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Development of RP-HPLC Method for the Determination of N-Methylurea Content in Methohexital Drug Substance and Its Validation

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M. V. Kishore[1], T.Sivarao[2*], K.Rama Srinivas[1], Ch.V.Satyendranath[1], Vundavilli Jagadeesh Kumar [1], K. S. R. Pavan Kumar [1]

[1] Aurobindo Pharma Limited, Survey No. 71&72, Indrakaran Village, Kandi Mandal, Sangareddy District-502329, Telangana, India.

[2] Department of Inorganic and Analytical Chemistry, AU College of Science and Technology, Andhra University, Visakhapatnam- 530003, Andhra Pradesh, India.

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ABSTRACT

A simple, sensitive and rapid RP- HPLC method developed and validated for the determination of residual impurity N-Methylurea at low level in Methohexital drug substance. In this method column conditions are established and optimized on Atlantis dC18, 250 mm × 4.6 mm, 5µm column with oven temperature maintaining at 40°C. 0.02M Potassium Phosphate buffer was chosen as mobile phase A and acetonitrile was selected as mobile phase B in gradient reverse phase mode. Chromatographic parameters such as flow rate of mobile phase was maintained at 0.7 ml/min, 50µl of injection volume, total runtime 35 min, were established and detected the analyte at 205 nm in the present methodology. Based on validation data, the method is found to be specific, sensitive, accurate and precise. The Limit of detection (LOD) and Limit of quantification (LOQ) for this impurity is found to be 0.002 %w/w and 0.005 %w/w respectively. The average recovery obtained was 96.9% at four levels in twelve determinations for N-Methylurea in Methohexital drug substance. This method can be used as good quality control tool for quantization of N-Methylurea at low level. The experimental results are discussed in detail in this research paper.

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. Methohexital has two asymmetric centers and four stereoisomers. The β-1-isomer is 4-5 times more potent than α-1-isomer, but produces excessive motor responses. Therefore, methohexital marketed as the racemic mixture of α-1-isomer [1]. Methohexital is a short-acting barbiturate anesthetic [2] and that has actions similar to those of Thiopental [3]. Methohexital molecular formula is $C_{14}H_{18}N_2O_3$ and molecular weight is 262.30. Methohexital remains the most commonly used induction agent and is regarded as the "golden standard" by the American Psychiatry Association [4]. It is favored due to its rapid onset and short duration of action, as well as its low cardiac toxicity [5]. A recent systematic review showed that methohexital was superior to other anesthetics with regard to motor seizure duration [6]. It has the advantage of being easily titrated. However, due to a lack of availability, other induction agents have begun to become more widely used [7]. Methohexital is given as the sodium salt under trade name Brevital Sodium. 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 [8]. Chemical structure of Methohexital has shown in Figure 1.

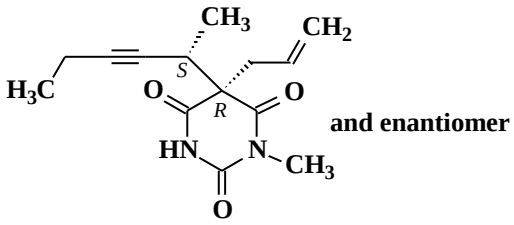


Figure 1: Chemical Structure of Methohexital

In the preparation of Methohexital, methyl 2-cyano-3-methyl-2-(2-propen-1-YL)-4-heptynoate intermediate was taken and condensed with N-methyl urea and followed by cyclization leads to formation of Imine intermediate. Further, this intermediate gets hydrolyzed and finally produced Methohexital. The reaction scheme as shown in Figure 2.

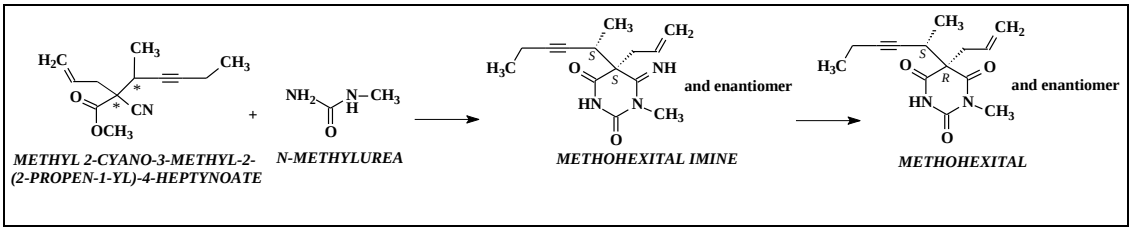


Figure 2: Reaction Scheme

During the synthetic procedure a little part of raw materials and intermediates are carry over into the final products, hence the accumulation of small quantities into the final drug substances causes risk for utilization. In view of usage of N-Methylurea in the preparation of Methohexital and as per regulatory agencies requirement, quantification of N-Methylurea is essential in Methohexital drug substance with a limit of 0.05%w/w which is lower than any unspecified impurity limit NMT 0.10%, [according to ICH Q3A (R2)][9]. To the best of our knowledge, determination of N-Methylurea by HPLC in Methohexital drug substance has not been reported in literature till date. Hence, we aimed to develop a RP-HPLC method for the determination and validation of N-Methylurea in Methohexital as per ICH guidelines [10] by using Atlantis dC18.

MATERIALS AND METHODS

Chemicals, reagents and samples

Methohexital drug substance, its related substances and N-Methylurea were procured from APL Research Centre-II (A division of Aurobindo Pharma Ltd., Hyderabad, India). Potassium dihydrogen orthophosphate (Analytical grade), Acetonitrile (HPLC grade) were procured from Merck, India and highly pure milli-Q water was obtained by using millipore purification system.

Instrumentation and Chromatographic conditions

Chromatographic separations were performed on HPLC (High Performance Liquid Chromatograph) system with Alliance –waters e2695 separation module with 2998 PDA detector using Empower software. Mobile phase A was prepared by dissolving 2.72 g of Potassium dihydrogen orthophosphate in 1000 ml of water. Mobile phase B was Acetonitrile. The analysis was carried out on Atlantis dC18, (250mm × 4.6 mm), 5 µm particle diameter column (Make: Waters), maintained at temperature 40°C. Flow rate was kept as 0.7 ml/min

and pump was in gradient mode was given in the below. The run time for the standard was kept as 12 min with initial gradient ratio and the sample was 35 min. The injection volume was 50 µl and the analyte was monitored at 205 nm. Water was used as diluent. The retention time of N-Methylurea peak is at about 5.5 min.

Gradient Programme		
Time (mins)	Mobile phase A(v/v)	Mobile phase B(v/v)
0.01	99	1
12	99	1
12.1	30	70
25	30	70
25.1	99	1
35	99	1

Preparation of solutions

Standard solution

Accurately weigh and transfer about 40 mg of N-Methylurea reference standard into a 100 ml clean, dry volumetric flask, add 70 ml of diluent and sonicate to dissolve. Makeup to volume with diluent. Dilute 5 ml of this solution to 100 ml with diluent. Further, dilute 5 ml of this solution to 50ml with diluent. Filter through 0.45 µ or finer porosity membrane filter.

Sample solution

Accurately weigh and transfer about 80 mg of sample into a 20 ml clean, dry volumetric flask, add 15 ml of diluent and sonicate for 5 minutes to dissolve (sample may not be soluble completely). Makeup to volume with diluent. Filter through 0.45 µ or finer porosity membrane filter.

System suitability criteria

The column efficiency as determined from the N-Methylurea peak is not less than 3000 USP plate count and USP tailing for the same peak is not more than 2.0 from N-Methylurea standard solution chromatogram.

RESULTS AND DISCUSSION

Method development and Optimization of RP-HPLC

The aim of this study is to develop simple, sensitive and rapid, and robust chromatographic method which can separate N-Methylurea from Methohexital. We tried a lot of isocratic combinations, but not get desired separation. So finally gradient program selected for method development.

Selection of stationary phase

Structural moiety of N-Methylurea indicates that molecule is polar in nature. So tried different column chemistries and finally selected Atlantis dC18, 250 mm × 4.6 mm, 5µm column for the method development and validation, because Atlantis dC18 columns that are used for polar compound retention. The Atlantis dC18 columns feature di-functionally bonded C18 ligands that have been optimized for use with highly aqueous mobile phases, including 100% water. Stationary Phase Information given in the below table.

Stationary Phase Information			
Brand	**Atlantis**	**Particle Size (dp)**	**5µm**
% Carbon Load	12	Pore size	100 Å
Bonding Technology	dC18	Particle Substrate	Silica
Chemistry	C18	Silinol Activity	Medium
Surface area	330	End capped	Yes
System	HPLC	End fitting Type	Waters
USP Classification	L1	Format	Column
ID	4.6 mm	Units for package	1/pkg
Length (mm)	250 mm	PH range	7-Mar
Mode	Reverse Phase	Particle Shape	spherical

Selection of Mobile phase:

Selection of mobile phase is depending on the pKa value of the main drug substance. Methohexital pKa is 8.73 strong acidic side, hence, it is optimal to use acidic buffer. Hence, used potassium phosphate buffer as mobile phase A and Acetonitrile used as mobile phase B due to method developed at 205 nm.

Method validation

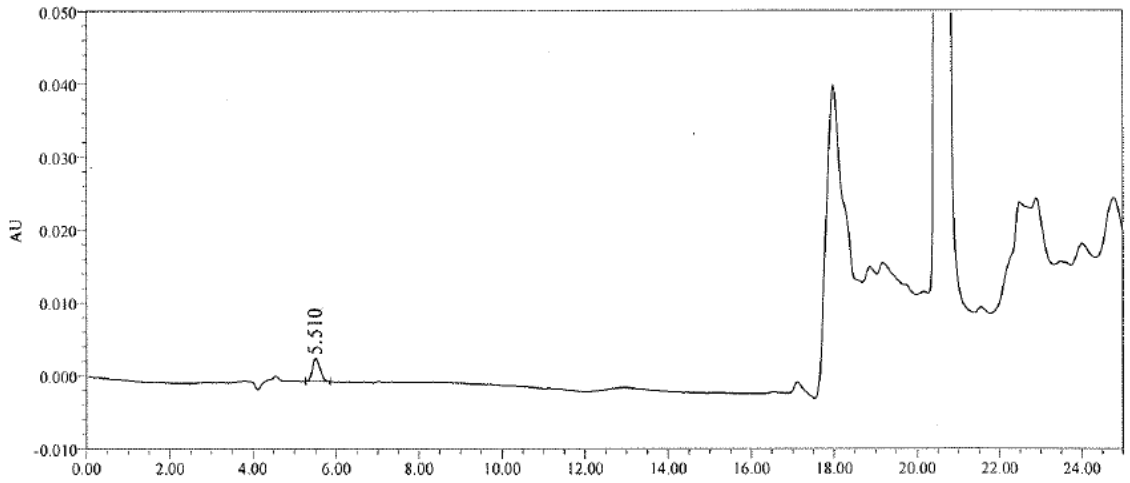
The developed and optimized method was then validated for its specificity, linearity, LOD and LOQ, accuracy, stability of solutions and precision to demonstrate that the method is suitable for its intended use per regular sample analysis to quantify the levels of N-Methylurea in Methohexital drug substance.

Specificity

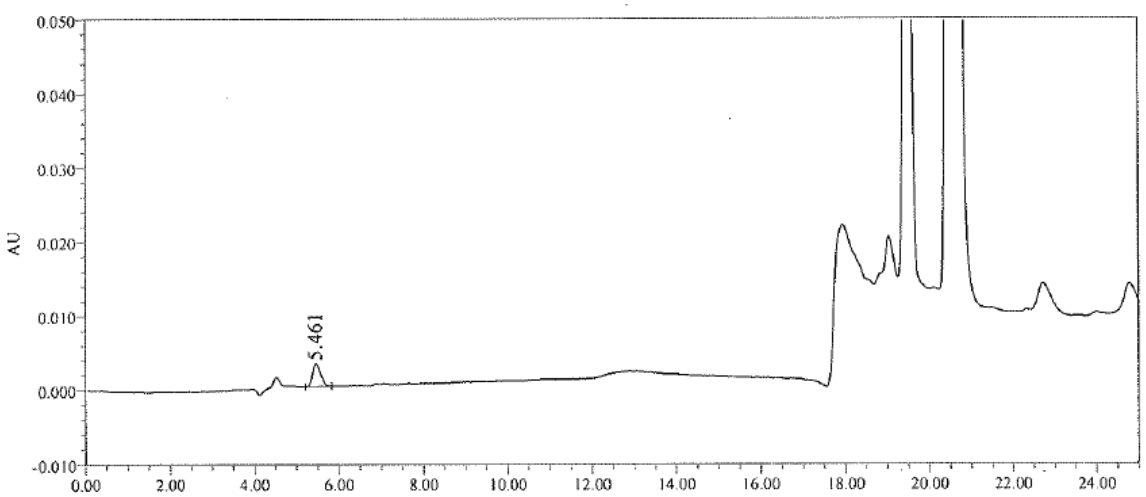
Specificity of the method is its ability to detect and separate all the related substances is present in the Methohexital drug substance. Specificity of the method is demonstrated in terms of spectral as well as peak purity data of the drug and its impurities are present in the drug. Peak passed the peak purity test. Diluent, Methohexital drug substance spiked with N-Methylurea (control sample) and Methohexital drug substance spiked with all known related substances including N-Methylurea (spiked sample) were injected to confirm any co-elution with N-Methylurea peak from any known related substances. Peak purity for N-Methylurea was established by using waters Empower software and found to be passed (Purity angle should be less than purity threshold). Moreover, No peak is observed at the retention time of N-Methylurea peak in the diluent chromatogram and all related substances are well separated from N-Methylurea peak. Hence, this method is specific and selective. The typical HPLC chromatograms of Methohexital spiked with N-Methylurea and Methohexital spiked with all known related substances including N-Methylurea are shown in Figure 3. The specificity experiments data is given in Table 1. Based on this experimental data, the peak purity data of N-Methylurea from control sample and spiked sample indicated that the peaks were homogeneous and have no co-eluting peaks. Hence, it can be concluded that there is no interference due to listed known related substances for the determination of N-Methylurea content in Methohexital drug substance.

Table 1: Specificity experiments data

Sample	N-Methylurea Retention Time (min)	Peak Purity	
		Purity angle	Purity threshold
Control sample	5.510	0.320	0.732
Spiked sample	5.461	0.713	0.935



Control sample (spiked with N-Methylurea)



Spiked sample (spiked with N-Methylurea along with known related substances)

Figure 3: Typical HPLC chromatograms of specificity experiment

LOD and LOQ

The sensitivity was demonstrated by determining the limit of detection (LOD) and limit of quantitation (LOQ). LOD/LOQ values of N-Methylurea were determined from based on signal to noise ratio data. The predicted concentrations of LOD and LOQ for N-Methylurea were verified for precision by preparing the solutions containing N-Methylurea at about predicted concentrations. Each of these solutions six times injected into the HPLC.

Linearity

Linearity of the method was checked by preparing solutions at nine concentration levels from LOQ to 150% of specification level (0.05%) by prepared using N-Methylurea standard solution and each solution was injected into HPLC. Linearity was established by using concentration (µg/ml) on X-axis, area on Y-axis and calculated statistical values like slope, intercept, residual sum of squares and correlation coefficient. The linearity, LOD and LOQ experiments data is shown in Table 2.

Table 2: LOD/LOQ and Linearity experiments data

LOD & LOQ		
LOD	0.002 %w/w	14.3 (% RSD)
LOQ	0.005 %w/w	1.0 (% RSD)
Linearity		
Slope	24455	
Intercept	-165	
STEYX	346	
Correlation coefficent	0.9999	

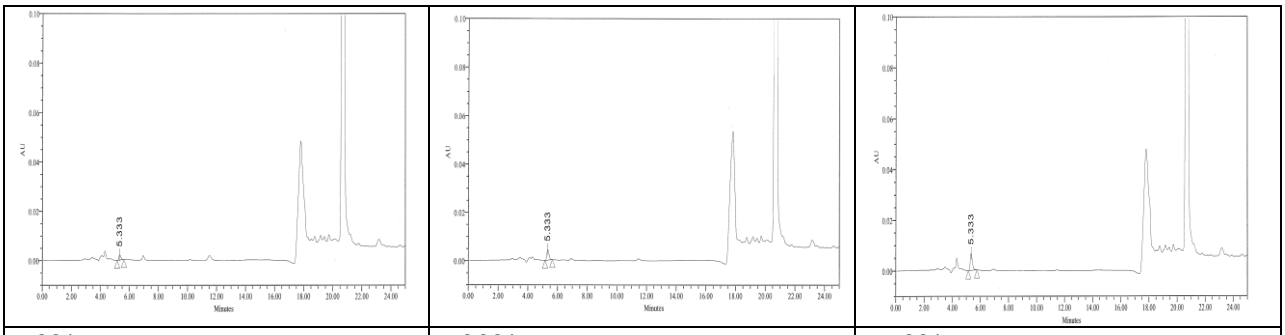
Accuracy

Accuracy of the method was performed by recovery experiments using standard addition technique. Sample solutions were prepared in triplicate by spiking N-Methylurea at levels of LOQ, 50%, 100% and 150% of specification limit as per test method and injected each solution into HPLC as per methodology and the percentage recoveries were calculated. The fully validated recovery results are shown in Table 3 and HPLC chromatograms are shown in Figure 4.

Table 3: Accuracy data

LOQ level							
% Level / Sample ID		**Amount Added (µg/g)**		**Amount Found (µg/g)**		**% Recovery**	
LOQ Level Sample - 1		48		40		83.3	
LOQ Level Sample - 2		48		41		85.4	
LOQ Level Sample - 3		48		41		85.4	
Statistical Analysis							
Mean	84.7	SD	1.21	% RSD	1.4	95% Confidence Interval (±)	3.0

(50% to 150% level)					
Concentration / Sample ID	**Amount Added (µg/g)**	**Amount Found (µg/g)**	**% Recovery**	**Statistical Analysis**	
50% Level Sample 1	249	226	90.8	Mean	90.9
50% Level Sample 2	249	225	90.4	SD	0.61
50% Level Sample 3	249	228	91.6	% RSD	0.7
100% Level Sample 1	498	522	104.8	Mean	103.5
100% Level Sample 2	498	515	103.4	SD	1.21
100% Level Sample 3	498	510	102.4	% RSD	1.2
150% Level Sample 1	743	812	109.3	Mean	108.4
150% Level Sample 2	743	799	107.5	SD	0.90
150% Level Sample 3	743	805	108.3	% RSD	0.8
Overall Statistical Analysis					
Mean	100.9	SD	7.84	% RSD	7.8	95% Confidence Interval (±)	6.0



50% 100% 150%

Figure 4: Typical HPLC chromatograms of Accuracy experiment

Precision

System precision was demonstrated by preparing the standard solution of N-Methylurea as per methodology and analyzed by injecting six replicates. Method precision experiments demonstrated by preparing six sample solutions individually using a single batch of Methohexital drug substance spiked with N-Methylurea at specification level and determined the N-Methylurea content by HPLC. Achieved results like % RSD and 95% confidence interval for six determinations are summarized in Table 4.

Table 4: Precision experiments data

System Precision	Injection ID	N-Methylurea area	Statistical Analysis	
	1	48014		
	2	48390		
	3	48012	Mean	48266
	4	48320	SD	202
	5	48384	% RSD	0.4
	6	48473	95% Confidence Interval (±)	212

Method Precision	Sample	N-Methylurea (%w/w)	Statistical Analysis	
	1	0.051		
	2	0.048		
	3	0.049	Mean	0.048
	4	0.050	SD	0.003
	5	0.042	% RSD	6.3
	6	0.049	95% Confidence Interval (±)	0.003

Intermediate Precision	Sample	N-Methylurea (%w/w)	Statistical Analysis		
	1	0.048			
	2	0.047		For ruggedness	overall
	3	0.048	Mean	0.048	0.048
	4	0.048	SD	0.000	0.002
	5	0.048	% RSD	0.0	4.2
	6	0.048	95% Confidence Interval (±)	0.000	0.001

Solution stability

For the determination of stability of the standard and sample solutions, standard solution and sample solution spiked with N-Methylurea at specification level were prepared as per test methodology and analyzed initially and at different time intervals by keeping the solution at room temperature (25°±2°C). The % difference in the peak area obtained at initial and after 13 hours time interval was found to be less than 8.2 for standard solution and 2.0 for sample solution at room temperature (25°±2°C). Based on data, it was concluded that the standard solution is stable at least 13 hours and sample solution is stable at least for 15 hours at 25°±2°C temperature. Summarized results are shown in Table 5.

Table 5: Stability of solutions experiment data

		N-Methylurea area	% Difference
Standard (at 25°±2°C)	Initial	47402	8.2
	After 13 hours	51274	
Sample (at 25°±2°C)	Initial	54851	2.0
	After 15 hours	53777	

CONCLUSION

The HPLC chromatography method was developed, optimized and validated for the determination of N-Methylurea content in Methohexital drug substance and the results of various validation parameters proved that the method is specific, sensitive, precise and accurate and the method can be introduced into routine testing.

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