Light scattering detector based on light-emitting diodes-Solar cells for a flow analysis of Warfarin in pure form and pharmaceutical formulations

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Abstract. Continuous turbidimetric analysis (CTA) for a distinctive analytical application by employing a homemade analyser (NAG Dual & Solo 0-180°) which contained two consecutive detection zones (measuring cells 1 & 2) is described. The analyser works based on light-emitting diodes as a light source and a set of solar cells as a light detector for turbidity measurements without needing further fibres or lenses. Formation of a turbid precipitated product with yellow colour due to the reaction between the warfarin and the precipitation reagent (Potassium dichromate) is what the developed method is based on. The CTA method was applied to determine the warfarin in pure form and pharmaceutical formulations in the concentration range from 2.0-16 & 0.7-16 mmol/L with 0.58 and 0.55 mmol/L of the limit of detections. The correlation coefficients (r) of the developed method were 0.9977 and 0.9981 for cell 1 and 2 respectively. For validation of proposed method, the ICH guidelines were followed. The developed method was successfully applied for the determination of Warfarin in pure and pharmaceutical preparations. In addition, the method can be considered as a quality control method and conveniently used for routine analysis in laboratories since the method permits quantitatively determination of 60 samples/h.

1. Introduction
\[3-(alpha-acetonylbenzyl)-4-hydroxycoumarin], (Figure 1). In early 1950, It was approved in the US for being used as an anticoagulant (preventing thrombosis and embolism), and has remained the most popular and prescribed anticoagulant drug ever since [1]. In spite of its effectiveness, warfarin interacts with many widely used medications and some foods. Thus, it has several shortcomings and must be accurately monitored in the blood to ensure the safe dosages are taken. Decreasing the blood coagulation by warfarin is performed by the inhibit vitamin k1 reductase, this enzyme recycles the oxidized form of vitamin k1 which participates in the carboxylation of many blood coagulation proteins like factor VII. and prothrombin to its reduced form. For this reason, warfarin is also used as a function of vitamin K1 antagonist [2]. In the literature, several analytical methods have been reported for qualitative determination of warfarin in the pharmaceutical preparation and biological fluids. For example, LC-MS-MS [3], UPLC-MS-MS [4], micellar electro kinetic mass spectrometry [5], chiral HPLC [6], and dissolution methods [7] have been applied for warfarin analysis. The determination of warfarin in plasma has also been reported by using direct HPLC methods [8-10], indirect methods where a chiral stationary phase is used [11-15]. Nowadays, the most common method is the direct injection of plasma into the reverse phase [16].
summarizes different types of HPLC methods for the quantification of warfarin which found in the literature. Majority of the methods used fluorescence, MS and UV absorbance as detectors.

![Figure 1. The chemical structure of Warfarin](image)

**Table 1. Shows previously reported HPLC methods for determination of Warfarin in tablets**

| Detection          | Internal standard          | Type          | Column (stationary phase) | Ref. |
|-------------------|---------------------------|---------------|---------------------------|------|
| Fluorescence      | Benoxaprofen              | Chiral RP     | BSA – ISRP                | [11] |
| Fluorescence      | Naproxen                  | Chiral RP     | AGP                       | [14] |
| UV absorbance     | Oxybenzone                | Chiral RP     | β-Cyclodextrin             | [17] |
| UV absorbance     | Naproxen                  | Achiral RP    | C18                       | [18] |
| Mass spectrometry (LC/MS) | deuterium-labeled 7- hydroxywarfarin | Achiral RP | Luna C18 | [19] |
| GC-MS             | p-chloro warfarin         | Achiral RP    | β-Cyclodextrin             | [20] |

The turbidity concept is an analytical technique which has a wide range application in the determination of lots of active ingredient in pharmaceutical formulation. A great number of drugs have been determined using turbidity technique like Ephedrine Hydrochloride [21], Ciprofloxacin HCl [22], Cypromeptidine hydrochloride [23] and Theophylline [24]. However, the determination of warfarin using turbidity method has never been reported in the literature. The main objective of the proposed method was to develop and validate a new approach that combines the flow analysis technique with turbidimetric detection allowing the simultaneous quantitative determination of warfarin in pharmaceutical formulations for the same injected sample. Therefore, in the present work, a new, two lines manifold system for flow injection analysis is developed in which two consecutive detection zones (cells 1 & 2) for the same injected sample were applied using NAG dual & solo (0-180°) detector. This type of manifold is used for the chemical reactions when the warfarin is injected into the carrier streamline (distilled water) prior to mixing with the precipitation reagent line (sodium persulfate). The two lines are merged at Y junction point to form a yellow ion association complex due to the reaction between the warfarin and the potassium dichromate, the proposed reaction is shown in Figure 2. The intensity of the complex is detected by two consecutive detection zones (cells 1 & 2). Thus, two peaks will be obtained for any injected sample. Therefore, a new manifold system for the flow injection spectrophotometric analysis was successfully presented and applied for the quantitative determination of warfarin in different commercial tablets. The proposed method is rapid, simple, economic and more sensitive than papers reported earlier.
2. Materials and Methods

2.1. Material
All the standards materials of Potassium dichromate, Potassium bromide, Potassium nitrate, Potassium nitrate, Sodium nitrite, Sodium chloride, ammonium chloride and the solvents such as sulfuric acid, nitric acid, acetic acid and hydrochloric acid were with purity higher than 98% and procured from Sigma Aldrich. Double-distilled water was used in all the preparations of aqueous solutions. Samara company for pharmaceutical industries, Ltd. Iraq supplied this study with the pure material of standard warfarin, while the warfarin tablets under their brand names were procured from the local market.

2.2. Preparation of experiment solutions (Standard and sample)
Warfarin stock solution 20 mmol/L was prepared by dissolving of 1.54 g in 250 ml volumetric flask and filled to the label with double distilled water. An amber bottle was used to keep the solution away from the light and use it in a further experiment. A series of warfarin standard solution were prepared by dilution appropriate volumes of stock solution with a double distilled water to obtain standard solutions ranging from 0.25-25 mmol/L. UV spectrophotometry measurements were performed to the stock solution before each experiment in order to monitor the stability of the warfarin since some drugs become unstable in their solutions. The results showed there is no decomposition (did not break into other substance which means the warfarin has high stability) in the warfarin during the proposed method.

2.3. Preparation of samples solutions (Tablets)
In this study, two of warfarin commercial companies (Warfarin ®, Bristol, UK) and (Orfarin ®, Orion, Finland) were investigated. Twenty tablets were precisely weighed, powdered and smoothly mixed. The average weight of the tablet was accurately dissolved in double distilled water, mechanically shaken for 30 min and filtered. The residue from the filtration was washed four times with the distilled water and transferred to a volumetric flask and diluted to the mark with distilled water. The prepared solution was used for further experiments by further dilution of previously prepared warfarin standard solution. Each of the commercial tablet types has followed the above procedure for sample solution preparation.

2.4. Apparatus
All the turbidimetric measurements were performed using a homemade optoelectronic detector integrating a flow injection system [25, 26]. The detector contains two identical twin cells i.e., cell no. 1 and cell no. 2, each one of them has 100 mm length, and in between of them there is a 20 mm without any detection as shown in Fig 3. The light source in the detector unit is white snow led, each measuring cell contains 10 white snow LED (WSLED) as an irradiation source (blue-violet 42.4%, Green 56.73% and 1.15% Red). Each one of the WSLED irradiates a circle spot of 3mm diameter for the flow cell Oϕ 4mm (Outer diameter of the flow cell) and Iϕ 2mm (Internal diameter of the flow cell) of the active total distance of the cell. The inlet distance for each cell unit (100 mm) is covered by

Figure 2. The proposed reaction between the warfarin and potassium dichromate
3-solar cells with (37.8 mm (L)x10mm (W)x1mm, thickness) as cell dimensions. Identical twins’ solar cells are used with minimum 2.5VDC at ambient light. The integrated manifold flow system composed of two-channel Ismatec (Switzerland) model 796 peristaltic pump supplied with Tygon pump tubing was used for the propulsion of the fluids. Different lengths of Teflon tubing (0.8 i.d.) were used to connect the manifold parts together, mix and carry the flow streams. Sample injection was performed by a 6-way selection valve (Type Upchurch Scientific® Medium Pressure Valves) that supplied with an external sample loop style. The reactants were mixed at the Y junction point with 0.8 i.d. which was made from methyl methacrylate. The obtained profiles of the S/N against the time from the detector are shown in a chart (Figure 4).

2.5. Recommended Procedure

Warfarin determination was performed using a manifold analysis system contains two consecutive detection zones (cells 1 & 2), which allows simultaneous detection for the same injected sample. A series of physical and chemical parameters like salts effect, precipitation reagent concentration, flow rates, light intensity, mixing coil, the volume of the sample and purge time were optimized under flow injection conditions. The proposed method based on the forming of an ion association complex, this product is white and turbid and can be determined by the detectors. The formation of the product is conducted by mixing the sample with the presentation reagent. In the beginning, 150 μL of sample solution contains 20 mmol/L of warfarin is injected into the injection valve at a place called an injection loop. Then, the direction of the injection valve is changed to the injection mode. The water stream is carried out the injected sample to the junction point where is mixed with the reagent and a yellow precipitate turbid solution is formed. The formed colored solution is transported to the detection units by the stream. The carrier streamlines (distilled water) and the reagent line solutions (6.0 mmol/L) were propelled using a two-channel peristaltic pump at flow rates 2.8 ml/min and 3.4 ml/min for the carrier stream and precipitation reagent lines respectively. The absorbance of the formed yellow product is continuously recorded on the chart paper represented (first peak for detection cell1 and the second peak for the detection cell 2). These two peaks are responsible for the determination of injected sample concentration by treated them mathematically. Figure 4 shows the shape of the peaks obtained from the recorder. The concentration of warfarin is determined by plotting the calibration graph (peaks height 1, 2).
3. Results and discussion

The warfarin is found to be forming an ion association complex with potassium dichromate, Fig 2. illustrates the proposed reaction of the product. The product is a yellow, turbid and can be detected using the optoelectronic detector. The analysis system working under flow injection conditions; therefore, a series of physical and chemical parameters will be optimized.

3.1. Optimization of chemical parameters

3.1.1. Potassium dichromate concentration (precipitation reagent)

The stock solution of potassium dichromate was prepared by dissolving potassium dichromate in distilled water to obtain the concentration of 25 mmol/L. Further dilution by distilled water to the previously prepared stock solution is conducted to obtain the concentrations range from (0.3-8.0 mmol/L). The experimental conditions of the measurements were: warfarin 20 mmol/L, sample volume 150μL, light intensity (I=1, 4 for cell 1 and cell 2 respectively), flow rate of 3.2 ml.min⁻¹ for carrier stream and 4.3 ml.min⁻¹ flow rate for reagent and open valve mode. The obtained results showed there is a gradual increase in the peak height during increasing in the reagent concentration reaching 6.0 mmol/L of potassium dichromate. However, above 6.0 mmol/L there was insignificantly increased in the peak height which probably indicates there is no increase in the concentration of formed product at these concentrations. Therefore, the 6.0 mmol/L of potassium dichromate (precipitation reagent) was chosen to be the optimum concentration for further experiment as shown in Figure 5.

![Figure 4. The signals profiles of the S/N against the time obtained for warfarin at 4.0 mmol/L](image-url)
3.1.2 Effect of aqueous salt solutions

Using different aqueous salts solutions (H₂O, NH₄Cl, NaCl, KBr, CH₃COONH₄, NaNO₂, KNO₃, HCl, HNO₃ and CH₃COOH) instead of the distilled water as a carrier stream were carried out to investigate whether using these solutions can enhance the concentration of the formed product. The experimental conditions of the experiment were: potassium dichromate 6.0 mmol/L, the flow rate of the carrier stream 3.3 ml.min⁻¹, while 4.3 ml.min⁻¹ flow rate for reagent with sample volume 150μL, light intensity (I=1, 4 for cell 1 and cell 2 respectively), and open valve mode. From the results, it can be observed that there is an increase in sensitivity of detector during using the distilled water as a carrier stream compared with these aqueous salts solutions which are caused to decrease in the detector sensitivity; and this might be attributed to disperse of precipitate particulate in the presence of salts. Therefore, distilled water was chosen as the optimum medium (carrier stream) (Figures 6 A and B).

3.2 Optimization of physical parameters

3.2.1 The effect of using variables intensities of white snow light emitting diodes (WSLEDs)

Each cell in the detector has 5 levels of light intensity ranging from 1-5 (lowest-highest) in addition to the off position for both cells which are individually controlled which means each one of the instrument cells has its own selector switch of the light source. In order to determine the optimum light
intensity for each cell (1 & 2), variables intensities of the incident light of cell 1 and 2 were carried out. Under applied following experimental conditions: 20 mmol/L of warfarin, 6.0 mmol/L of potassium dichromate, 3.3 & 4.3 ml.min$^{-1}$ flow rates for carrier stream (distilled water) and reagent respectively with sample volume 150 µL and open valve mode, the light intensity experiment was performed. From the obtained results it was observed that, for the cell 1, the favored light intensity was I=1, and this is may be attributed to the nature of the formed precipitate particles which means the well-distributed particles with small size can leave very short spaces between them during passing through the cell 1, therefore, using a low intensity of light source can detect these particles and obtaining a signal instead of using high intensity of the light source which cannot detect these small sizes of particles. On the other hand, cell 2 preferred intensity of light I= 5, and this is can be explained by the same fact, during passing through the cell 1, the particles will start to growing up and suffering from conglomeration and as a result of that, the particle size will be increased. At this point using a low intensity of light source cannot be useful therefore, the light intensity of the light source must be high. Thus, the intensity of light (I=1, 5) was chosen to be the optimum intensity for further experiment for cell1 and 2 respectively as shown in Figure 7.

3.2.2. Effect of flow rate

In order to choose the optimum flow rate for both of the carrier stream-line and the precipitation reagent line 6.0 mmol/L potassium dichromate, ranges of flow rates (0.5-7.0 ml min$^{-1}$) have been applied for the carrier stream and the precipitation reagent lines. While the other experimental conditions i.e.; warfarin 20 mmol/L, 150µL sample volume, light intensity (I=1, 5 for cell 1 and cell 2 respectively) and open valve mode are kept constant. The obtained results have shown that at a low flow rate for both of the carrier stream-line and the reagent line there is a gradual increase in detector signal for cell 1 & 2 up to (2.8 & 3.4 ml min$^{-1}$) for stream-line and reagent line respectively and the bases of these responses are wide. At a high flow rate (i.e.; more than 2.8 and 3.4 for both lines carrier stream and precipitation reagent respectively), the detector signal of these flow rates have decreased and became very sharp with the base of these peaks became narrow, because the precipitated particulates are moved faster and take only a very short time to passing in front of the measuring cells. Therefore, the flow rates 2.8 & 3.4 ml/min for stream-line and reagent line respectively were chosen to be the optimum flow rates (Figure 8).
The effect of the sample volume in CFIA

In this experiment, variable of sample volumes ranges from (32-250 µL) using Teflon tubes (4.07-38.17 cm) with 0.5 mm as a diameter were used. The results showed there is an increase in the peak high during the increase in the sample volume up to 150 µL, at this point, the maxima peak high was recorded as sample volume 150 µL (22.45 cm as a length of sample segment). Therefore, 150 µL has chosen to be the optimum sample volume and use it in further experiments as shown in Figure 9.

![Figure 8](image1.png)

**Figure 8.** The effect of flow rates

![Figure 9](image2.png)

**Figure 9.** The effect of sample volumes
3.2.4. The effect of mixing coil

In order to check whether the flow system requires adding a mixing coil to ensure a maximum reaction between the warfarin and potassium dichromate or not, under optimization conditions, mixing coils (made from Teflon, 0.5 mm i.d.) with different length of 10, 15, and 20 cm were used. The results showed there is no improvement in the sensitivity of the peak height has recorded in comparison with the system without using the mixing coil which indicates that the reaction between the warfarin and the potassium dichromate is already complete and the manifold system does not need any mixing coil. (See Figure 10).

3.2.5. Purge time of the sample segment

The purge time by definition, is the required time that the sample segment needs to leave from the injection valve, mixing with the reagent and reaching the detection units. Therefore, periods of times ranged from (3-30 sec and open valve mode) were studied. The results have shown that 10 sec is the time that the sample segment requires to leave from the injection valve towards the Y-junction. Therefore, from 10- open valve mode considered as the optimum purge time of the sample segment and it can choose any one of those times that fall in that range as shown in Figure 11.
3.3. **Validation parameters of proposed methods**

The proposed method has been validated based on ICH guidelines [27]. Therefore, the developed method was validated for precision, accuracy, specificity, linearity, and LOD.

3.3.1. **Calibration curve and the limit of detection (LOD)**

It is a general procedure which is used for determine the concentration of a chemical substance in an unknown sample by establishment a set of known concentration and comparing with the unknown. By applying all of the optimized analytical parameters of the proposed method, the calibration curve was constructed by recording the attenuated light peak heights for various concentrations of the warfarin. The linear region of the curve for warfarin has shown in the range of 2.0-16 mmol/L, 0.7-16 mmol/L with ($R^2=0.9963$ & 0.9956) for both cell 1 & 2 respectively as shown in Fig 12. While the limits of detection were calculated based on the following formula:

$$\text{LOD} = 3.3 \frac{S_a}{b}$$

Where: $S_a$ = the standard deviation ($S_D$) of the Y-intercept and $b$ = the slope which can be estimated from the calibration curve. Note that, the $S_a$ is usually meant (RMSE) the root mean squared error or SD of the residuals which can be taken from the regression line. Table 2 describes the summary of linear regression lines.

**Figure 12.** The linear calibration curves for determination of warfarin for measuring cell 1 & 2 (A&B) respectively.

**Table 2.** The summary of linear regression lines of the proposed method

| Parameter                  | Obtained Value | Proposed method (cell 1) | Proposed method (cell 2) |
|----------------------------|----------------|--------------------------|--------------------------|
| Linearity (mmol/L)         | 2.0-16         | 0.7-16                   |
| Regression equation        | $33.177+15.534 [War.] (mmol/L)$ | $63.629+46.534 [War.] (mmol/L)$ |
| Slope                     | 15.534         | 46.534                   |
| Intercept                 | 33.177         | 63.629                   |
| Correlation coefficient, $r$ | 0.9981         | 0.9977                   |
| coefficient of determination, $R^2$ | 0.9963         | 0.9956                   |
| LOD (mmol/L)               | 0.5773         | 0.5511                   |

3.3.2. **Specificity & interferences**

Based on the official guideline of the ICH Q2 in method validation, the specificity can be defined as the ability of the method to assess the analyte in its matrix in the other words, in the presence of other components. The effect of interference of excipients on the analysis of warfarin in the tablet’s dosage was carried out by the analysis of sample prepared with all potential the excipients that present in the tablets (a mixture of excipients) such as Lactose, Sucrose, Starch, Gelatin and Magnesium Stearate but without the warfarin. The response of the instrument (peak height) did not show any potential interference of the tablet’s excipients (See table 3).
Table 3. The effect of the interferences on the determination of warfarin (5.0 mg)

| Excipients        | Using 8.0 mmol/L of warfarin (measuring cell 1) | Using 8.0 mmol/L of warfarin (measuring cell 2) |
|-------------------|-----------------------------------------------|-----------------------------------------------|
|                   | Fold added (mmol/L) | % E (mmo/L) | Added % E | Fold added (mmol/L) | % E (mmo/L) | Added % E |
| Gelatin           | 2.0 | 8.02±0.14 | 0.33 | 2.0 | 8.08±0.10 | 1.33 |
| Magnesium stearate| 2.0 | 8.05±0.12 | 0.83 | 2.0 | 8.02±0.18 | 0.33 |
| Lactose           | 2.0 | 8.10±0.18 | 1.66 | 2.0 | 8.10±0.19 | 1.66 |
| Sucrose           | 2.0 | 7.99±0.15 | -0.16 | 2.0 | 8.12±0.18 | 2.0 |
| Starch            | 2.0 | 7.98±0.02 | -1.5 | 2.0 | 7.95±0.03 | -0.83 |
| Calcium carbonate | 2.0 | 8.10±0.18 | 1.66 | 2.0 | 8.10±0.19 | 1.66 |
| All above         | 2.0 | 8.05±0.11 | 1.16 | 2.0 | 8.13±0.06 | 2.16 |

3.3.3. Precision and accuracy

The accuracy can be defined as the closeness of the agreement in the values between the found value and an accepted reference or true value in an analytical procedure. While the precision knows as the closeness of the agreement between the values obtained from multiple measurements of the same homogeneous sample using the prescribed conditions. For the proposed method, three sets of diluted concentrations (6.0 and 8.0 mmol/L) were prepared and each one of them was injected and determined on 5 consecutive days. These values represented the inter-day precision, while the intra-day precision was evaluated by determination of three concentrations of each drug on five successive occasions. The RSD values of both inter-day and intra-day have been used for expressing the precision. RSD % of the obtained data have found to be 0.14-1.95 % and 0.24-1.61 for inter and intraday precision respectively. The error % was used for expressing the accuracy which was ranged from 1.5-3.16 % and 1.63-3.16 % for intra-day and inter-day respectively, as shown in Table 4.

Table 4. The inter- and intra-day assay of precision and accuracy of the proposed method for the determination of warfarin

| Taken of warfarin (mmol/L) | Intra-day (n=5) | Inter-day (n=5) |
|---------------------------|----------------|----------------|
|                           | Found [Warfarin ±SD] (mmol/L) | RSD % | Accuracy as E % | Found [Warfarin ±SD] (mmol/L) | RSD % | Accuracy as E % |
|                           | Cell 1 | Cell 2 |        |                  | Cell 1 | Cell 2 |        |
| 6.0                       | 6.07±0.005 | 0.14 | 1.91 | 6.08 | 0.27 | 2.10 |
|                           | 6.09±0.02 | 0.56 | 2.33 | 6.09 | 0.40 | 2.33 |
| 8.0                       | 8.09±0.01 | 0.16 | 1.50 | 8.09 | 0.24 | 1.63 |
|                           | 8.10±0.02 | 0.43 | 1.66 | 8.10 | 0.33 | 1.66 |

3.4. Application of the proposed method to determine of warfarin in tablets

The proposed method was successfully applied for the determination of warfarin in its commercial tablets of two different companies (Warfarin ®, Bristol, UK) and (Orfarin ®, Orion, Finland) which comprise (5.0 mg of warfarin). The standard addition method was followed by injection of six samples and the results were satisfied and in the excellent an agreement with the label claims and with the official USP method as shown in Tables 5. The one-sample t-test and F-test were carried out for the obtained results. The t-test analysis has shown that there is no a significant difference were found between the results obtained by proposed method and the label claims for the same batch. The given values by the proposed method were also compared with the official method using the F-test. The statistical evaluation (F-test) has shown that there was no significant difference between the methods used as shown in Table 5.
Table 5. Determination of commercial tablets containing 5.0 mg warfarin using the proposed and official methods

| Brand name (tablets) | Nominal amount, mg/tablet | UV-spectrophotometry | Found b (n=3) |
|----------------------|---------------------------|----------------------|---------------|
|                      |                           | Official method [28]  | developed method | developed method |
|                      |                           | developed method 1st cell | 2nd cell |
| Warfarin ® (Bristol, UK) | 5.0 | 0.160 | 1.245 | 100.04±0.03 | 100.63±0.12 | 101.36±0.26 |
| Orfarin ® (Orion, Finland) | 5.0 | 0.010 | 1.885 | 101.12±0.24 | 103.39±0.03 | 103.49±0.06 |

* 2.056 and 2.57 are the tabulated values of both t and F at P=0.05 (95%C.I) respectively [29].
* b mean found ± SD

4. Conclusion
A simple and sensitive turbidimetric method was developed and validated for the determination of warfarin. The warfarin has been determined in tablets using two consecutive detection units (cell 1 & 2). The detection units have given accuracy and trust ability to the method since each of injected sample is determined twice at the same time. The proposed method is rapid, accurate, economics and does not require any pre-treatment in comparison with the reported methods. By virtue of its rapidity and simplicity, the proposed method can be applied for routine analysis in a laboratory as a quality control method since the method permits quantitively determination of warfarin (60 samples/hr.). The method also does not require any toxic chemicals or organic solvents, free from the interference, and its procedure meets the requirements of green chemistry. Generally, the proposed method should be used for the determination of warfarin in tablets.

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