

A headspace-gas chromatography method for isopropanol determination in warfarin sodium products as a measure of drug crystallinity

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Coumadin® and several generic products of warfarin sodium (WS) contain the crystalline form (clathrate) in which WS and isopropanol (IPA) are associated in a 2:1 molar ratio. IPA is critical in maintaining the WS crystalline structure. Physicochemical properties of the drug and drug product may change when the crystalline drug transforms to amorphous form. A headspace-gas chromatography (HS-GC) method was developed and validated for IPA determination in the WS drug product. *n*-propanol (NPA) was used as internal standard and the method was validated for specificity, system suitability, linearity, accuracy, precision, range, limits of detection and quantification, and robustness. The method was specific, with good resolution between IPA and NPA peaks. Chromatographic parameters (retention time, IPA/NPA area ratio, tailing factor, theoretical plates, USP symmetry, capacity factor, selectivity and resolution) were consistent over three days of validation. The analytical method was linear from 2–200 µg mL⁻¹ (0.1–10 % IPA present in the drug product). LOD and LOQ were 0.1 and 2 µg mL⁻¹, respectively. Accuracy at low (2 µg mL⁻¹) and high (200 µg mL⁻¹) IPA concentrations of the calibration curve was 103.3–113.3 and 98.9–102.2 % of the nominal value, resp. The validated method was precise, as indicated by the RSD value of less than 2 % at three concentration levels of the calibration curve. The method reported here was utilized to determine accurately and precisely the IPA content in in-house formulations and commercial products. In summary, IPA determination by HS-GC provides an indirect measure of WS crystallinity in the drug product. Nevertheless, it should be confirmed by another analytical method since IPA from the drug substance is not distinguishable from IPA that may be present outside the drug crystals in a dosage form when prepared by wet granulation with IPA.

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Narrow therapeutic index (NTI) drugs are those for which small differences in dose or concentration can lead to serious concentration-dependent therapeutic failures or adverse drug events (1, 2). NTI drugs defined in the Code of Federal Regulations (3) are those drugs which exhibit less than a 2-fold difference in median lethal dose (LD_{50}) and median effective dose (ED_{50}) values, or have less than a 2-fold difference in the minimum toxic concentrations and minimum effective concentrations in the blood. These drugs are also called critical dose drugs, which often require therapeutic or pharmacodynamics monitoring (4). Examples of NTI drugs are digoxin (5), warfarin sodium (WS) (6), carbamazepine (7), theophylline (8), phenytoin (9), *etc.* There are growing concerns regarding substitution of brand NTI drug products with generic ones due to safety and efficacy issues (9, 10). Concerns are further substantiated by recall data, which is significantly higher than that of overall drug products (2). For these reasons, some states in the USA do not allow substitution of a brand NTI drug product with a generic one without prescriber consent (2, 11, 12). Some regulatory agencies even tighten bioequivalence requirements for generic NTI products in order to increase confidence in the quality of such products, *e.g.*, by tightening C_{max} and AUC limit from 85–125 % to 90–111.11 % (2, 13). Furthermore, clinical performance of NTI drug products may be ensured by maintaining product quality throughout their shelf life and usage period.

WS is a NTI class drug (6) and is used to control hypercoagulable conditions such as venous thrombosis and its extension, pulmonary embolism, thromboembolic complications associated with atrial fibrillation and/or cardiac valve disease, recurrent myocardial infarction, and thromboembolic events such as stroke or systemic embolization after myocardial infarction (14). WS commercial products contain its crystalline form. Crystalline WS is a clathrate molecule in which isopropanol (IPA) is a guest molecule trapped in the crystal lattice of WS. IPA provides stability to WS crystalline structure by interacting with oxygen atoms of the coumarin ring by hydrogen bonding and with sodium through a coordination bond. The crystalline structure would collapse without IPA (15). The US Pharmacopoeia (16) defined 2:1 molecular association between WS and IPA and its specification of 8.0–8.5 % in the drug substance. However, crystalline WS can convert to its amorphous form through loss of IPA. Amorphous and crystalline WS forms have distinct physicochemical properties. The change of crystalline to amorphous WS may influence product quality, *e.g.*, hardness, disintegration time and dissolution rate and extent (17–21). IPA measurement provides an indirect method of measuring the crystallinity of the WS drug substance and its drug product since the amorphous form does not contain IPA. There is no literature report on a gas chromatography (GC) method for quantifying IPA in WS products. IPA is an important critical quality attribute of WS products as its presence or absence changes WS from the crystalline to amorphous form or *vice versa*. Thus, it assures the optimal therapeutic performance of the product by influencing its bioavailability. For example, an increase in hardness and disintegration time may result in a decrease in dissolution and bioavailability, and hence affect clinical performance (17, 18). The US Pharmacopoeia (16) IPA quantification method is intended for the drug substance not for the products and it uses the older packed column technology with liquid injection. This paper provides a validated headspace gas chromatography (HS-GC) method using a capillary column for determination of IPA in commercial products. Such a method would allow sponsors to better monitor the quality of WS products during manufacture and throughout their life cycle. The advantages of capillary columns *vs.* packed ones are well known. These include higher separation efficiency and sensitivity (22, 23). In addition, headspace

analysis provides cleaner injected samples with higher sensitivity to volatile compounds compared to liquid injection (24). Moreover, our group has already reported on crystallinity determination of WS in pharmaceutical products using X-ray powder diffraction, near infrared, infrared, Raman and solid-state nuclear magnetic resonance spectroscopy (19–21). None of these methods directly measure IPA, which further provides assurance of WS crystallinity in the product.

EXPERIMENTAL

Materials

Crystalline WS clathrate (Ark Pharma Inc., USA), HPLC grade IPA, dimethylsulfoxide (DMSO) (Fisher Scientific, USA), ACS grade *n*-propanol (NPA) (J. T. Baker, USA), lactose monohydrate (LM), anhydrous lactose (LA) (Foremost Farms, USA), pregelatinized starch (Starch 1500®, Colorcon, USA), magnesium stearate (MgSt), hydroxypropyl cellulose (HPC) (MW 80K, Sigma-Aldrich, USA) were used as received. 18 MW deionized water was obtained from an in-house Milli-Q Gradient A-10 water purification system (Millipore, USA).

Headspace-gas chromatography operating conditions

IPA content in the formulations was determined by the HS-GC method. The headspace sampler was a pressure-loop HT3 model (Teledyne Tekmar, USA). The HS parameters used were: GC cycle time 19.25 min, valve oven temperature 105 °C, transfer line temperature 110 °C, platen temperature 80 °C, platen temperature equilibration time 0.5 min, sample equilibration time 25 min, vial pressure 204.7 kPa, pressurization time 1 min, pressure equilibration time 0.5 min, loop fill pressure 170.3 kPa, loop fill time 1 min and inject time 1 min.

The GC system used was a 7890 model (Agilent Technologies, USA), equipped with split/splitless injector (8:1 split ratio, 150 °C) and a flame ionization detector maintained at 300 °C, with gas flows of 450 mL min⁻¹ air, 50 mL min⁻¹ hydrogen and 25 mL min⁻¹ helium makeup. The capillary column used was a 30 m × 0.32 mm × 1.8 µm Rxi®-624Sil MS (Restek Corporation, USA). Column initial temperature was 50 °C maintained for 3.5 min, then ramped to 120 °C at 40 °C min⁻¹ and kept for 6 min. Helium was used as a carrier gas flowing at a rate of 5.695 mL min⁻¹.

Preparation of isopropanol solutions

Two stock solutions of 20 mg mL⁻¹ IPA were prepared by weighing accurately 1 g IPA in a volumetric flask and making up the volume to 50 mL with water. One stock solution was used for calibration and the other for accuracy (quality control) samples. Standard solutions of 10, 7.5, 5, 2.5, 1, 0.5, 0.25 and 0.1 mg mL⁻¹ were used for calibration. Separately, another set of solutions of 10, 5 and 0.1 mg mL⁻¹ were used for accuracy evaluation.

Similarly, the internal standard (IS) solution (5 mg mL⁻¹) was prepared by weighing 125 mg NPA into a 25-mL volumetric flask and making up the volume with water. Calibration (2–200 µg mL⁻¹), quality control (2, 100 and 200 µg mL⁻¹) and system suitability (20 µg mL⁻¹) samples were prepared by adding 4.8 mL water, 100 µL IS and 100 µL of standard IPA solu-

tion into vials and vortexing for 15 s. Concentrations of calibration samples were 2, 5, 10, 20, 50, 100, 150 and 200 $\mu\text{g mL}^{-1}$ (0.1–10 % IPA present in the drug product). The 20 $\mu\text{g mL}^{-1}$ IPA solution was used for the system suitability test; 2, 100 and 200 $\mu\text{g mL}^{-1}$ concentrations were used for accuracy testing.

Preparation of in-house formulations

Two WS tablet formulations (dose: 10 mg) were manufactured by direct compression. The excipients chosen were based on excipients used in the commercial WS tablets. The formulation composition was as follows: 5 % WS (crystalline form), 86 % LM (product LM) or LA (product LA), 5 % pregelatinized starch, 2.5 % HPC and 1 % MgSt. All formulation components (except MgSt) were passed through a 0.425 mm sieve and blended in a MINI-BLEND™ (Globe Pharma, USA) for 15 min at 10 rpm. MgSt was passed through a 0.250 mm sieve, added to the formulation blend and mixed for another 5 min in the blender. Final blends were compressed into tablets using a Mini Press-1 (Globe Pharma, USA) 10-station tableting machine with 8-mm flat die and punches (Natoli Engineering Company, USA). Placebo formulations were prepared in the same way.

Sample preparation

Sample preparation involved addition of one formulation tablet into a vial containing 4.9 mL water and 100 μL IS. Contents were placed on a horizontal shaker for 60 min at 120 rpm and vortexed for 1 min before being placed in the HS-GC autosampler. Similarly, the IPA content in three commercial products was also determined to show the potential use of the validated method. Three commercial products of 5 mg strength were purchased from a local pharmacy and coded as product A, B and C (commercial products). HS-GC samples of commercial products were prepared in the same way as those of in-house formulations, except that two tablets were used in sample preparation instead of one tablet. This was due to the half potency of commercial products (5 mg WS) in comparison with the in-house formulations (10 mg WS).

Method validation

The method was validated for specificity, system suitability, linearity, accuracy, precision (repeatability and intermediate precision), linearity range, limits of quantification and detection (LOQ and LOD) and robustness, to demonstrate the suitability of the method as per ICH and USP guidance documents (16, 25).

Specificity. – Specificity was checked by injecting a blank (water), internal standard (NPA), analyte (IPA) and a sample containing both. In addition, placebo and WS formulations which contained excipients were tested. All chromatograms were analyzed for interferences in the region where IPA and NPA peaks would elute.

Linearity. – Linearity of the method was evaluated by running calibration standard solutions in duplicate in an eight-concentration range for three days. The concentration ranged from 2–200 $\mu\text{g mL}^{-1}$ of IPA present in the drug product (10 mg warfarin sodium). Regression analysis using the least squares method was performed on IPA/NPA area ratios and IPA concentrations. Linearity was evaluated by the following parameters of the re-

gression line: correlation coefficient, slope and its intercept, 95 % confidence interval of the intercept, residual sum of the squares and the residual plot. Slope fluctuations are indicative of the method inaccuracy. The slope of the developed line was consistent in inter-day analysis and varied from 0.99×10^{-2} to 1.01×10^{-2} mL μg^{-1} .

Accuracy. – Accuracy was determined with IPA standards at three concentrations, namely, 2, 100 and 200 $\mu\text{g mL}^{-1}$, which represented low, medium and high IPA concentrations in the calibration curve. Five replicate sample injections of each concentration were injected and IPA was calculated.

Accuracy was also determined in the drug products in an attempt to account for the influence of formulation on the estimation of IPA. The method of standard additions was used (26). Commercial product samples containing one tablet were spiked with 100 μL of 100 $\mu\text{g mL}^{-1}$ IPA standard and 100 μL IS in 4.8 mL water.

Precision. – Precision of the method was established as repeatability and intermediate precision. Repeatability was performed by injecting five samples of 2, 100 and 200 $\mu\text{g mL}^{-1}$ solution containing IS. Intermediate precision was determined by comparing RSD on different days. Precision of in-house and commercial products analysis was also determined by injecting three samples.

Limit of quantification and limit of detection. – LOQ was the lowest concentration of analyte within the linearity range that could be determined with acceptable accuracy and

Table I. Factors and experiments matrix

Factor	Low	Middle	High	DOE	Colum temperature (°C)	Carrier gas flow (mL min ⁻¹)	Injector temperature (°C)	Platen temperature (°C)	Equilibration time (min)	Vial pressure (kPa)
GC factor				DOE-1	47.5	5.410	157.5	84	23.75	96.5
Colum temperature (°C)	47.5	50.0	52.5	DOE-2	50.0	5.695	150.0	80	25.00	103.4
Carrier gas flow (mL min ⁻¹)	5.410	5.695	5.980	DOE-3	52.5	5.410	157.5	76	23.75	110.3
				DOE-4	47.5	5.980	157.5	76	26.25	96.5
Injector temperature (°C)	142.5	150.0	157.5	DOE-5	52.5	5.410	142.5	84	26.25	96.5
				DOE-6	52.5	5.980	157.5	84	26.25	110.3
HS factor				DOE-7	47.5	5.410	142.5	76	26.25	110.3
Platen temperature (°C)	76	80	84	DOE-8	52.5	5.980	142.5	76	23.75	96.5
Equilibration time (min)	23.75	25	26.25	DOE-9	50.0	5.695	150.0	80	25.00	103.4
				DOE-10	50.0	5.695	150.0	80	25.00	103.4
Vial pressure (kPa)	96.5	103.4	110.3	DOE-11	47.5	5.980	142.5	84	23.75	110.3

DOE – design of experiment, GC – gas chromatography, HS – headspace

precision under the stated conditions. A signal-to-noise ratio (S/N) of 10:1 can also provide an estimate of LOQ . LOD was calculated from S/N of at least 3:1 (16, 25).

Robustness. – Method robustness was studied by the design of experiment method (DOE) (27). Six independent variables of GC and HS were selected based on the literature and instrument parameters. The independent GC variables studied were: column temperature, carrier gas flow and injector temperature. Independent variables selected for HS were platen temperature, equilibration time and vial pressure. Upper and lower values of variables were 95 and 105 % of the center value (optimized), resp. A fractional factorial DOE was run with 11 experiments that contained a triplicate center point (three-day validation) (Table I). The selected parameters were: retention time (t_R), IPA/NPA area ratio, peak width, tailing factor, peak symmetry, number of theoretical plates, selectivity, resolution and IPA content in the in-house formulations.

System suitability. – The purpose of the system suitability test was to confirm that critical chromatographic parameters were adequate for analyses. System suitability was determined by running six injections of a sample containing 20 $\mu\text{g mL}^{-1}$ IPA before other sample analyses. System suitability should meet FDA and US Pharmacopeia requirements (16, 28).

RESULTS AND DISCUSSION

Method optimization

Headspace-gas chromatography. – Initially, liquid injection with a capillary GC column was used. Eventual loss of precision in system suitability samples was observed. This was likely due to non-volatile components in the samples accumulating in the injection liner and front of the column. This required frequent liner changes and column cutting to recover precision to acceptable levels. In addition, the range of liquid injection did not go below 20 $\mu\text{g mL}^{-1}$ IPA. Switching to the headspace technique greatly reduced the need for liner changes and column cutting. Furthermore, headspace analysis lowered the LOQ by at least one order of magnitude, *i.e.*, 2 $\mu\text{g mL}^{-1}$ IPA.

Sample preparation. – Samples were shaken on a mechanical shaker because the results for IPA for these samples reached higher precision compared to sonicated samples. Water was selected as the diluent because it provided cleaner chromatograms compared to DMSO. Water samples also had peak areas approximately twice as large for IPA and three times for NPA. This might be due to the lower miscibility of IPA and NPA with water compared to DMSO. A salt was added to the water to further lower the solubility of analytes (IPA and NPA). Use of sodium sulfate resulted in an increase in IPA and NPA peak areas of up to 2.5–3 times. In addition, crimp top headspace vials yielded slightly larger chromatographic peaks compared to screw caps. This was presumably due to the crimp cap vials having a tighter seal. However, the method already provided the necessary sensitivity. For method simplicity, a salt was not added and screw cap vials were employed because of their ease of use. Crushing the tablets or heating the samples while shaking did not result in increased recovery of IPA.

Method validation

Specificity. – Blank samples did not show any peaks while NPA and IPA showed peaks at distinguished t_R . Baseline resolution of IPA and NPA peaks could be achieved when chromatographic resolution (R_s) was equal or greater than 1.5. IPA and NPA were well separated and eluted at 1.4 and 2.0 min, resp.; chromatographic resolution was higher than 9.20 (Fig. 1). Placebo and WS formulations containing either LM or LA as a major component did not show interfering peaks in the region of 1.2 to 2.5 min, which was the region of IPA and NPA peaks (Fig. 2).

System suitability. – The method met system suitability parameter requirements as outlined in FDA and US Pharmacopeia documents (16, 31). Variability in the IPA/NPA area ratio was very low, as indicated by the RSD value of less than 0.1 %. Tailing factor was less than 1.12, USP symmetry was between 0.85 and 0.92 and theoretical plates were more than 9266 and 14003 for IPA and NPA peaks, resp. Selectivity (α) between two peaks was 1.81 and chromatographic resolution between the peaks was more than 9.20. The suitability parameters data were consistent in inter-day validation (Table II).

Linearity. – The correlation coefficient between the IPA/NPA area ratio and the chosen concentration range was 0.9999, which indicated a very good linear correlation between

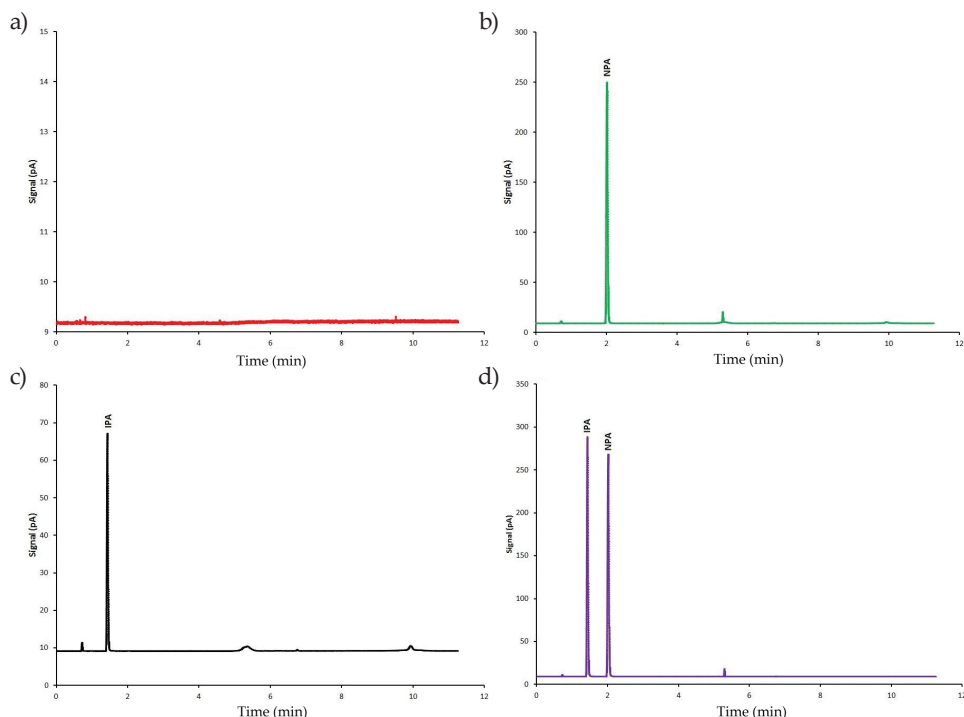


Fig. 1. Chromatograms of: a) water, b) *n*-propanol, c) isopropanol and d) *n*-propanol + isopropanol.

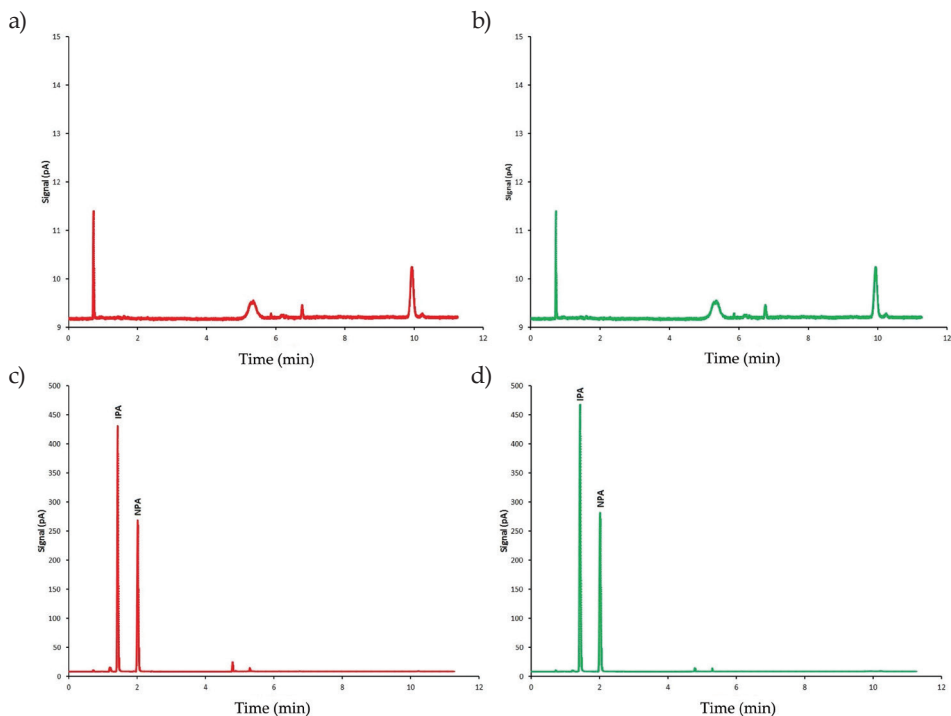


Fig. 2. Chromatograms of formulations: a) product LM placebo, b) product LA placebo, c) product LM, d) product LA. LA, LM – in-house formulations.

two variables (Table III, Fig. 3a). The regression model predicted values were very close to the actual values and the residuals between the actual and model predicted values were very low (Fig. 3b). Fitness of the regression model was further determined by the residual sum of the squares (RSS), which measures the discrepancy between the actual and model estimated values. Lower RSS values indicated good fit of the model to the data. In addition, the intercept was very low, indicating a low constant error in the model.

Range. – The results of the studies indicated that the linearity range of the calibration curve was from 2 to 200 $\mu\text{g mL}^{-1}$ (Table III).

Accuracy and matrix effect. – Accuracy data is shown in Table III. Accuracy of the method was measured through IPA solutions at three concentrations (2, 100 and 200 $\mu\text{g mL}^{-1}$; LOQ, medium and high level of linearity range). It varied from 103.3–113.3, 98.6–101.0 and 98.9–102.2 % of the nominal values, resp., in three-day validation studies. In addition, the effects of excipients on IPA results were indirectly estimated since no placebo tablets of commercial products are available. Samples containing one commercial product tablet, IS and IPA solution (100 $\mu\text{g mL}^{-1}$) were tested for the matrix effect of the formulation. The results of analyte spike indicated that the matrix had no effects on IPA values. Measured recovery of the added IPA was within 2 % of the nominal amount added (Fig. 4).

Table II. Chromatographic parameter/system suitability data of HS-GC method^a

Parameter	IPA peak	NPA peak	IPA peak	NPA peak	IPA peak	NPA peak
	Day 1		Day 2		Day 3	
Retention time (min)	1.43 ± 0.11	2.01 ± 0.09	1.43 ± 0.11	2.01 ± 0.07	1.43 ± 0.12	2.01 ± 0.07
Tailing factor	1.12 ± 0.76	1.07 ± 0.27	1.12 ± 0.87	1.07 ± 0.97	1.12 ± 0.54	1.08 ± 0.50
USP symmetry	0.86 ± 0.95	0.92 ± 0.44	0.85 ± 1.66	0.92 ± 1.34	0.85 ± 1.22	0.91 ± 1.28
Theoretical plates, <i>N</i>	9417.53 ± 2.11	14192.51 ± 2.34	9266.33 ± 2.17	14003.93 ± 2.09	9332.47 ± 2.52	14099.72 ± 2.05
HETP (mean, mm)	3.19	2.11	3.24	2.14	3.21	2.13
Capacity factor, <i>k'</i>	1.03 ± 0.18	1.87 ± 0.00	1.03 ± 0.17	1.87 ± 0.00	1.03 ± 0.20	1.87 ± 0.00
Selectivity (α)	1.81 ± 0.00		1.81 ± 0.00		1.81 ± 0.00	
Chromatographic resolution (<i>R_s</i>)	9.27 ± 1.06		9.20 ± 1.15		9.23 ± 1.22	
IPA/NPA area ratio ^b	0.20 ± 0.20		0.20 ± 0.63		0.20 ± 0.55	

IPA – isopropanol, NPA – *n*-propanol

^a Mean ± RSD, *n* = 6.

^b IPA: 20 µg mL⁻¹; NPA: 100 µg mL⁻¹

Precision. – The RSD of repeatability measurements of the samples of 2, 100 and 200 µg mL⁻¹ concentrations were 1. 9, 1.1 and 1.4 %, resp. Similarly, intermediate precision RSD of the same samples on different days was in the range of 0.4–1. 9, 0.9–1.6 and 0.8–1.4 %, resp. (Table III).

LOQ and LOD. – 2 µg mL⁻¹ was chosen as LOQ based on its acceptable linearity, accuracy and precision (Table III). *S/N* was 100 at this concentration, which was an order of

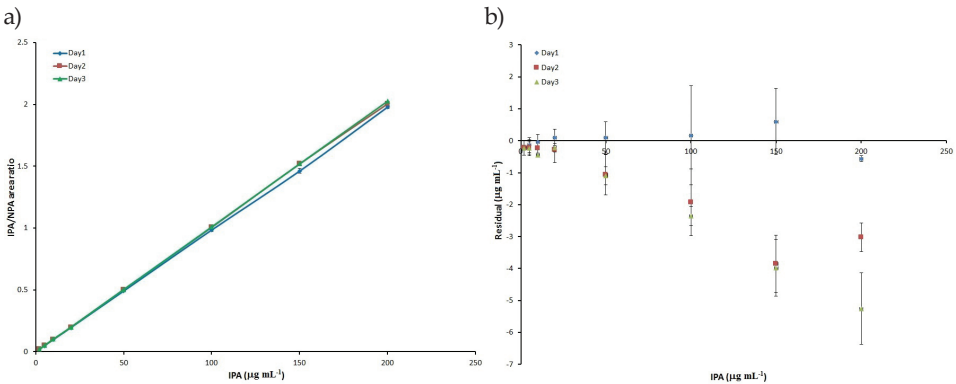


Fig. 3. a) Calibration curves and b) residual plots of actual and predicted values of isopropanol (IPA).

Table III. Validation results

Parameter	Day 1	Day 2	Day 3	Mean/mean $\pm$ SD
Linearity ^a				
Correlation coefficient	0.9999	0.9999	0.9999	0.9999
Slope ($\mu\text{g mL}^{-1}$)	0.99×10^{-2}	1.01×10^{-2}	1.01×10^{-2}	$1.00 \times 10^{-2} \pm 0.15 \times 10^{-3}$ ^{a,c}
Y-intercept	-0.13×10^{-2}	-0.07×10^{-2}	-0.23×10^{-2}	$-0.14 \times 10^{-2} \pm 0.08 \times 10^{-2}$ ^{a,c}
Y-intercept confidence interval	-0.34×10^{-2} to -0.10×10^{-2}	-0.18×10^{-2} to 0.03×10^{-2}	-0.26×10^{-2} to -0.20×10^{-2}	-0.36×10^{-2} to 0.02×10^{-2}
Residual sum of squares	2.79	30.46	51.77	27.28 ± 24.56 ^{a,c}
Concentration range ($\mu\text{g mL}^{-1}$)	2–200	2–200	2–200	2–200
LOD ($\mu\text{g mL}^{-1}$) ^a	0.1	0.1	0.1	0.1
LOQ ($\mu\text{g mL}^{-1}$) ^a	2	2	2	2
Accuracy, mean (%)				
2 $\mu\text{g mL}^{-1}$ ^b	111.0	103.3	113.3	109.2
100 $\mu\text{g mL}^{-1}$ ^b	101.0	98.6	100.3	100.0
200 $\mu\text{g mL}^{-1}$ ^b	102.2	98.9	100.8	100.6
Product A + 100 $\mu\text{g mL}^{-1}$ IPA spike ^a	101.9	–	–	101.9
Product B + 100 $\mu\text{g mL}^{-1}$ IPA spike ^a	101.1	–	–	101.1
Product C + 100 $\mu\text{g mL}^{-1}$ IPA spike ^a	101.80	–	–	101.8
Precision, RSD (%)				
2 $\mu\text{g mL}^{-1}$ ^b	1.9	0.9	0.4	1.1
100 $\mu\text{g mL}^{-1}$ ^b	1.1	1.6	0.9	1.2
200 $\mu\text{g mL}^{-1}$ ^b	1.4	0.8	1.0	1.0
Product LM ^a	1.9	1.7	2.4	2.0
Product LA ^a	0.4	1.3	0.1	0.6
Product A ^a	0.8	–	–	0.8
Product B ^a	0.6	–	–	0.6

LOD – limit of detection, LOQ – limit of quantitation, A, B, C – commercial formulations, IPA – isopropanol, LA, LM – in house formulations

^a $n = 3$, ^b $n = 5$, ^c mean $\pm$ SD

magnitude above the typical S/N requirement of 10 for LOQ. LOD was found to be $0.1 \mu\text{g mL}^{-1}$ with a S/N of 5.

Robustness. – Robustness results are shown in Fig. 5 and Table IV. The t_R , peak width, tailing factor and theoretical plates for IPA and NPA ranged between 1.33 and 1.54 min,

Table IV. Robustness of the calibration curve and IPA content in in-house products

DOE	Robustness										
	DOE-1	DOE-2	DOE-3	DOE-4	DOE-5	DOE-6	DOE-7	DOE-8	DOE-9	DOE-10	DOE-11
Calibration	R	0.9999	0.9996	0.9997	0.9997	0.9999	0.9999	0.9999	0.9999	0.9999	0.9995
	Slope	0.99×10^{-2}	0.98×10^{-2}	0.98×10^{-2}	0.98×10^{-2}	0.96×10^{-2}	0.95×10^{-2}	0.99×10^{-2}	1.01×10^{-2}	1.01×10^{-2}	0.99×10^{-2}
	Intercept	-0.13×10^{-2}	-0.13×10^{-2}	0.02×10^{-2}	0.36×10^{-2}	-0.08×10^{-2}	0.30×10^{-2}	-0.31×10^{-2}	-0.07×10^{-2}	-0.23×10^{-2}	-0.27×10^{-2}
IPA ($\mu\text{g mL}^{-1}$, mean $\pm$ RSD) ^a	Product LM	154.8 ± 2.2	162.0 ± 1.9	158.8 ± 2.1	154.6 ± 0.8	161.2 ± 1.4	157.8 ± 0.8	153.8 ± 1.0	155.6 ± 1.7	158.6 ± 2.4	157.4 ± 0.5
	Product LA	156.4 ± 1.2	157.4 ± 0.4	154.6 ± 1.0	156.0 ± 0.8	159.0 ± 2.5	157.2 ± 1.1	153.2 ± 1.8	155.0 ± 1.3	155.2 ± 0.1	160.2 ± 1.4
IPA (% mean $\pm$ RSD) ^a	Product LM	7.7 ± 2.2	8.1 ± 1.9	7.9 ± 2.1	7.7 ± 0.8	8.1 ± 1.4	7.9 ± 0.8	7.7 ± 1.0	7.8 ± 1.7	7.9 ± 2.4	7.9 ± 0.5
	Product LA	7.8 ± 1.2	7.9 ± 0.4	7.7 ± 1.0	7.8 ± 0.8	8.0 ± 2.5	7.9 ± 2.5	7.7 ± 1.8	7.8 ± 1.3	7.8 ± 0.1	8.0 ± 1.4
Crystallinity (% mean $\pm$ RSD) ^a	Product LM	92.8 ± 2.2	97.1 ± 1.9	95.2 ± 2.1	92.7 ± 0.8	96.7 ± 1.4	94.6 ± 0.8	92.2 ± 1.0	93.3 ± 1.7	95.1 ± 2.4	94.4 ± 0.5
	Product LA	93.8 ± 1.2	94.4 ± 0.4	92.7 ± 1.0	93.5 ± 0.8	95.3 ± 2.5	94.3 ± 1.2	91.9 ± 1.89	92.9 ± 1.3	93.1 ± 0.1	96.1 ± 1.4

Response	IPA				ANOVA				NPA			
	R^2	R^2 adjust	F-ratio	Prob > F ⁵	R^2	R^2 adjust	F-ratio	Prob > F ⁵	R^2	R^2 adjust	F-ratio	Prob > F ⁵
Retention time	0.999	0.998	838.09	<0.0001	0.998	0.995	273.09	0.0003	0.995	0.954	30.57	0.0086
Peak width	0.999	0.967	42.31	0.0054	0.986	0.915	16.32	0.0214	0.954	0.930	20.11	0.0159
Tailing factor	0.942	0.807	6.98	0.0700	0.974	0.930	20.11	0.0159	0.915	0.985	95.45	0.0016
Plates	0.964	0.879	11.39	0.0355	0.979	0.985	95.45	0.0016	0.979	0.985	95.45	0.0016
Resolution	–	–	–	–	0.996	–	–	–	–	–	–	–
Product LM	0.604	–0.318	0.65	0.71	–	–	–	–	–	–	–	–
Product LA	0.873	0.576	2.94	0.20	–	–	–	–	–	–	–	–

DOE – design of experiment, IPA – isopropanol, LA, LM – in-house formulations, NPA – *n*-propanol; Prob > F is alpha level (probability of rejecting null hypothesis when it is true) and the value less than 0.05 is considered significant

^a *n* = 3

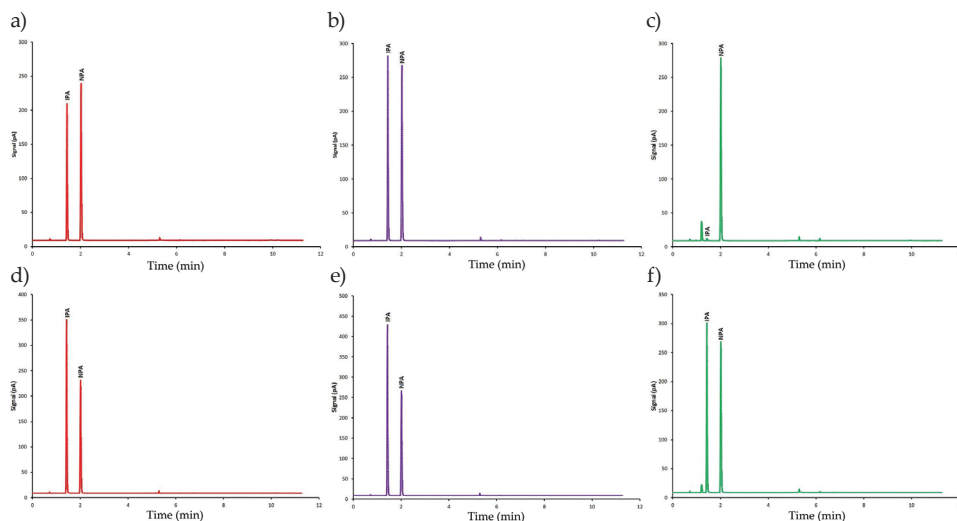


Fig. 4. Chromatograms of: a) product A, b) product A + 100 $\mu\text{g mL}^{-1}$ IPA, c) product B, d) product B + 100 $\mu\text{g mL}^{-1}$ IPA, e) product C and f) product C + 100 $\mu\text{g mL}^{-1}$ IPA. A, B, C – commercial formulations, IPA – isopropanol.

0.0344–0.0386 min, 1.10–1.25 and 8352–9978, and 1.84–2.22 min, 0.0382–0.0439 min, 1.04–1.10 and 12182–15367, resp. In addition, resolution between IPA and NPA peaks ranged from 8.24–10.04. Column temperature had a statistically significant ($p < 0.05$) effect on all chromatographic parameters (t_R , tailing factor, theoretical plates, resolution). The t_R , symmetry and tailing factor of IPA and NPA were significantly ($p < 0.05$) affected by the carrier gas flow rate, but the IPA/NPA area ratio was not affected by the studied variables (Fig. 5). Similarly, the result for IPA content in in-house formulations was not significantly affected by the selected HS-GC independent variables (Table III). Since the measurement of IPA was not affected, the method can be considered robust. However, it should be noted that column temperature and flow rate should be strictly controlled to maintain system suitability.

Linearity was not affected by the independent factors studied, as indicated by the correlation coefficient > 0.9995 between the IPA/NPA area ratio and actual IPA content and negligible intercept (Table IV). Furthermore, the predicted models and actual IPA values were in good agreement, as indicated by R^2 -predicted > 0.942 for all the studied responses. R^2 -adjusted and R^2 -predicted were in reasonable agreement, as indicated by the difference of less than 0.2. Furthermore, ANOVA analysis of the data indicated that t_R , peak width, tailing factor, theoretical plates and resolution were significantly ($p < 0.05$) affected by the studied variables for NPA (Table IV). IPA showed the same trend ($p < 0.05$), except for the tailing factor.

Analytical application

Accuracy and precision of the validated method were evaluated by measuring the IPA content in a known sample (quality control samples). Accuracy was found to be very close

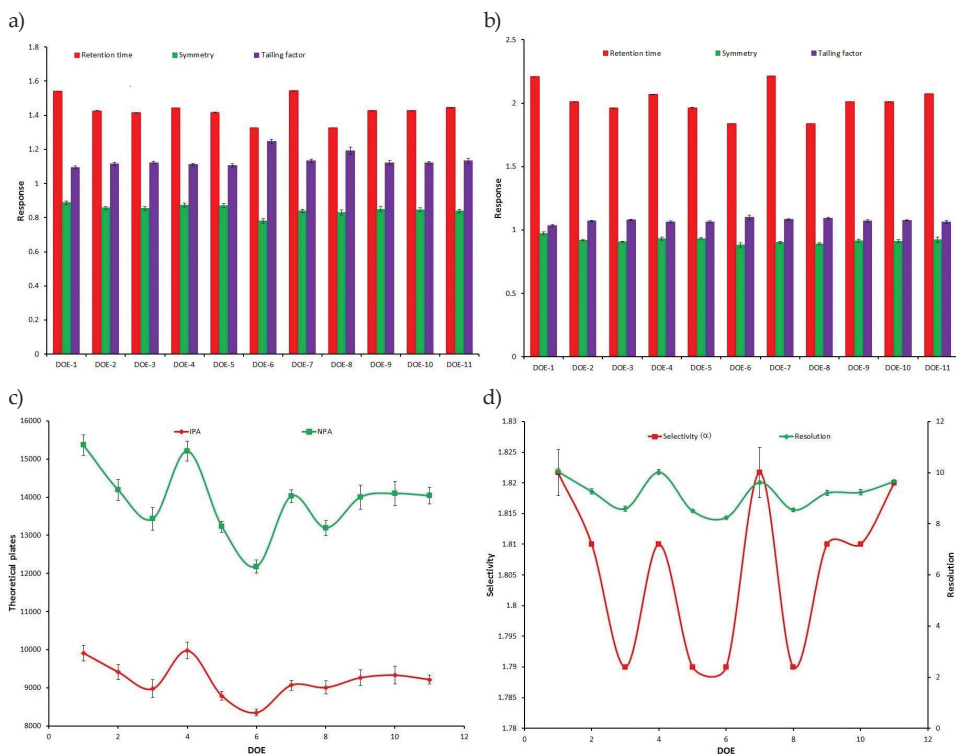


Fig. 5. Robustness of: a) retention time (min), symmetry and tailing factor of IPA peak, b) retention time (min), symmetry and tailing factor of NPA peak, c) theoretical plates of IPA and NPA, d) selectivity (a) and resolution factor. IPA – isopropanol, NPA – *n*-propanol.

to the actual values, with high precision indicated by low RSD values (Table III). Application of the validated method was further tested in in-house and commercial formulations and calculation for WS crystallinity was based on the 2:1 molecular mass ratio between WS and IPA. IPA contents of in-house formulation products LM and LA were $158.73 \pm 1.97 \mu\text{g mL}^{-1}$ ($7.94 \pm 1.97\%$ IPA or WS crystallinity $95.18 \pm 1.97\%$) and $155.87 \pm 0.60 \mu\text{g mL}^{-1}$ ($7.79 \pm 0.60\%$ IPA or WS crystallinity $93.46 \pm 0.60\%$) (average of three days) (formulations DOE-2, DOE-9 and DOE-10, Table IV), respectively. The results were accurate, as indicated by three-day values. Similarly, IPA contents in commercial products A, B and C were $79.00 \pm 0.76 \mu\text{g mL}^{-1}$ ($3.95 \pm 0.76\%$ IPA or WS crystallinity $47.37 \pm 0.76\%$), $97.40 \pm 0.76 \mu\text{g mL}^{-1}$ ($4.87 \pm 0.62\%$ IPA or crystallinity $58.40 \pm 0.62\%$) and below *LOQ* ($1.2 \mu\text{g mL}^{-1}$), resp. The RSD of in-house LM and LA formulations varied from 0.5 to 2.4 % and 0.1 to 2.5 %, resp. Similarly, RSD varied from 0.6 to 0.8 % for commercial formulations. Thus, the method was precise, as indicated by low RSD values (Table III and IV).

The IPA and crystallinity values of the commercial products were low compared to the in-house products probably due to the use of unsealed bottles that were purchased six months before testing. Furthermore, there were variations in IPA contents among the products, which may be due to the differences in composition or manufacturing method,

since product A, product B and product C were manufactured by dry granulation, direct compression and wet granulation, resp. In conclusion, the developed method can be used to determine IPA in warfarin sodium products as no literature or pharmacopeial method for this purpose is available.

CONCLUSIONS

Measuring IPA in the WS product provides an indirect means of measuring drug crystallinity in the product. Validated HS-GC method provides a reasonably fast and accurate method to monitor IPA. However, the results for IPA obtained by HS-GC may not distinguish between IPA of the drug substance and that of the excipients present in the formulation. It needs to be confirmed by a secondary method, which may be spectroscopy or X-ray powder diffraction, as indicated in our previous works (19–21).

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