RESEARCH PAPER

Head-Space Gas Chromatography Method for the Determination of Residual Solvents and Solvent Impurity in Amifampridine Phosphate

Karunakar A[1,2] and Lakshmi B[3]*

[1,3]*Department of Chemistry, GSS, GITAM University, Visakhapatnam-530045, A.P., India*
[2]*Research & Development, Shilpa Pharma Lifesciences Ltd, Raichur-584134, Karnataka, India*
** Author for Correspondence: lbaviset@gitam.edu; rakskk@gmail.com*

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ABSTRACT
A new headspace gas chromatographic method utilizing flame-ionisation detection is presented for the first time, aimed at quantifying residual solvents and solvent impurity in the amifampridine phosphate drug substance. The method successfully separated six analytes: methanol, acetone, methylene chloride, ethyl acetate, mesityl oxide, and dimethyl sulfoxide on a DB-624 capillary column (30 m × 0.32 mm, 1.8 µm), employing nitrogen as the carrier gas and N-methyl-2-pyrrolidone as the sample diluent. Adjustments to the split ratio, carrier flow, and oven temperature program were made to enhance resolution and reduce detection limits. All six analytes were eluted at consistent, well-defined retention times without any interference in the blank, thereby confirming the method's specificity. The method demonstrated high sensitivity, with limits of quantification below 30% of the specification limits set by the ICH. Additionally, it exhibited precision, with relative standard deviation values not exceeding 15.0%, linearity, as indicated by correlation coefficient values greater than 0.990 for all calibration curves, and accuracy, with average recoveries ranging from 87.7% to 114.9%. Minor, intentional modifications to the chromatographic parameters did not affect the system-suitability criteria, indicating robustness.

Keywords: *Amifampridine phosphate, residual solvents, solvent impurity, gas chromatography, method development, method validation*
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INTRODUCTION

Amifampridine phosphate (AMP, commonly marketed as Firdapse) is a potassium channel blocker used to treat Lambert-Eaton myasthenic syndrome in adults and children 6 years and older. It enhances muscle strength by boosting the release of acetylcholine at the neuromuscular junction. It is chemically known as 3,4-diaminopyridine phosphate[1] with a molecular weight of 207.1 and a molecular formula of $C_5H_7N_3 \cdot H_3PO_4$.

Organic solvents are indispensable at almost every stage of drug substance manufacture as reaction media and in crystallization, purification and washing and the solvent chosen often governs the yield as well as properties such as crystal form, purity and solubility. Thus, these solvents inevitably remains entrapped in the isolated solid and are termed residual solvents. Distinct from them is the solvent impurity, a contaminant already present within a solvent that is carried into the drug substance along with it. Every residual solvent is thus an impurity, although the converse does not hold. Because these volatiles confer no therapeutic benefit yet may pose a toxicological hazard to the patient, their concentrations must be held within the limits laid down in the ICH Q3C guideline[2].

In the synthesis of AMP, methanol, acetone and dimethyl sulfoxide were utilized. The primary starting material for AMP is 4-Aminopyridine (4-AP). During the production of 4-AP, methylene chloride and ethyl acetate were employed. Mesityl oxide is generated through the aldol condensation of two acetone molecules facilitated by an acid or base catalyst followed by a dehydration process[3]. Therefore, these six solvents were considered as residual solvents in the synthesis of AMP and were subsequently evaluated. Mesityl oxide is regulated to a maximum of 50 ppm (1/10 of ICH Class 3 solvents), while the permissible levels for the other residual solvents were established in accordance with ICH Q3C guidelines.

Several literature sources have outlined the determination of related substances of AMP[4-6] and quantification of AMP in human plasma and serum[7-10] by HPLC. Furthermore, a few methods have been reported for determining residual solvents[11-13] and mesityl oxide in different drug substances[14, 15]. None of these methods, however, were specifically tailored for the determination

**Author for Correspondence: lbaviset@gitam.edu*

of residual solvents in AMP. The current research therefore aimed to develop and validate[16] a gas chromatography method specifically designed for this purpose.

MATERIALS AND METHODS

Instruments and Materials: A gas chromatograph equipped with a flame ionization detector (7890A) and headspace sampler (G1888) procured from Agilent Technologies, 2850 Centerville Rd, Wilmington, DE 19808, United States was used for the analysis. It was operated with Empower 3 software (Waters Corporation, Milford, MA 01757, USA). AMP sample was procured from Shilpa Pharma Lifesciences Ltd. R&D Centre, Raichur, India. All the solvents used in this study were procured from Merck Life Science Pvt. Ltd., Mumbai, India.

Chromatographic Conditions: The DB-624 column (30 m x 0.32 mm, 1.8 mm) obtained from Agilent Technologies, located in Santa Clara, CA 95051, USA. was utilized for the analysis. The programmed temperature rise was established as follows: the initial temperature was maintained at 40 ℃ for 10 minutes, followed by a temperature increase at a rate of 10 °C/min to reach 100 °C. Subsequently, the temperature was raised at a rate of 30 °C/min until it reached 250 °C, where it was held for 9 minutes. Consequently, the total runtime amounted to 30 minutes. Nitrogen served as the carrier gas at a flow rate of 2.0 ml/min, with a split ratio of 1:8. The temperatures for the injection port and detector were set at 140 °C and 250 °C, respectively. The flow rates for hydrogen, air, and nitrogen (make-up flow) were established at 40 ml/min, 400 ml/min, and 25 ml/min, respectively. The headspace sampler conditions included a vial oven temperature, loop temperature, and transfer line temperature set at 100 °C, 110 °C and 115 °C, respectively. The injection time, pressure equilibration time, vial equilibration time and GC cycle time were configured to 1.0 min, 1.0 min, 30 min and 45 min, respectively.

Preparation of Solutions: NMP was used as a diluent. Individual analyte stock solutions were prepared by transferring separately about 50 mg of mesityl oxide, 75 mg of methylene chloride, 375 mg of methanol, 625 mg each of acetone, DMSO and ethyl acetate into 50 ml volumetric flasks containing about 20 ml of diluent, mixed and made up to volume with diluent. Standard solution for each analyte was prepared by diluting a specific volume of the respective stock solution with diluent. LOD and LOQ solutions (blend) were prepared by diluting adequate volumes of individual analyte stock solutions to 50 ml with diluent. For the linearity study, standard stock solution A was prepared by transferring about 75 mg of methylene chloride, 375 mg of methanol, 625 mg each of acetone, DMSO and ethyl acetate into a 50 ml volumetric flask, mixed and made up to volume with diluent. Standard stock solution B was prepared by dissolving 50 mg of mesityl oxide in 50 ml of diluent. Standard solution (blend) was prepared by transferring accurately 4.0 ml of standard stock solution A and 0.50 ml standard stock solution B into a 50 ml volumetric flask, followed by mixing and diluting to the mark with a diluent. Linearity solutions covering 50%, 80%, 100%, 120% and 150% to limit level were prepared by taking 2.0 ml, 3.2 ml, 4.0 ml, 4.8 ml and 6.0 ml each of standard stock solution A and 0.25 ml, 0.40 ml, 0.50 ml, 0.60 ml and 0.75 ml each of standard stock solution B, respectively in 50 ml volumetric flasks and made up to the mark with diluent. 1.0 ml each of the linearity solution was taken separately into 20 ml headspace vial. For accuracy study, spiked sample solutions at LOQ level, 100% and 150% of the limit were prepared by transferring about 200 mg of sample into separate 20 ml headspace vials followed by the addition of 1.0 ml of the respective linearity solution. Blank solution was prepared by taking 1.0 ml of diluent into 20 ml headspace vial. Sample solution was prepared by transferring about 200 mg of sample into a 20 ml headspace vial followed by the addition of 1.0 ml of diluent. Spiked sample solution was prepared by transferring about 200 mg of sample into a 20 ml headspace vial followed by the addition of 1.0 ml of standard solution. Each vial was sealed with a septum and crimp-capped immediately.

RESULTS AND DISCUSSION

Method Development: A flame ionization detector (FID) was employed for this method due to its high sensitivity. Nitrogen was preferred over helium as the carrier gas on grounds of cost. The injection system is chosen based on the boiling points of selected analytes. Headspace injection is opted when analytes are highly volatile and the test sample is low-volatile or non-volatile. Current method development was initiated based on the boiling points of selected analytes (Methanol: 64.7 ºC, Acetone: 56 ºC, Methylene chloride: 39.6 ºC, Ethyl acetate: 77.1 ºC, Mesityl oxide: 129.5 ºC and DMSO: 189 ºC). Headspace analysis was opted as most of the analytes have low boiling points. As 624 column (6% cyanopropyl/phenyl, 94% polydimethylsiloxane, equivalent to USP phase G43) is a widely used mid-polar column for analyzing residual solvents, the DB-624 capillary column (30 m x 0.53 mm, 3.0 µm) was selected in the initial trial. Its improved phase technology reduces bleed and increases signal-to-noise ratios of analytes. NMP, having a boiling point of 202 ºC was selected as a diluent as it is an inert solvent and can dissolve a wide variety of drug substances and gives a smooth base line. Injector temperature was selected as 180 ºC in view of high boiling analytes. As low and high boiling solvents and high boiling diluent were involved, it was planned to maintain low isothermal temperature initially, then gradient elution followed by isothermal elution. Thus, oven temperature was set at 50 °C initially and held for 5 min., then increased to 240 °C with a ramp rate of 20 °C / min and held for 5.5 min. Then the standard solution was injected. In the initial oven temperature, methanol, acetone, and methylene chloride, and in gradient ethyl acetate, mesityl oxide, DMSO and NMP (diluent) were eluted. However, the diluent peak eluted

closely with methylene chloride (Fig. 1). Thus, a low-micron column was used to improve resolution (DB-624 capillary column, 30 m x 0.32 mm, 1.8 µm). Accordingly, the oven program was modified based on the column dimensions. In addition, the injector temperature was also decreased to 140 ºC to reduce the impact of the sample solvent (diluent). The sensitivity of the method was impacted directly by headspace (vial) oven temperature. It was kept at 100 °C because the boiling point of selected analytes ranges between 39.6 to 189 ºC. To minimize the carryover problems, the loop temperature was kept 10 °C higher than the vial oven temperature, and the transfer line temperature was kept 5 °C higher than the loop temperature. Therefore, the headspace vial incubation temperature, loop temperature and transfer line temperature were kept at 100 °C, 110 °C and 115 °C, respectively. The vial equilibration time was set to 30 min. In this trial, blank peaks were suppressed and peak shapes and resolutions between solvent and blank peaks were improved. Hence, these conditions were finalized (Fig. 2).

Method Validation: The developed method was validated following ICH guidelines[16] and the results were shown in Table 1.

System Suitability: **To establish system suitability, six replicate preparations of standard solution were injected. The system suitability criteria was set as a) USP tailing factor for all the analytes should not be more than 2.0 and b) %RSD for the peak areas of each analyte for six injections of the standard solution (1st to 6th and 2nd to 6th and bracketing standard) should be not more than 15.0. The findings indicate that the method satisfies the criteria for system suitability.**

Specificity: **Individual standard solutions of analytes, their blend (mix), unspiked test sample and spiked test sample solutions with analytes were analyzed. The analytes were sufficiently separated from one another and eluted at distinct retention times. In addition, no interference was found in the blank at the retention times of analytes confirming that the method is specific for the determination of analytes in AMP substance.**

Limit of Detection and Limit of Quantitation: Standard solution was injected into GC system for determining the limit of detection (LOD) and limit of quantitation (LOQ) values. The LOD and LOQ values of each analyte were predicted from the Signal-to-Noise (S/N) ratio method. The precision of each predicted LOQ concentration was verified. The established LOD and LOQ values of each analyte met the acceptance criteria (S/N ratio values should be a minimum 3 and 10 for LOD and LOQ solutions, respectively), indicating that the method is precise for the quantification of all selected analytes in AMP.

Linearity and Range: Linearity was established by injecting a series of analyte solutions at concentration levels ranging from LOQ to 150% of the limit. The data underwent statistical analysis through the application of a linear regression model. The statistical parameters, including the slope, intercept and correlation coefficient values were computed. The correlation coefficient values were found to be more than 0.990 for each analyte indicating that the response of each analyte is linear from LOQ to 150% of limit.

Precision: System precision was ensured by injecting standard solution six times into GC system on the day tested. RSD for the peak area of each analyte was found to be less than 15.0% showing the excellent performance of the used GC system. Method precision was studied by injecting six spiked sample solutions prepared individually. RSD values for the results of each analyte obtained below 15.0% indicated that the developed method is repeatable. Intermediate precision was established by injecting six spiked sample solutions prepared individually into GC system using a different column, system and by another analyst on a different day. RSD values or the results of each analyte obtained from the analysis of six individual spiked sample preparations were found to be less than 15.0%. In addition, overall RSD for the results of each analyte obtained from method precision and intermediate precision experiments was also found to be less than 15.0% demonstrating the ruggedness of the method for analyst-to-analyst, system-to-system, column-to-column and day-to-day variation.

Accuracy: Accuracy experiments were performed by preparing spiked sample solutions at LOQ level, 100% and 150% of limit in triplicate, injected each solution into GC system and the percentage recoveries were calculated. These recovery results indicated that the developed method has an acceptable level of accuracy for the determination of selected analytes in AMP from LOQ level to 150% of limit.

Robustness: To assess the robustness, standard solution (for the evaluation of system suitability) and spiked sample solution were prepared and injected into GC at different deliberately varied chromatographic conditions. These varied conditions include variation in carrier gas flow (± 0.5 mL), initial temperature of column oven (± 5°C) and vial oven temperature (± 5°C). The system suitability criteria were met in all the variable conditions and much variation was not observed in retention times of analytes. Thus, the developed method is robust for all variable conditions.

CONCLUSIONS

A highly effective gas chromatography technique has been established for the identification of six residual solvents and solvent impurities in the amifampridine drug substance. This technique consistently yields symmetric peak shapes and appropriate retention times with sufficient resolutions. Validation of the method, performed in accordance with ICH guidelines, confirms that it is simple, sensitive, specific, linear, precise, accurate, and robust.

The chosen analytes can be reliably quantified within the defined limits in the amifampridine drug substance. Therefore, this method is ideally suited for routine analysis in quality control laboratories involved in bulk drug manufacturing.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this article.

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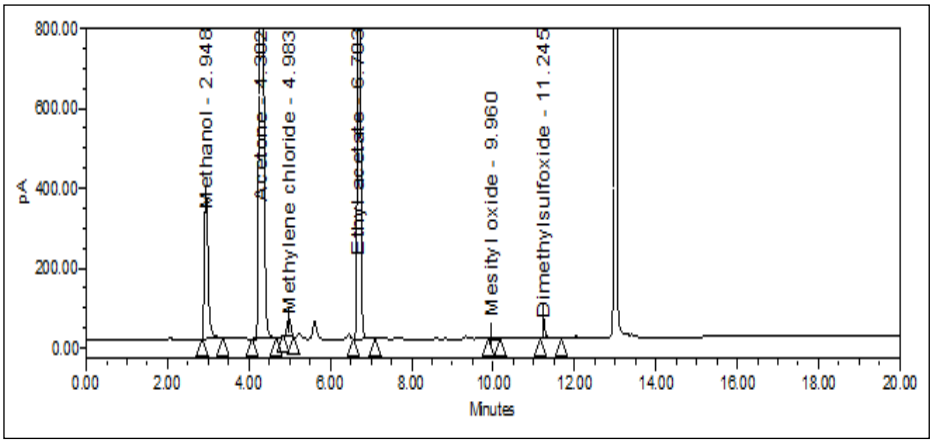

Figure No 1: Standard Solution Chromatogram (Trial-I)

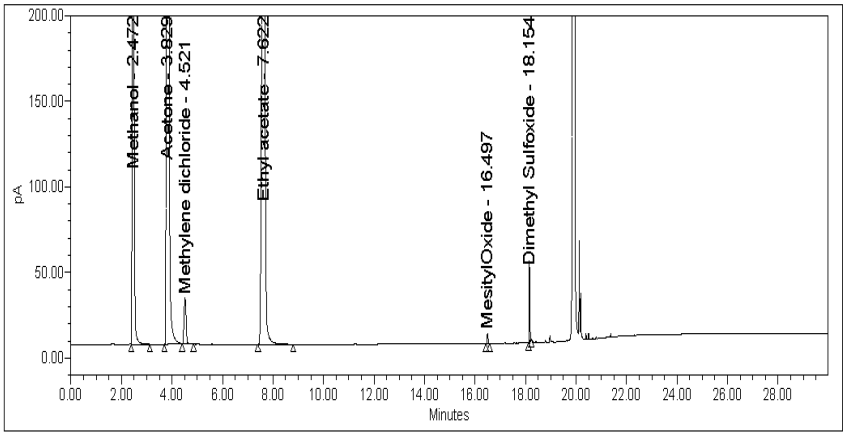

Figure No 2: Standard Solution Chromatogram (Finalized Conditions)

Table 1: Method Validation Results

Parameter	Results					
	Methanol	**Acetone**	**Methylene chloride**	**Ethyl acetate**	**Mesityl oxide**	**Dimethyl sulfoxide**
Limit (ppm)	3000	5000	600	5000	50	5000
RT (min)	2.4	3.8	4.5	7.5	16.5	18.2
System suitability						
Tailing factor	1.1	1.1	1.1	1.0	1.0	1.4
%RSD (1st to 6th standard)	1.5	2.0	1.8	1.9	2.0	5.0
%RSD (2nd to 6th and bracketing standard)	1.5	1.7	1.6	1.7	1.9	4.7
LOD (ppm)	7.5	2.1	10.0	3.3	4.6	81.5
LOQ (ppm)	24.5	6.2	30.2	10.0	10.7	370.5
Linearity						
Slope	0.4064	1.3159	0.2434	0.8912	0.3254	0.0208
Intercept	-2.0818	12.9198	1.0452	10.6593	0.1427	-7.1883
Correlation coefficient	0.9999	0.9998	0.9998	0.9998	0.9995	0.9964
Precision (% RSD)						
LOQ precision	1.8	1.4	1.3	1.5	2.2	9.3
System precision	2.1	2.1	2.5	2.4	5.3	5.3
Method precision	3.7	4.3	3.6	4.3	2.7	2.4
Intermediate precision	1.9	2.3	2.2	2.3	2.8	2.9
Accuracy (% Mean)						
LOQ recovery	109.4	103.6	113.5	114.9	87.7	100.6

100% recovery	98.7	99.2	98.7	99.6	100.1	96.8
150% recovery	100.6	100.0	99.3	100.9	104.6	98.0

* * *