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Validation of Stability Indicating Reverse Phase HPLC Method for the Determination of Related Substances in Methohexital Drug Substance

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N. Sreenivas[1*], K. Raghu Babu [2], S. Paul Douglas [2], Uttam Kumar Ray [1], Vundavilli Jagadeesh Kumar [1*], K. S. R. Pavan Kumar [1] and Hemant Kumar Sharma[1]

[1]Analytical Research Department, Aurobindo Pharma Limited Research Centre-II, Indrakaran (V), Sangareddy (M), Medak Dist-502329, Telangana, India.

[2]Department of Engineering Chemistry, Andhra University, Vishakapatnam-530003, Andhra Pradesh, India.

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ABSTRACT

A gradient reversed phase high performance liquid chromatography (RP-HPLC) method has been developed and validated for the determination of related substances of Methohexital drug substance. The well chromatographic separation of Methohexital from its process related impurities was achieved on ACE C18-PFP, 3µ (150mm × 4.6mm) column *i.e* Octadecyl silane with pentafluoro phenyl groups chemically bonded to porous silica particles of 3µm diameter at temperature of 30°C by using mixture of phosphate buffer pH 4.6 and methanol as mobile phase A and acetonitrile as a mobile phase B. Wavelength for UV detection: 225nm, flow rate: 0.8ml/min and Injection volume: 20µl. The method suitability checked and validated according to the ICH guidelines for specificity, linearity, accuracy, precision, limit of quantification, limit of detection robustness and ruggedness and also Methohexital was subjected to stress conditions of thermal, hydrolysis, humidity, peroxide and photolytic to observe the degradation products. Limit of detection of each impurity is 0.006 indicating that the developed method is highly sensitive. The experimental results are given in detailed in this research article.

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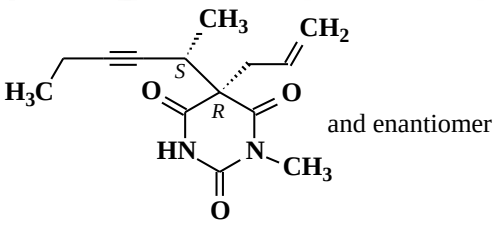
1.0 INTRODUCTION

Methohexital is chemically known as α-(±)-1-Methyl-5-(1-methyl-2-pentyn-1-YL)-5-(2-propen-1-YL) 2,4,6 (1*H*,3*H*,5*H*)-pyrimidinetrione, molecular formula is $C_{14}H_{18}N_2O_3$ and molecular weight is 262.30. Methohexital is a short-acting barbiturate anesthetic [1] and that has actions similar to those of Thiopental [2]. Methohexital remains the most commonly used induction agent and is regarded as the "golden standard" by the American Psychiatry Association [3]. It is favored due to its rapid onset and short duration of action, as well as its low cardiac toxicity [4]. A recent systematic review showed that methohexital was superior to other anesthetics with regard to motor seizure duration [5]. Methohexital has the advantage of being easily titrated. However, due to a lack of availability, other induction agents have begun to become more widely used [6]. Methohexital is given as the sodium salt under trade name Brevital Sodium [7]. Methohexital sodium for injection is a freeze-dried, sterile, nonpyrogenic mixture of methohexital sodium with 6% anhydrous sodium carbonate added as a buffer. It contains not less than 90% and not more than 110% of the labeled amount of methohexital sodium, which is administered by direct intravenous injection or continuous intravenous drip, intramuscular or rectal routes [7]. A typical dose of methohexital for induction of anesthesia is 50 to 120mg given at a rate about 10mg (1ml of a 1% solution) every 5 seconds. For maintenance of general anesthesia, methohexital sodium may be given by intravenous injection in doses of 20 to 40mg every 4 to 7 minutes as required [8]. The chemical structures of Methohexital and its impurities are shown in Figure 1.



Methohexital

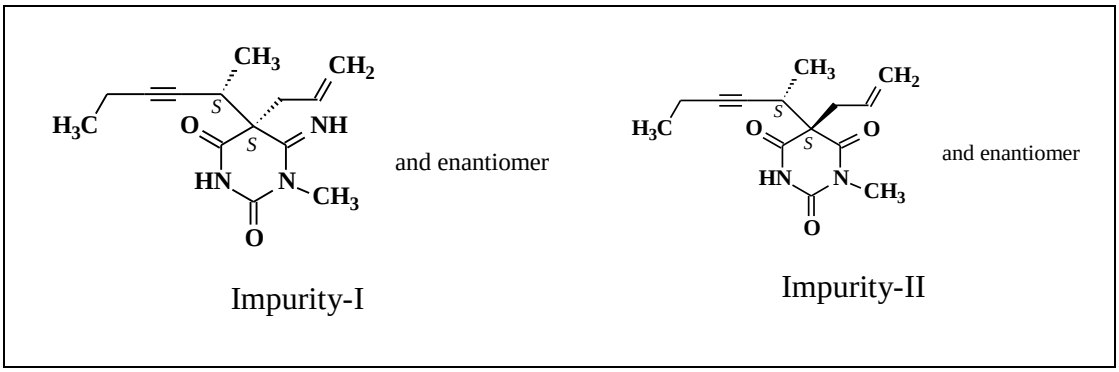


Fig. 1: Chemical structures of Methohexital and its impurities

There is no HPLC method was specified for determination of Methohexital and its related substances in available literature official Pharmacopoeias (i.e. USP, European Pharmacopoeia). There are USP monographs available for Methohexital and Methohexital sodium for injection [9, 10], but in which there is no HPLC procedures are available for determination of related substances. In this research paper, we report the development of a new gradient stability indicating HPLC method for the simultaneous detection and quantitative determination of the impurities I and II in Methohexital drug substance. Forced degradation studies according to ICH stability guidelines [11] were carried out to establish stability indicating nature of the method. System suitability, limit of detection (LOD), limit of quantification (LOQ) and linearity were established as per ICH Guidelines. The limit of each impurity is considered 0.15% level of accordance with ICH guidelines based on maximum daily dose [12]. The developed chromatographic method can resolve with two impurities with acceptable resolution to achieve good chromatography and the optimized methodology have been validated to accomplish ICH guidelines on validations [13].

2.0 MATERIALS AND METHODS

Experimental

2.1 Chemicals, reagents, standards and samples

The investigated samples of Methohexital drug substance, its related impurities and Methohexital for system suitability (Methohexital enriched with Impurity-II) were gifted from APL Research Centre-II Laboratories (A division of Aurobindo Pharma Ltd., Hyderabad). AR grade of Sodium dihydrogen phosphate monohydrate, Acetonitrile, Methanol and Orthophosphoric acid (~88%)

were procured from Merck, India and pure milli-Q water was used with the help of millipore purification system (Millipore®, Milford, MA, USA).

2.2 Instrumentation and methodology

The HPLC system used for method development, method validations as well as forced degradation studies were Waters Alliance 2695 separation module equipped with 2996 photodiode array detector with Empower data handling system i.e Empower 2 software, Build No: 2154 [Waters Corporation, MILFORD, MA 01757, USA] was used.

HPLC column: A stainless steel column 150mm long, 4.6mm internal diameter filled with Octadecyl silane with pentafluoro phenyl groups chemically bonded to porous silica particles of 3µm diameter. [ACE C18, 3µ (150mm × 4.6mm) (Make: ACE)], column oven temperature: 30°C. Mobile phase A: degassed mixture of buffer pH and methanol in the ratio of 720: 280 v/v. (Buffer: dissolve 3.45g of Sodium dihydrogen phosphate monohydrate in 1000ml of water, adjust pH to 4.60±0.05 with orthophosphoric acid and filter this solution through 0.45µ or finer porosity membrane filter. Mobile phase B: Acetonitrile. Diluent: water, acetonitrile and methanol in the ratio of 50:25:25%v/v/v. Flow rate: 0.8ml/min, injection volume: 20µl, data acquisition time: 50min and UV detection: 225nm. Retention time of Methohexital: about 28 minutes. The pump is in gradient mode and the program is as follows: Time (min)/ A (v/v): B (v/v); $T_{0.01}$/90:10, T_{30}/75:25, T_{45}/45:55,T_{50}/45:55,T_{52}/90:10, T_{60}/90:10

2.3 Preparation of solutions

2.3.1 System suitability solution

1 mg/ml concentration of Methohexital for system suitability (Methohexital enriched with Impurity-II) in diluent.

System suitability evaluation:

USP Plate count of Methohexital: Not less than 25000; USP Tailing NMT 1.5 from standard solution.

The USP resolution between Methohexital and Impurity –II peaks is not less than 2.0 from system suitability solution.

2.3.2 Standard solution

0.0015 mg/ml concentration of solution using Methohexital standard in diluent.

Sample solution

1.0 mg/ml concentration of solution using Methohexital sample in diluent.

3.0 RESULTS AND DISCUSSION

3.1 Method Validation

3.1.1 Specificity

Specificity is the ability to assess unequivocally of analytic in the presence of components which may be expected to be present. For determination of specificity, injection of blank, impurities solutions were prepared and injected to confirm the individual retention times. The solutions of Methohexital drug substance (Control Sample) and Methohexital spiked with known related substances at specification level (Spiked Sample) were prepared and injected into HPLC. Peak purity was established by using Empower Software. A typical representative HPLC chromatogram of Methohexital drug substance spiked with all impurities is shown in Fig. 2. The specificity results are tabulated in Table 1.

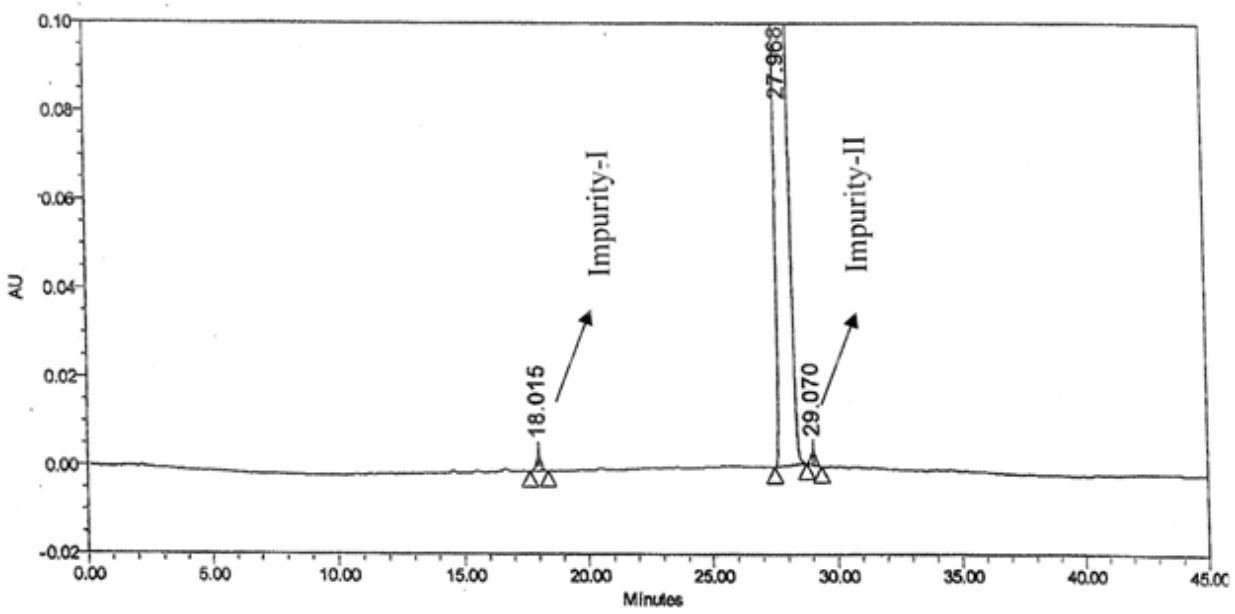


Fig. 2: A typical representative HPLC chromatogram of Methohexital drug substance spiked with impurities

Tab. 1: Specificity experiment from spiked sample

Name	Retention time (min)	RRT	Peak purity	
			Purity angle	Purity threshold
Impurity-I	18.015	0.64	0.373	1.456
Impurity-II	29.070	1.04	0.418	1.789
Methohexital Control sample / diluted	--	--	0.068	0.278
Methohexital Control sample / diluted	--	--	0.063	0.287

3.1.2 Forced degradation

The degradation activities of Methohexital have been studied by performing forced degradation experiments. Methohexital was subjected to different stress conditions [13] i.e. acid/base hydrolysis [5M HCl/85°C/120 min & 5M NaOH/85°C/45 min], peroxide degradation under oxidative stress [30% H_2O_2 / 85°C / 120 min], thermal degradation [70°C/120Hours], humidity degradation study (90% RH/25°C/120 hrs) and photolytic degradation [white Fluorescent light, 1.2 million Lux hours and UV light, 200 watt-hours / m^2] w.r.t ICH option 2 of Q1B [14]. Peak purity of Methohexital peak was established by using PDA detector in these stress samples. The forced degradation results are tabulated in Table 2.

In all of the above degradation conditions, there was no significant change observed w.r.t known impurities. However, in base degradation (5M NaOH/85°C/45 min), unknown impurity at RRT about 0.42 was observed up to 2.54%. In peroxide degradation (30% H_2O_2 / 85°C / 120 min), two unknown impurities at RRTs about 0.59 & 0.88 detected up to 2.19 & 0.39% respectively w.r.t undegraded sample. The above results of various stress conditions employed to degrade Methohexital indicate that drug substance is susceptible to degrade under acidic, basic hydrolysis and oxidative conditions and moderately sensitive to heat whereas, it is found to be stable to photolytic and humidity stress conditions. Experimental data are shown in Table 2.

Tab. 2: Specificity experiment –forced degradation studies

Degradation mechanism	Degradation condition	Degradation (%)	Peak purity of Methohexital	
			Purity angle	Purity threshold
-	Undegraded Sample	-	0.063	0.276
Acid	5M HCl / 85°C / 120 min	1.6	0.066	0.257
Base	5M NaOH / 85°C / 45 min	7.7	0.058	0.258
Peroxide	30% H_2O_2 / 85°C / 120 min	6.1	0.058	0.257
Thermal	70°C / 120 hours	3.8	0.053	0.257
Photolytic	White Fluorescent light, 1.2 million Lux hours and UV light, 200 watt hours / meter square	0.6	0.065	0.256
Humidity	90% RH / 25°C / 120 hours	1.2	0.066	0.256

3.1.3 Limit of Detection (LOD)/ Limit of Quantification (LOQ)

LOD and LOQ were calculated on the basis of response and slope of the regression equation. These are calculated from the formula 3.3 δ/S and 10 δ/S respectively where 'δ is standard deviation of the y-intercept of the regression line and 'S' is slope of the calibration curve which was predicted from linearity experiment. The precision study was carried out at about predicted LOD and LOQ levels by injecting six replicates and calculating the % RSD of the area of each impurity.

3.1.4 Linearity

A series of solutions were prepared using Methohexital and its impurities at concentration levels from LOQ to 150% of specification level and each solution was injected and calculated the

statistical values like slope, intercept, STEYX and correlation coefficient from linearity plot drawn for concentration versus area. The statistical values are presented in Table 3.

Tab. 3: Statistical evaluation of linearity and LOD/LOQ experiments

	Impurity-I	**Impurity-II**
Concentration range (µg/mL)	0.180-2.273	0.185-2.310
Slope	35073	33351
Intercept	24	-453
STEYX	546	428
RF	1.00	1.05
Correlation Coefficient	0.9998	0.9998
LOD		
(% w/w)	0.006	0.006
% RSD	13.4	5.0
LOQ		
(% w/w)	0.180	0.019
% RSD	3.1	6.4

3.1.5 Precision

Precision (system precision) was evaluated by injecting six injections of Methohexital standard solution and calculating the % relative standard deviation. The method precision was checked by injecting six individual preparations of Methohexital spiked with each impurity with 0.15% with respect to sample concentration. % RSD of content of each impurity was calculated. Intermediate precision of the method was also evaluated using different analyst, different instrument, and different lot of column on different day. The interday variations were calculated. The precision experiments results are given in Table 4.

Tab. 4: Precision experiment results

System Precision										
	Inj-1	Inj-2	Inj-3	Inj-4	Inj-5	Inj-6	Mean	SD	% RSD	95% Confidence Interval (±)
Methohexital Peak area	53264	53391	54151	54208	54453	55401	54145	777	1.4	816

Method Precision										
	Inj-1	Inj-2	Inj-3	Inj-4	Inj-5	Inj-6	Mean (% w/w) *n=6*]	SD	% RSD	95% Confidence Interval (±)
Impurity-I	0.147	0.148	0.148	0.148	0.148	0.148	0.148	0.000	0.0	0.0
Impurity-II	0.144	0.145	0.144	0.145	0.145	0.145	0.145	0.001	0.7	0.001

Ruggedness										
	Inj-1	Inj-2	Inj-3	Inj-4	Inj-5	Inj-6	Mean (% w/w) *n=6*]	SD	% RSD	95% Confidence Interval (±)
Impurity-I	0.146	0.147	0.147	0.146	0.147	0.147	0.147	0.001	0.7	0.001
Impurity-II	0.141	0.141	0.142	0.140	0.142	0.141	0.141	0.001	0.7	0.001

3.1.6 Accuracy

The accuracy of the method was determined by analyzing Methohexital (n=3) samples spiked with impurities at different levels (LOQ, 50, 100 and 150% of specification, i.e 0.15%). The percentage recovery values for all the impurities are calculated and tabulated in Table.5.

Tab. 5: Accuracy experiment results

Recovery details (average of 3 replicates)	**Impurity- I**			**Impurity- II**		
	Added (%w/w)	Recovered (%w/w)	Recovery (%)	Added (%w/w)	Recovered (%w/w)	Recovery (%)
LOQ	0.0177	0.0176	99.4	0.0186	0.0157	84.4
50	0.076	0.074	97.4	0.075	0.070	93.3
100	0.151	0.148	98.0	0.150	0.144	96.0
150	0.227	0.222	97.8	0.225	0.221	98.2

3.1.7 Robustness

To determine the robustness of the method, experimental conditions were deliberately changed and to evaluate system suitability requirement as per methodology. For this evaluation, system suitability solution and sample solution spiked with impurities at specification level were prepared as per test method and injected into HPLC. To study the effect of flow rate, 10% variation (±0.1 units) of flow rate was changed. The effect of column temperature was studied by keeping 25°C and 35°C instead of 30°C. The effect of pH was studied by varying ±0.2 units of methodology value. In the same manner, detection wavelength (±3nm) and organic in mobile phase (±2% absolute in Gradient Composition) have been verified and the results obtained from these experiments are summarized in Table 6.

Tab. 6: Robustness experiment results

Condition	Variation	System Suitability			Spiked Sample (RRT)	
		USP Resolution	USP Plate count	USP Tailing	Imp-I	Imp-II
STP	-	2.5	73133	1.0	0.64	1.04
Flow	-10%	2.4	76949	1.0	0.65	1.04
	+10%	2.5	70591	1.0	0.63	1.04
Wavelength	-3 nm	2.6	77418	1.0	0.64	1.04
	+3 nm	2.6	76907	1.0	0.64	1.04
% Organic in gradient variation	-2% absolute	2.6	87494	1.0	0.66	1.04
	+2% absolute	2.5	65981	1.0	0.63	1.04
% Organic in mobile phase A	-2 % absolute	2.6	79997	1.0	0.65	1.04
	+2 % absolute	2.5	64865	1.0	0.63	1.04
pH of Buffer	-0.2 units	2.5	69800	1.0	0.64	1.04
	+0.2 units	2.6	78865	1.0	0.65	1.04
Column Oven Temperature	-5°C	2.6	79602	1.0	0.64	1.04
	+5°C	2.5	71521	1.0	0.65	1.04

3.1.8 Stability of solutions

Standard solution and sample solution spiked with impurities were prepared and analyzed initially and at different time intervals by keeping the solutions at room temperature (~ 25°C). To be removed. Test results show that standard and sample solutions are stable up to 24 hours at 25°C±2°C. The experimental results are shown in Table 7.

Tab. 7: Stability of solutions- Experiment results

At 25°C+2°C - Standard solution						
Time	**Methohexital area**	**% Difference**	**Impurity-I area**	**% Difference**	**Impurity-II area**	**% Difference**
Initial	53264	-	50344	-	46279	-
After 1 hour	53391	0.2	51064	1.4	47890	3.5
After 2 hours	54151	1.7	50359	0.0	46866	1.3
After 3 hours	54208	1.8	49795	1.1	47443	2.5
After 4 hours	54453	2.2	50741	0.8	47280	2.2
After 5 hours	55401	4.0	50506	0.3	48309	4.4
After 6 hours	55495	4.2	51186	1.7	48529	4.9
After 7 hours	55305	3.8	50313	0.1	47720	3.1
After 8 hours	55808	4.8	50497	0.3	47610	2.9
After 9 hours	55979	5.1	50368	0.0	47638	2.9
After 10 hours	57533	8.0	50672	0.7	47679	3.0
After 11 hours	56270	5.6	50883	1.1	47630	2.9
After 12 hours	56412	5.9	49833	1.0	47805	3.3
After 13 hours	57106	7.2	50502	0.3	47766	3.2
After 14 hours	57220	7.4	50820	0.9	47627	2.9
After 15 hours	56806	6.6	50648	0.6	48217	4.2
After 20 hours	56379	5.8	50145	0.4	47400	2.4
After 24 hours	57175	7.3	49842	1.0	45751	1.1

4.0 CONCLUSION

A reverse phase stability indicating HPLC method was developed and validated for the quantitative determination of impurities of Methohexital. The present research work will help the manufacturers and suppliers of Methohexital to quantify and qualify the quality in terms of purity based on experimental results. Thus, it can be used for routine analysis, quality control and for determining the quality during stability studies of pharmaceutical analysis.

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