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Method Development And Validation For Hydroxyurea In Fixed Oral Solid Dosage Forms Using RP-HPLC With DAD Detection

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ABSTRACT:

A simple isocratic Reverse Phase High Performance Liquid Chromatography (RP-HPLC) method was developed and validated for the determination of Hydroxyurea in two different oral solid dosage forms (tablets and Capsules). The strength of samples were 100 mg, 200 mg, 300 mg and 400 mg. The method consists of a mobile phase combination of methanol (HPLC grade) and a salt solution (1.7 g quantity of tetrabutylammonium hydrogen sulphate and 1.74 g of dibasic potassium phosphate anhydrous diluted to 1L with pH adjusted to 5.0 with 0.2 M phosphoric acid) in a ratio of 5:95. Phenomenex Luna 5-µm C18 (2)-150 x 4.6-mm, 5-µm) was used as the stationary phase. The column oven was set to a temperature of 30±1°C. Quantification was achieved with a DAD detector set at 210 nm. Resolution was achieved at a short run time of 3 minutes. With a flow rate of 1.0 mL per minute. Hydroxyurea eluted at 1.557±0.024 minutes. The method was found to be specific, robust, accurate and precise for the estimation of Hydroxyurea in fixed oral dosage tablets over the concentration ranges of 0.09 mg/mL - 1.064 mg/mL. The Correlation Coefficient (r^2) was observed to be 0.9998. The LOD and LOQ were found to be $4.4x10^{-4}$ mg/mL and 1.33 $x10^{-3}$ mg/mL respectively. The proposed method is precise, specific, accurate and robust for the estimation of Hydroxyurea in oral solid dosage form.

KEYWORD: *validation, Hydroxyurea, HPLC*

INTRODUCTION

Hydroxyurea was first synthesized in 1869 in Germany by Dressler and Stein. A century later, phase I and II trials began testing the safety of this drug in humans with solid tumors. It was first approved by the FDA in 1967 for the treatment of neoplastic diseases. In subsequent years, clinical trials demonstrated the efficacy of this drug for the treatment of CML, psoriasis, and polycythemia vera. Although there have been reformulations of this drug, there were no labeling revisions until 1996. In February 1998, Hydroxyurea received a new indication, for the treatment of sickle cell disease. It is approved for use in reducing the frequency of painful crises and the need for blood transfusions in adult patients with recurrent moderate-to-severe painful crises (generally at least three during the preceding 12 months) [1]. Hydroxyurea is also approved for use in the treatment of melanoma, resistant CML, and recurrent, metastatic, or inoperable carcinoma of the ovary [2].

Few chromatographic methods for the determination of hydroxyurea have been reported including those in the compendia [3,4].

This study was done in accordance with the International Conference on Harmonization (ICH) guidelines [5,6].

MATERIALS AND METHODS

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A. Materials and Reagents:

Chemicals / Reagents: Tetra-n-butylammonium-Hydrogen sulfate, Manufacturer: Merck, Batch number: S5304158, Purity: 98 %. Di-Potassium hydrogen orthophosphate, Manufacturer: BDH, lot number: A755275, Purity 98.0. Sodium hydroxide pellets, Manufacturer: Merck, Batch number: MD0M601069, Purity: 95 %. Orthophosphoric acid, Batch number: 29420, Purity: 85%. Methanol, Manufacturer: Merck, Batch number: SE0SF60450, Purity: 99.7 %. Doubly distilled water.

Analytical Reference Standards: Hydroxyurea working standard, Manufacturer: Ria international LLC China, Batch #: 090600111, Potency (OAB): 99.21 %.

Glassware / Filters: 10 mL, 25 mL, 50 mL, 100 mL and 1000 mL Volumetric flasks, 5 mL and 2 mL graduated pipette, 100 and 1000 mL Measuring cylinders and 0.45 µm Membrane filter.

Instrumentation and Chromatographic conditions: Agilent Technologies 1200 series HPLC modules (G1315D, G1315A, G1329A, G1311A, G1332A and organizer, with ChemStation data processing software) Hitachi U-2810 Spectrophotometer (with data processing system unit, having a 1.5 nm band slit),

Mobile phase and diluent consist of 95 portion solution "A" was mixed with 10 portion of methanol. Solution "A" was prepared by mixing a quantity of tetrabutylammonium hydrogen sulphate weighing 1.7 g and 1.74 g of dibasic potassium phosphate anhydrous and dissolved to a 1000 mL. The pH was adjusted to 5.0 with 2M phosphoric acid. The DAD detector was set at 210 nm. Column oven was set at 30±1 ^{o}C and the run time was 10 minutes.

B. TEST SAMPLES

Hydroxyurea oral dosage forms consist of the various strengths: 100 mg tablets, 200 mg Capsule, 300 mg Capsule and 400 mg Capsule. Samples were analysed using the developed RP-HPLC method. The results were reported as means ± S.D (standard error of the mean). Results were statistically analysed for significant differences.

C. Validation Parameters

The validation exercise was performed as per ICH guidelines.

Specificity: This was performed by injecting 10 µL aliquot of the Mobile phase (diluent), Placebo solution, diluent spiked with Hydroxyurea working standard having an approximate concentration of 0.4 mg/mL. Formulated tablets and capsules also having approximately 0.4 mg/mL concentration were injected. System suitability test was determined by making six replicate injections of the standard solution. The respective peak responses and the RSD for six replicate injections were recorded. The retention time, RSD of relative peak areas were recorded.

The precision and accuracy: This was evaluated at three concentration levels, 0.10 mg/mL, 0.4 mg/mL and 0.70 mg/mL for hydroxy urea standard, grounded capsule content and tablets. In each case, six different masses of samples were transferred and dissolved to the mark with diluent and sonicated for two minutes. 1.0 mL each were taken and dilutes separately to 10 mL. The Percentage contents and relative standard deviation (RSD) were determined in each case. The results were subjected to statistical

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analysis at 95 % confidence interval to determine any significant differences.

The stability of the mixture: This was determine for 6 replicate sample preparations for standard solutions, Hydroxyurea tablets and capsule prepared at a concentration of approximately 0.4 mg/mL. The replicate preparations were analysed at various time points (0 (initial), 3, 6, 9, 24 hours). The recovery were compared with the freshly prepared samples and the results were analysed statistically.

Ruggedness and/or robustness: This was performed on 6 replicated injection. The prepared solutions of approximately 0.4 mg/mL of Hydroxyurea content in tablet were then analysed with the developed method with deliberated changes. The change in parameters include flow rate, mobile phase composition, pH, temperature and column.

Intermediate precision (interday precision:. Interday precision was performed on six replicates at intervals of one day by two different analysts over a period of six days. The Percentage contents and relative standard deviation (RSD) were determined in each case. The results were subjected to statistical analysis at 95 % confidence interval to determine any significant differences.

Linearity, LOD and LOQ: Working Concentration range was determine through a calibration curve preparation. LOD and LOQ were subsequently determined from the calibration curve.

RESULTS AND DISCUSSION

An isocratic HPLC with a DAD was developed for the quantification of Hydroxyurea in single dose oral solid dosage form, Hydroxyurea tablets and capsules. The mobile phase includes the use of 95 portion of solution A to 5 portion of methanol. Solution A was prepared as follows (A quantity of tetrabutylammonium hydrogen sulphate weighing 1.7 g and 1.74 g of dibasic potassium phosphate anhydrous were quantitatively transferred into a 1000 mL of beaker. Water was added to 800 mL and sample was sonicated to dissolve. The pH was adjusted to 5.0 with 2M phosphoric acid)

Phenomenex Luna 5-µm C18 (2)-(150 x 4.6-mm, 5-µm) was used as the stationary phase and wavelength of 210 nm was adopted for appreciable peak area for the analyte. A flow rate of 1mL/min, run time of 5 minutes, injection volume of 10 µL and a column oven temperature of 30 ^{o}C were adopted for the analysis.

A. Method Validation

In evaluating specificity: there were no interferences. Injections of the placebo and the mobile phase (diluent) gave no peaks. Mean retention times are shown in table 1. Figure 1 to figure 6 are the various chromatograms.

Table 1: Results of specificity

No	Solution	Mean retention time (minutes)
1	Diluent	no peak
2	Placebo	no peak
3	Hydroxyurea	1.570±0.003
4	Tablet	1.571±0.002
5	Capsules	1.569±0.003

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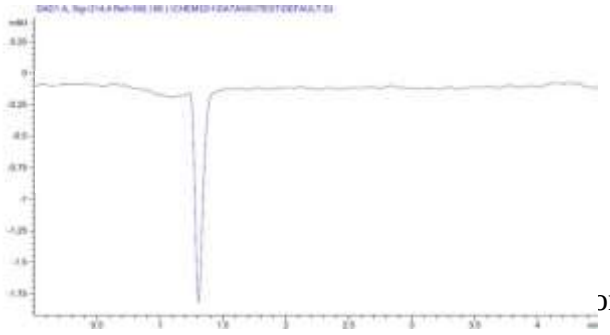

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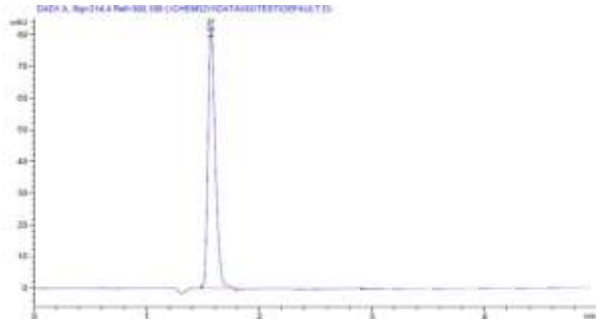

Fig. 2: Sample Chromatogram of Standard Hydroxyurea

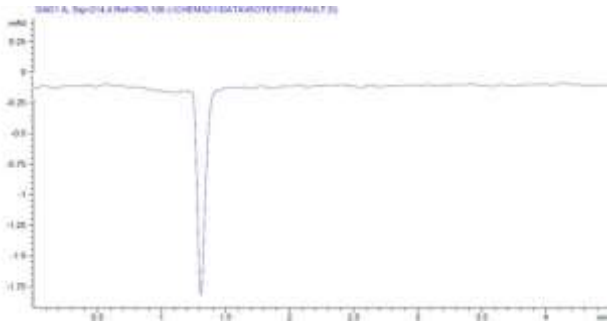

Fig. 3: Chromatogram of placebo in Hydroxyurea tablets

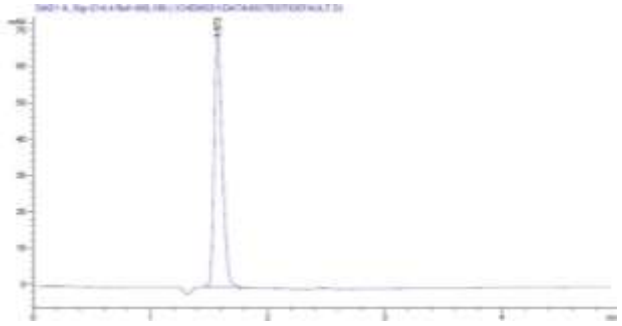

Fig. 4: Sample Chromatogram of Hydroxyurea in tablet

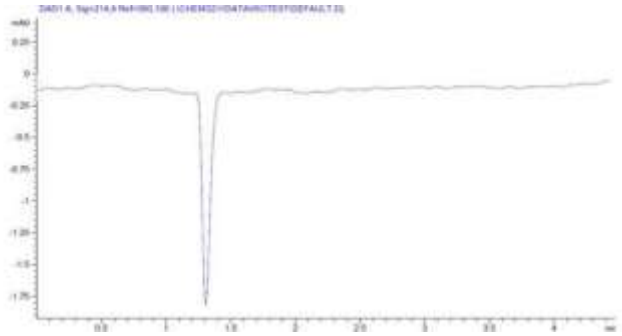

Fig. 5: Sample Chromatogram of placebo in Capsules

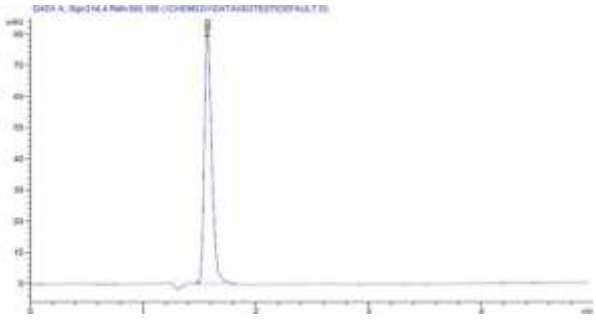

Fig. 6: Sample Chromatogram of Hydroxyurea in capsules

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From the chromatograms observed, no extraneous peak was observed in either the blank or placebo which could interfere with the hydroxyurea peak obtained from the assay. Thus the hydroxyurea peak was well separated from any potential peak or valley which could interfere with the peak area of hydroxyurea test.

System suitability: The RSD, Mean and SD were evaluated for system suitability at 100% concentration for the method developed.

Table 2: System Suitability test

ID	Peak area	Retention time(min)
Mean	361.2625	1.5680
SD	0.3062	0.0011
RSD	0.2848	0.0019

The peak area precision and retention time precision were observed to be within the limits of less than 2 %.

Precision and Accuracy: The precision and accuracy evaluated at three concentration levels were performed with API, formulated tablets and capsules.

Table 3: Precision and accuracy at different concentration levels

	Concentration levels		
	0.1 mg/mL	0.4 mg/mL	0.7 mg/mL
	Mean Recovery		
API	99.45±0.34	100.2±0.24	101.12±0.31
RSD	0.34	0.25	0.3
Tablet	99.23±0.65	99.95±0.61	99.00±0.48
RSD	0.64	0.6	0.49
Capsule	100.41±0.64	99.42±0.53	99.14 ± 0.12
RSD	0.64	0.53	0.12

The mean percentage recovery for all samples at 0.10 mg/mL, 0.4 mg/mL and 0.7 mg/mL were within a range of 99.00 % to 101.12 %. Their corresponding standard deviation observed at all the concentration levels were less than 1.00 %. The results obtained shows good precision and accuracy.

Analysis of variance (ANOVA) performed at 95 % confidence interval reveals that there were no statistically reliable difference between the amounts recovered for the various active compounds analysed in each sample and across the various concentrations used. P value observed were greater than 0.05, ($p > 0.05$).

Stability studies of solution: Stability studies of solution containing working standard, formulated tablets and capsules were studied within a period of 6 hours. The Mean, SD and RSD recorded.

Table 4: Results of % Stable analyte in sample solution over a study period of 6 Hours

Time	Sample	Mean (%)	SD	RSD
0 HOUR	API	99.95	0.61	0.61
	Tablet	99.45	0.46	0.45

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3rd HOUR	Capsule	99.56	0.56	0.56
	API	99.23	0.46	0.46
	Tablet	99.33	0.37	0.35
	Capsule	99.36	0.36	0.36
6th HOUR	API	99.58	0.38	0.38
	Tablet	100.18	0.18	0.17
	Capsule	99.12	0.12	0.13

The recovery of analyte were within a range of 99.12 % to 100.18 % across all samples. Statistical analysis performed on each analyte after the 6 hour period shows that there were no statistical reliable difference in the recovery of sample at 95 % confidence interval. P value obtained in each case were > 0.05.

Ruggedness: For ruggedness, deliberate changes were made to flow rate, pH of Mobile phase composition and temperature.

Table 5: Results of % Stable analyte in sample solution over a study period of 6 Hours.

Change Factors	level	Hydroxyurea
Flow Rate		
0.8 mL/min	-2	99.87±0.74
1.0 mL/min	0	99.95±0.61
1.2 mL/min	2	100.52±0.44
pH		
2	-3	99.02±0.61
5	0	99.95±0.60
8	3	99.99±0.81
Mobile phase composition		
Buffer (pH 5.0):Acetonitrile	92:8	99.89±0.76
Buffer (pH 5.0):Acetonitrile	95:5	100.46±0.61
Buffer (pH 5.0):Acetonitrile	98:2	100.09 ±0.61
Temperature		
25 ^{O}C	-5	99.98±0.64
30 ^{O}C	0	100.46±0.62
35 ^{O}C	5	100.13±0.73

The mean percentage recovery at the various parameters used for the robustness were all within the range of 100% ±2%. Their corresponding standard deviation observed were also less than one. The results obtained shows good precision and accuracy even after deliberate alteration of the parameters used for the analysis. The % RSD observed were less than 1%. .

Intermediate precision (interday precision): *Interday* precision was performed on six replicates at intervals of one day by two different analysts over a period of six days.

Table 6: Intermediate Precision and accuracy for two analyst over a six day period.

Analyst	Column used	Mean (%)	SD	RSD
Analyst	Eclipse XDB	100.07	0.67	0.67

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one	5u C18, 150 x 4.6mm Phenomenex luna 5u C18, 150 x4.6mm	100.20	0.62	0.61
Analyst two	Eclipse XDB 5u C18, 150 x 4.6mm	100.38	0.66	0.65
	Phenomenex luna 5u C18, 150 x4.6mm	99.49	0.60	0.60

The results analysed at 95 % confidence interval proved to have no statistical reliable difference since the p value was less than 0.05.

Linearity, LOD and LOQ: Linearity and Working Concentration range, LOD and LOQ were determine for hydroxyurea. The working concentration range from 0.09 mg/mL to 1.06 mg/mL.

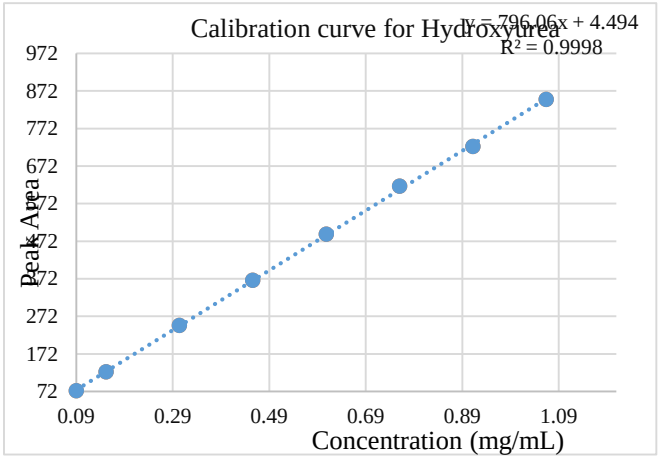

Fig 7: calibration curve for hydroxyurea

The Correlation co-efficient was observed was 0.9998. The limit of detection (LOD) of the instrument for the assay was observed to be 0.00044 mg/mL and the limit of quantification (LOQ) was 0.00133 mg/mL. The limits obtained proved that acceptable concentrations higher than the limit of Quantification were used for the calibration curve.

CONCLUSION

The proposed RP-HPLC method for the estimation of hydroxyurea in model tablets, capsules and API was validated as per ICH guidelines. The method was found to be specific, robust, accurate and precise over the concentration ranges of 0.01 mg/mL to 1.06 mg/mL. The r^2 value obtained was 0.9998. LOD and LOQ calculated were 0.00044 mg/mL and 0.00133 mg/mL respectively

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