IDENTIFICATION AND QUANTITATIVE ANALYSIS OF WARFARIN FORMS IN ORAL SUSPENSION USING RAMAN SPECTROSCOPY

Evangelia Dimitrokalli[1], Malvina Orkoula [1], Christos Kontoyannis[1, 2]

[1]Department of Pharmacy, University of Patras, [2]ICE/HT-FORTH, Patras, Greece

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ABSTRACT

In the present study micro-Raman Spectroscopy was employed in order to establish a novel, fast and reliable method for quantitative analysis of the API (Active Pharmaceutical Ingredient) Warfarin Sodium which is present in commercial oral suspension (syrup).

It was found that the amorphous Warfarin sodium precipitates as a crystalline form after its dissolution in either water or in the mixture of the liquids that constitute the placebo of the oral suspension. The existence of the new crystalline form was verified by recording the respective Raman spectrum and XRPD pattern. A quantitative method, using Raman spectroscopy, for the precipitated new crystalline form in the commercial syrup was also developed. The ratios of warfarin's crystalline form vibration peaks' areas at 1573 and 1612 cm^{-1} to the area of a strong placebo peak at 923 cm^{-1} were calculated. A calibration line was constructed, using as standards, dispersions of syrup and placebo. Limits of Detection (L.O.D) and Quantification (L.O.D) were calculated and found to be 0.0414 and 0.1243 mg/mL respectively.

INTRODUCTION

Active Pharmaceutical Ingredient (API) Warfarin Sodium can be found in three formulations: as a tablet, oral suspension (syrup) with strength of 1 mg/mL and dilution for injections. Warfarin Sodium is the sodium salt of 3-(α-acetonylbenzyl)-4-hydroxycoumarin. The pharmacologic function of the compound is an anticoagulant that inhibits the synthesis of warfarin K-dependent coagulation factors. The treatment aims at preventing further extension of the formed clots and secondary thromboelic complications that may result in serious and possible fatal sequelae [1].

Two solid forms of Warfarin sodium, amorphous and crystalline clathrate [2], are known to exist. The crystalline clathrate form is an Warfarin Sodium-isopropyl alcohol complex. It is has been also reported that Warfarin Sodium is a true 2-propanol solvate, not a clathrate, which it is transformed to the amorphous state through an intermediate crystalline step [3]. Both forms are used as APIs in pharmaceutical formulations and the most common method for quantitative analysis, adopted also by Pharmacopoeia [4], is High Pressure Liquid Chromatography (HPLC), which is not capable to identify if the measured quantity refers to crystalline or to amorphous warfarin sodium.

In this work it is suggested that Raman Spectroscopy, a vibration technique, can be successfully used for quantitative determination of the API being much less cumbersome and time consuming than the suggested HPLC method. Also as oppose to XRPD, it can differentiate between the amorphous and the crystalline warfarin.

The present work focus in: (a) developing a methodology for quantitative analysis of the oral suspension of Warfarin Sodium using Raman Spectroscopy, (b) investigating the presence of a new crystal form of the compound which is not yet characterized.

EXPERIMENTAL

Chemicals and reagents

Warfarin sodium amorphous, the final oral dispersion having strength of 1 mg/mL, the placebo mixture as well as placebo's individual excipients were provided by a Greek Pharmaceutical company. All aquatic solutions were prepared in Milli-Q distilled water that had resistance of greater than 17 MΩ-cm.

Preparing the mixtures

For the construction of the calibration line five dispersions were prepared. The first four were made by adding placebo to commercially available syrup (25% syrup-75% placebo, 50% syrup- 50% placebo, 75% syrup- 25% placebo and 100% syrup) and the last one by enriching the placebo with solid API in order to achieve a concentration 120% compared to syrup's concentration.

For the experiments of API's dissolution and subsequent re-crystallization, a pseudo-placebo was used which was prepared by mixing, in the appropriate ratios, the three major liquid excipients of the oral suspension (water 66%-liquid maltitol 27%-propylene glycol 4%).

Instrumentation

***Raman Spectroscopy*:**

A Renishaw, InVia, Raman microscope equipped with a laser with a 785 nm excitation line was used. The laser line was focused by Olympus oblective lens (20x) onto the samples surface. The system was equipped with a CCD detector. The power of the incident laser was 250mW. The typical spectral resolution was 2 cm^{-1}. A Windows-based software was used (WiRE© 2.0) to obtain the spectra. Instrument response (laser power and the wavenumber) was checked by recording the peak of Si.

***Recording the spectra*:**

Raman spectra were acquired by focusing a laser line at through the objective lens (20x) of an optic microscope onto the sample's surface. Prior to each Raman recording one drop of each dispersion was placed with a Pasteur pipette on a slide with gold substrate which served as reflectance surface (mirror). Each drop placed on the golden substrate was approximately 0.30 mL. The material deposited on the slide had a diameter of approximately 1.5 cm (Fig 1a). The drops were dried in the oven for 30 minutes at 100 °C. After solvents' evaporation (water and part of propylene glycol and water in liquid maltitol) a gel formation was obtained in which the strength of warfarin was approximately triple. The slide was placed on a home-made apparatus (Fig. 1b) and the sample was rotated. In this way it was possible to collect the Raman signal from the circumference of a circle formed during sample's rotation, minimizing the under-sampling problems. The recorded spectra were the sum of 5 scans which were acquired in the region 250-2000 cm^{-1}.

Fig 1a. On the left the apparatus with the gold slide on, under the objective lens of micro Raman; on the right the gold slide with the drop of syrup on.

Fig 1b. Home-made rotating apparatus for minimizing the under-sampling problems

XRPD

PXRD patterns were obtained at room temperature using an X-ray diffractometer (Bruker AXS D8 Advance with Bragg-Bretano geometry), LynxEye detector and Cu Ka spectral line (l=1,540562 A) with the generator voltage and current set at 40 kV and 40 mA, respectively. The sample holder, had a central circular cavity of diameter 2 cm in which the filtered crystals being on the filter, were placed. Samples were scanned from 5 to 30° 2θ at a step size of 0.02(θ) and scan speed 1s/step. Instrument performance was checked against the recording of corundum reference sample A13-B73 provided by Bruker.

Optic Microscope

A Leica (DM 2500 M) optic microscope was used to obtain pictures of Warfarin Sodium, in reflection mode, using the appropriate Leica oblective lens. A color video camera (Leica,model DFC 420C) was used to relay the microscope image to a computer image processor, which in turn employed a Windows based software (LAS© V3.8) for image acquisition and analysis.

RESULTS AND DISCUSSION

API identification and stability

The spectrum of solid form of the amorphous Warfarin Sodium added in the suspension exhibits a single strong peak at 1606 cm-1 (Fig. 2; red line). After its dispersion in placebo, being mostly a mixture of water, propylene glycol and liquid maltitol along with other solid excipients, two API peaks at 1573 and 1612 cm^{-1} appear. The presence of the two peaks indicates a possible transformation of the amorphous Warfarin Sodium to another state.

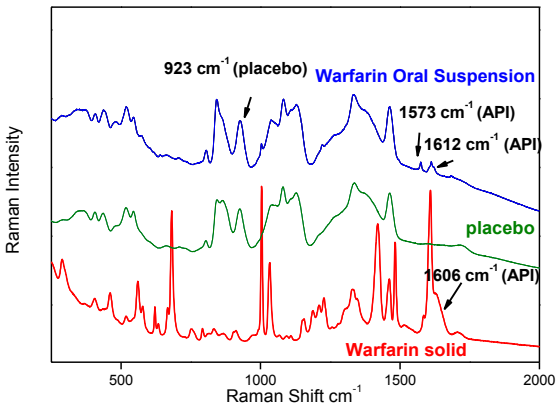

Fig. 2. Raman spectra of Warfarin Oral Suspension and placebo after evaporation, and Warfarin Sodium amorphous (solid)

In order to investigate this transformation, a quantity of Warfarin sodium amorphous was dissolved, at room temperature, in both distilled water and to pseudo-placebo (experimental section). After the initial fast dissolution of the amorphous material, it was observed that after approximately 2 hours crystals were precipitated.

The crystals were collected, through filtration, and the crystals were photographed under the optic microscope while their Raman spectra and XRPD patterns were recorded and compared against the XRPD patterns of the amorphous API, warfarin isopropanol solvate [3].

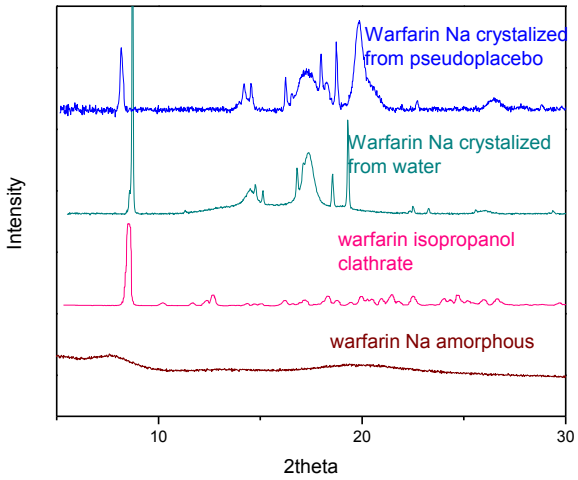

Fig. 3. PXRD patterns of Warfarin Sodium amorphous, warfarin isopropanol solvate, and API precipitated from water and pseudo-placebo. The pattern of warfarin isopropanol solvate was digitized from [3] using the UNSCAN© software.

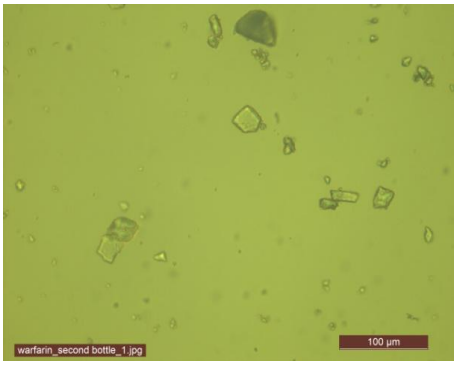

Fig. 4a. Particles of amorphous warfarin Na.

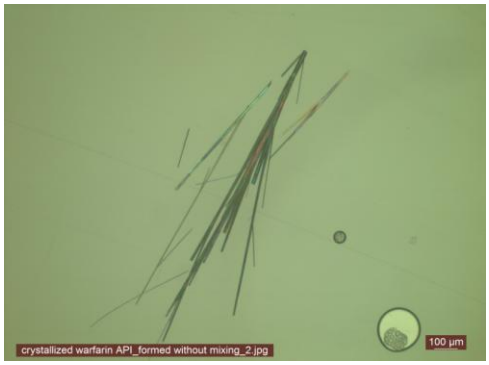

Fig. 4b Needle shape crystals of warfarin Sodium after precipitation from pseudo-placebo.

Fig. 4c. Needle shape crystals of warfarin Sodium after precipitation from pseudo-placebo

From the XRPD patterns and the images obtained from the optical microscope it is apparent that the API was re-crystallized to another form. This finding was also verified by recording the Raman spectra (Fig.5) from the

filtrated material after re-crystallization. It was found to match the spectrum obtained from the commercial oral suspension and not that of the initially added warfarin sodium amorphous.

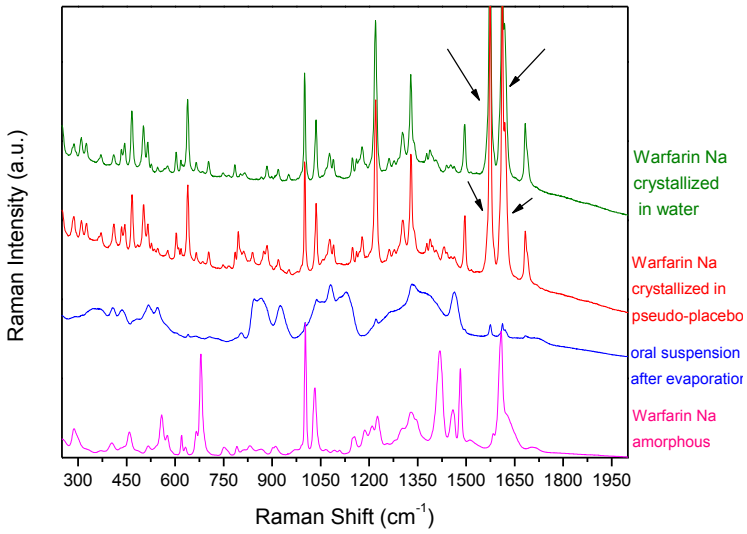

Fig.5. Raman spectra of the filtated crystalline warfarin sodium after being its dissolution and subsequent precipitation from water and the pseudo-placebo

The oral suspension was then centrifuged and the Raman spectra of both of the precipitated solid and the supernatant liquid were recorded. As shown in Fig. 6 a weak peak appears at 1604 cm^{-1} in the liquid, as well as the two strong peaks of the new crystalline form appear in the solid. This finding indicates that the API exists in the commercial suspension in both dissolved and precipitated state.

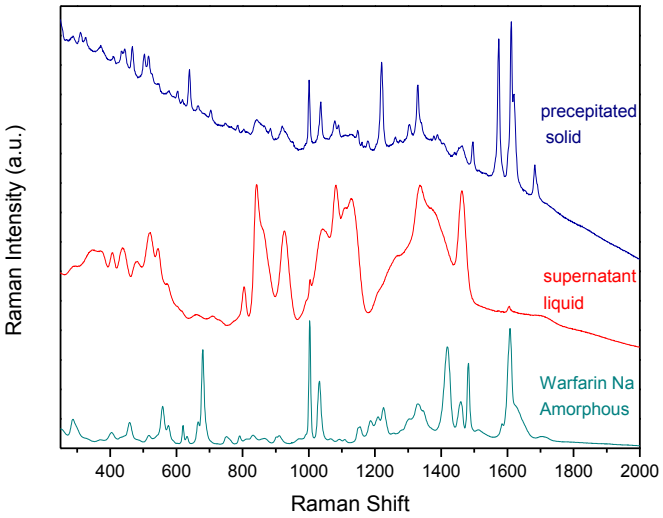

Fig.6. Raman spectra of the precipitated solid and liquid supernatant of oral suspension after its centrifugion.

API quantitative analysis

Constructing the calibration curve

The intensity of a Raman line depends on a number of factors including incident laser power, frequency of scattering and the response of the detection system. Thus, the measured Raman intensity, I(v), can be represented as:

$$I_{(v)} = I_0 K_{(v)} C \quad (1)$$

Where I_o is the intensity of the excitation laser line, v is the Raman shift, and K(v) is a factor which includes the frequency dependent terms: the overall spectrophotometer response, the self-absorption of the medium and the molecular scattering properties. C is the concentration of the Raman active species.

After the evaporation of suspension's (see experimental section) volatile solvents it is secured that: a) even the small quantities of the partially dissolved API were also precipitated and b) the composition of the remaining placebo ingredients is stable while c) the concentration of the API was increased to approximately 33 mg/mL. For the quantitative analysis the ratios of vibration peaks' areas of re-crystallized API (1573 and 1612 cm^{-1}) to the area of placebo peak at 923 cm^{-1} were calculated. Thus, using equation (1), a plot of the ratio of the Raman areas of warfarin vibrations to the placebo peak against the concentration of API, is expected to yield a straight line:

$$\frac{A_{warfarin}^{1573,1612}}{A_{placebo}^{923}} = \frac{I_o k_{1573+1612}^{warfarin} C_{warfarin}}{I_o k_{923} C_{placebo}} \quad (2)$$

And assuming that placebo concentration in the final gel formation is constant

$$\frac{A_{warfarin}^{1573,1612}}{A_{placebo}^{923}} = k_{total} C_{warfarin} \quad (3)$$

The obtained Raman spectra from the standard solutions are in Fig. 7. Ensuring the API's peaks for the quantitative analysis and using also a strong excipient peak at 923 cm^{-1} seven Raman spectra were recorded from each standard dispersion for the construction of the calibration line. After the integration of these peaks with the appropriate software (OriginPro© 8), and by calculating the averages of each area a calibration line was constructed (Fig. 7A).

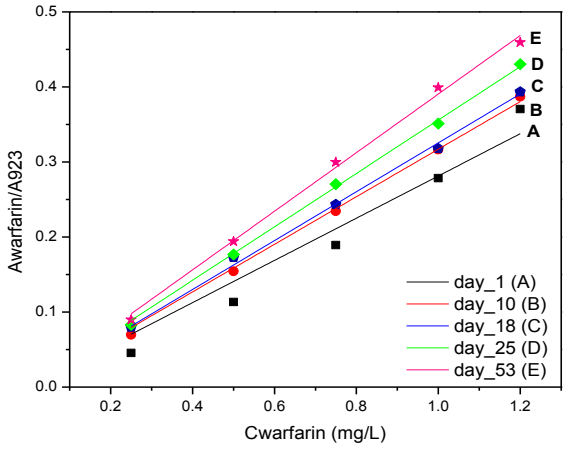

Fig. 7. Calibration lines from the standards suspensions after the formation of a gel through solvent evaporation. A. day 1; B. day 10; C. day 18; D. day 25; E. day 53.

The resulting calibration line can be represented as:

$$\frac{A_{warfarin}}{A_{923}} = \{0.26433(\pm 0.00851)\} C_{warfarin}, R^2 = 0.98169 \quad (4)$$

The Limit of Detection (LoD) was calculated at 99.9% confidence level using the following:

ΔXmin (minimum detectable quantity) is given by:

$$\Delta X \min > t \times S_b \times \sqrt{\frac{N_1 + N_2}{N_1 \times N_2}} \quad (5)$$

where $S_b = \sqrt{\frac{\sum x_1^2 - \left(\sum x_i\right)^2 / N_2}{N_2 - 1}}$ (6) is the standard deviation of blank measurements, N_1 the repeats of a measurement,N_2 is the number of blank measurements and t is the statistical parameter t known as Student's t-value defined as: $t = \left(x - \mu\right) / s$, where s is the standard deviation of measurements and $\left(x - \mu\right)$ represents the absolute deviation from the mean value [5]. For this case 10 measurements of the placebo spectra were calculated.

$$\Delta X \min > 3.922 \times 0.0236 \times 0.4472 > 0.0414$$

$$\Delta X \min > 0.0414 \quad (7)$$

So $L.O.D. = 4.14\% = 0.0414$ mg/mL

The Limit of Quantification (LoQ) of Warfarin Sodium is: $L.O.Q = 3 \times L.O.D = 12.43\% = 0.1243$ mg/mL

During the intraday measurements it was observed that the ratios were varied resulting in a shifting calibration line (Fig. 7A-E). This is caused probably due to continuous gradual evaporation of remaining solvents from the gel at a much lower evaporation kinetic resulting in a gradually increased API concentration. The calibration line seemed to be stabilized after day 10. So it important to note that for reliable results the evaporation time and the elapsed time after the formation of gel and the recording of Raman should be controlled.

CONCLUSIONS

Warfarin sodium amorphous after its addition to oral suspension placebo initially dissolved and then transforms to a crystalline form while a part of the API remain in the liquid state as dissolved material. Raman spectroscopy can be used for both identification of the crystalline form and for quantitative analysis. Initial results indicate that there is a linear correlation between the concentration of the precipitated crystalline API and Raman's signal (ratio of vibration peaks' areas of API to the area of the strong excipients peak). Calibration line was also found to depend on the days that the samples, after gel formation, remain on the slide before the recording of the Raman measurement, probably due to the continuous slow evaporation of remaining excipients into the gel. Despite this shortcoming Raman spectroscopy can be easily applied for routine quantitative analysis of the oral suspensions since it is easier and much faster as oppose to currently used HPLC method, provided that the parameters such as gel formation temperature and time as well as the time elapsed until the Raman recording are carefully controlled.

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