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Bio-analytical Validated Method Development and Bioequivalence Study of Serotonin Receptor 5-HT4 Agonist Dihydrobenzofurancarboxamide Derivative used for Chronic Constipation Drug Prucalopride by LC-ESI-MS/MS in Indian Human Plasma

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Abstract

Aim and objective: Clinically, prucalopride is used to treat persistent constipation since it is a serotonin receptor agonist. According to US-FDA and European Medicines Agency (EMA) requirements, a validated bioanalytical technique for prucalopride quantification from human plasma was developed and utilized in a comparative investigation of pharmacokinetics and bioequivalence of the drug.

Method: Prucalopride's protonated precursor ion was 368.1/196.0 m/z, while propranolol's was 260.1/116.0 m/z. When it came to plasma, it was extracted using liquid-liquid technology. It combined with 10 mM ammonium acetate as an aqueous solvent with an apparent pH of 1.20 and 1% formic acid in acetonitrile as an organic solvent at a flow rate of 0.5000 ml/min as the mobile phase. 0.25 ng/ml, 0.50 ng/ml, 1 ng/ml, 2 ng/ml, 4 ng/ml, 8 ng/ml, and 16 ng/ml is used as calibration concentrations.

Result: Peak Concentration Cmax was 4.98 ng/ml ± 0.95 ng/ml for reference preparation at 2.71 hour ± 0.33 hour. (Tmax) and 5.01 ± 1.05 ng/ml for test preparation at 2.64 hour ± 0.28 hour (Tmax).

Conclusion: Using this technique for comparative pharmacokinetics and bioequivalence studies has proven to be a highly selective, sensitive, high recovery, low ion suppression, repeatable, and cost effective approach.

Keywords: Prucalopride; LC-MS/MS; Bioanalytical method; Serotonin receptor agonist

Abbreviation: GABA: Gamma-Aminobutyric Acid; HT: Hydroxytryptamine; hERG: human Ether-Ago-Go Related Gene; USFDA: United States Food and Drug Administration; CPU: Clinical Pharmacological Unit; CDSCO: Central Drugs Standard Control Organization; ISTD: Internal Standard; DMSO: Dimethyl Sulfoxide; LLOQ: Lower Limit of Quantification; LQC: Low-Quality Control; MQC: Middle-Quality Control; HQC: High-Quality Control; EMA: European Medicine Agency; API: Active Pharmaceutical Ingredient; LOD: Limit of Detection; ESI: Electrospray Ionization; ME: Matrix Effect; LLE: Liquid-Liquid Extraction; MTBE: Methyl Tert-Butyl Ether

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Introduction

Serotonin receptors, activated by the neurotransmitter serotonin, are G-protein coupled receptors and ligand-gated ion channels found in the peripheral and central nervous systems [1]. But these receptors modulate releases of many neurotransmitters and hormones like GABA, dopamine, acetylcholine, epinephrine or norepinephrine, glutamate, and hormone-like oxytocin, prolactin, vasopressin, corticotrophin, and substances P [2,3].

All 5-HT receptors have excitatory activity except 5-HT1 and 5-HT5, which have inhibitory activity [4]. The pharmacological functions are anxiety, appetite, GI motility, learning, memory, mood respiration [5,6]. Constipation is a symptom which causes divided into congenital, primary (intrinsic problems of colonic or anorectal function), and secondary (related to organic disease, systemic disease, or medications) [7-11]. Prucalopride is a dihydro benzofuran carboxamide derivative from the benzofuran family that is a serotonin 5-HT4 receptor agonist and has enterokinetic properties that are it has potent prokinetic activity [12]. This drug changes the colonic motility pattern *via* serotonin 5-HT4 receptor stimulation and stimulates colonic mass movements, providing the main propulsive force for defecation [13-15]. Prucalopride is well absorbed in the GI tract. It reaches maximum plasma concentration around 3 hrs after initial administration, an overdose may cause severe diarrhea, and bioavailability is over 90% and not influenced by the ingestion of food. Still, plasma protein binding is 30% [16-20]. After reaching maximum plasma concentration, prucalopride is mainly excreted by the urine (84%) and remaining excreted by feces [21].

Ultra-high-performance mass spectrometry was used to measure prucalopride in human plasma after a literature analysis revealed one or two techniques. There was no mention of a precise bioanalytical approach, mass spectrometric fragmentation of the medication, or a complete process for validating the results.

As part of the bioequivalence study conducted by Estradiol Valerate (EV), we developed and validated a fast, rapid, sensitive, and specific LC-MS/MS (API-4000) method for quantifying prucalopride in Indian human plasma. This method was successfully applied to several pharmacokinetic studies on healthy human volunteers.

Materials and Methods

Chemical and reagents

Acetonitrile purchased from Merck (MERCK India Ltd., Mumbai), isopropyl alcohol, formic acid was AR grade, and solvents were HPLC grade. Milli-Q water was used in the study (Elix, Milli-Q A10 Academic, Bedford, MA, USA) until a resistivity of 18.2 MΩ was achieved. The blank human plasma with EDTA-K3 anticoagulant was collected from the Clinical Pharmacological Unit (CPU) of TAAB Biostudy Services, Kolkata, and stored at 20°C until analysis.

Ethical clearance

The protocol of prucalopride study and related documents like volunteers informed consent form, case record form, subject information sheet submitted to the HURIP Independent Bioethics committee, Kolkata, India (Central Drugs Standard Control Organization (CDSCO) registration: ECR/103/Indt/WB/2013/RR-19 which is valid up to 21st Nov, 2024) and the ethical clearance obtained before initiation of the study.

Drug information and study design

It is a randomized, open-label, balanced, laboratory blind, two treatment, two-period, two sequences, single-dose, truncated, two way, crossover, bioequivalence study of Prucalopride 2 mg tablet as a test with Pruvict 2 (Prucalopride 2 mg) tablet of Torrent pharmaceutical Ltd as a reference product, in healthy adult human subjects under fasting condition. The volunteers have received either test or reference product with 240 ml drinking water based on the randomization code in each clinical period as specified in **Table 1**.

Table 1: Randomization schedule.

Subject No.	Period I	Period II
1	A1	A2
2	A2	A1
3	A2	A1
4	A1	A2
5	A1	A2
6	A2	A1
7	A1	A2
8	A2	A1
9	A2	A1
10	A1	A2
11	A1	A2
12	A2	A1
13	A1	A2
14	A2	A1
15	A1	A2
16	A1	A2
17	A2	A1
18	A1	A2
19	A2	A1
20	A2	A1
21	A1	A2
22	A1	A2
23	A2	A1
24	A2	A1

A1:Reference preparation; A2:Test preparation

Test product Prucalopride 2 mg tablets were compared to a reference product under fasting conditions with the primary goal being to compare the rate and amount of absorption. Prucalopride 2 mg tablet will be administered orally to healthy adult human volunteers under fasting conditions with the secondary goal of monitoring safety and acceptability. Total 21 blood sampling time point were 0 hour (before drug administration) and 0.50 hours, 1.00 hours, 1.33 hours, 1.67 hours, 2.00 hours, 2.33 hours, 2.67 hours, 3.00 hours, 3.33 hours, 3.67 hours, 4.00 hours, 4.50 hours, 5.00 hours, 6.00 hours, 8.00 hours, 10.00 hours, 12.00 hours, 24.00 hours, 48.00 hours, 72.00 hours, which were collected from cubital vein and separated plasma by centrifugation and stored in -20˚C. 5 ml of blood taken at each time point. The demographic data of each volunteer is specified in **Table 2.**

Table 2: Demographic data of 24 volunteers.

Vol. No.	Sex	Age	Height (cm)	Weight (kg)	BMI (kg/m²)
1	M	28	156	52	21.37
2	M	42	156	55	22.6
3	M	39	160	60	23.44
4	M	34	163	65	24.46
5	M	42	165	58	21.3
6	M	24	179	65	20.29
7	M	24	170	56	19.38
8	M	32	166	59	21.41
9	M	42	163	65	24.46
10	M	30	158	53	21.23
11	M	28	173	61	20.38
12	M	40	165	52	19.1
13	M	30	155	51	21.23
14	M	22	167	53	19
15	M	29	163	52	19.57
16	M	35	173	72	24.06
17	M	45	157	56	22.72
18	M	35	158	60	24.03
19	M	42	166	62	22.5
20	M	41	155	52	21.64
21	M	30	154	56	23.61
22	M	38	167	56	20.08
23	M	27	165	55	20.2
24	M	27	164	57	21.19
Mean		33.58	163.25	57.62	21.63
S.D.		7.08	6.39	5.38	1.7

Bio-analytical method development by LC-MS/MS (API-4000)

Prucalopride is a dihydro benzofuran carboxamide derivative from the benzofurane family, with chemical name 4-Amino-5-chloro-2methoxybenzoic acid, 3-dihydro-N-[1-(3-methoxypropyl)-4-piperidinyl]-7-benzofurancarboxamide group and represented in **Figure 1** [22]**.** It acts as an agonist of the 5-HT4 receptor. In addition, it acts as a prokinetic drug that stimulates these receptors and colonic mass movements, providing the primary propulsive force for defecation [23].

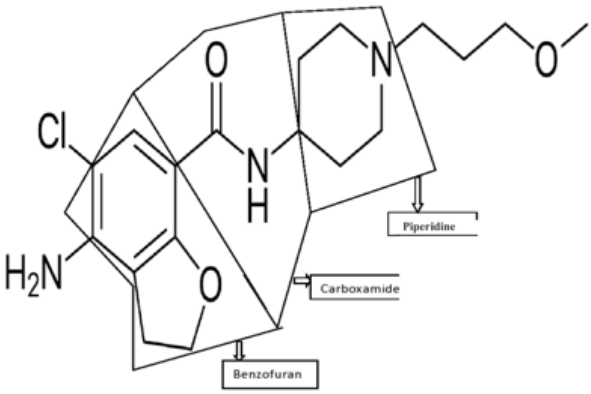

Figure 1: Chemical structure of Prucalopride.

The exact mass of prucalopride is 367.16 (molecular weight 367.88) and the PKa value of prucalopride is 14.64 (conjugate base) and 8.98 (strongest basic) for benzofuran and piperidine heterocyclic structure. Benzofuran is a fragile basic compound depending on its PKa value -2.9 that is strongly conjugate basic and used as psychoactive substances which interacted with 5-HT receptors and activated 5-HT4 receptors. Piperidine is an azacycloalkane that is cyclohexane. A more substantial base replaces one carbon because the lone pair of electrons in piperidine is in an sp3 hybrid orbital and PKa value 11.28, which is used as an analgesic in prucalopride. So drug prucalopride itself a basic [24,25].

Due to lower protein binding, optimum conditions of plasma extraction and method development are required for a different type of pH value of the analyte. Therefore, propranolol is used as an Internal Standard (IS) for quantitation positive polarity to achieve adequate response for their simultaneous analysis. Moreover, the positive ionization mode is selective and highly sensitive for compounds with low electron affinity. Thus positive ionization mode was selected to fragment the analyte and IS to obtain intense and consistent product ions.

The protonated precursor ions $(M+H)^+$ at 368.1 m/z (highest peak), 370.1 (2^{nd} peak), were observed in Q1 MS in which selected parent ion 368.1 for prucalopride and characteristic product ions or fragment ions found in Q3 MS were at m/z 196.0, 278.8, 156.1. However, the most stable and consistent fragment ion selected was m/z 196.0 $(M\text{-}C_{19}H_{20}N_2O+H)^+$ for releasing nonanohydrazide and the product ion chemical name 2-[(4-chlorophenyl) methylideneamino]acetate, which monoisotopic mass 196.0 and displayed in **Figure 2.**

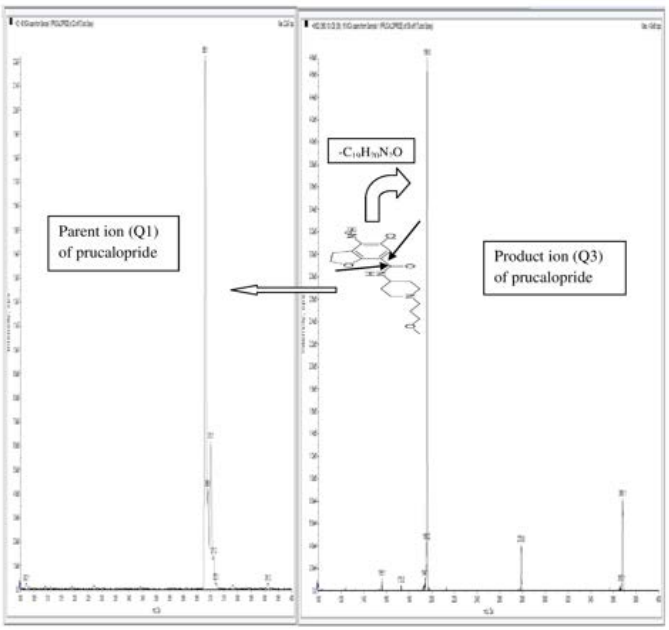

Figure 2: Parent (Q1) and product ion (Q3) scan of Prucalopride.

For the internal standard, the protonated precursor ions $[M+H]^+$ at m/z 260.1 (highest peak) were observed in Q1 MS for propranolol, and characteristic product ions or fragment ions found in Q3 MS were m/z 116.0, 182.9. However, the most stable and consistent fragment ion selected was m/z 116.0[$(M-C_{10}H_8O+H)^+$] forreleasingnaphthalen-2-ol and the product ion chemical name hexanamide monoisotopic mass 116.0. The chromatographic elution of the analytes on a Phenomenex Kinetex 5µ C18 100A 50 × 3 mm column was initiated as a rapid, susceptible, and rugged bioanalytical method covering the dynamic linear range. Mobile phase selection was necessary for the analysis of the drug depending on its pka value. Thus, the pH of the mobile phase, buffer concentration, and choice and proportion of diluents were necessary for chromatographic resolution with adequate response to achieve the desired sensitivity. Optimized instrumental (mass) parameters for prucalopride and IS are illustrated in **Table 3.**

Table 3: Optimized instrumental (mass) parameters for Prucalopride and IS.

Parameter(s)	Value
Ionization mode	MRM (positive)
Source temperature (ºC)	400
Dwell time per transition (msec)	100
Curtain gas (psi)	35
CAD gas (psi)	8
Ion spray voltage (V)	5500
Ion source gas 1 (psi)	55
Ion source gas 2 (psi)	45
Focussing potential (V)	400
Declustering potential (V)	23 (Prucalopride) and 30 (IS)
Entrance potential (V)	11
Collision energy (V)	39 (Prucalopride) and 29 (IS)
Collision cell exit potential (V)	15 (Prucalopride and IS)

Parameter(s)	Value
Transition pair of Prucalopride (analyte)	368.1/196.0
Transition pair of Propranolol (IS)	260.0/116.0

Initially, acetonitrile/methanol with one mM ammonium acetate buffer (pH 6.5) gave a response for prucalopride. However, the response was not reproducible. The signal of the lower limit of quantification concentration was changed when buffer concentration increased from 1 mM to 10 mM. Further, the chromatography was better with a higher response using an acetonitrile buffer than a methanol-buffer combination. At pH above 5.0 that is basic, the resolution of prucalopride was affected, which further deteriorated with an increase in PH. Thus, to achieve more excellent reproducibility and better chromatography, low pH buffers were tried.

The lower drug concentration's reproducibility and peak shape were better in 1.0% formic acid, but the inadequate signal-noise ratio. So as a final solvent was used, 1% formic Acid was in Milli Q water and mixed with 10 mM ammonium acetate as an aqueous solvent having apparent pH 1.20 and 1% formic acid in acetonitrile as an organic solvent at a flow rate of 0.5000 ml/min then a superior signal to noise ratio (≥ 22), and baseline resolution achieved. So the selected mobile phase was 1% formic acid in Milli Q water and mixed with 10 Mm ammonium acetate as an aqueous solvent, and 1% formic acid in acetonitrile as an organic solvent drug was totally basic, so for better ionization and more negligible matrix effect, the acidic solvent was necessary. For analysis of prucalopride, the bioanalytical method was performed by gradient technique in which 90% aqueous solvent was used for 0.01 min to 0.50 min. From 0.50 min to 3.50 min of total run time, 10% aqueous solvent was used, and from 3.50 min to rest of the entire run time (7.00 min) for washing purposes used 90% aqueous solvent was. The gradient curve is illustrated in **Figure 3**. For better intensity and a narrower peak, the acidic aqueous solvent runs for a long time, and in this timing, the analyte and IS eluted. The chromatographic elution time for prucalopride and IS (propranolol) was found at 1.76 and 1.82 min respectively, in a total run time of 7.00 min. The representative MRM chromatograms were showed in **Figures 4-9.**

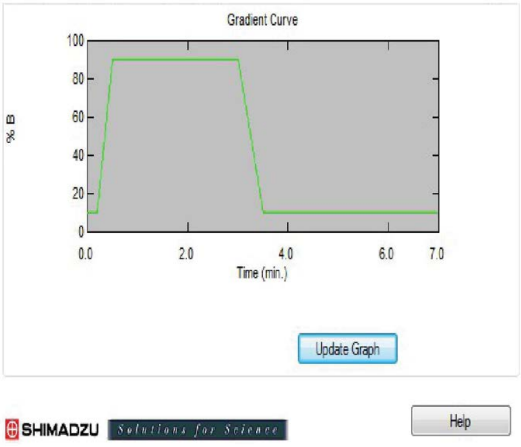

Figure 3: Gradient curve of method development.

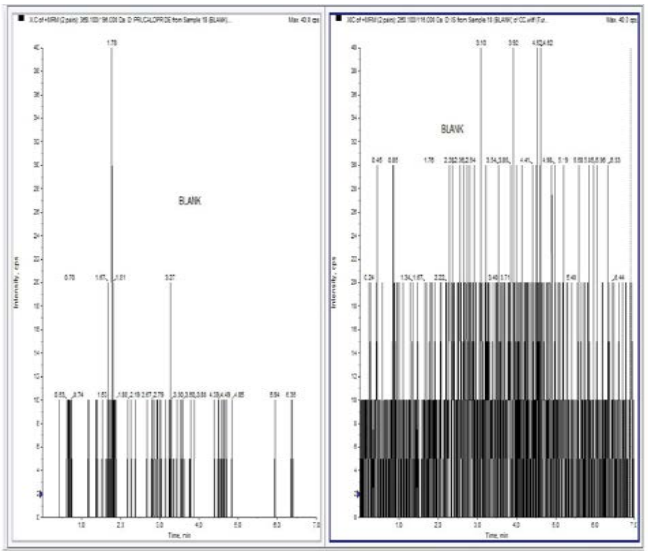

Figure 4: Blank sample chromatogram of prucalopride.

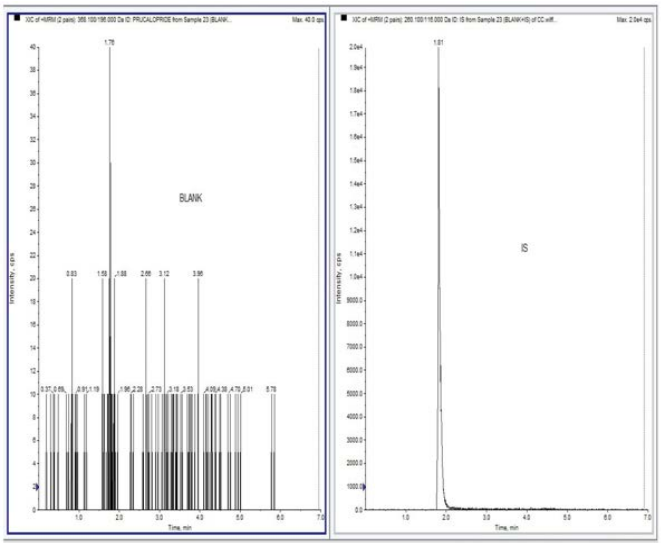

Figure 5: Blank IS sample chromatogram of Prucalopride.

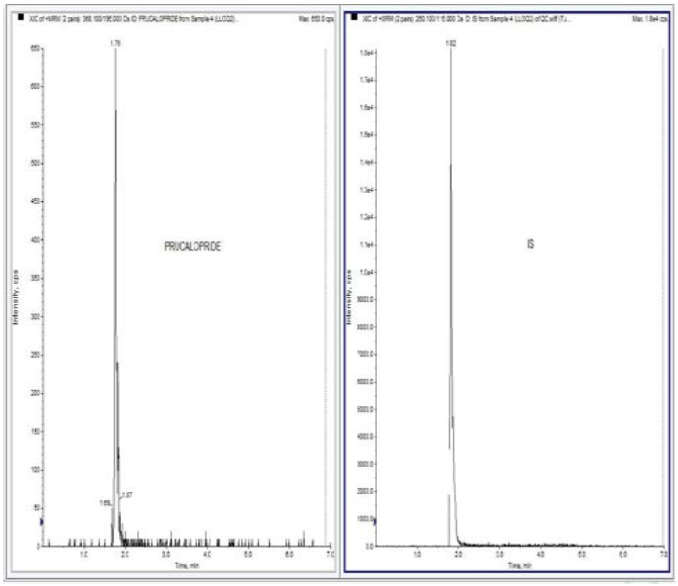

Figure 6: LLOQ sample chromatogram of Prucalopride.

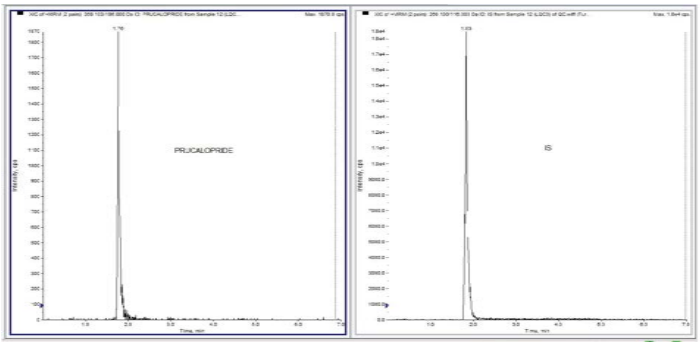

Figure 7: LOC sample chromatogram of Prucalopride.

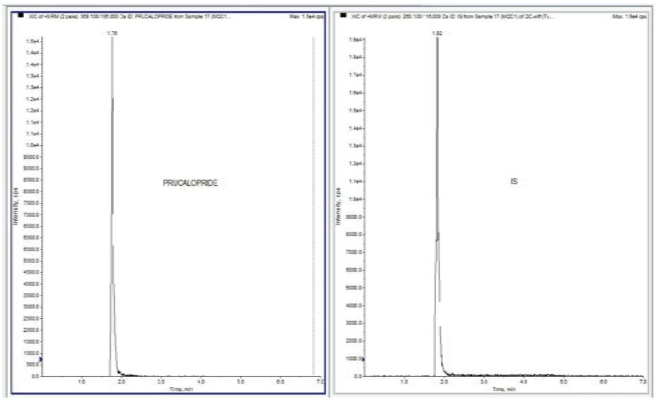

Figure 8: MQC sample chromatogram of Prucalopride.

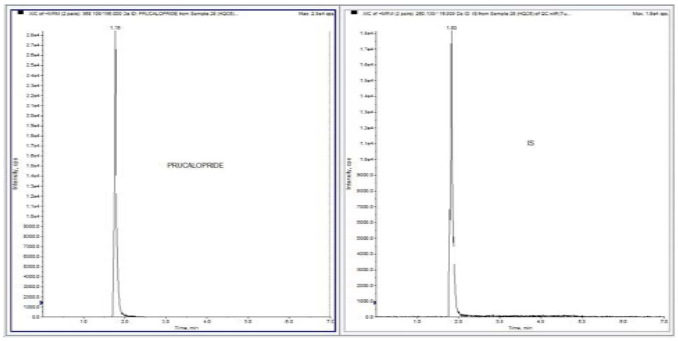

Figure 9: HQC sample chromatogram of prucalopride.

Plasma extraction procedure of prucalopride

The liquid-liquid extraction technique performed plasma extraction. 400 µl volume of plasma sample transferred to a 15 ml plastic Carson tube. Then 100 µl of internal standard propranolol (25 µg) spiked for getting 5 µg/ml of standard internal concentration in the final prepared sample and added 500 µl n-Hexane then vortex 1 min added 1.0 ml of Diethyl Ether and vortex for 1 min then 3 ml TBME added to the sample tubes. After that, the sample was vortex mixed for 5 min and then centrifuged at 5000 r.p.m for 10 min. Then obtained supernatant organic layer 3.5 ml was transferred to a 15 ml plastic Carson tube and evaporated to dryness at 400°C under a stream of nitrogen. Then the dried extract was reconstituted in 200 µl of diluents (mobile phase) acetonitrile: water (50:50) and vortex for 2 min, then taken into autosampler vial, and 10 µl aliquot injected into chromatographic system.

Stock solution and calibration standards preparation

Preparation of prucalopride and propranolol stock solution (W/V): Weighted about 1.0 mg of prucalopride and propanolol separately and dissolved in 1.0 ml DMSO in a separate tube then mixed well by a vortex. So this each of the two stocked solution concentrations was 1mg/ml and stored in the refrigerator at 2˚C-8˚C. This stock solution was used to prepare an intermediate concentrated solution of prucalopride and internal standard, respectively.

Preparation of calibration concentrations in plasma: To prepare calibration concentrations in plasma of prucalopride, use a liquid-liquid extraction procedure to extract prucalopride from human plasma. So from prepared stock solutions of prucalopride and IS (propranolol) diluted with ACN: water 50: 50 (v/v) and prepared intermediate concentrations. The intermediate each of the corresponding concentrated solutions 500 µl of Prucalopride transfer into 500 µl blank human plasma to achieve calibration concentrated points 0.25 ng/ml, 0.50 ng/ml, 1.00 ng/ml, 2.00 ng/ml, 4.00 ng/ml, 8.00 ng/ml, 16.00 ng/ml including LLOQ 0.25 ng/ml, LQC 0.75 ng/ml, MQC 6.0 ng/ml, HQC 12.0 ng/ml.

Method validation: The method validation performed as per guidelines for bioavailability and bioequivalence studies published by Central Drugs Standard Control Organization (CDSCO) and EMA [26].

Results and Discussion

Method validation

Specificity, selectivity, and linearity: The prepared plasma calibration concentrations of prucalopride were 0.25 ng/ml, 0.50 ng/ml, 1.00 ng/ml, 2.00 ng/ml, 4.00 ng/ml, 8.00 ng/ml and 16.00 ng/ml and LLOQ was 0.25 ng/ml, LQC was 0.75 ng/ml, MQC and HQC were 6 ng/ml and 12 ng/ml respectively. The concentrations range of the proposed assay was linear, and it was 0.25 ng/ml to 16 ng/ml in plasma. The calibration curve of linearity was showed in **Figure 10**, which codes no LIN-2. The Limit of Detection (LOD) of lower concentrations of prucalopride in plasma was 0.10 ng/ml. The mean regression value of three days linearity was 0.9985, and the %of CV was 0.16. Pre-study linearity of detector response with statistics represented in **Table 4 and Table 5.**

Table 4: Pre-study linearity of detector response (n=3).

Linearity	Concentration (ng/ml)						
	0.25	0.5	1	2	4	8	16
LIN 1	0.24	0.54	1	1.98	3.92	7.86	16.09
LIN 2	0.25	0.5	0.98	1.98	4.04	8.35	15.48
LIN 3	0.23	0.56	1.02	2.04	3.78	7.98	15.35
Average	0.24	0.533	1	2	3.913	8.063	15.64

Linearity	Concentration (ng/ml)						
	0.25	0.5	1	2	4	8	16
S.D	0.01	0.031	0.02	0.035	0.13	0.255	0.395
% C.V.	4.167	5.728	2	1.732	3.325	3.168	2.526
Nominal %	96	106.67	100	100	97.83	100.79	97.75

Table 5: Pre-study linearity of detector response statistics (n=3).

Linearity	Statistics		
Linearitycode	Slope (m)	Intercept (c)	R square
LIN 1	0.123	-0.00524	0.9991
LIN 2	0.118	0.002	0.9996
LIN 3	0.123	-0.00322	0.9967
MEAN	0.1213	Not Aplicable	0.9985
S.D.	0.0029		0.0016
C.V.%	2.38		0.16

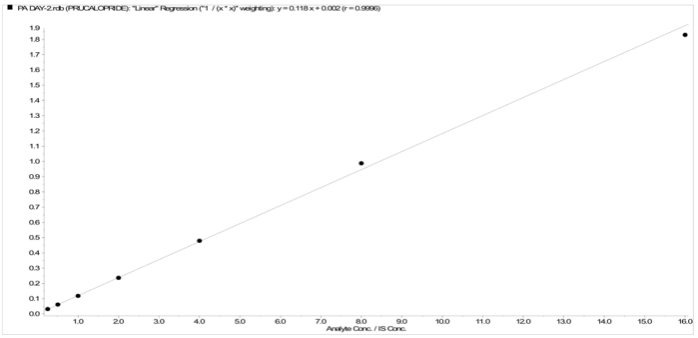

Figure 10: Plasma calibration curve of prucalopride.

Precision and accuracy: In the case of between-run precision and accuracy, the percent of coefficient of variation range was 3.825 to 5.516 for precision, and the percent of absolute bias was 101.33% for LLOQ, 102.58% for LQC, 99.99% for MQC, and 96.14% for HQC for accuracy In the case of within-run precision and accuracy, the percent of coefficient of variation range was 2.645 to 3.683 for precision, and percent of absolute bias range was 94.92 to 100.00 for LLOQ to HQC range accuracy. The obtained result of precision and accuracy illustrated in **Table 6.**

Table 6: Precision and accuracy (n=5).

	Between run			Within run		
Sample	Mean ± SD	C.V.%	Absolute bias (%)	Mean ± SD	C.V.%	Absolute bias (%)
LLOQ (0.25 ng/ml)	0.253 ± 0.014	5.516	101.33	0.250 ± 0.007	2.828	100
LQC (0.75 ng/ml)	0.769 ± 0.032	4.182	102.58	0.750 ± 0.020	2.667	100
MQC (6.00 ng/ml)	5.999 ± 0.229	3.825	99.99	5.956 ± 0.219	3.683	99.27
HQC (12.00 ng/ml)	11.537 ± 0.533	4.616	96.14	11.390 ± 0.301	2.645	94.92

Stability: The result of the stability study illustrated in **Table 7.**

Table 7: Stability Study (Freeze thaw, Short term, Auto sampler, Bench top stability, Long term stability)

		Inj No.	LQC (0.75 ng/ml)	MQC (6.00 ng/ml)	HQC (12.00 ng/ml)
Freshly Thawed		1	0.81	6.06	11.73
		2	0.81	6.37	11.03
		3	0.8	6.13	11.61
		4	0.82	6.09	12.32
		5	0.78	5.85	11.63
		Mean	0.8	6.1	11.66
Freeze Thaw Stability	After 3 cycle in 2-8˚C in freeze	1	0.75	6.6	12.37
		2	0.69	6.19	11.72
		3	0.78	5.49	11.17
		4	0.82	6.58	12.62
		5	0.84	5.38	11.44
		Mean	0.78	6.05	11.86
% Stability			96.52	99.15	101.71
Short term stability	After 24 hours in 2˚C-8˚C in freeze	1	0.74	5.9	10.68
		2	0.73	5.95	13.17
		3	0.78	5.36	11.16
		4	0.75	5.51	12.75
		5	0.76	6.73	11.55
		Mean	0.75	5.89	11.86
% Stability			93.53	96.56	101.7
Auto Sampler Stability	After 24 hours in auto sampler (15˚C)	1	0.79	5.39	10.9
		2	0.73	6.55	12.92
		3	0.73	5.75	11.05
		4	0.83	5.66	11.78
		5	0.72	5.43	10.98
		Mean	0.76	5.76	11.53
% Stability			94.53	94.36	98.82
Bench top stability	After 24 hours in laboratory room temperature	1	0.79	5.62	11.12
		2	0.78	6.16	11.09
		3	0.79	6.5	11
		4	0.79	5.7	11.56
		5	0.7	5.37	11.03
		Mean	0.77	5.87	11.16
% Stability			95.77	96.23	95.68
Long term Stability	After 7 days in 2˚C-8˚C in freeze	1	0.73	6.35	11.76
		2	0.73	6.16	12.23
		3	0.78	5.97	11.35
		4	0.8	5.92	12.89
		5	0.8	6.11	13.14
		Mean	0.77	6.1	12.27
% Stability			95.52	100.03	105.23

- **Short term stability:** The stability samples of LQC, MQC and HQC analyzed after keeping the samples in the refrigerator at 2˚C-8˚C for 24 hours and compared these samples with fresh samples of the same concentration. The result of short term stability of prucalopride obtained from 93.53%-101.70%
- **Freeze-thaw stability:** The stability samples of LQC, MQC and HQC analyzed after keeping the samples in the refrigerator at 2˚C-8˚C for three cycles and compared these samples with fresh samples of the same concentration. The result of freeze-thaw stability of prucalopride obtained from 96.52%-101.71%
- **Auto-sampler stability:** The stability samples of LQC, MQC and HQC analyzed after keeping the samples in an auto sample at 15˚C and compared with fresh samples of the same concentration. The result of autosampler stability of prucalopride obtained from 94.36%-98.82%
- **Benchtop stability:** The stability samples of LQC, MQC and HQC analyzed after keeping the samples on the sample preparation bench at laboratory environmental room temperature and compared these samples with fresh samples of the same concentration. The result of benchtop stability of prucalopride obtained from 95.68%-96.23%
- **Long term stability:** The stability samples of LQC, MQC and HQC analyzed after keeping the samples in the refrigerator at 2˚C-8˚C for seven days and compared these samples with fresh samples of the same concentration. The result of long term stability of prucalopride obtained from 95.52%-105.23%

Matrix effect: The matrix effect of mass analysis of the analyte is the suppression of ionization of the analyte and IS in mass spectrometry. This parameter calculates by the peak area of the analyte and IS of the plasma extracted samples of LQC, MQC and HQC concentrations comparing with the unextracted samples of the same concentrations. The matrix factor of prucalopride was 0.92-0.95, and matrix effect ranges were 92.31%-94.82%, and for IS matrix factor was 0.95 and matrix effect was 94.79%-95.18%. The matrix effect of prucalopride illustrated in **Table 8.**

Table 8: Matrix effect (area) (N=5)

Sample	Matrix effect IS		Matrix effect Prucalopride	
	% of ME	Matrix factor	% of ME	Matrix factor
LQC (0.75 ng/ml)	94.95 ± 3.66	0.95 ± 0.4	92.31 ± 2.32	0.92 ± 0.02
MQC (6.00 ng/ml)	94.79 ± 4.04	0.95 ± 0.4	94.82 ± 2.70	0.95 ± 0.03
HQC (12.00 ng/ml)	95.18 ± 2.62	0.95 ± 0.3	94.74 ± 3.86	0.95 ± 0.04

Recovery: Recovery of mass analysis of the analyte and IS calculated by the peak area of the analyte and IS of the plasma extracted samples of LQC, MQC, and HQC concentrations comparing with the unextracted samples of the same concentrations. The recovery of prucalopride was 100.43%-111.83%, and for IS, it was 95.66%-106.02%. The recovery result of prucalopride is illustrated in **Table 9 and Table 10.**

Table 9: Recovery of IS.

INJ No.	Diluent Sample			In Plasma		
	L 0.75 ng/ml	MQC 6.00 ng/ml	H 12 ng/ml	LQC 0.75 ng/ml	MQC 6.00 ng/ml	HQC 12 ng/ml
1	70132.46	68283.9	73732.73	75982.59	68029.45	67391.87
2	95865.56	67887.55	67707.4	65385.23	67865.24	88859.28
3	63211.14	94384.67	65609.54	81186.64	85221.24	74671.35
4	78147.19	75667.52	112860.53	85800.22	62345.28	88445.57
5	60634.57	79912.52	67686.94	81776.07	85926.6	66348.09
Mean	73598.18	77227.23	77519.43	78026.15	73877.56	77143.23
% Recovery				106.02	95.66	99.51

Table 10: Recovery of Prucalopride.

INJ No.	Diluent Sample			In Plasma		
	LQC 0.75 ng/ml	MQC 6.00 ng/ml	HQC 12.00 ng/ml	LQC 0.75 ng/ml	MQC 6.00 ng/ml	HQC 12.00 ng/ml
1	5951.24	42134.83	92371.6	6120.5	44304.5	105546.93
2	8629.33	44551.84	85602.85	6193.08	42910.1	117985.14
3	6072.98	69771.72	85820.57	7908.51	56142.68	108367.84
4	6507.96	47394.4	148659.48	7975.65	44709.54	112446.6
5	4877.86	49325.84	100658.96	7630.86	66211.11	84466.55
Mean	6407.87	50635.73	102622.69	7165.72	50855.59	105762.61
% Recovery				111.83	100.43	103.06

Result of comparative pharmacokinetic study of prucalopride in human volunteers

From the analysis of unknown concentrations of prucalopride 2 mg in human plasma by LCMS/MS, itobserved that after administration of reference film-coated tablet of prucalopride 2 mg as a single dose in the fasting state reached maximum plasma concentration 4.98 ng/ml ± 0.95 ng/ml (Cmax) at the time of 2.71 hours ± 0.33 hours. (Tmax) and on the other hand, after administration of test preparation of film-coated prucalopride 2 mg in same volunteers, it observed that the maximum plasma concentration of prucalopride 2 mg reached 5.01 ng/ml ± 1.05 ng/ml (Cmax) at the time of 2.64 hours ± 0.28 hours (Tmax). The area under the curve of plasma concentration time of reference preparation was 30.34 ng.h/ml ± 8.07 ng.h/ml (AUC_{0-t}) and 31.96 ng.h/ml ± 8.09 ng.h/ml ($AUC_{0-\infty}$), but in test preparation, it was 30.44 ng.h/ml ± 9.78 ng.h/ml (AUC_{0-t}) and 31.82 ng.h/ml ± 10.10 ng.h/ml ($AUC_{0-\infty}$) shown in **Figure 11**. The elimination constant (Kel) value in reference preparation was 0.057 hour ± 0.014 hour. 1 hour and 0.058 hour ± 0.011 hour. (1 hour for test preparation). The elimination half life (T1/T2) of reference preparation was 12.84 hour ± 2.78 hour and 12.37 hour ± 2.28 hour for test preparation. From the above comparative pharmacokinetic study of prucalopride 2 mg, the relative bioavailability of the prucalopride test sample was 100.35% compared to the reference tablet prucalopride. The comparative pharmacokinetic parameters illustrated in **Table 11.**

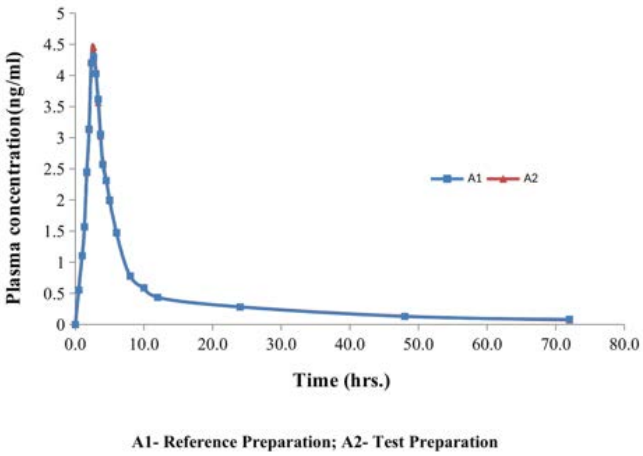

Figure 11: Mean pharmacokinetics plasma concentration time profile of prucalopride.

Table 11: Pharmacokinetic parameters of prucalopride (n=24).

Pharmacokinetic parameter		Prucalopride	
		Reference preparation (A1)	Test preparation (A2)
Cmax (ng./ml.)	Mean	4.98	5.01
	S.D.	0.95	1.05
Tmax (hour.)	Mean	2.71	2.64
	S.D.	0.33	0.28
AUC 0-t (ng. hour./ml.)	Mean	30.34	30.44
	S.D.	8.07	9.78
AUC 0-inf (ng. hour./ml.)	Mean	31.96	31.82
	S.D.	8.09	10.1
kel ($hr.^{-1}$)	Mean	0.057	0.058
	S.D.	0.014	0.011
T1/2 (hr.)	Mean	12.84	12.37
	S.D.	2.78	2.28
Relative Bioavailability (%)		100%	100.35%

Result of statistical analysis

Statistical ANOVA test using SAS 9.1.3 Version-Grizzles Model by PROC GLM was applied to calculate Ln Cmax, AUC_{0-t} and Ln AUC_{0-t} values.

The results of Cmax, AUC_{0-t}, $AUC_{0-\infty}$, Tmax, Kel, T1/2 of ANOVA for untransformed and logtransformed data of Cmax show that the parameters like Period and Treatment are not statistically significant at 5% level in both untransformed and log-transformed data. Still, subject and sequence are statistically significant at 5% in both untransformed and log-transformed data i.e. $p<0.05$.

The 90% confidence interval for the ratio (Test/Reference) of geometric means, based on the log-transformed data for Cmax, was found to be 97.12%-103.98% relative to Test Preparation with Reference Preparation, for AUC0-t found to be 97.95%-101.04% relative to Test Preparation with Reference Preparation, for AUC_{0-inf} found to be 97.53%-101.02% close to Test Preparation with Reference Preparation.

The 95% confidence interval for the ratio (Test/Reference) of geometric means, based on the log-transformed data for Cmax, was found to be 97.63%-101.36% relative to Test Preparation with Reference Preparation, for AUC_{0-t} found to be 97.63%-101.36% relative to test preparation with reference preparation, for AUC_{0-inf} found to be 97.16%-101.38% close to test preparation with reference preparation.

Application of paired t-test that the Cmax (untransformed data) for test preparation and reference preparation is not statistically significant at 5% level and p-value=0.874. Both the trials are similar effectson the body. The summarised statistical analysis report shown in **Table 12.**

Table 12: Representation of class level information.

		Class Level Information
Class	**Levels**	**Values**
Sequence	2	12
Treatment	2	12
Subject	24	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24
Period	2	12
Number of Observations Read		48
Number of Observations Used		48

Dependent variable: Cmax in Tables 13-16.

Table 13: Annova for response of the "model."

Source	DF	Sum of Squares	Mean Square	F Value	pr>F
Model	25	39.13519167	1.56540767	4.73	0.0002
Error	22	7.27373333	0.33062424	-	-
Corrected Total	47	46.408925	-	-	-

Table 14: Representation of R2 , Coefficient variable, Root MSE, and Cmax Mean.

R-Square	Coeff Var	Root MSE	Cmax Mean
0.843269	11.51438	0.574999	4.99375

Table 15: Fit statistics response for the "model" of type I.

Source	DF	Type I (SS)	Mean Square	F Value	pr>F
Period	1	1.29363333	1.29363333	3.91	0.0606
Sequence	1	6.885675	6.885675	20.83	0.0002
Subject (sequence)	22	30.94625	1.40664773	4.25	0.0006
Treatment	1	0.00963333	0.00963333	0.03	0.866

Table 16: Fit statistics response for the model of type III.

Source	DF	Type III (SS)	Mean Square	F Value	pr>F
Period	1	1.29363333	1.29363333	3.91	0.0606
Sequence	1	6.885675	6.885675	20.83	0.0002
Subject (sequence)	22	30.94625	1.40664773	4.25	0.0006
Treatment	1	0.00963333	0.00963333	0.03	0.866

Dependent variable: AUC0-t in Tables 17-20.

Table 17: Annova for response of the "model."

Source	DF	Sum of Squares	Mean Square	F Value	pr>F
Model	25	3549.0339	141.96136	21.23	<.0001
Error	22	147.08151	6.685523	-	-
Corrected Total	47	3696.1155	-	-	-

Table 18: Representation of R2, Coefficient variable, Root MSE, and AUC0-t Mean.

R-Square	Coeff Var	Root MSE	AUCt Mean
0.960206	8.50828	2.585638	30.38966

Table 19: Fit statistics response for the "model" of type I.

Source	DF	Type I (SS)	Mean Square	F Value	pr>F
Period	1	18.372297	18.372297	2.75	0.1116
Sequence	1	937.680901	937.680901	140.26	<.0001
Subject (sequence)	22	2592.848306	117.856741	17.63	<.0001
Treatment	1	0.132437	0.132437	0.02	0.8894

Table 20: Fit statistics response for the model of type III.

Source	DF	Type III (SS)	Mean Square	F Value	pr>F
Period	1	18.372297	18.372297	2.75	0.1116
Sequence	1	937.680901	937.680901	140.26	<.0001
Subject (sequence)	22	2592.848306	117.856741	17.63	<.0001
Treatment	1	0.132437	0.132437	0.02	0.8894

Dependent variable : AUC0-∞ in Tables 21-24.

Table 21: Annova for response of the "model."

Source	DF	Sum of Squares	Mean Square	F Value	pr>F
Model	25	3629.820281	145.192811	14.12	<.0001
Error	22	226.288472	10.28584	-	-
Corrected Total	47	3856.108753	-	-	-

Table 22: Representation of R2 , Coefficient variable, Root MSE, and AUCinf Mean.

R-Square	Coeff Var	Root MSE	AUCinf Mean
0.941317	10.05244	3.207154	31.90425

Table 23: Fit statistics response for the "model" of type I.

Source	DF	Type I (SS)	Mean Square	F Value	pr>F
period	1	21.736016	21.736016	2.11	0.1602
sequence	1	904.770337	904.770337	87.96	<.0001
subject (sequence)	22	2703.092221	122.867828	11.95	<.0001
treatment	1	0.221707	0.221707	0.02	0.8846

Table 24: Fit statistics response for the "model" of type I.

Source	DF	Type III (SS)	Mean Square	F Value	pr>F
Period	1	21.736016	21.736016	2.11	0.1602
Sequence	1	904.770337	904.770337	87.96	<.0001
Subject (sequence)	22	2703.092221	122.867828	11.95	<.0001
Treatment	1	0.221707	0.221707	0.02	0.8846

Dependent variable: Ln Cmax in Tables 25-28.

Table 25: Annova for response of the "model."

Source	DF	Sum of Squares	Mean Square	F Value	pr>F
Model	25	1.65321574	0.06612863	5.32	<.0001
Error	22	0.27326483	0.01242113	-	-
Corrected Total	47	1.92648057	-	-	-

Table 26: Representation of R2, Coefficient variable, Root MSE, and LnCmax Mean.

R-Square	Coeff Var	Root MSE	LnCmax Mean
0.858153	7.016677	0.11145	1.58836

Table 27: Fit statistics response for the "model" of type I

Source	DF	Type I (SS)	Mean Square	F Value	pr>F
Period	1	0.04650698	0.04650698	3.74	0.066
Sequence	1	0.31598188	0.31598188	25.44	<.0001
Subject (sequence)	22	1.29064962	0.05866589	4.72	0.0003
Treatment	1	0.00007727	0.00007727	0.01	0.9378

Table 28: Fit statistics response for the "model" of type I.

Source	DF	Type III (SS)	Mean square	F Value	pr>F
Period	1	0.04650698	0.04650698	3.74	0.066
Sequence	1	0.31598188	0.31598188	25.44	<.0001
Subject (sequence)	22	1.29064962	0.05866589	4.72	0.0003
Treatment	1	0.00007727	0.00007727	0.01	0.9378

Dependent variable: Ln AUC0-t in Tables 29-32.

Table 29: Annova for response of the "model."

Source	DF	Sum of squares	Mean square	F Value	pr>F
Model	25	5.0952268	0.20380907	21.12	<.0001
Error	22	0.2123498	0.00965226	-	-
Corrected Total	47	5.3075766	-	-	-

Table 30: Representation of R2, Coefficient variable, Root MSE, and LnAUCt Mean.

R-Square	Coeff Var	Root MSE	LnAUCt mean
0.959991	2.920634	0.098246	3.363856

Table 31: Fit statistics response for the "model" of type I.

Source	DF	Type I (SS)	Mean square	F Value	pr>F
Period	1	0.0158377	0.0158377	1.64	0.2136
Sequence	1	1.2210363	1.2210363	126.5	<.0001
Subject (sequence)	22	3.85589462	0.17526794	18.16	<.0001
Treatment	1	0.00245817	0.00245817	0.25	0.6188

Table 32: Fit statistics response for the "model" of type I.

Source	DF	Type III (SS)	Mean square	F Value	pr>F
Period	1	0.0158377	0.0158377	1.64	0.2136
Sequence	1	1.2210363	1.2210363	126.5	<.0001
Subject (sequence)	22	3.85589462	0.17526794	18.16	<.0001
Treatment	1	0.00245817	0.00245817	0.25	0.6188

Dependent variable : Ln AUC0-∞ Tables 33-36.

Table 33: Annova for response of the "model."

Source	DF	Sum of squares	Mean square	F Value	pr>F
Model	25	4.68997647	0.18759906	14.5	<.0001
Error	22	0.28463085	0.01293777	-	-
Corrected Total	47	4.97460732	-	-	-

Table 34: Representation of R2, Coefficient variable, Root MSE, and LnAUCinf Mean.

R-Square	Coeff Var	Root MSE	LnAUCinf Mean
0.942783	3.330096	0.113744	3.415646

Table 35: Representation of R2, Coefficient variable, Root MSE, and LnAUCinf Mean.

Source	DF	Type I SS	Mean square	F Value	pr>F
Period	1	0.014672	0.014672	1.13	0.2985
Sequence	1	1.035116	1.035116	80.01	<.0001

Source	DF	Type I SS	Mean square	F Value	pr>F
Subject (sequence)	22	3.633799	0.165173	12.77	<.0001
Treatment	1	0.006389	0.006389	0.49	0.4896

Table 36: Fit statistics response for the model of type III.

Source	DF	Type III (SS)	Mean square	F Value	pr>F
Period	1	0.01467201	0.01467201	1.13	0.2985
Sequence	1	1.03511628	1.03511628	80.01	<.0001
Subject (sequence)	22	3.63379871	0.16517267	12.77	<.0001
Treatment	1	0.00638947	0.00638947	0.49	0.4896

Dependent variable : Tmax Tables 37-40.

Table 37: Annova for response of the "model."

Source	DF	Sum of squares	Mean square	F Value	pr>F
Model	25	4.14468333	0.16578733	16.9	<.0001
Error	22	0.21578333	0.00980833	-	-
Corrected Total	47	4.36046667	-	-	-

Table 38: Representation of R2 , Coefficient variable, Root MSE, and Tmax Mean.

R-Square	Coeff Var	Root MSE	Tmax Mean
0.950514	3.704627	0.099037	2.673333

Table 39: Fit statistics response for the "model" of type I.

Source	DF	Type I (SS)	Mean square	F Value	pr>F
Period	1	0.00240833	0.00240833	0.25	0.6251
Sequence	1	0.68163333	0.68163333	69.5	<.0001
Subject (sequence)	22	3.40323333	0.15469242	15.77	<.0001
Treatment	1	0.05740833	0.05740833	5.85	0.0243

Table 40: Fit statistics response for the model of type III.

Source	DF	Type III (SS)	Mean square	F Value	pr>F
Period	1	0.00240833	0.00240833	0.25	0.6251
Sequence	1	0.68163333	0.68163333	69.5	<.0001
Subject (sequence)	22	3.40323333	0.15469242	15.77	<.0001
Treatment	1	0.05740833	0.05740833	5.85	0.0243

Dependent variable : Kel in Tables 41-44.

Table 41: Annova for response of the "model."

Source	DF	Sum of Squares	Mean Square	F Value	pr>F
Model	25	0.005737	0.00022948	2.93	0.0065
Error	22	0.00172067	0.00007821	-	-
Corrected Total	47	0.00745767	-	-	-

Source	DF	Sum of Squares	Mean Square	F Value	pr>F
Model	25	0.005737	0.000229	2.93	0.0065
Error	22	0.001721	7.82E-05	-	-
Corrected Total	47	0.007458	-	-	-

Table 42: Representation of R2 , Coefficient variable, Root MSE, and Kel Mean.

R-Square	Coeff Var	Root MSE	Kele Mean
0.769275	15.53809	0.008844	0.056917

Table 43: Fit statistics response for the "model" of type I.

Source	DF	Type I (SS)	Mean Square	F Value	pr>F
Period	1	0.00028033	0.00028033	3.58	0.0716
Sequence	1	0.00028033	0.00028033	3.58	0.0716
Subject (sequence)	22	0.00516433	0.00023474	3	0.0064
Treatment	1	0.000012	0.000012	0.15	0.699

Table 44: Fit statistics response for the model of type III.

Source	DF	Type III (SS)	Mean Square	F Value	pr>F
Period	1	0.00028033	0.00028033	3.58	0.0716
Sequence	1	0.00028033	0.00028033	3.58	0.0716
Subject (sequence)	22	0.00516433	0.00023474	3	0.0064
Treatment	1	0.000012	0.000012	0.15	0.699

Dependent variable : T1/2 in Tables 45-51.

Table 45: Annova for response of the "model".

Source	DF	Sum of Squares	Mean Square	F Value	pr>F
Model	25	253.0316482	10.1212659	3.85	0.0011
Error	22	57.7888073	2.626764	-	-
Corrected Total	47	310.8204555	-	-	-

Table 46: Representation of R2 , Coefficient variable, Root MSE, and Thalf Mean.

R-Square	Coeff Var	Root MSE	Thalf Mean
0.814077	12.74987	1.620729	12.71173

Table 47: Fit statistics response for the "model" of type I.

Source	DF	Type I (SS)	Mean Square	F Value	pr>F
Period	1	2.4430675	2.4430675	0.93	0.3453
Sequence	1	29.816345	29.816345	11.35	0.0028
Subject (sequence)	22	218.148158	9.9158254	3.77	0.0015
Treatment	1	2.6240777	2.6240777	1	0.3284

Table 48 : Fit statistics response for the model of type III.

Source	DF	Type III (SS)	Mean Square	F Value	pr>F
Period	1	2.4430675	2.4430675	0.93	0.3453
Sequence	1	29.816345	29.816345	11.35	0.0028
Subject (sequence)	22	218.148158	9.9158254	3.77	0.0015
Treatment	1	2.6240777	2.6240777	1	0.3284

Table 49: Statistical report.

Level of period	N	Cmax		AUCt		AUCinf		LnCmax	
		Mean	Std Dev	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev
1	24	5.157917	1.059914	31.00834	9.286691	32.57718	9.621599	1.619488	0.211704
2	24	4.829583	0.915487	29.77099	8.582497	31.23133	8.610267	1.557233	0.192145

Table 50: Version-Grizzles model by PROC GLM.

Level of period	N	LnAUCt		LnAUCinf		Tmax		Kele	
		Mean	Std Dev	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev
1	24	3.382021	0.348156	3.433129	0.343011	2.66625	0.31193	0.05933	0.016048
2	24	3.345692	0.329944	3.398163	0.313038	2.68042	0.30361	0.0545	0.007384

Table 51: The GLM procedure.

Level of period	N	T half	
		Mean	Std Dev
1	24	12.486125	3.2262967
2	24	12.937333	1.731682

Overall Conclusion

According to the criteria, it was sensitive, selective, had minimal ion suppression, was highly recoverable, and was repeatable. Aside from that, this technique successfully utilised to quantify prucalopride in unidentified volunteer plasma samples and an *in-vivo* pharmacokinetic and bioequivalence investigation. There were just a few articles on bioanalytical technique development, based on the literature review. LC-MS/MS API-4000 was utilised in this work to establish bioanalytical methods to quantify prucalopride in human plasma.

Conflict of Interest

None

Acknowledgement

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Ethical Considerations

The protocol of prucalopride study and related documents like volunteers informed consent form, case record form, subject information sheet was submitted to the HURIP Independent Bioethics committee, Kolkata, India (Central Drugs Standard Control Organization (CDSCO) registration: ECR/103/Indt/WB/2013/RR-19 which is valid up to 21-Nov, 2024) and the ethical clearance obtained before initiation of the study.

Safety Assessment

Clinical examinations were done at the time of screening, check-in and checked out from the clinical facility in each period. All vital signs like blood pressure, pulse ratemeasured, and wellbeing enquiry performed before check-in, before dosing, at 3 hours, 6 hours, 9 hours and 12 hours post-dose (± 60 minute) and check Fout of each period whenever felt necessary by the investigators or medical doctors Body temperature was monitored during the pre-dose and at the time of check out. In addition to the above well being, the assessment performed during each ambulatory sample collection. Clinical laboratory tests performed during screening and at the end of the study. The subjects did not report any adverse events and toxic effects at the time of the survey of prucalopride.

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