractopamine for Calibration standards

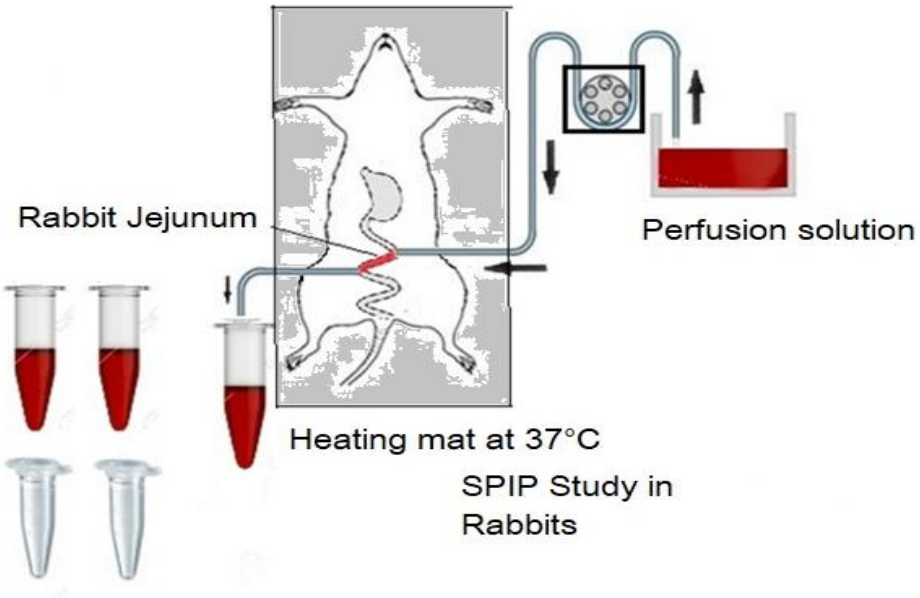

Figure 4: Perfusion study and sample collection of Ractopamine in rabbit

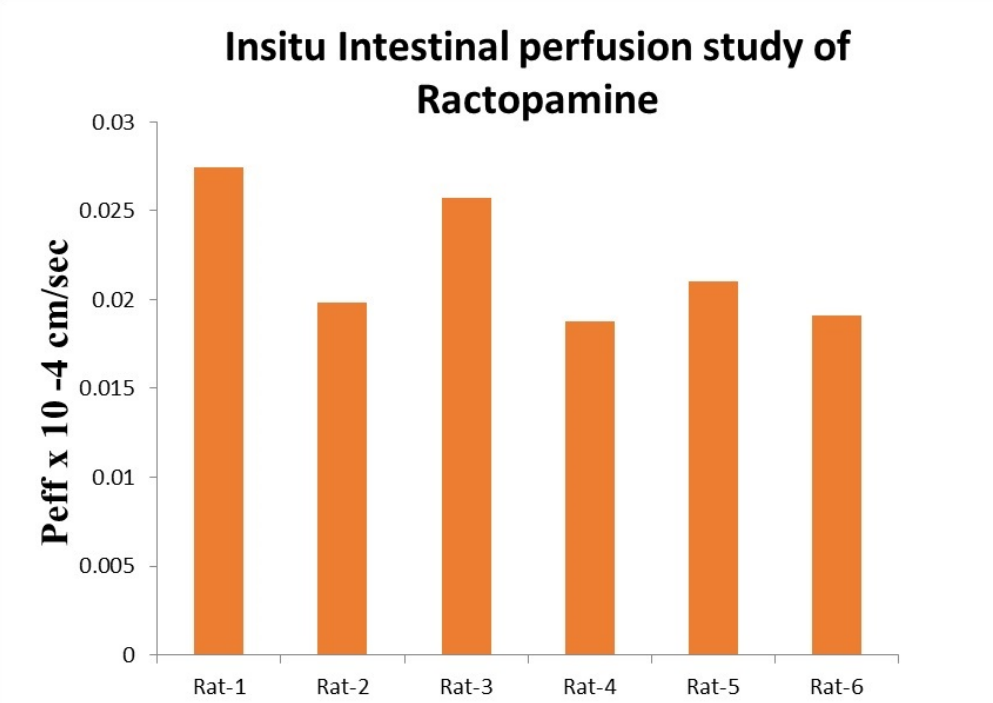

Figure 5: Mean concentrations of Ractopamine in rabbit Perfusion study(n=6)

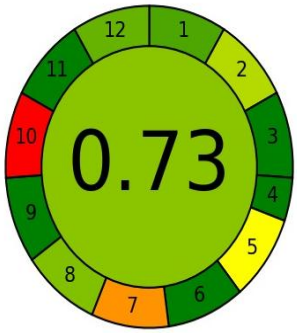

Figure 6: Greenness of the proposed UPLC method

LIST OF TABLES

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**		**LOW QC**		**MID QC**		**HIGH QC**	
	50 ng/mL		**200 ng/mL**		**500 ng/mL**		**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**