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LC-ESI-MS/MS techniques for Method Development and Validation for Estimation of Propofol In plasma matrix

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Abstract

A compound can often be measured by several methods and the choice of analytical method involves many considerations. Analysis of drugs and their metabolites in a biological matrix is carried out using different extraction techniques like liquid- liquid extraction, solid phase extraction (SPE) and protein precipitation from these extraction methods samples are spiked with calibration (reference) standards and using quality control (QC) samples. These methods and choice of analytical method describes the process of method development and includes sampling, sample preparation, separation, detection and evaluation of the results. The developed process is then validated. These Bioanalytical validations play a significant role in evaluation and interpretation of bioavailability, bioequivalence, pharmacokinetic, and toxicokinetic studies. In which different parameters like accuracy, precision, selectivity, sensitivity, reproducibility, and stability are performed.

Keywords: - LLE, SPE, Quality control samples, Bioequivalence, Bioavailability, Validation.

1. INTRODUCTION:

To investigate the pharmacokinetic of new drug candidates. To compare pharmacokinetic profiles of different formulations. To monitor drug levels to establish the appropriate dose or frequency of administration. For fast and reliable measurement of the compounds in biological matrices. A bio-analytical method consists of two main components: Sample Preparation. Determination of drug and its metabolites [1].

Sample preparation is a method used to clean up a sample before analysis and to concentrate a sample to improve its detection. Sample preparation in the Bio-analysis: it is the "last frontier" and starting point for the accurate LC-MS/MS analysis. Processing step includes; Method Development, Validation, Assay Performance and Work Flow [2].

1.1 Criteria for Bio-analytical method;

- Simplicity of the developed method
- Short Method Development time and
- Validation failures kept at the minimum
- Develop the most selective sample preparation method.

1.2 How to develop Bioanalytical Method:

Peak Plasma Concentration (C_{max}): In Cmax, maximum concentration of analyte in biological fluid to be determined from literature survey, which is helps in establishment of target sensitivity of the developing method.

Physicochemical Property: Physicochemical Properties of an analyte of interest such as solubility, molecular weight, structure, melting point, dissociation constant (pKa) which is helps in selection of the suitable extraction method.

Determination of Lowe r and Upper Limit of Quantification: The lowest concentration of an analyte in a sample that can be quantitatively determined with an acceptable precision and accuracy is usually 1/20$^{th\ of}$ the Cmax value. After calculating ULOQ and LLOQ value have to prepare standard stock solution from which solution of different concentration are prepared [3].

Selection of drug volume to be spiked: The volume of analyte of interest is depends upon the volume of plasma spiked. Analyte concentration is normally 5% of the spiked plasma volume. For Example; if spiked plasma volume is 500 µL, so the volume of analyte to be added will be 25µL.

Sample preparation: Sample preparation technique is used to the clean up a sample by removing endogenous material as

well as to concentrate a sample before analysis to exclude errors in its detection [4].

1.3 Application of Bio-Analytical Method:

- The determination of drug concentrations in biological fluids yields the data used to understand the time course of drug action, or pharmacokinetics, in animals and man and is an essential component of the drug discovery and development process.
- Bio-analysis, employed for the quantitative determination of drugs and their metabolites in biological fluids, plays a significant role in the evaluation and interpretation of Bioequivalence [5], Pharmacokinetic (PK), and Toxicokinetic studies.
- Selective and sensitive analytical methods for quantitative evaluation of drugs and their metabolites are critical for the successful conduct of pre-clinical and/or bio- pharmaceutics and clinical pharmacology studies.

1.4 Bio-Analytical Method Validation

- Validation may be defined as documented evidence that provides a high degree of assurance that a specific process will consistently produce a product meeting its pre-determined specifications and quality attributes.
- Method validation is the process to confirm that the analytical procedure employed for a specific test is suitable for its intended use.

1.5 Drug profile: Propofol

- Propofol is an intravenous anesthetic with a phenolic structure. It is used for both induction and maintenance of anaesthesia.
- Propofol (Diprivan) [7] is rapidly acting, has a short recovery time, and possesses antiemetic properties.
- Rapid recovery and its antiemetic properties make Propofol anesthesia very popular as an induction agent for anesthesia. Propofol can also be used to supplement inhalational anaesthesia in longer procedures.
- The recent advent of computer assisted IV drug administration has made more practical the maintenance of anaesthesia with anaesthetic with relatively short half - life. (E.g., Propofol & short acting Phenyl piperidine opioids).
- The technique is called TIVA [8] (total intravenous anaesthesia) is done with short acting drug so that rapid recovery occurs even after long infusion.

2. MATERIALS AND METHODS:

Table 1: Major Equipment Used

Equipment	Make	Model
HPLC	Shimadzu	LC10-AD series
Autosampler	Shimadzu	SIL-HTC
ESI	AB Sciex	API 5500
MS-MS	AB Sciex	API 5500

Table 2: Working and Reference Standard Details

Details	Drug	Internal Standard
Name	Propofol	Propofol D18
Standard Type	Working Standard	Working Standard
Manufactured By	Splenddid Lab	Splenddid Lab
Storage Condition	At 2 - 8 ºC In Refrigerator & Protect from Normal Light	At 2 - 8 ºC In Refrigerator & Protect from Normal Light

Table 3: Materials Used

Sr. No.	Material	Purpose	Grade
1.	Propofol	Drug	Working Standard
2.	Propofol D18	Internal Standard	Working Standard
3.	Methanol	Solvent	HPLC
4.	Acetonitrile	Solvent	HPLC
5.	Mili-Q Water	Solvent	In-House
6.	Formic Acid	Extraction Buffer	Emparta
7.	Extraction Cartridges	Method Development	Strata-X 33µm Polymeric Reversed Phase30mg/1mL
8.	Column	Method Development	Kinetex®, 5µm,EVO C18 100 *4. 6 mm
9.	K3EDTA and Sodium Heparinized Human Plasma	Blank Plasma	In-house

2.1 Preparation of Stock Solutions:

Drug Stock Solution for Propofol (10.000 mg/ml)

Weigh accurately Propofol standard equivalent to 10.0 mg of Propofol and add appropriate volume of Methanol to make final concentration of Propofol equivalent to 10.000 mg/ml.

ISTD Stock Solution for Propofol D18 (1.000 mg/ml)

Weigh accurately Propofol D18 standard equivalent to 2.0 mg of Propofol D18 and add appropriate volume of Methanol [9] to make final concentration of Propofol D18 equivalent to 1.000 mg/ml.

2.2 Preparation of spiking solutions for ISTD and drug:

- **Preparation of ISTD Stock Solution, 1.000 mg/mL:**

Weigh accurately Propofol D18 standard equivalent to 2 mg of Propofol D_{18} and add appropriate volume of Methanol to make final concentration of Propofol D_{18} equivalent to mg/mL Correct the final concentration for Propofol D18 accounting for its potency and the actual amount weighed. Provide the batch number and store in refrigerator at 5±3°C [10]. Use this solution within 7 days from the date of preparation.

- **ISTD dilution (2000.000 ng/mL):**

Pipette out 200µL of ISTD stock solution (1.000 mg/mL) and dilute up to 100.0 mL with methanol. Provide the batch number and store in refrigerator at 5 ± 3°C. Use this solution within 2 days from the date of preparation.

- **Preparation of Drug Stock Solution, 10.000 mg/mL:**

Weigh accurately Propofol standard equivalent to 10 mg of Propofol and add appropriate volume of Methanol to make final concentration of Propofol equivalent to 10.000 mg/mL Correct the final concentration for Propofol accounting for its potency and the actual amount weighed. Store in refrigerator at 5±3°C [11]. Use this solution within 7 days from the date of preparation.

- **Preparation of Drug Intermediate Solution: (500000.000ng/mL):**

Take 0.500mL of Drug Stock Solution, 10.000 mg/mL in polypropylene tube. Make up the volume to 10.0mL with Methanol. Store in refrigerator at 5 ± 3°C. Use this solution within 7 days from the date of preparation.

- **Preparation of DI Spiking Solution:**

Prepare the working solution (SS) for DISS [12, 13] in Methanol using Drug Stock solution, 10.000/mL, as described in the table below.

- **Preparation of Spiked CC Standards:**

Prepare the CC standards by spiking the respective CC spiking solutions in screenedK_3EDTA human Plasma [14] as described in the table below:

1. For storage into deep freezer, aliquot either 0.200 mL or appropriate required volume of each sample into separate pre-labeled tubes cap them and store in deep freezer at -20±5°C and at -78±8°C.
2. After aliquoting CC Standards, discard the leftover quantity of samples if any.

SS ID	SS Concentration (ng/mL)	SpikingVolume (mL)	Volume of Plasma(mL)	Final Volume (mL)	Spiked Concentration (ng/mL)	STD ID
Methanol	0.000	0.200	9.800	10.000	0.000	STD BL
SS STD 1	250000.000	0.200	9.800	10.000	5000.000	STD 1
SS STD 2	125000.000	0.200	9.800	10.000	2500.000	STD 2
SS STD 3	50000.000	0.200	9.800	10.000	1000.000	STD 3
SS STD 4	25000.000	0.200	9.800	10.000	500.000	STD 4
SS STD 5	12500.000	0.200	9.800	10.000	250.000	STD 5
SS STD 6	5000.000	0.200	9.800	10.000	100.000	STD 6
SS STD 7	2500.000	0.200	9.800	10.000	50.000	STD 7
SS STD 8	500.000	0.200	9.800	10.000	10.000	STD 8
SS STD 9	250.000	0.200	9.800	10.000	5.000	STD 9

- **Preparation of Spiked QC Samples**:

Prepare the QC samples by spiking the respective QC spiking solutions [15] in screened K3EDTAhuman Plasma as described in the table below:

500000.000	1.600	2.400	4.000	200000.000	SS HQC
200000.000	2.000	2.000	4.000	100000.000	SS MQC-1
100000.000	0.500	4.500	5.000	10000.000	SS MQC-2
10000.000	0.750	9.250	10.000	750.000	SS LQC
750.000	3.000	6.000	9.000	250.000	SS LLOQ QC

SS ID	SS Concentration (ng/mL)	Spikingvolume (mL)	Volume ofPlasma (mL)	Final volume(mL)	Spiked Concentration (ng/mL)	QC ID
SS HQC	200000.000	0.200	9.800	10.000	4000.000	HQC
SS MQC1	100000.000	0.200	9.800	10.000	2000.000	MQC1
SS MQC2	10000.000	0.200	9.800	10.000	200.000	MQC2
SS LQC	750.000	0.200	9.800	10.000	15.000	LQC
SS LLOQQC	250.000	0.200	9.800	10.000	5.000	LLOQQC

Aliquot 0.100mL of each standard into separate pre-labeled tubes for processing. Note:

1. For storage into deep freezer, aliquot either 0.200mL or appropriate required volume of each sample into separate pre-labeled tubes cap them and store in deep freezer at -20±5°C and at -78±8°C.
2. After aliquoting QC Samples, discard the leftover quantity of samples if any.

Preparation of Mobile Phase (Acetonitrile: Mobile Phase Buffer: 70:30v/v,):

Separately measure 700mL of Acetonitrile and 300mL of Mobile Phase Buffer in measuringcylinder. Transfer both the contents into a reagent bottle and mix the contents thoroughly. Store at ambient temperature [16]. Use this solution within 3 days from the date of preparation.

Table 4: Trials for Optimization of Chromatographic Condition

Sr. No.	Trials	Ratio (% V/V)	Column	Result
1.	Ammonium Carbonate : Methanol	20 : 80	Cosmosil C8	Drug retained in void volume (R_t 0.58)
2.	AmmoniumCarbonate :Acetonitrile	10 : 90	CosmosilC18	Peak shape was not proper and less response observedat MQC level
3.	Ammonium Carbonate :Acetonitrile	20 : 80	Gemini C18	Good response but tailing wasobserved
4.	10 mM Ammonium Formate in Water :Acetonitrile	20 : 80	Gemini C18	Poor chromatography and less responsewas observed at MQC level
5.	10 mM AmmoniumAcetate in Water : Methanol	30 : 70	Gemini C18	Good chromatographybut less responsewas observes at MQC level
6.	10 mM AmmoniumAcetate in Water : Methanol	40 : 60	Gemini C18	Good chromatography but less responsewas observes at

2.3 Trials for Optimization of Column

Table 5: Trials for Optimization of Column

Sr. No.	Trials	Ratio	Column	Result
9.	10 mM AmmoniumAcetate in Water : Acetonitrile	30 : 70	Gemini C18	Good response and good hromatographywas observed (R_t 4.3)
10.	10 mM Ammonium Acetate in Water : Acetonitrile	30 : 70	Cosmosil C18	Tailing was observed(R_t 2.8)
11.	10 mM AmmoniumAcetate in Water : Acetonitrile	30 : 70	EVO C18	Good response and good chromatography was observed (R_t 2.0)

2.4 Extracted sample preparation:

Step 1: Aliquot 0.100 ml of sample into pre-labelled tubes.

Step 2: Add 50 µl of ISTD dilution (2000.000ng/ml) to all the samples except STD BL and vortex for about 30 seconds. Add 50 µl Methanol to STD BL sample.

Step 3: Add 500 µl of Formic Acid in water, 2 % v/v to all samples and vortex to mix.

Step 4: Arrange the required number of pre- labelled Strata-X 33µm Polymeric Reversed Phase 30mg/1ml extraction cartridges [17, 18] on EZYPRESS 48-48 Position Positive Pressure Processor. Condition the cartridges with 1.0ml Methanol followed by 1.0 ml Water.

Step 5: Load the prepared samples on conditioned cartridges carefully.

Step 6: Wash the cartridges with 1.0 ml of Water, followed by Methanol in water, 10 % v/v and dry the cartridges for about 5 minutes by applying positive pressure at maximum flow rate or by applying full vacuum.

Step 7: Elute the contents from the cartridges with 0.300 ml Acetonitrile into pre- labelled tubes.

Step 8: Transfer appropriate volume of each sample into separate pre- labelled Autosampler vials, arrange them in the Autosampler and inject by using LC- ESI-MS/MS.

3. RESULT AND DISCUSSION:

3.1 Identification of Drug and ISTD

Identification of drug and internal standard was done by using Mass spectrometer.Parent ion and product ion of drug and ISTD.

Table 6: Q1 and Q3 Mass for Drug and ISTD

Parameter	Drug (Propofol)	ISTD (Propofol D18)
Molecular Weight	178.27	196.38
Q1 Mass	177.0	194.1
Q3 Mass	177.1	194.1

Table 7: Optimized Chromatographic Condition

Sr. No.	Chromatographic Parameter	Condition
1.	Mobile Phase	10 mM Ammonium Acetate in Water : Acetonitrile
2.	Column	Kinetex®, 5µm, EVO C18 100 * 4. 6mm
3.	Flow Rate	1.0 ml/minute
4.	Retention Time	At about 2.0 minutes
5.	Run Time	3.0 minutes
6.	Injection Volume	20 µl
7.	Column Oven Temperature	45 ± 3 °C
8.	Autosampler Rinsing Solution	Acetonitrile : Water (50 : 50)
9.	Autosampler Rinsing Volume	1000 µl
10.	Pressure Range	0 – 6200 psi
11.	Purging Time	1.0 minute
12.	Autosampler Temperature	5 ± 3 °C

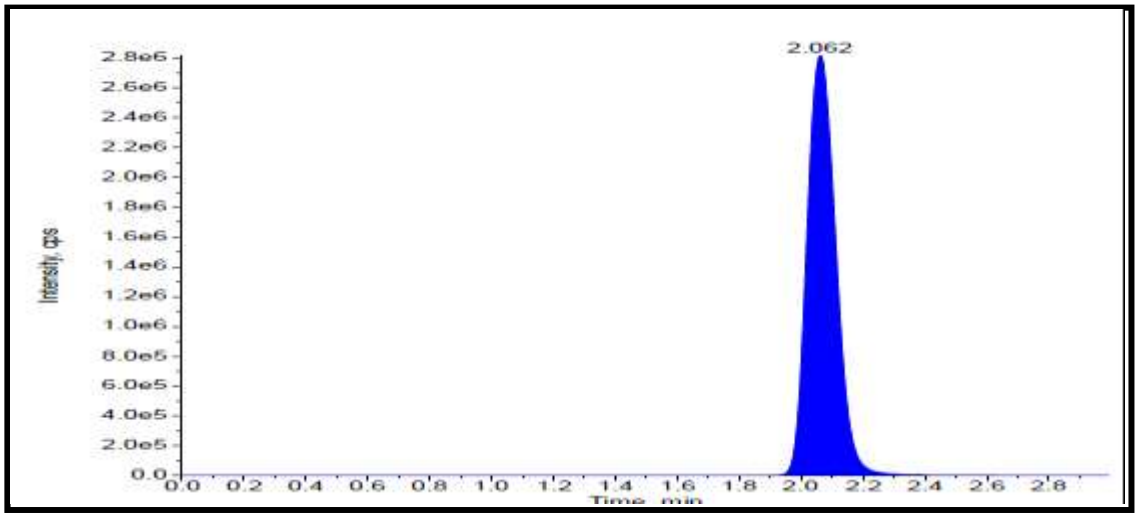

Figure 1: Chromatogram for Optimized Chromatographic Condition

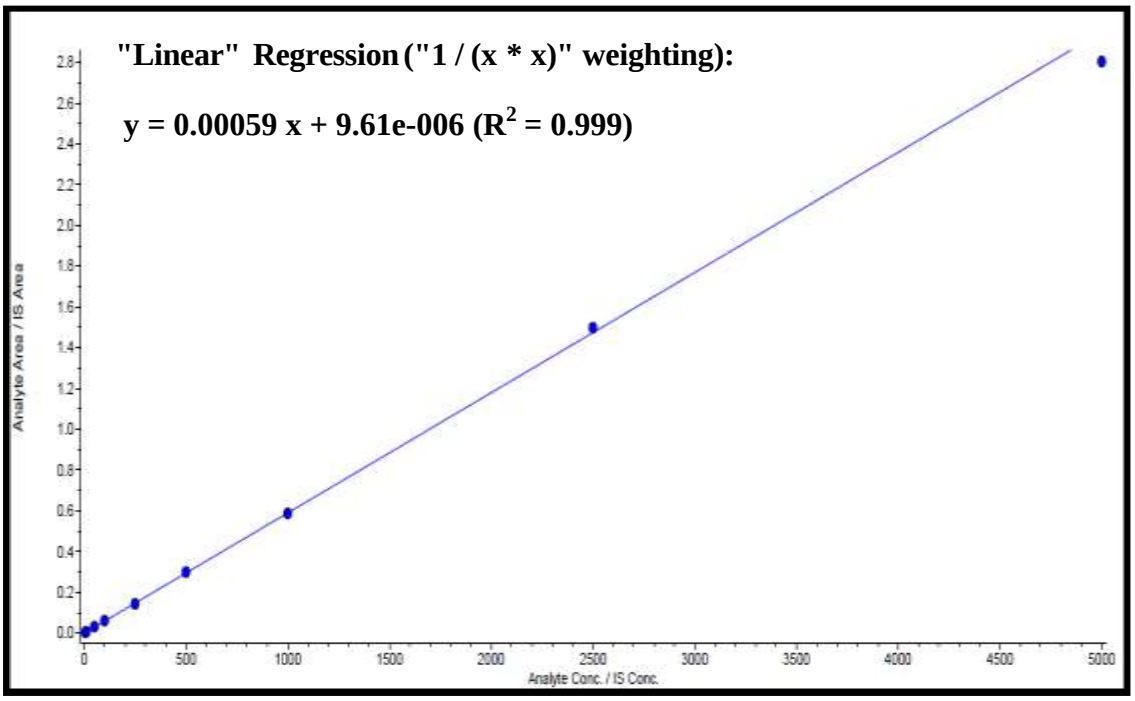

Figure 2: Representative Calibration Curve for Propofol

3.2 Precision and Accuracy:

Table 8: Result Table for Intra - run Precision & Accuracy I

P & A I	LLOQ QC (5 ng/ml)	LQC (15 ng/ml)	MQC 2 (200 ng/ml)	MQC 1 (2000 ng/ml)	HQC (4000 ng/ml)
1.	5.946	13.473	204.792	2084.803	4281.441
2.	4.907	14.123	201.278	2121.824	4227.684
3.	5.748	14.341	203.028	2055.893	4211.849
4.	5.678	14.995	207.765	2166.652	4202.627
5.	6.211	14.504	199.764	2054.608	4140.933
6.	6.206	13.953	192.831	2096.753	4257.130
Mean	5.783	14.232	201.576	2096.756	4220.277
SD	0.483	0.516	5.111	42.668	48.646
% CV	8.36	3.63	2.54	2.03	1.15
% Mean Accuracy	86.47	105.40	99.22	95.39	94.78

Table 8: Result Table for Recovery

Sr. No.	HQC		MQC		LQC	
	ExtractedPeak Area	Un- extracted Peak Area	ExtractedPeak Area	Un- extracted Peak Area	ExtractedPeak Area	Un-extracted Peak Area
1.	18879018	25311751	9094726	12953262	56614	88400
2.	18999513	25461086	9317325	13194222	57251	91886
3.	19158229	25898294	9456934	13329272	58574	92893
4.	19426356	26000707	9582754	13369845	58767	93093
5.	19973295	26065522	9799694	13373909	60608	94143
6.	20543467	26234063	9995869	13871317	62415	94575
Mean	19496646.3	25828570.5	9541217.0	13348637.8	59038.2	92498.3
SD	643599.38	362549.25	326137.88	301603.46	2155.31	2222.53
% CV	3.30	1.40	3.42	2.26	3.65	2.40
% Mean Recovery	75.48		71.48		63.83	
Correction Factor	1.00					
% Mean Recovery With Correction Factor	75.48		71.48		63.83	
% Overall Recovery	78.84					
% Overall Recovery With Correction Factor	70.26					
% Overall CV	8.43					

3.3 Selectivity or Specificity:

Table 9: Result Table for Specificity

Matrix batch / LotNo.	Drug Response			ISTD Response		
	STD BL	LLOQ	% Interference	STD BL	LLOQ	% Interference
1.	2	10144	0.02	62	3067912	0.00
2.	17	10367	0.16	270	3197020	0.01
3.	49	9870	0.50	370	3275672	0.01
4.	85	11325	0.75	305	3325910	0.01
5.	72	9872	0.73	312	3578945	0.01
6.	91	10342	0.88	125	3975892	0.00
Haemolysed	82	7373	1.11	63	2304796	0.00
Lipemic	139	11380	1.22	423	3293992	0.01
Na Heparin	75	14207	0.52	51	3498215	0.00

3.4 Ruggedness:

Table 10: Result Table for Ruggedness (Different Equipment)

P & A	LLOQ QC (5 ng/ml)	LQC (15 ng/ml)	MQC 2 (200 ng/ml)	MQC 1 (2000 ng/ml)	HQC (4000 ng/ml)
Mean	2.247	14.625	196.844	1989.120	3843.883
SD	0.225	0.635	4.250	15.831	65.852
% CV	4.29	4.34	2.16	0.83	1.71
% Mean Accuracy	104.94	97.50	98.42	94.91	96.10

4. CONCLUSION:

Inter - run precision for all high, middle and low quality control samples was found to be within acceptance limit of 15.00 %.The method is considered valid for extraction and analysis of Propofol in K_3EDTA human samples within investigated concentration range of 5.000 - 5000.000 ng/ml using 0.1 ml processing volume.The validation criteria used in present study is instrument stability, sample preparation strategy, calibration, precision and accuracy, specificity and selectivity of drugs. Upon injection of spiked plasma after a valid extraction procedure into LC-MS/MS instrument at LLOQ, specificity and selectivity shows acceptable results which means that instrument at this lowest concentration is capable to give a reproducible result. There was no significant peak observed from endogenous compounds at retention time of analyte and internal standard which means chromatographic conditions were quite perfect for satisfactory separation within chromatographic run time (2 minutes).The overall matrix effect value: % CV obtained from all 10 plasma lots were < 15 % for both analytes including their ISTD and did not show any peak area differences which means matrix effect does not affect this test procedure and sample preparation strategy used.Further, good results were obtained in plas ma calibration curves. The results obtained from their intra and inter-run precision and accuracy was determined using six sample replicates at four different concentration QC levels (LLOQ QC, LQC, MQC and HQC) that reveals acceptability of data included in accuracy within ± 15.0 % deviation from nominal values and precision of < 15% relative standard deviation(RSD) % CV , except for LLOQ, where it should not exceed < 20 % of deviation which shows that method validated to meet acceptance criteria of industrial guidance for bio-analytical method validation.Hence, it can be concluded that simple, rapid and sensitive isocratic reverse phase liquid chromatographic-tandem mass spectrometric method was devised to quantify Propofol in human plasma and can be successfully applied for bioequivalence studies in human subjects.

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