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Development and validation of a bioanalytical RP-HPLC method for quantification of ifenprodil in rat plasma

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ABSTRACT

This research aimed to develop a simple, reliable, efficient, and precise Reverse phase high-performance liquid chromatography technique for measuring or determining the quantity of Ifenprodil concentration in rat plasma. Dextromethorphan served as the internal standard (IS). The separation was achieved using the isocratic method with a Luna Phenyl hexyl column (dimensions: 150 mm × 4.6 mm, particle size: 3.5 µm) on a Waters 2,695 equipment with Empower software version 2.0. The analyte was extracted utilizing a liquid–liquid extraction technique, employing acetonitrile (ACN) as an additive. For the measurement of the drug in rat plasma, a mobile phase was utilized, consisting of a mixture of ammonium formate (pH 2.5) with formic acid (FA) and ACN, in a volumetric ratio of 60:40%. The separation procedure was performed utilizing a flow rate of 1 ml/minute and a detection wavelength of 220 nm. The retention times of Ifenprodil and IS were 2.362, and 5.603 minutes, respectively. The linearity of the separation is observed within the concentration range of 200–4,000 ng/ml, exhibiting a robust correlation coefficient of 0.9998. The validation and stability study was conducted by following the guidelines outlined by the US Food and Drug Administration. The findings obtained from the study imply that the proposed approach can be effectively employed for the pharmacokinetics and bioequivalence profiling of Ifenprodil in rat plasma.

INTRODUCTION

Bioanalysis is a widely utilized technique for quantifying the levels of pharmaceutical substances and their metabolic byproducts in diverse biological samples, i.e., serum, plasma, cerebrospinal fluid, saliva, and urine [1–3]. Bioanalytical methodologies are employed to determine the suitability of implementing a quantitative analytical technique in the context of a biochemical process [4]. Validation refers to the systematic process of documenting laboratory experiments to ascertain the suitability and reliability of the method being employed for its intended uses [5–6]. This approach is utilized to assess bio availability and conduct bio equivalence studies, quantitatively analyze pharmaceuticals and their metabolites, facilitate drug development, explore clinical pharmacokinetics, engage in scientific research, and monitor therapeutic drug levels [7]. Bio analytical techniques are considered to be at the forefront of technological advancements as a result of their continuous evolution and continual enhancements [8]. Ifenprodil (Fig. 1) is a potential neuromodulatory drug, which has been recently discovered category of N-methyl-D-aspartame (NMDA) receptor antagonist [9–12]. This medication exhibits a specific inhibitory effect on NMDA receptors that incorporate the NR2B subunit [9,13,14]. Ifenprodil is employed in the treatment of cerebrovascular diseases and peripheral artery obliterative illness[15–16]. Furthermore, the safety and efficacy of Ifenprodil are being still assessed in confirmed COVID-19 patients by Novotech (Australia) sponsored by Algernon Pharmaceuticals with clinical trial ID: NCT04382924. The efficacy of this drug is yet to be established [17].

A limited range of analytical methodologies has been established for the analysis of Ifenprodil. The detection of Ifenprodil in animal plasma has been established in the literature using a liquid chromatographic approach [10]. The approach is distinguished by its extreme sensitivity, simplicity, specificity, rapidity, and cost effectiveness. The main objective

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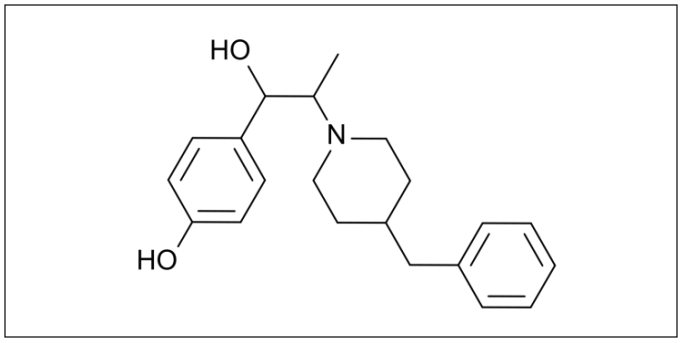

Figure 1. Chemical structure of Ifenprodil.

of the development of a bioanalytical method was to establish its appropriateness for the intended use and evaluate its possible advantages for researchers.

MATERIALS AND METHODS

Reagents and materials

The injectable Ifenprodil tartrate and the reference standard dextromethorphan were purchased from Supriya Life Science, Mumbai. HPLC-grade FA, ammonium formate, and acetonitrile (ACN) were procured from the Merck chemical division located in Mumbai. During the experiment, we employed the water sourced from the Milli-Q water purifying system [18].

Preparation of buffer solution

Accurately weighed 0.77 g of ammonium formate in a 1,000 ml volumetric flask add about 900 ml of Milli-Q water added and degas to sonicate and finally make up the volume with water then pH adjusted to 2.5 with a FA solution.

Preparation of mobile phase

Mixture of ammonium formate (pH 2.5) and CAN in the ratio of 60:40% W/V.

Ifenprodil standard solution

In the developmental phase of the bioanalytical reverse phase high-performance liquid chromatography (RP-HPLC) process, the preparation of the Ifenprodil stock solution involved dissolving the precisely weighed standard drug in ACN. Ifenprodil standard solution had a concentration of 2,000 ng/ml.

Preparation

A working standard of Ifenprodil, measuring 8 mg, was diluted in a 10 ml solution of diluent. Subsequently, a volume of 0.1 ml from the aforementioned solution underwent additional dilution, resulting in a final volume of 10 ml. Then, mix 500 µl with plasma to achieve a final volume of 2 ml. To create working standard solutions for method development, calibration curves, and quality control (QC) samples, suitable dilutions were made using the mobile phase from the stock solution. The resulting solution and standard solutions were transferred to polypropylene vials and stored in a freezer maintained at −20°C [19].

Extraction procedure

By using a micropipette, transfer 200 µl of plasma into an Eppendorf microtube. Following this, mix the contents by adding 300 µl of ACN and vortexing the tubes. In the same tube, introduce 500 µl of Ifenprodil and IS, vortexing again to ensure thorough mixing. Subsequently, add 500 µl of diluent and vortex for 5 minutes. Centrifuge the tubes at 5,000 rpm for 20 minutes, separate the supernatant liquid, and immediately inject 10 µl of the eluate into the HPLC system [20].

METHOD VALIDATION

The validation procedure was confirmed to meet the specifications set by the US FDA.

System suitability

The purpose of this indicator is to find out the operational status of the instrument and provide authorization to proceed with the analysis of the subsequent set of samples. System appropriateness tests are commonly employed in chromatographic procedures to verify that the system possesses enough sensitivity, specificity, and reproducibility for the ongoing analytical run and its application in a specific study. To identify the appropriateness of the system, samples that were representative of the batch were incorporated at the commencement, and conclusion of each batch. The concentration of Ifenprodil in the system suitability samples was adjusted to 2,000 ng/ml. The concentration of 2,000 ng/ml was present in the mobile phase. The %CV values for both peak area and retention time were computed for Ifenprodil and the IS over six consecutive injections. The objective was to determine if these %CV values were within the range.

Specificity and selectivity

The study entailed the examination of plasma samples acquired from six samples to detect any potential chromatographic interference that may occur at the retention periods of Ifenprodil and IS.

Calibration curve

A calibration curve was constructed using eight data points by introducing accurate volumes of a working solution into a blank plasma sample. The aforementioned procedure yielded ultimate concentrations of Ifenprodil of 200, 500, 1,000, 1,500, 2,000, 2,500, 3,000, and 4,000 ng/ml. The calibration curve was created by plotting the ratio of peak areas for the transition pair of Ifenprodil and the IS against the nominal concentration of the calibration standards. Linear regression analysis was utilized for data interpretation. Standards were considered acceptable if their back-calculated concentrations fell within a deviation range of ±15% (SD) from the nominal value. However, a slightly wider range of ±20% was set for the Lower limit of quantification (LLOQ).

Precision and accuracy

The assessment of intraday precision encompassed the examination of six duplicate samples with varying concentrations,

encompassing the LLOQ, Low-quality control (LQC), Medium quality control (MQC), and high-quality control (HQC) samples. The assessment of the method's reproducibility involved the validation of its day-to-day variance, with a specific focus on inter-day precision. The analysis involved the examination of six sets of samples with varying quantities, encompassing the LLOQ, LQC, MQC, and HQC samples. The analysis was conducted on three distinct occasions.

The precision of intra and inter-day assays was assessed by computing the coefficient of variation (%CV), a measure obtained by dividing the SD by the mean and presenting the result as a percentage.

To assess both intra-assay and inter-assay precision, six replicates were analyzed at four levels of samples, namely LQC, MQC, HQC, and LLOQ. Intra-assay accuracy was evaluated by analyzing on the same day, while inter-assay accuracy was assessed over three distinct days. The accuracy of measurements was assessed by computing the percentage ratio between the determined concentration and the known concentration.

Recovery

The determination of analyte recovery was conducted by comparing the analytical outcomes of extracted samples at three distinct concentrations LQC, MQC, and HQC in six repetitions with the outcomes of extracted standards (without any processing), which serve as a reference for 100% recovery. An experimental study was undertaken to assess the recovery of IS at a specific dose of 2,000 ng/ml.

Dilution integrity

The process of evaluating dilution integrity entails the procedure of introducing a concentration of analyte within the upper limit of quantification control through the addition of analyte to the matrix. It was subsequently followed by diluting the resulting mixture using a blank matrix.

Ruggedness

The recovery percentages and %CV for Ifenprodil were determined by two separate analysts using two different columns (Sonoma C18, Luna silica). The results obtained for the LQC, MQC, and HQC samples were found to fall within acceptable ranges. The results showed that the approach utilized demonstrated a significant level of resilience.

Matrix effect

The matrix effect refers to changes in the analyte response caused by interference from unidentified components in the sample matrix. This effect is assessed at LQC and HQC concentration levels. The peak area response is examined in the presence and absence of matrix ions, and the % matrix factor is determined. When the IS is introduced with the analyte, a normalized factor is calculated. The acceptable variability in the matrix factor should be below 15%.

Stability

The assessment of the stability research was carried out as an essential element of the process of validating the approach. A stability test was done to assess the possible disintegration of Ifenprodil under different conditions.

Freeze-thaw stability

A temperature of −20°C was maintained for the storage of six replicates each for the LQC, MQC, and HQC samples. The samples were subsequently thawed completely at ambient temperature and promptly refrozen back to a temperature of −20°C. The aforementioned procedure was repeated twice, and thereafter, the samples were directly injected into RP-HPLC.

Bench-top stability

The primary aim of the bench-top stability experiment was to evaluate the stability of Ifenprodil in rat plasma during 8 hours of exposure on the laboratory bench. The assessment was carried out using three distinct concentrations, specifically the LQC, MQC, and HQC Each concentration was reproduced six times.

Auto sampler stability

Determination of Ifenprodil concentrations in plasma was performed through the analysis of samples representing LQC, MQC, and HQC. The specimens were administered at regular intervals of 1 hour for 24 hours, as a component of stability investigation. The samples were compared to the freshly generated samples at the initial time point (0 hours) in six repetitions to assess various methods. The evaluation of sample stability was based on assessing the assay data against predefined criteria for accuracy, specifically within a standard deviation range of ±15%, as well as precision.

Short-term stability

The temporal or spectral stability of a time or frequency signal within a short measurement period is usually not exceeding 100 seconds.

Long-term stability

The examination of stability within a certain setting. This study entails the execution of studies aimed at evaluating the many physical, chemical, biological, bio pharmaceutical, and microbiological characteristics of a pharmaceutical compound. The investigations are conducted both during and after the projected shelf-life and storage duration of the samples. The storage conditions employed in these studies are indicative of the conditions anticipated in the target market [21].

RESULTS AND DISCUSSION

Optimized method

Several studies were undertaken to examine the effects of different ACN compositions in the mobile phase on the resolution of Ifenprodil. The most favorable peak shape was seen while employing a mobile phase used as a mixture of ammonium formate pH−2.5/ FA and ACN (60:40%). This specific composition led to minor deviations in the retention time of Ifenprodil while keeping its peak response unaltered. The optimized conditions and chromatograms for blank,

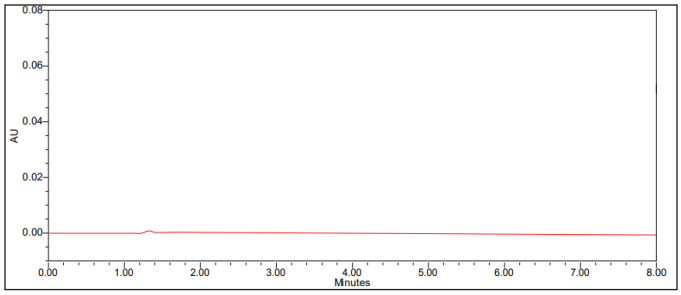

Figure 2. Blank (rat plasma) chromatogram.

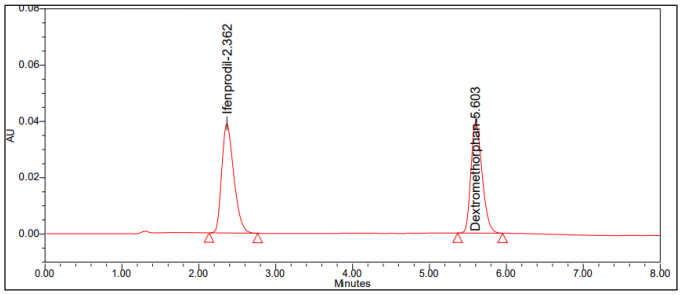

Figure 3. Chromatogram of Ifenprodil and dextromethorphan IS.

Table 1. Summary of optimized chromatographic conditions.

Parameters	Description
Equipment	The separation module of the Waters HPLC Alliance 2,695, is fitted with a PDA detector and operates on Empower 2 software.
Column	Luna phenyl hexyl column with dimensions of 150 mm × 4.6 mm and a particle size of 5 µm.
Mobile Phase	Ammonium formate pH–2.5/ FA: ACN in the ratio of 60:40% *v/v*
Flow rate	1.0 ml/minute
Run time	8 minutes
Retention time	2.362 minutes
Injection volume	10µl

ifenprodil, and dextromethorphan (IS) are given in Table 1 and Figures 2 and 3 respectively.

Specificity

The assessment of specificity entailed the usage of six separate rat plasma samples. Figures 2 and 3 depict the chromatograms obtained from plasma samples collected from rats. No co-eluting peaks originating from naturally occurring substances were seen at the specific retention time associated with Ifenprodil. The analyte Ifenprodil was eluted with a retention time of 2.362 minutes. However, the execution duration of the sample was optimized to 8 minutes.

Sensitivity

The percentage coefficient of variation values for the area ratio of Ifenprodil and the IS were found to be 0.10% and 99.43%, respectively. Hence, it effectively exhibited a capacity for sensitivity.

Table 2. Linearity data of Ifenprodil in rat plasma.

Name	Ifenprodil Conc. (ng/ml)	Ifenprodil Response	IS Response	Area res ratio
Linearity-1	200	4,223	43,556	0.097
Linearity-2	500	10,737	43,614	0.246
Linearity-3	1,000	21,504	43,289	0.497
Linearity-4	1,500	30,867	43,602	0.708
Linearity-5	2,000	42,187	43,261	0.975
Linearity-6	2500	52,931	43,745	1.210
Linearity-7	3,000	63,722	43,680	1.459
Linearity-8	4,000	84,349	43,611	1.934
Slope				0.0005
Intercept				0.00047
r^2				0.9998

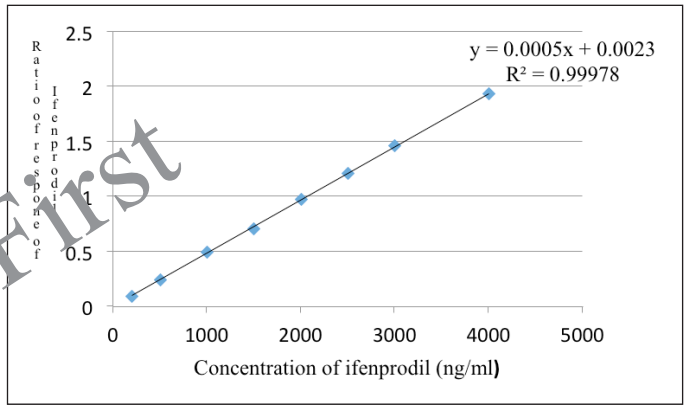

Figure 4. Linearity curve of Ifenprodil in rat plasma.

Table 3. LOD and LOQ data for Ifenprodil.

Name	LOD		LOQ	
	Concentration (ng/ml)	S/N	Concentration (ng/ml)	S/N
Ifenprodil	60	3	200	10

Linearity

The standard curves exhibited linearity in the concentration range of 200–4,000 ng/ml for Ifenprodil, with an average correlation coefficient (r^2) of 0.9998. Quantification of samples involved determining the ratio of the analyte peak area to the peak area of the IS. The plotted data comprised ratios of peak areas to plasma concentrations Table 2 provides the linearity data of ifenprodil and Figure 4 shows the linearity curve of ifenprodil.

LOD and LOQ

The determination of LOD and LOQ was conducted by independent investigations utilizing the calibration curve approach. The bioanalytical RP-HPLC method established the determination of the LOD and LOQ for the compound

Table 4. Accuracy and precision data of the Ifenprodil.

	Intra day precision				Inter day precision			
QC samples	**LLOQ**	**LQC**	**MQC**	**HQC**	**LLOQ**	**LQC**	**MQC**	**HQC**
Mean	4197	21201	42267	63685	4220	21214	42257	63677
SD	0.34	0.71	0.93	0.98	0.52	0.26	0.61	0.89
% CV	0.08	0.003	0.002	0.002	0.012	0.001	0.001	0.001
% Mean accuracy	99.62	99.95	99.63	99.99	99.41	99.92	99.53	99.89

Table 5. Recovery data of Ifenprodil.

Replicate number	**HQC**		**MQC**		**LQC**	
	Extracted response	**Un Extracted response**	**Extracted response**	**Un extracted response**	**Extracted response**	**Un extracted response**
1	63,404	63,654	42,165	42,354	21,112	21,001
2	63,612	63,711	42,287	42,331	21,056	21,389
3	63,524	63,648	42,132	42,588	21,243	21,152
4	63,398	63,600	42,408	42,276	21,139	21,210
5	63,465	63,598	42,266	42,493	20,987	21,070
6	63,418	63,632	42,153	42,265	21,018	21,286
N	6	6	6	6	6	6
Mean	63,470	63,641	42,235	42,[illegible]	21,093	21,185
SD	84.082	41.779	105.6[illegible]6	128.864	92.958	141.853
%CV	0.13	0.07	0.25	0.30	0.44	0.67
%Mean recovery	99.67%	99.94%	99.49%	99.84%	99.37%	99.81%
Overall % mean recovery			99.69%			
Overall SD			99.20			
Overall %CV			0.31			

by incrementally injecting decreasing amounts of standard solutions. For Ifenprodil, the LOD is 60 ng/ml, with a corresponding signal-to-noise (S/N) value of 3. The LOQ, representing the reliable quantification threshold, is 200 ng/ml, and the observed S/N ratio at this level is 10 Table 3 provides LOD and LOQ data for ifenprodil.

Precision and accuracy

The evaluation of precision and accuracy within a single assay was carried out by analyzing six replicates, each consisting of Ifenprodil at six different QC levels. The concept of accuracy is commonly understood as the condition in which data is confined inside a certain range of 85%–115% of the true values. In contrast, precision was assessed through the calculation of the %CV, with an acceptable range of ±15%, except for the minimum quantifiable level. In the context of LLQC, it is anticipated that the level of accuracy will fall between the range of 80%–120%, while the RSD is required to be below 20%. The data for precision and accuracy were provided in Table 4.

Recovery of analyte

The evaluation of the recovery of both the drug and IS was conducted at three distinct concentration levels, specifically

Table 6. Bench top stability data of Ifenprodil.

Replicate no.	**HQC**	**LQC**	**MQC**
	Nominal concentration (ng/ml)		
	3,000.0	**1,000.0**	**2,000.0**
	Area of analyte		
1	63,652	21,245	42,356
2	63,714	21,032	42,222
3	63,620	21,169	42,181
4	63,542	21,178	42,486
5	63,715	21,032	42,232
6	63,546	21,194	42,304
N	6	6	6
Mean	63,632	21,142	42,297
SD	77.008	88.924	111.854
% CV	0.12	0.42	0.26
% Mean accuracy	99.93%	99.60%	99.63%

Table 7. Freeze thaw stability data of Ifenprodil.

Replicate no.	HQC	LQC	MQC
	Nominal concentration (ng/ml)		
	3,000.0	1,000.0	2,000.0
	Area of analyte		
1	63,714	21,216	42,165
2	63,285	21,135	42,174
3	63,512	21,285	42,256
4	63,247	21,053	42,285
5	63,385	21,235	42,351
6	63,231	21,158	42,337
N	6	6	6
Mean	63,396	21,180	42,261
SD	187.855	82.442	79.071
% CV	0.30	0.39	0.19
% Mean accuracy	99.56%	99.78%	99.55%

Table 8. Short term stability results for Ifenprodil.

Replicate no.	HQC	LQC	MQC
	Nominal concentration (ng/ml)		
	3,000.0	1,000.0	2,000.0
	Area of analyte		
1	61,301	20,964	40,525
2	61,541	20,858	40,384
3	61,329	20,942	40,736
4	61,253	20,89[illegible]	40,521
5	61,345	[illegible]0,057	40,384
6	61,184	20,195	40,426
N	6	6	6
Mean	61,326	20,652	40,579
SD	120.591	411.410	192.555
% CV	0.20	1.99	0.47
% Mean accuracy	96.31%	97.30%	95.59%

at low, medium, and HQC levels. The determination of recovery was conducted by comparing the reaction of duplicate samples to that of the undiluted standard solution. The assessment of extraction efficiency, which involves recovering an analyte from a sample matrix, requires comparing the analytical response obtained from a known quantity of added analyte with the response obtained from the sample matrix. ACN was chosen for the extraction process due to the specific properties of Ifenprodil The recovery of analyte data is given in Table 5.

Stability

Bench top stability

Percentage mean accuracy values for Ifenprodil HQC, LQC, and MQC were found to be 99.63%, 99.60%, and 99.63%. Benchtop stability findings are presented in Table 6.

Table 9. Long-term stability results for Ifenprodil.

Replicate no.	HQC	LQC	MQC
	Nominal concentration (ng/ml)		
	3,000.0	1,000.0	2,000.0
	Area of analyte		
1	61,251	20,404	40,956
2	61,417	20,707	40,784
3	61,136	20,749	40,962
4	61,112	20,223	40,853
5	61,204	20,524	40,762
6	61,084	20,336	40,589
N	6	6	6
Mean	61,201	20,491	40,818
SD	122.474	208.675	139.812
% CV	0.20	1.02	0.34
% Mean accuracy	96.11%	96.54%	96.15%

Freeze-thaw at −20°C

The values for mean accuracy of Ifenprodil were found to be 99.56%, 99.78%, and 99.59% respectively. Freeze-thaw stability findings were presented in Table 7.

Short-term stability

Percentage mean accuracy values for Ifenprodil HQC, LQC, and MQC were found to be 99.63%, 99.60%, and 99.63%. Short-term stability results were found in Table 8.

Long-term stability (28 days)

The % CV and mean accuracy for Ifenprodil were found to be within the acceptable limit, which passed the long-term stability Long-term stability results were found in Table 8.

DISCUSSION

The developed RP-HPLC method was optimized for estimating Ifenprodil in rat plasma, employing dextromethorphan was used as an IS. Analytes were separated using ammonium formate pH 2.5/ FA and ACN (60:40% v/v) at 1 ml/minute flow rate on a Luna phenyl hexyl Column (150 mm × 4.6 nm: particle size 3.5 µm). The method was also validated following United states food and drug administration (USFDA) guidelines. The retention time of drug and IS was discovered at 2.362 and 5.603 minutes, respectively. In the rat plasma matrix, liquid–liquid extraction yielded clean samples with a consistent mean recovery of 99.69%. The method was linear over the ranges of 200–4,000 ng/ml, with r^2 of 0.9998. Therefore, the proposed method could be considered valid for routine drug analysis in biological matrices.

CONCLUSION

The suggested RP-HPLC method for the determination of Ifenprodil in rat plasma is rapid, sensitive, and repeatable, with linear dynamic ranges of 200–4,000 ng/ml, respectively. It has been validated and adheres to the USFDA standards, demonstrating

a high level of accuracy and precision following the guidelines. The selected method was suitable for pharmacokinetics and bioequivalence studies of Ifenprodil samples.

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LIST OF ABBREVIATIONS

HQC, High-quality control; IS, Internal standard; LQC, Low-quality control; LLOQ, Lower limit of quantification; MQC, Medium quality control; RP-HPLC, Reverse phase high-performance liquid chromatography; S/N, Signal-to-noise; USFDA, United states food and drug administration.

AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

FUNDING

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CONFLICT OF INTERESTS

The authors report no financial or any other conflicts of interest in this work.

ETHICAL APPROVAL

The study protocol was approved by the Institutional Animal Ethics Committee of KL College of Pharmacy, Andhra Pradesh, India (Approval No: 1250/PO/RcBi/S/11/CPCSEA, Date: 24/06/2023).

DATA AVAILABILITY

All data generated and analyzed are included in this research article.

USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declares that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

PUBLISHER'S NOTE

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