Development and validation of three colorimetric charge transfer complexes for estimation of fingolimod as an antineoplastic drug in pharmaceutical and biological samples

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Abstract
An inexpensive, simple, sensitive and validated approach is developed for estimation of fingolimod through production of colored charge transfer complexes of fingolimod with different electron acceptor reagents, including a reaction of fingolimod as n-donor with 7,7,8,8-tetracyanoquinodimethane, tetrachloro 1,4-benzoquinone and tetracyanoethylene and as n-acceptors, yielding colored and stable anions which were measured spectrophotometrically. The range that obeyed Beer’s law is 50–300 µg mL−1 for fingolimod with all the studied reagents. The various parameters that affect the reaction were studied and optimized. The results were statistically compared with a reported method showing equal precision and accuracy. The researched approaches were utilized to determine the cited drug in its pharmaceutical form and spiked human plasma with accepted accuracy and precision.

Keywords: Fingolimod · TCNQ · TCNE · CL · CTC · Dosage form · Human plasma

Introduction
Fingolimod is an immune modulating medication which is utilized to treat multiple sclerosis, that is considered as the most familiar inflammatory anarchy of the central nervous system (Brinkmann et al. 2010, 2002; Chiba 2005; Salm et al. 2006) with nomenclature of 2-amino-2-[2-(4-octylphenyl)ethyl]propane-1,3-diol (Fig. 1). Fingolimod metabolism is phosphorylated, that can cause a decline in lymphocytes in the periphery, while increasing sphingosine-1-phosphate (SIP) receptors on lymphocytes (Brinkmann et al. 2002). SIP receptors activation is required for the dispersion of 99 of leukocytes to other organs from lymph nodes (Chiba 2005). In the literature survey for the determination of fingolimod, few methods are reported, including LC- (Salm et al. 2006; Zollinger et al. 2011; Ferreiros et al. 2012; Emotte et al. 2012), HPLC (Chhabda et al. 2013; Narasimha et al. 2014; Effat et al. 2015; Somsubhra and Ravikumar 2015; Islam and Emad 2016; Ravi et al. 2016; Suneethaa and Rajeswari 2016), only three spectrophotometric methods (Zhila et al. 2016; Jian-Rong et al. 2015; Somsubhra et al. 2014) and only one spectrofluorimetric method (Salem et al. 2021). In terms of the availability of regents and handy and relatively inexpensive apparatus, one of the simplest and most cost-effective approaches of chemical analysis is colorimetric assay (Fernandes 2020). Colorimetric estimation of chemicals and pharmaceuticals has recently resurfaced as a point of interest for researchers, as it can be utilized for the detection and the quantification of dosage forms, forensic and environmental purposes (Shrivas et al. 2017; Lantam et al. 2020; Kailasa et al. 2019; Son et al. 2021). That charge transfer reaction, in which the analyte interacts with various reagents to yield a charge transfer complex, which is stable with absorption in a range of 400–800 nm (visible area) is the most infamous colorimetric interactions (El-Adl et al. 2019).

In this work, four colorimetric approaches for the estimation of fingolimod were designed and validated, through the reaction with the charge acceptor reagents, including tetrachloro 1,4-benzoquinone (CL), tetracyanoethylene (TCNE), 7,7,8,8-tetracyanoquinodimethane or (TCNQ), (Fig. 1) in pharmaceutical form and biological specimens.
Experimental

Apparatus

Shimadzu – UV 1800 double beam UV–Visible spectrophotometer (Japan) having a glass cuvettes of 1 cm (a pair) was utilized for required operations. An Eppendorf micro-pipette (Germany) that was calibrated was utilized for the spiking of plasma.

Materials

The reagents utilized were of analytical grade. Fingolimod of 99.98% purity (Salem et al. 2021) applied form Novartis Pharmaceutical Corp (USA). Gilenya® Capsules (batch No: 0385) manufactured by Novartis pharmaceutical dorp, USA, that is defined to have 0.5 mg fingolimod for one capsule. TCNQ, CL, TCNE and methanol were bought from Sigma Chemical Co. USA. Human collective plasma was taken from VACERA (Egypt).

Preparation of stock and standard solutions and reagents

50 mg of fingolimod, TCNQ, CL and TCNE was precisely weighed and quantitatively transferred into a set of different volumetric flask of 100-mL, dissolved in methanol and finished to the line utilizing same solvent. 50 mg of fingolimod was dissolved in DMSO (dimethylsulfoxide) and sonicated for 15 min. for plasma spiking.

Preparation of pharmaceutical samples

Twenty Gilenya® capsules content was unfilled and mixed well; the powder equal to 25 mg was weighed and blended in methanol via stirring by a magnetic stirrer for 20 min.; then, the solution was filtered via a filter paper, that has been cleaned with methanol. After that the filtrate, in volumetric flask of 50 mL, was finished to the line with same solvent. The finished solution contained 500 mg mL⁻¹ of fingolimod was obtained.

Preparation of spiked plasma samples

A spiked plasma of 0.2 mL combined with 50 mL of the stock solution of fingolimod dissolved in DMSO, mixed well with vortex for 20 s before being held in a 37 °C water bath for 10 min. For protein precipitation and liberation of drug, 0.5 mL of methanol was added. The mixture was centrifuged for 10 min at 4000 rpm, then 4 mL of the centrifuge was transferred to volumetric flask of 10 mL and processed as previously described down general procedure. The blank was made in the same design, and except instead of fingolimod solution, DMSO was utilized.

General procedure

Certain volumes of each of the following—standard solution, dosage form solution or biological sample—were relocated to a volumetric flask of 10-mL, then 3, 4 and 6 mL of each of the reagents; TCNQ, CL and TCNE, respectively, were mixed well with the sample, after that the volume finished with methanol to obtain the desired concentration. The mixture left for 30 s and the resulted color measured at 843, 640 and 418 nm for TCNQ, CL and TCNE, respectively. The blank was made under the same condition. The blank of spiked plasma was previously cited.

Effect of diluting solvent

The reaction of CL, TCNE and TCNQ with the examined drug was carried out in several solvents as ethanol, chloroform, acetonitrile and methanol, to determine the best for charge transfer reaction. According to the solubility of the drug and reagents as well as the increased absorbance reported in comparison to the other solvents, it was found that methanol is the optimum solvent in those reactions.
Effect of reaction time

The optimum time is established by spectrophotometrically examining the intensity of produced color developed over TCNQ, TCNE and CL reagents at specific time intervals at room temperature (25 °C).

Effect of reagent volume

The reagent volume is examined by utilizing diverse volumes of 50 mg% CL and 100 mg% of either TCNQ or TCNE reagents that combined with a rigid concentration of the researched drug in methanol to study its effect and to know the select the proper reagent volume.

Determination of reaction stoichiometry

To define the ratio of different reagents and the cited drug in each complex, the equimolar solutions of TCNQ, CL or TCNE and fingolimod were produced, and Job’s method of continuous variation (Basavaiah and Zenita 2011) was utilized.

Results and discussion

Drug complexes spectra

TCNQ reacts with fingolimod, at room temperature (25 ± 5 °C) giving bluish green colored complex that absorb maximally at 843 nm; CL reacts with fingolimod, at room temperature (25 ± 5 °C) giving color complex that absorb at 640 nm maximally although TCNE acts with fingolimod yielding a brownish yellow colored complex that absorb at new 418 nm maximally. The spectra of fingolimod-complex with either TCNQ, CL or TCNE are displayed in Fig. 2. Peaks of the generated complexes are put in the visible range where it noticed that there will be no interference with the medication or reagents spectra. Colors produced are simply detectable by the naked eye, making them valuable in the creation of portable quantification or detection probes.

Effect of the reaction time

The colorful complexes are generated instantly with the three reagents, and they stay for 30 min, stable at room temperature at least, according to the reaction time research. This rapid production of the colored complex could be advantageous in using this method to determine the cited drug in quality control or pharmacokinetics researches, as well as if it is employed as a base of a portable analysis or detection purposes. Time effect on TCNQ, TCNE and CL complexes with the chosen drug is shown (Fig. 3).

Effect of reagent volume

By increasing the volume of TCNQ, CL and TCNE till 3, 4 and 6 mL, respectively, the intensity of the absorbance of
the produced complex is gradually increased, and after that nothing changed in the intensity (Fig. 4). It is clear that 3, 4 and 6 mL of TCNQ, CL and TCNE reagent solutions were satisfactory for the formation of maximum intensity of reproducible color with the studied drug solutions.

**Effect of temperature**

The disintegration of the generated complexes induced color fading at temperatures over 40 °C when the ensuing charge transfer complex of TCNQ, TCNE or CL was heated in a water bath. This suggests that at high temperatures, the complex dissociates back into its constituents. The complexes, however, were stable at ambient temperature and up to 30 °C. As a result, lower temperature is preferred for those reactions. For quantitative work, room temperature was determined to be sufficient.

**Reaction stoichiometry between drug and TCNQ, TCNE and CL**

Job’s continuous variation approach was used to determine the stoichiometry of the reactions, yielding the drug reagent ratios 1:1 for each of the cited reagents (Fig. 5).

**Analytical method validation**

The proposed approaches were validated in accordance with the guidelines of International Conference of Harmonization (ICH) (Moffat et al. 2000). The validation results were as follows.

**Linearity and range**

By plotting each produced complex's absorbance, at 843, 418 and 640 nm for TCNQ, TCNE and CL, respectively, versus the selected concentration, calibration curves for colored complexes generated between TCNQ, TCNA and CL with fingolimod were obtained (Fig. 6). Table 1 summarizes the parameters of the derived calibration curves. The high correlation coefficient obtained greatly aided linearity. The determination coefficient ($r^2$) ranged from 0.9997 to 0.9999, suggesting good linearity.

![Fig. 6 Absorbance of the formed complexes between fingolimod with TCNQ, CL or TCNE as a function of drug concentration. Complexes were formed in methanol and absorbance is corrected against blank prepared without drug](image)

**Table 1 Validation parameters of the proposed determination methods of fingolimod by charge transfer complex formation with TCNQ (determination at 000 nm), TCNE (determination at 000 nm) and CL (determination at 000 nm)**

| Parameter                  | TCNQ | TCNE | CL |
|----------------------------|------|------|----|
| Linearity range (µg mL$^{-1}$) | 50–300 | 50–300 | 50–300 |
| LOD (µg mL$^{-1}$)         | 4.54 | 3.98 | 4.09 |
| LOQ (µg mL$^{-1}$)         | 13.62 | 11.94 | 12.27 |
| Slope                     | 0.0060 | 0.0031 | 0.0045 |
| Intercept                 | −0.1993 | −0.1008 | −0.1458 |
| $r^2$                     | 0.9999 | 0.9997 | 0.9999 |
| $r$                       | 0.9999 | 0.9998 | 0.9999 |
| Determination accuracy*   | 100.15 ± 1.11 | 100.3 ± 1.03 | 99.81 ± 0.95 |
| RSD of accuracy           | 1.11 | 1.03 | 1.01 |
| Interday                  | 100.85 ± 1.55 | 100.00 ± 1.33 | 98.67 ± 0.95 |
| RSD of interday           | 1.55 | 1.33 | 1.01 |
| Intraday                  | 100.28 ± 1.41 | 100.51 ± 0.99 | 100.58 ± 1.33 |
| RSD of intraday           | 1.41 | 0.99 | 1.33 |

*Mean ± SD on nine values (triplicate of three different concentrations)
Linearity and range

Three-time analyses of three distinct concentrations of pure sample were utilized to assess the accuracy. The results demonstrate a high degree of agreement between the measured values and true ones, showing that the proposed approaches are accurate. Three replications of three concentrations were used to test intraday and inter-day precision. The standard deviation was detected to be small under 2, indicating that the proposed approaches are repeatable and reliable. Table 1 summaries the findings and their statistical analysis.

Robustness

During the development phase, the robustness of the proposed approaches was evaluated by examining the impact of small variations in various variables, as time spent standing or volume of the reagent, on the researched approaches. With each change in each single parameter, the % recovery was recorded. The recovered standard deviations and % recoveries show that any tiny change in any researched variables has no major impact on the suggested procedure’s results, Table 2. This evaluation revealed the proposed charge transfer approach’s reliability in everyday operation, making them acceptable for quality control laboratories or for biological samples.

Table 2 Robustness of the proposed colorimetric methods using TCNQ, TCNE and CL for determination of fingolimod at 000 nm, 000 nm and 000 nm, respectively

| Parameter                           | TCNQ  | TCNE  | CL    |
|-------------------------------------|-------|-------|-------|
| Standard general procedure          | 100.28±0.75 | 99.87±0.99 | 100.15±1.34 |
| Variation in volume reagent ± 0.2 mL | 99.71±1.15 | 98.54±1.37 | 100.02±0.99 |
| Variation in standing time 1 min    | 99.44±0.77 | 100.51±1.53 | 98.89±0.96 |
| Variation in standing time 10 min   | 100.37±0.94 | 99.71±0.94 | 100.76±1.11 |

All values are mean± SD of 5 determinations
Sample concentration 100 µg mL⁻¹

Application to pharmaceutical dosage form

The proposed approaches were successfully implemented for the examination of certain dosage forms of the researched drug, and the recovered data were compared with the reported method (Salem et al. 2021) for validation, using student’s t test at a confidence level of 95%. The outcome is shown in Table 3. There was no discernible contrast among the results produced using the researched and reported approaches. Excipients and additives had no effect on the analysis, and the results were as accurate, precise and confident as the reported and validated approaches. By applying the standard addition technique, the validity of the proposed approaches regarding handling, extraction and so on was tested. A certain quantity of each single concentration was combined to the researched pharmaceutical form, powder Gilenya® capsules, for comparing the acquired data from the proposed approaches to the for-real combined standard. The standard addition technique data, shown in Table 3, ensure the validity of the proposed approach for determining the cited drug in its dosage form without any interference from utilized additives. The approaches demonstrated to be advisable for determining fingolimod in its dosage form with sufficient precision and accuracy.

Determination of fingolimod in spiked plasma

After adding organic solvent to plasma for precipitation of its proteins and liberation of the bound drug, fingolimod was measured in spiking plasma samples. The proposed approaches were successful in accurately and precisely determining the cited drug in plasma Table 3. To reduce the impact of the plasma matrix on the findings, a blank plasma solution was treated as the same.

Conclusion

The charge transfer reaction offers a suitable foundation for sensitive, cost-effective and practical analysis approaches for determining the anticancer agent, fingolimod. TCNQ, CL and TCNE, three charge transfer reagents, were profitably

Table 3 Results of application of the proposed colorimetric methods using TCNQ, TCNE and CL for determination of fingolimod at 843 nm, 640 nm and 418 nm, respectively, in pharmaceutical dosage form, standard addition to dosage form and spiked plasma

| Method              | TCNQ          | TCNE          | CL             |
|---------------------|---------------|---------------|----------------|
| Reported method     | Proposed method | t value 2.306 | Proposed method | t value 2.306 | Proposed method | t value 2.306 |
| Gilenya® Capsules   | 100.97±1.01   | 100.53±0.97   | 1.31           |
| Standard addition   | 99.75±1.32    | 101.76±1.14   | 1.75           |
| Spiked plasma       | 101.05±1.12   | 98.01±1.94    | 0.79           |

All results are mean and standard
utilized to determine the researched drug in its pharmaceutical dosage form and spiked plasma in a speedy, accurate and precise manner. These methods of determination can be employed in quality control for various dosage forms of the drug, as well as in pharmacokinetics and preclinical researches. Colorimetric assays are utilized as an acceptable tool for the yielding of estimation kit or automated determination probes.

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**Declarations**

**Conflict of interest** The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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