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Method Development and Validation for the Estimation of Amifostine in Pharmaceutical Dosage form by using RP-HPLC

Nagaraju Pappula*, T. Sowjanya Jyothi , T. Anil Kumar

Department of Pharmaceutical Analysis, Hindu College of Pharmacy, Guntur – 522002, Andhra Pradesh, India

A B S T R A C T

A rapid, simple, precise, accurate, and isocratic high performance liquid chromatography (HPLC) method has been developed for routine quality control of amifostine in pharmaceutical formulations. Separation was carried out by C18 column. The mobile phase was a mixture of acetonitrile and water in ratio of 70:30 v/v at a flow rate of 1 $mLmin^{-1}$. The ultraviolet (UV) detection and column temperature were 220 nm, and ambient in nature. The run time was 10 min under these chromatographic conditions. Excellent linear relationship between peak area and amifostine concentration in the range of 80-120 µg mL^{-1} has been observed (r^2, 0.999). Developed method has been found to be sensitive, precise (the interday and intraday relative standard deviation (RSD) values for peak area and retention time were less than 0.4), accurate (recovery, 100.2-102.24%), specific and robust (% RSD were less than 1.00, for system suitability parameters). Proposed method has been successfully applied for quantification of amifostinein pharmaceutical formulations.

Keywords: Amifostine, RP-HPLC, Cancer, UV-Vis

1. Introduction

Amifostine, 2(3-aminopyopylamino) ethylsulfanyl phosphonic acid, is an organic thiophosphate cytoprotective1 agent indicated to reduce the cumulative renal toxicity associated with repeated administration of cisplatin in patients with advanced ovarian cancer or non-small cell lung cancer and also to reduce the incidence of moderate to severe xerostomia in patients undergoing post-operative radiation treatment for head and neck cancer. Amifostine is a prodrug2 that is dephosphorylated by alkaline phosphatase in tissues to a pharmacologically active free thiol metabolite. Healthy cells are preferentially protected because amifostine and metabolites are present in healthy cells at 100-fold greater concentrations than in tumour cells. Chemically it is 2-(3-aminopropylamino)ethylsulfanylphosphonic acid trihydrate and soluble3 in PBS [Lead(II) sulphide], pH 7.2, is approximately 5 mg/ml. It is rapidly cleared from plasma by biphasic decay with an alpha half-life (T1/2 alpha) of 0.88 min and a T1/2 beta of 8.8 min (Fig.1).

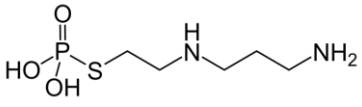

Figure 1- Chemical structure of amifostine

According to the literature search, there are few published high performance liquid chromatography (HPLC) methods for estimation of amifostine in dosage forms4-7

* *Corresponding author*
E-mail address: *pappulanagaraju@gmail.com*

2. Experimental

Chemicals: Analytical grade chemicals were used in this study. HPLC-grade acetonitrile and methanol were purchased from fisher scientific. Ultra-pure water was obtained from water purification unit (Millipore Elix^{R}). Amifostine pure drug was obtained as a gift sample from Natco parenterals division, Nagarjuna nagar, india. Amifostine vial (Ethyol, 500 mg) were obtained from Actiza pharmaceutical private limited, Uttan, Surat, Gujarat.

Stock standard solution

36mg of pure drug was accurately weighed, dissolved in about 10mL of deionized water and transferred to a 100 mL dry cleaned volumetric flask. The resulting stock solution was sonicated for 5min and filtered through a 0.45 mm filter. The stock solution was further diluted with deionized water to obtain the required concentration of standard solutions before being injected into the system for analysis.

Sample solution

The amount of lyophililized active ingredient supposed to be present in 5 vials each having 100mg was accurately weighed and transferred in to 100ml volumetric flask and 100ml of diluent was added and sonicated for about 30min.

Chromatographic conditions

Chromatographic analysis was performed on a column of Lunar C8 (4.6 mm X 250 mm X 5.0 mm). The mobile phase consisted of acetonitrile: water in ratio of 70:30. The mobile phase was filtered and degassed through a 0.45 mm membrane filter before use and then pumped at a flow rate of 1 mL min^{-1}. The column temperature has been ambient in nature. The run time was 10 min under these conditions.

Method validation

The analytical method validation has been performed as per ICH guidelines of Validation of Analytical Procedure: Q2 (R1) [8,9]. The validation parameters such as system suitability, linearity, the limit of detection (LOD), the limit of quantification (LOQ), accuracy, specificity, precision, and robustness were addressed.

Linearity

Standard calibration has been prepared using six standard solutions within the concentration range of 80-120µg/ml has been observed (r^2, 0.999). In optimized chromatographic conditions, each standard solution was chromatographed for 10 min three times. Least squares linear regression analysis of the average peak area versus concentration data were used to evaluate the linearity of the method.

Specificity/selectivity

Selectivity is the ability of the analytical method to produce a response for the analyte in the presence of other interference. The selectivity of the method was tested by comparing the chromatograms obtained for amifostine standard, injection, and blank solutions. The parameters retention time and tailing factor were calculated in order to prove that the method chosen was specific.

Limit of detection and limit of quantification

These values were determined using the standard error (s) and slope of the regression line (m) as shown in following equations:

LOD = 3.3*s/m

LOQ = 10*s/m

Precision

Precision was analysed by calculating variations of the method in intraday (repeatability performed by analysing standard solution on the same day) and inter-day (repeatability carried out by analysing standard solution on three different days).

Accuracy

Recovery studies were conducted by the standard addition technique to confirm the accuracy of the proposed method. In this method, 50, 100, 150% of three different levels of pure drug were added to the previously analysed sample solutions, and amifostine recovery was calculated for each concentration.

Robustness

A robustness analysis was performed to determine the impact of minor yet systematic differences in chromatographic conditions. The modifications include different flow rate, composition of mobile phase and temperature. After each change, System suitability parameters were checked by injecting the sample solution into the chromatographic system and the results were compared with those under the original chromatographic conditions.

3. Result and Discussion

Determination of λmax

The wavelength corresponding to maximum absorbance (λmax) was determined as 220 nm from the UV spectrum of standard solution.

Method development

Several preliminary studies were conducted to optimize the chromatographic conditions for the quantification of amifostine. Mobile phases consisting of acetonitrile and water in ratio of 70:30 v/v due to its peak being well shaped and symmetrical using this system and provided stronger theoretical plates (>2,000) and peak tailing factor (<1.0).

Optimised chromatographic conditions were achieved using an isocratic mobile phase comprising acetonitrile and water in ration of 70:30 v/v at a flow rate of 1.0 mL min^{-1} on an Lunar C8 (4.6 mm X 250 mm X 5.0 mm) that was kept at 30°C. The analysis was conducted which offers a lot of advantages such as good chromatographic peak shape, enhanced column efficiency, and low-column pressure, in addition to being economic. The eluate was monitored using a UV detector set at 220 nm. Under the chromatographic conditions amifostine was eluted at retention time 4.51 min.

4. Method Validation

Linearity: The stock standard solution of etodolac was diluted appropriately with deionized water to obtain standard solutions within the concentration range of µg mL^{-1} has been observed (r^2, 0.999). Each standard solution was injected three times into the HPLC system under the above-mentioned chromatographic working conditions. Linearity of the proposed method has been estimated at 5 concentration levels in the range of µg $mL^{-1}$by regression analysis. The calibration curve was developed by plotting average peak area versus standard concentration (Fig. 2). The correlation coefficient, slope, and intercept of the regression line were determined using the least squares method. The relation between mean peak area Y (n = 3) and concentration, X expressed by equation Y = a+bX, was linear. Values of slope, intercept, and correlation coefficient (r) were 9468, 5376.4 and 0.999, respectively as shown in Table 1. Lack of fit test was performed to evaluate linearity.

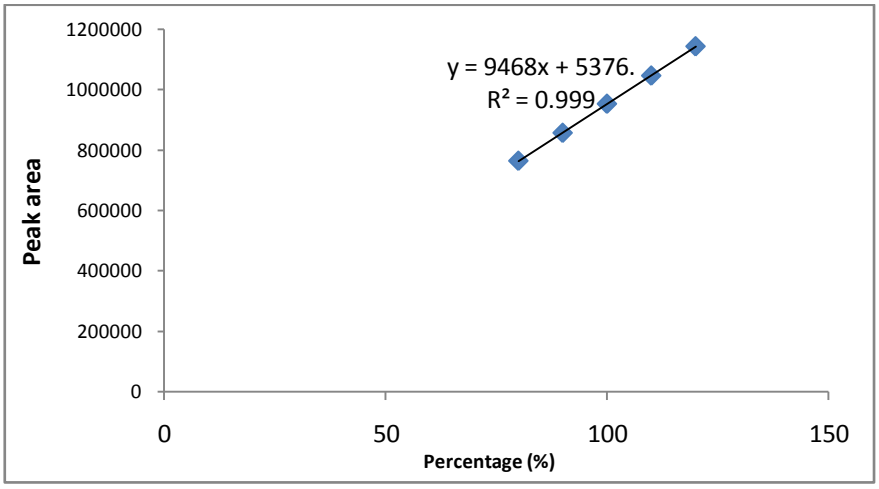

Table 1: Statistical data of amifostine

Parameters	Values
Slope	9468
Intercept	5376.4
Correlation coefficient	0.999
LOD	0.44µg/ml
LOQ	1.32 µg/ml

Specificity/selectivity

The chromatogram of amifostine standard solution has been given in Fig. 3A. There is only one peak at the retention time of 4.51 min. The chromatogram of the tablet solution has been given in Fig. 3B. There is only one peak at the retention time of 4.51 min in this chromatogram. There are no other peaks caused by excipients and additives in this chromatogram. The chromatogram of the mobile phase has also given in Fig. 3C. There are no other peaks caused by contents of the mobile phase in this chromatogram. This indicates that the analytical method is specific. The parameters retention time and tailing factor were calculated in order to prove that the method chosen was specific. Retention time and area values were 4.51 and928598 respectively. All of the values were within the accepted level.

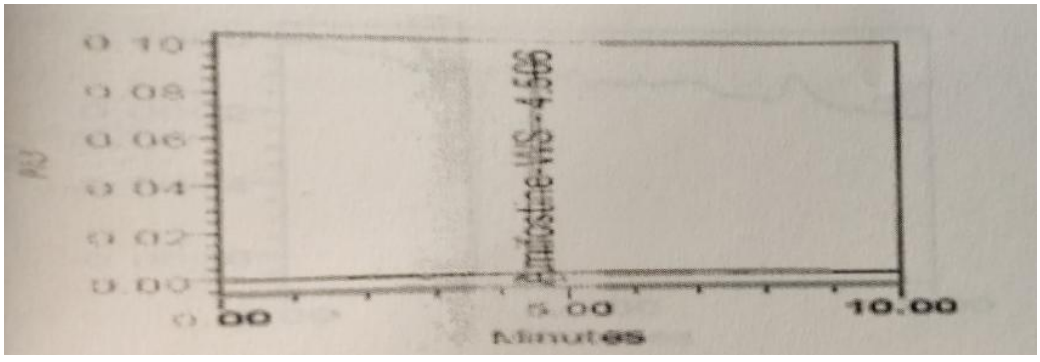

Fig 3A: Typical chromatogram of standard drug

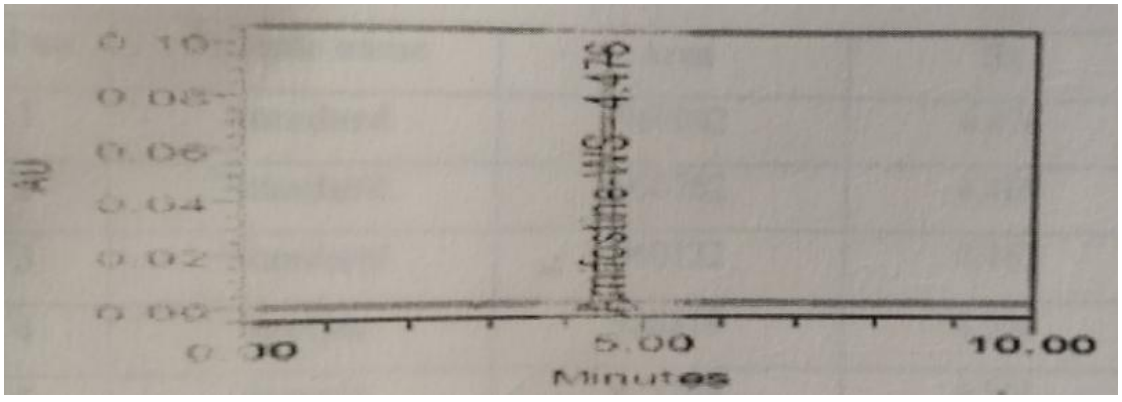

Fig 3B: Typical chromatogram of sample drug

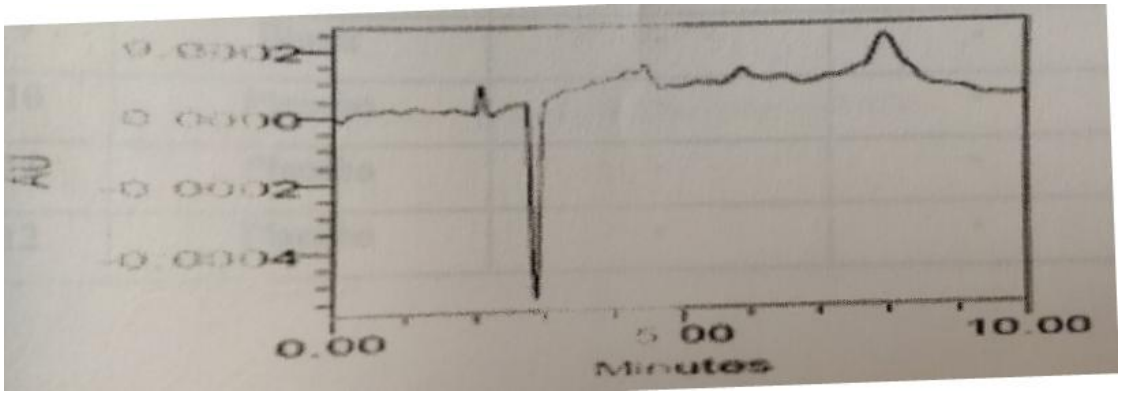

Fig 3C: Typical chromatogram of blank

Precision

Precision study was performed by injecting six times of standard solution at three different concentrations. All RSD values for retention time and peak area for selected amifostine concentrations were less than 0.5 and 2.0%, respectively. In this case, the method is precise and can be used for our intended purpose. The data was summarized in Table 2.

Table 2: Precision values of Amifostine

Inter day			**Intra day**		
Inj. No.	**Area**	**Acceptance criteria**	**Inj. No.**	**Area**	**Acceptance criteria**
1	923456	The % RSD peak areas of amifostine should not be more than 2.0%	1	963616	The % RSD peak areas of amifostine should not be more than 2.0%
2	929763		2	963335	
3	931142		3	962136	
4	928692		4	962955	
5	929578		5	962208	
6	928953		6	963058	
Mean	925855		Mean	942885	
SD	2689.5		SD	599	
% RSD	0.286		% RSD	0.063	

Accuracy study

A known quantity of standard solution has been added to the sample solutions previously analyzed at three different levels (50%, 100% and 150%). The amount recovered for amifostine has been calculated for three concentrations. The recovery data were summarized in Table 3. Percent RSD values for all analyses were less than 2% indicating that excipients found in pharmaceutical formulations do not interfere and analytical method is very accurate.

Table 3: Recovery data of amifostine

% level	Amount added	Amount recovered	% recovery	Mean % recovery
50	50	50.0	100	100.2
	50	50.12	100.4	
	50	50.1	100.2	
100	100	100.12	100.12	100.17
	100	100.22	100.22	
	100	100.17	100.17	
150	150	152.89	101.92	102.24
	150	153.12	102.08	
	150	153.0	102.0	

Robustness

The results showed that the change in flow rate, mobile phase concentration and column temperature did not have a significant effect on the method. The results of this study, expressed as % RSD, were presented in Table 4.

Table 4: Robustness data of amifostine

Condition	Mean area	% Assay
Flow rate		
At 0.9ml/min	1055020	547.39
At 1.1ml/min	863354	447.98
pH		
At 2.9	987828	512.69
At 3.1	998623	507.92
Temperature		
At 30°C	965041	499.47
At 32°C	932673	481.87

Application of the method to the marketed formulation

The developed and validated method has been applied successfully for determination of amifostine in pharmaceutical formulations. The result of assay of the marketed injection of amifostine is shown in Table 5. The results obtained are closely related to the amount indicated on the labels of the tablets. This shows that the method for content evaluation is useful.

Table 5: Assay of amifostine

S. No.	Sample peak area	Standard peak area	Labelled amount (mg/vial)	Amount found	% Assay (95-105%)
1	960042	928598	500	491.28	98.25
2	960762	928598	500	491.64	98.32
Statistical Analysis			Mean	491.46	98.29
			SD	0.25	0.0509
			% RSD	0.05	0.0517

5. Conclusion

A very quick, cost-effective, precise and accurate HPLC method for the determination of amifostine has been developed and validated in compliance with ICH guidance Q2. Besides the short run time (8min), retention time (4.51) and flow rate of mobile phase (1 mL min^{-1}) made the method attractive because these features save analysis time and cost. In short, this method is sensitive, selective, reproducible and rapid for amifostine in bulk and formulations.

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