Newly developed method for the determination of sitagliptin phosphate using rubeanic acid as a precipitating agent via the use of ISNAG continues flow fluorimeter

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ABSTRACT

A new, a simple, sensitive and fast developed method for the estimation of sitagliptin phosphate in pure and pharmaceutical drugs (tablet). This method based on the reaction between sitagliptin phosphate and rubeanic acid to form reddish-black ppt, using ISNAG-Fluorimeter analyzer via the measure of diverged light at 90° from radiation source which is used a range of high intensity ultraviolet 184.9 nm & 253.7 nm by low-pressure mercury lamp while detector cell can detect at 410-1150 nm. The resultant of diverged light by an interaction between incident photons and precipitated particles giving rise to a longer wavelength that the detector can detected. Chemical and physical parameters were studied and optimized. The calibration graph was linear in the range of (0.03-13 ) mmol.L$^{-1}$, with correlation coefficient (r) = 0.9881, linearity percentage $r^2\% = 97.64$, L.O.D = 0.7848μg/sample and RSD lower than 0.6% for (3&7) mmol.L$^{-1}$ (eight replicates) concentration of sitagliptin phosphate. This method was successfully applied for the determination of sitagliptin phosphate in two different companies of pharmaceutical drugs. A comparison was made between the newly developed method analysis with the classical method (UV-spectrophotometer by measuring of absorbance) using standard addition method via the use of t-test, it was noticed that there was no significant difference between two methods at %95 confidence level.

INTRODUCTION

Sitagliptin Phosphate is described chemically 1,2,4triazolo[4,3-a] pyrazine, 7-[[3R]-3amino-1-oxo-4-(2,4,5triﬂuorophenyl)butyl]-5,6,7tetrahydro-3-(trifluoromethyl) phosphate. The structure is given has been illustrated in Figure 1.

Molecular weight-523.32 gm/mol

It is the first and simplest prescription medicinal drug in a novel magnificence of the oral antihyperglycemic agents, enhancing the ability of the body in lowering the glucose of the blood in the case where it is elevated. The therapeutic aggregate in the second Type is orally using lively inhibitors Dipeptidyl Peptidase-4 (DPP-IV) (Herman et al., 2007; Green et al., 2006) such as the Sitagliptin Phosphate. It’s an oral anti-diabetic medication (Gallwitz, 2007; Aschner et al., 2006) that allow to govern the levels of the blood sugar through the regulation of the levels of insulin within the body. (Raz et al., 2006; Koenig et al., 1976).

Literature review revealed the estimation of STP in dosage form both alone or combined form by spectrophotometric (Mohanty et al., 2017; Lotfy et al., 2015), derivative spectroscopy (Jeyabalan et al,
This study offers a new method into determining the STP in Pharmaceutical preparations using Continuous Flow injection through the use of ISNAG Fluorimeter Instrument (Turkie and Abd-Alrazack, 2018, 2019).

**EXPERIMENTAL**

**Reagent and chemical**

Every chemical has been used of the analytical-reagent and the distilled water turned into used for the preparation of every solution. A standard solution 0.1mol/L of sitagliptin phosphate molecular formula C_{16}H_{20}F_{6}N_{5}O_{6}P, M.wt. 523.23 g/mole and SDI-Iraq become prepared through dissolving 5.2323 g in 100 ml of the distilled water. A stock solution 50mmol/L of rubeanic acid molecular formula C_{2}H_{4}N_{2}S_{2} M.wt. 120.2 g/mole has been produced through the dissolving 3.005 g in 500 ml of sodium hydroxide (0.01N).

**Sample preparation**

20 tablets have been weight and later crushed and ground. Tablets which contain 100mg of sitagliptin phosphate have been weighted 2.8911 g, 2.17307 g (equivalent to 0.5232 g of the active ingredient, 10 mmol/L) for sitavi- Iraq and Januvia- MSD-UK, respectively. Everyone from the two types of drug which have been dissolved in distilled water. This solution has been filtered in order to cast off the undissolved materials, the residue has been washed by the distilled water and the quantity has been completed to 100 ml by distilled water.

**Apparatus**

The response was measured by a homemade ISNAG-fluorimeter. A low-pressure mercury lamp is used, which is characterized by two lambdas (184.9 & 253.7) nm. While the detector that is been used was a 2[4 x 2.5cm] solar cell. The flow device used to determination of sitagliptin phosphate be seen in Figure 2. Peristaltic pump 2 channels with different speeds (Ismatec, Switzerland). Valve six-port medium pressure injection valve (I D E X corporation, U.S.A.) with the loop of the sample (1 mm i.d. Teflon, different length )2[4 x 2.5cm] solar cells are utilized as a detector to collect the signal via sample travel through a line of 2mm optical aperture extended for 100mm distance. The signals of the output have been recorded through the potentiometric recorder (Siemens, Germany) (1V- 5 V, 1000 mV-5000 mV). The peak height has been evaluated for every one of the signals. UV-Spectrophotometer digital double beam type (UV-Vis spectrophotometer, UV-1800, Shimadzu, Japan) has been applied as well for scanning the sitagliptin phosphate spectrum, 4cm quartz.

**METHODOLOGY**

Using the optimum parameters for sitagliptin phosphate (7 mmol.L^{-1}, 150 μL, open valve mode) and rubeanic acid (1mmol.L^{-1})as precipitating reagent. A design system of manifold consisting of two lines (Figure 2) was used.

The 1st line is the carrier stream (KCl 0.07 mmol.L^{-1}, 1.5 ml/min) that will introduce the sample segment into the reaction stream by combined with the second line (rubeanic acid 1.6 ml/min) to form a reddish-black ppt at Y-junction.

The precipitate measured by using ISNAG-Fluorimeter via low-pressure mercury lamp it gives two main wavelengths, namely 184.9nm and 253.7nm. These both two lines are easily diverged due to its high frequency. The divergence of this beam of the incident light will be detected at 90° through a flow cell of 2mm path length that extends for 100mm distance by using 2[4 x 2.5cm] solar cell. Scheme 1 indicates a suggested mechanism for the reaction of sitagliptin phosphate–rubeanic acid.

**RESULTS AND DISCUSSION**

**Chemical and physical variations**

**Chemical parameters**

**Rubeanic acid concentration**
Table 1: Effect of rubeanic acid concentration on response function expressed as an average peak height

| Concentration of Rubeanic acid in mmol.L⁻¹ | Υ̇_{im}V (n=3) | R.S.D% | C.I | Υ̇_{im}V ± t_{0.025, 2} σn-1/√n |
|-------------------------------------------|---------------|--------|-----|--------------------------------|
| 0.1                                       | 422           | 0.29   | 422± 3.04 |
| 0.5                                       | 540           | 0.1    | 540±1.34 |
| 0.7                                       | 704           | 0      | 704±0    |
| 1                                         | 830           | 0.07   | 830±1.44 |
| 3                                         | 422           | 0.13   | 422±1.36 |

Υ̇_{im}V = Output of response, C.I = confidence interval

Table 2: Effect of various salt as a carrier stream on response function expressed as an average peak height

| Salt as carrier stream | Υ̇_{im}V (n=3) | R.S.D% | C.I | Υ̇_{im}V ± t_{0.025, 2} σn-1/√n |
|------------------------|---------------|--------|-----|--------------------------------|
| H2O                    | 832           | 0.09   | 832±1.86 |
| NaCl                   | 225           | 0.25   | 225±1.40 |
| NaNO3                  | 423           | 0.14   | 423±1.47 |
| KCl                    | 600           | 0.15   | 600±2.24 |
| KBr                    | 522           | 0.13   | 522±1.69 |
| NH₄Cl                  | 432           | 0.22   | 432±2.36 |

Table 3: Effect of potassium chloride on response function expressed as an average peak height

| [KCl] mmol.L⁻¹ | Υ̇_{im}V (n=3) | R.S.D% | C.I | Υ̇_{im}V ± t_{0.025, 2} σn-1/√n |
|---------------|---------------|--------|-----|--------------------------------|
| 0.05          | 530           | 0.12   | 530±1.58 |
| 0.07          | 840           | 0.10   | 840±1.99 |
| 0.1           | 600           | 0.11   | 600±1.64 |
| 0.3           | 432           | 0.31   | 432±3.33 |
| 0.5           | 298           | 0.23   | 298±1.70 |

Table 4: Effect of flow rate on the response function expressed as an average peak height

| Flow rate (ml/min) | Υ̇_{im}V (n=3) | R.S.D % | C.I | Υ̇_{im}V ± t_{0.025, 2} σn-1/√n | Δt₀ (Sec) |
|--------------------|---------------|--------|-----|--------------------------------|-----------|
| 0.9                | 542           | 0.17   | 542±2.29 |                             | 80        |
| 1.2                | 840           | 0.32   | 840±6.68 |                             | 50        |
| 1.3                | 1090          | 0.20   | 1090±5.416 |                          | 30        |
| 1.5                | 1080          | 0.16   | 1080±4.293 |                           | 25        |
| 1.7                | 998           | 0.20   | 998±4.95 |                             | 20        |
| 2                  | 840           | 0.17   | 840±3.55 |                             | 18        |
Table 5: Effect of sample volume & purge time on the response function expressed as an average peak height

| Sample volume (μL) | The output of response ($\bar{Y}_{imV}$) (n=3) |
|-------------------|-----------------------------------------------|
|                   | Lag time (Sec)                                |
| r= 0.5 mm         |                                              |
| 50                | 10 518 30                                   |
| 75                | 910 865 1070                                 |
| 100               | 1030 1130 1150                               |
| 150               | 1200 1289 1320                               |
| 200               | 1170 1288 1300                               |

Table 6: Summary of results for the determination of sitagliptin phosphate via the use of ISNAG-fluorimeter.

| Method              | [STP] mmol/L | r      | r²     | t-value at 95% , n-2 | Linear regression equation at 95% , n-2 | $\bar{Y}_{i(mV)}$ = a±s_a t + b±s_b t [x] |
|---------------------|--------------|--------|--------|----------------------|------------------------------------------|------------------------------------------|
| ISNAG-fluorimeter   |              |        |        |                      |                                           | 163.334±132.10+147.018±17.785[X]         |
| Measured Linear range | 0.03-15     | 0.9862 | 0.9776 | 2.28 <<               | 18.8403                                  |                                           |
|                     | n= 12        |        |        |                      |                                           |                                           |
|                     | 0.03-13      | 0.9881 | 0.9764 | 2.26 <<               | 19.2963                                  |                                           |
|                     | n= 11        |        |        |                      |                                           |                                           |

[x] = STP mmol.L⁻¹

Table 7: Repeatability & detection limit for STP at optimum parameters via diverged of light measured at 90° expressed in mV.

| [STP] mmol/L | Repeatability at 95% confidence interval | RSD% | Detection limit |
|--------------|-------------------------------------------|------|-----------------|
|              | $\bar{Y}_{i(mV)}$ ± t₀.₀²₅ , 2 σn⁻¹/√n |      | Theoretically based upon the value of the slope x=3S_B/slope for n=13 |
| 3            | 642±3.02                                   | 0.58 | 0.5227 μg/150μL |
| 7            | 1326±4.61                                  | 0.43 | 0.7848 μg/150μL |

X: Represents the value of the L.O.D. based on the slope, $S_B$: Standard deviation of the blank repeated for 13
Table 8: Results of Standard addition for the estimation of STP in two pharmaceutical preparation

| No. of sample | Commercial name | Sample weight equivalent to 0.5233gm (10 mmol.L\(^{-1}\)) of the active ingredient (g) | A new method using ISNAC-Fluorimeter UV-Spectrophotometer (classical method absorbance measurement at \(\lambda_{\text{max}}=267\) nm) |
|---------------|-----------------|---------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------|
|               | Company         | Country                                                                         | STP-phosphate [mmol.L\(^{-1}\)]                                                                 | Equation of standard addition at 95% for n-2 practical concentration mmol.L\(^{-1}\) |
|               | Content         |                                                                                  | \(\hat{Y}_{i}(mV)=a\pm s_{a}t+b\pm s_{b}[x]\) \(\hat{Y}_{i}=a\pm s_{a}t+b\pm s_{b}[x]\) | \(r,r^{2}\)% \(r^{2}\)% |
| 0             | 1               | 1.5 2 2.5                                                                         | 0 0.5 0.7 0.9 1                                                                                 | 10 ml 100ml 25ml 100ml |
| 1             | Sitavia         | 2.8911 200 418 520 590 700                                                       | 209.838±39.495+196.973±24.034[X]                                                                 | 0.9978 0.9956 0.9956 0.9956 |
|               | 100 mg          | Iraq                                                                             | 0.514±0.1001+1.041±0.141[X]                                                                     | 0.9973 0.9946 0.9946 0.9946 |
|               | Pioneer         | 99.56                                                                             | 99.46                                                                                           | 99.46 99.46 |
| 2             | Januvia         | 2.1731 160 344 423 510 600                                                       | 162.703±12.006+174.784±7.306[X]                                                                  | 0.9997 0.9995 0.9995 0.9995 |
|               | 100 mg          | MSD 160 344 423 510 600                                                          | 0.604±0.079+1.1834±0.112[X]                                                                       | 0.9987 0.9973 0.9987 0.9973 |
|               | Januvia         | 99.95                                                                             | 99.73                                                                                           | 99.73 99.73 |

\(\hat{Y}_{i}\) = Estimated response in mV for ISNAC-Fluorimeter and absorbance for UV-SP method, 
\([x] = [\text{STP}] \text{ mmol/L}\) 
\(r\) = Correlation coefficient, \(r^{2}\)% = Linearity percentage
Table 9: Paired t-test, practical content and efficiency of determination for estimation of STP in two samples of pharmaceutical preparation.

| Sample no. | A confidence interval for the average weight wt | The theoretical content of the active ingredient at 95% (mg) | Practical content wt (mg) | Efficiency of determination (Rec. %) | Individual comparison (Rec.-μ) tcal at 95% | Paired t-test Compared between two methods |
|------------|-----------------------------------------------|------------------------------------------------------------|--------------------------|-------------------------------------|------------------------------------------|--------------------------------------------|
|            | 3.840/1.96 at 95% (g)                         | μ±1.96/σn-1/√n                                           | 3.603/1.96               | ISNAG-Fluorimeter Analyser with a Quoted value t = 0.05/2 = 4.303 |
|            | UV-spectrophotometer (classical method)       |                                                           |                          |                                     |                                         |
| 1          | 0.5432±0.0032                                 | 106.506±5.135                                            | 106.53                   | 2.602< 7.706                        |                                         |
|            | 0.0032 0.7932                                 | 98.800±8.599                                            | 98.80                    | -0.6004/ < 0.6144 << 12.706        |                                         |
| 2          | 0.4083±0.0051                                 | 93.0952±11.892                                          | 93.09                    | -2.254/ < -                      |                                         |
|            | 0.0051 0.3101                                 | 102.030±15.860                                          | 102.03                   | 0.550 << 4.303                     |                                         |

Xd: Difference between two methods, Xd: difference mean, σn-1: Difference standard deviation, n= no. of sample =2

t0.025, ∞ = 1.96 at 95 %, μ: quoted value

A different concentration of rubeanic acid was used (0.1-3 mmol/L) as a precipitating agent at a flow rate of 1.2 ml/min for each line, using 75 μL sample volume and water as a carrier stream. Figure 3-A shows the response profile and the result obtained in Table 1, it was observed that an increase of diverged light expressing of peak height up to 1mmol/L for rubeanic acid (Figure 3-A). More than 1mmol/L lead to form a small size of particles, which in turn lead to a decrease in the amount of diverged light. Therefore, 1mmol/L for rubeanic acid (Figure 3-B) is the most suitable concentration.

Reaction medium used as carrier stream

A different solutions media were study as a carrier stream (NaCl, NaNO₃, KCl, KBr, NH₄Cl& H₂O ) in the reaction between STP (7 mmol/L) and rubeanic acid(1 mmol/L ) to form reddish-black ppt using sample volume 75 μL and flow rate 1.2 ml/min for carrier stream & reagent. Figure 4-A, B shows that the best media is KCl compared with other salts as a carrier stream, it might be due to aggregation precipitating of particles and increase of size lead to increase of scattered light on the detector. But other salts as a carrier stream lead to a decrease of light might be due to the form of small-sized particulate because of uncompleted growth form of particles. The obtained results were summarized in Table 2.

Effect of Potassium chloride concentration

Using experimental parameters (7mmol.L⁻¹ sitagliptin phosphate and 1mmol.L⁻¹ rubeanic acid at 1.2 ml/min flow rate of every line with 75 μL sample volume ).A variable concentration (0.05-0.5)mmol.L⁻¹ of potassium chloride solution were prepared to obtain the prefect concentration of it as a carrier stream. It was noticed that 0.07 mmol.L⁻¹ of KCl gives a height response; above that causes a decrease in the height of response(Figure 5-A, B). This effect might be due to the dissociation of
Figure 3: A) Time - profile of sitagliptin phosphate using a variable concentration of rubeanic acid B) Average peak heights vs. concentration of rubeanic acid

Figure 4: A) Time – profile using different salt solutions  B) Pie percentage explanation of the contribution of every salt
Scheme 1: A probable mechanism for the reaction of STP with rubeanic acid

Figure 5: A) Effect of potassium chloride utilized as carrier stream on response profile B) Average peak heights vs. concentration of potassium chloride
Figure 6: A) Diverged light vs. time profile using a variable flow rate B) Plot average peak heights vs. flow rate of the carrier stream

Figure 7: Effect of lag time & sample volume on, A) Response profile B) The output of a diverged beam of incident light using STP [7mmol.L\(^{-1}\)] - rubeanic acid [1mmol.L\(^{-1}\)] system
large particulate into small size than the falling wavelength, which leads to not detected by solar cells. The results were tabulated in Table 3.

Effect of flow rate

A changble of flow rates ranging (0.9- 2)ml/min for a carrier line and ( 1.0-2.1) ml/min for reagent line were used for sitagliptin phosphate (7mmol/L,75µL  )- rubeanic acid (1mmol.L⁻¹) system and open valve mode. Figure 6-A shows at low flow rate wide profile response were obtained, this might be attributed to the increased dispersion which in turn will increase the area of precipitated particle segment in a flow cell . while at higher flow rate; an increased peak response were obtained up to 1.3 ml/min followed by a decrease in response as not enough time is given to detect the variation in carrier stream composition. Figure 6-B shows that the flow rate 1.5, 1.6 ml/min for carrier & reagent line will be used to obtain a regular response. The acquired results were summarized in Table 4.

Physical parameters

Effect of sample loop & lag time

The study was carried out using variable sample volume (50,75,100, 150 & 200 µL) and lag time (10 sec, 30 sec & open valve mode), keeping all other variables were unchanged. It was noticed that an increase of peak height with an increase of sample volume up to 150 µL (Figure 7 A, B) at a similar time using the different lag time. The effect increases with an increasing either sample volume & lag time reaching up to 150µL and open valve due to irregular agglomerate formation of precipitate particulate, which leads to an increase of light at 0-90°to the solar cells. The obtained outcome were summarized in Table 5.

Calibration graph

Using the optimum conditions, a set of STP concentrations vary from (0.03-15) mmol/L were prepared to require to prepare a scatter plot graph followed by choice of calibration graph, Figure 8-A shows response profile for this study. While Figure 7-B shows the variation of scattered diverged light using ISNAG–fluorimeter with STP concentration. It was noticed that the linear calibration graph ranging from (0.03-13) mmol/L with correlation coefficient r: 0.9881 in which that an increase of STP concentration leadsto an increase of solid crystalline precipitate having a smooth surface which works as a reflecting mirror to word the detector that will be measured at 90° according to instrument design. This method compared with the classical method through measurement of absorbance by UV-spectrophotometer at 267 nm (Anudeepa et al., 2015),Figure 8-C. The results tabulated as Table 6.

Repeatability & Detection limit
The repeatability was studied for the determination of STP via the measurement of diverged light at 90° for the reaction of STP with rubaneic acid at the concentration (3 & 7 mmol/L), as shown in Figure 9.

The L.O.D of STP calculates through two methods. Gradual dilution of low concentration in the calibration graph or primarily based on the numerical value of slope as to be seen in Table 7.

**The use of ISNAG-fluorimeter for the determination of STP in the pharmaceutical preparation**

The technique that has been adopted has been utilized to determine the STP in two different companies of pharmaceutical drugs (Sitavia, Januvia). Continuous flow injection analysis using homemade ISNAG-fluorimeter using a mercury lamp tube of low-pressure (UV-Light) and detection of diverged scattered lights (visible light) At 2 X 90° using multi solar cells that cover 2 X 100mm distance of 2mm path length. A set of solutions have been prepared for each one of the pharmaceutical drugs (0.01 mol/L).

Through the transfer of 1ml to every 5 volumetric flasks (10 ml), followed with the aid of the addition of gradual standard STP volumes (0, 0.20, 0.30, 0.40, and 0.50) ml of 0.05mol/L for the sake of obtaining (0, 1, 0.5, 0.7, 0.9 and 1) mmol/L when use ISNAG-fluorimeter, while transferring 1.25ml to every 5 volumetric flasks (25ml), followed with gradually adding standard STP volumes. (0, 0.25, 0.35, 0.45 and 0.5) ml of 0.05mol/L for obtaining (0, 0.5, 0.7, 0.9 and 1) mmol/L for using UV-spectrophotometer instrument (the conventional approach). The measures have been carried out
with the two approaches. Figure 10-A&B&C has shown the response profile for this research and the calibration graphs of the standard addition with the use of the ISNAG-fluorimeter. The results have been treated mathematically for the approach of the standard addition. Results have been listed in Table 8 at a 95% confidence level, which shows the practical concentration of the STP in every one of the pharmaceutical drugs with the use of 2 analysis approaches. Table 9 has illustrated a practical content of the active ingredient at a confidence level equal to 95% & efficiency of determination besides the paired t-test indicating a comparison at 2 different paths (Miller and Miller, 1988; Bluman, 1997).

First-The individual t-test
Comparing between a newly proposed approach with the use of ISNAG-Fluorimeter with a (100mg) official quoted value through the calculation of the t-value as has been listed in Table 9 column 6. It is worth to notice that calculating the t-value is less compared to the critical tabulated t-value, which implies the fact that there has not been any considerable difference between each individual company’s quoted value with t_{cal} at 95% confidence interval.

Second-The paired t-test has been utilized efficiently for comparing between the developed approach the utilization of the ISNAG-Fluorimeter CFIA with the classical approach, the result that has been obtained, indicated that there has been simply no considerable difference between the 2 methods because the calculation of the t-value (-0.0732) has been less than t_{tab}(4.303) for determining the STP in the pharmaceutical preparations at a confidence level of 95% as can be seen in Table 9.

CONCLUSIONS
The suggested method for the estimation of STP based on the formation of reddish-black for ion-pair between the drug(STP) and rubeanic acid in KCl medium and measured the diverged light at 90° via the use of a homemade ISNAG-fluorimeter. The technique is easy, sensitive, does not require reaction coil, costly chemical compounds and without involving any specific sample treatment. In addition to clean and inexpensive carryout. This technique used for determination of STP in nanograms for 150 μl sample volume in pure and pharmaceutical preparation.

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Conflict of Interest
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