

The British University in Egypt

BUE Scholar

Pharmacy

Health Sciences

Fall 9-26-2021

New Tb³⁺-simvastatin optical biosensor for sensitive determination of folic acid, progesterone, testosterone and vitamin D₃ in biological fluids

Ekram Hany Mohamed

The British University in Egypt, ekram.hany@bue.edu.eg

Mohamed S. S. Attia

Chemistry Department, Faculty of Science, Ain Shams University, Abbassia, Cairo, 11566, Egypt

Ahmed O. Youssef

Analytical Chemistry Department, Faculty of Science, Ain Shams University, Cairo, Egypt

Mona N. Abou-Omar,

Department of Chemistry, Ain Shams University, Faculty of Women for Arts, Science and Education, Cairo, Egypt

Amal M. Ahmed,

Chemistry Department, Faculty of Science, Ain Shams University, Cairo, 11566, Egypt

Follow this and additional works at: <https://buescholar.bue.edu.eg/pharmacy>

See next page for additional authors

 Part of the [Laboratory and Basic Science Research Commons](#)

Recommended Citation

Mohamed, Ekram Hany; Attia, Mohamed S. S.; Youssef, Ahmed O.; Abou-Omar,, Mona N.; Ahmed,, Amal M.; Mahmoud,, Safwat A.; Amin, Mohammed A.; and Mahmoud,, tarek A., "New Tb³⁺-simvastatin optical biosensor for sensitive determination of folic acid, progesterone, testosterone and vitamin D₃ in biological fluids" (2021). *Pharmacy*. 698.

<https://buescholar.bue.edu.eg/pharmacy/698>

This Article is brought to you for free and open access by the Health Sciences at BUE Scholar. It has been accepted for inclusion in Pharmacy by an authorized administrator of BUE Scholar. For more information, please contact bue.scholar@gmail.com.

Authors

Ekram Hany Mohamed; Mohamed S. S. Attia; Ahmed O. Youssef; Mona N. Abou-Omar;; Amal M. Ahmed;; Safwat A. Mahmoud;; Mohammed A. Amin; and tarek A. Mahmoud,


 Cite this: *RSC Adv.*, 2021, 11, 32861

New Tb³⁺–simvastatin optical biosensor for sensitive determination of folic acid, progesterone, testosterone and vitamin D₃ in biological fluids

 Mohamed S. Attia,^a Amal M. Ahmed,^a Tarek A. Amin,^a Ahmed. O. Youssef,^a Mohammed A. Amin,^b Ekram H. Mohamed,^c Safwat A. Mahmoud^{*d} and Mona N. Abou-Omar^e

An innovative, simple and cost effective Tb³⁺–simvastatin photo probe was designed and used as a core for a spectrofluorometric approach to sensitively determine four vital biological compounds in different matrices. A Tb³⁺–simvastatin complex displays a characteristic electrical band with λ_{em} at 545 nm with significant luminescence intensity, which is quenched in the presence of folic acid, progesterone, testosterone and vitamin D₃ at four variant sets of pH: 5.0, 6.2, 7.5 and 9.0, respectively. The conditions were optimized and the best solvent for operation was found to be acetonitrile at λ_{ex} at 340 nm. Folic acid was successfully estimated in tablet dosage form, urine and serum in the concentration range of 2.49×10^{-9} to 1.28×10^{-6} mol L⁻¹. Progesterone, testosterone and vitamin D₃ were also assessed in serum samples using the same optimal conditions within concentration ranges of 5×10^{-9} to 1.9×10^{-6} , 5×10^{-9} to 2.8×10^{-6} and 5×10^{-9} to 4.2×10^{-6} mol L⁻¹, respectively. The proposed luminescence method was validated in accordance with ICH guidelines and found to be accurate, precise, and specific and free from any interference at the working pH for each analyte. The cost effectiveness and applicability of the method make it a good choice for routine analysis of the four compounds and early diagnosis of chronic diseases associated with abnormalities in their physiological levels.

Received 13th July 2021

Accepted 26th September 2021

DOI: 10.1039/d1ra05368j

rsc.li/rsc-advances

1. Introduction

Folic acid (FCA), vitamin B9, is a water-soluble vitamin¹ found naturally in various types of foods as legumes, leafy green vegetables, wheat germs, beets, broccoli, citrus fruits, fermented products, beef liver and eggs. FCA is an essential supplement for pregnant women in the first trimester to avoid birth abnormalities including congenital heart diseases and neural tube defects and autism.² FCA is essential for DNA and RNA production and amino acid metabolism.³ Untreated deficiency of FCA is linked with different health problems, including neurological and psychological manifestations like psychosis, depression, insomnia, and Alzheimer's disease, increase risk of cancer and osteoporosis.⁴

Elevated levels of homocysteine, a biomarker for arteriosclerosis, is also associated with FCA deficiency. Other symptoms include poor cognitive performance, hearing loss and other symptoms including fatigue, heart palpitations, shortness of breath, hair and skin discoloration, mouth sores, and swollen tongue.⁵ Different analytical techniques were reported in the literature for FCA determination in dosage form, dietary supplements, beverages and biological samples including spectroscopy⁶ and chromatography⁷ and electrochemistry.⁸ The chemical structure of folic acid is presented in Fig. 1.

Progesterone (PGS) is a member of progestogen steroid hormones group, secreted mainly during the menstrual cycle by the corpus luteum preparing the body for conception in case of ova fertilization.⁹ PGS is used in oral contraception either in single form or combined with estrogen and as hormonal replacement therapy to alleviate menopause symptoms. Low levels of progesterone may lead to abnormal bleeding during menstruation, premature labor and miscarriage during pregnancy and considered as sign for poly-cystic ovarian syndrome. While PGS elevated level may increase the risk of breast cancer development and marker for adrenal hyperplasia. PGS concentrations was recently estimated using different analytical approaches; spectroscopic,¹⁰ chromatographic,¹¹ electrochemical methods¹² and immunological assay.¹³ The chemical structure of PGS is presented in Fig. 1.

^aChemistry Department, Faculty of Science, Ain Shams University, Cairo 11566, Egypt. E-mail: Mohd_mostafa@sci.asu.edu.eg; Mohamed_sam@yahoo.com; Tel: +202 1229867311; +202 1060819022

^bDepartment of Chemistry, Collage of Science, Taif University, P. O. BOX 11099, Taif 21944, Saudi Arabia

^cPharmaceutical Analytical, Chemistry Department, Faculty of Pharmacy, The British University in Egypt, 11837, El Sherouk City, Egypt

^dPhysics Department, Faculty of Science, Northern Border University, Arar, Saudi Arabia. E-mail: samahmoud2002@yahoo.com

^eDepartment of Chemistry, Faculty of Women for Arts, Science and Education, Ain Shams University, Cairo, Egypt



Testosterone (TST), an anabolic steroid, is the primary male sex hormone where it regulates RBCs production, libido, fertility, spermatogenesis, fat distribution, bone and muscle mass.¹⁴ Imbalance in levels TST may cause serious body dysfunctions where diminished levels have an inverse impact sexual drive, erection, sperm count and muscle strength. Abnormal high TST levels may trigger early puberty in males and menstrual irregularities and baldness in females.¹⁵ TST could also be as a medication to replenish its insufficiency, manage breast cancer in women and enhance physique and performance, for instance in athletes.¹⁶ Its concentration in different matrices as plasma, serum, saliva was recently measured through spectroscopic¹⁷ chromatographic¹⁸ electrochemical methods¹⁹ and capillary electrophoresis.²⁰ Fig. 1 shows the structure of TST.

Vitamin D₃, one of fat-soluble vitamins, is naturally found in different types of foods as oily or fatty fish, dairy products, beefy liver and egg yolk and synthesized endogenously in human body upon exposure to sun. Vit. D₃ is converted to its active form through two successive hydroxylation steps forming calcidiol (25-hydroxyvitamin D) in liver followed by calcitriol (1,25-dihydroxyvitamin D) in kidney. It has a major role in regulation concentration of phosphate and calcium in serum and essential in bone remodeling and growth.²¹ It is also used to improve the cognitive functions and in treatment of specific type of psoriasis. In addition, it contributed to the management of Covid-19 by reducing the cytokine storms and thrombotic episodes associated with the infection.²² The deficiency of Vit. D may lead to serious conditions as rickets and osteomalacia in young and adults, respectively.²³ Low levels of Vit. D is also associated with increased risk of colon and pancreatic cancer respiratory acute infections.²⁴ On the other hand, the excessive intake of Vit. D may increase the levels of calcium both in soft tissues (calcinosis) and blood (hypercalcemia). To evaluate the status of Vit. D in human body, calcidiol level in blood is used as best indicator. The chemical structure of Vit. D is displayed in Fig. 1. In the last decade, quantification of Vit. D and/or its metabolites was established through chemiluminescent assay,²⁵ chromatography.²⁶ The reported methods showed relatively high limits of detection which restricts their practical applications. Moreover, the measurement of low concentrations of folic acid, progesterone, testosterone and vitamin D₃ in biological samples along with interference from some biomolecules such as uric acid (UA), ascorbic acid (AA), and different hormones requires to efficiently improve the sensitivity of chromatographic methods and the electrochemical sensors for practical applications. Therefore, developing a simple method for accurate determination of folic acid, progesterone, testosterone and vitamin D₃ in the presence of each other in the same sample is still of great significance. Today, the research field in which the lanthanide complexes were used as biosensors has a great interest.^{27–42} Luminescent optical biosensor Tb(simvastatin)₃ (Tb-SIM) complex embedded in PEG matrix have many advantages over the mentioned traditional methods. Terbium ion has sharp and precise emission bands in green light region. The terbium ion is used as photo probe for many analytes with a high selectivity depends on the excitation wavelength of terbium-analyte complex, pH and the type of solvent of the test

solution. Doping of the optical sensors in the polymer matrix increases its stability and durability.^{30–36} The sensor can provide a constant signal response for two years, which makes it 24-fold better balance compared to the lifetime warranted for the chromatographic and electrochemical methods. The source of error of the present work eliminated as it more stables for a long time; it gives a low standard deviation value. The higher stability of the current sensor can be attributed to the doping of the optical sensor in the polymer matrix.

2. Experimental

2.1. Instrumentation

A double beam UV-Visible spectrophotometer (PerkinElmer Lambda 25), fluorescence Spectrometer (Thermo Scientific Lumina, Meslo-PN; 222-263000). pH meter (Jenway; 33300)

2.2. Materials and reagents

Pure folic acid standard was kindly supplied by the National Organization for Drug control and Research (Giza, Egypt). Pharmaceutical preparation of folic acid tablets dosage form labelled to contain 500 µg manufactured by Mepaco-Medifood (Arab Company for Pharmaceutical and Medicinal plants, Egypt) was purchased from community pharmacy in the Egyptian market.

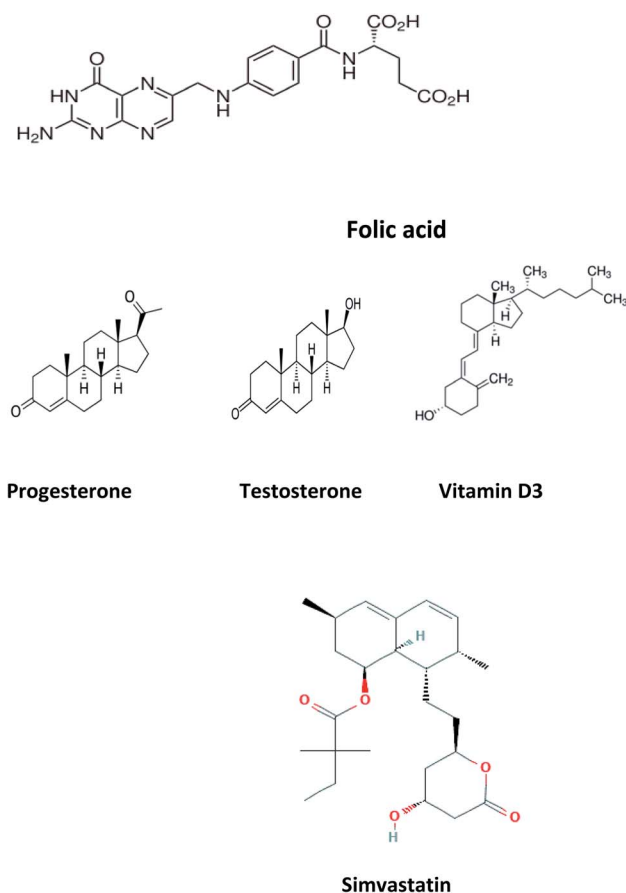


Fig. 1 Chemical structure of folic acid, progesterone, testosterone, vitamin D₃ and simvastatin.

Progesterone, testosterone, vitamin D₃, solvents including ethanol, acetonitrile, dimethylformamide (DMF), chloroform and dimethyl sulfoxide (DMSO) were purchased from Sigma Aldrich. Analytical grade ammonium hydroxide (NH₄OH), hydrochloric acid (HCl), Tb(NO₃)₃·5H₂O, simvastatin and polyethylene glycol (PEG) were purchased from Sigma Aldrich.

The Human real samples were gathered from both Ain Shams Specialized and Teaching New Al-Kasr-El-Aini Hospitals, Cairo, Egypt in accordance with the approved protocol of World Health Organization (WHO) for the collection of human specimens and the use of the clinically related information and data for the purpose of research. The patients approved and were all consented before using their samples.

2.3. Preparation of standard solutions

Stock solutions of Tb(NO₃)₃·5H₂O and simvastatin; were prepared separately by accurately weighing and transferring 0.11 g and 0.039 g, respectively of their authentic pure forms into separate 25 mL volumetric flasks by the aid of the least amount of ethanol till dissolution and completing the volume with the same solvent to obtain final concentration of (10⁻² mol L⁻¹) for each of them.

Tb³⁺-simvastatin complex solution; was prepared by mixing 0.1 mL of Tb(NO₃)₃ stock solution with 0.3 mL of simvastatin (Fig. 1) stock solution in 10 mL volumetric flask and completing the volume to the mark with acetonitrile.

For the four compounds under study, all stock solutions were separately prepared in 10 mL volumetric flasks in concentration of solution (10⁻² mol L⁻¹). This was achieved by dissolving 0.044 g of FCA in least amount of DMF and then completing the volume to the mark using acetonitrile. For PGS, TST and Vit. D 0.031 g, 0.0288 g, 0.033 g, were dissolved, respectively in small amount of ethanol and then volume was diluted to the mark with acetonitrile. Further dilutions for the stock solutions using acetonitrile were performed to obtain working solutions with concentrations of 1.0 × 10⁻⁴ to 1.0 × 10⁻⁹ mol L⁻¹ of FCA, PGS, TST and Vit. D.

0.1 mol L⁻¹ of NH₄OH and HCl were used to adjust the pH to 9.0, 5.0, 6.2, 7.5 for FCA, PGS, TST and Vit. D, respectively. All the

prepared solutions should be kept at low temperature (2–8 °C) to remain stable.

2.4. Preparation of FCA pharmaceutical dosage form solution

Ten tablets of Folic acid® 500 µg were weighed and grinded into fine homogenous powder. The average weight of one tablet was calculated and dissolved in few mL of DMF and sonicated for 20 minutes. The solution was then filtered using whatman filter papers (12 mm) into 10 mL volumetric flask to obtain final concentration of FCA equivalent to 1.1 × 10⁻³ mol L⁻¹. Further dilution was performed to obtain different solutions with concentration range of (1.0 × 10⁻⁴ to 1.0 × 10⁻⁷ mol L⁻¹) was prepared by appropriate dilution with acetonitrile.

2.5. Preparation of urine sample spiked with FCA

The urine sample was collected from a healthy volunteer who didn't administer any previous medications, it was then manipulated in the lab as follows; 10 mL of the collected urine sample were centrifuged at 4000 rpm for 15 min to remove all interferants including crystals, salts, pus and red blood cells. 1.0 mL of urine was spiked with 1.0 mL of previously prepared drug solution with concentration of 1.0 × 10⁻⁶ mol L⁻¹ and completed by acetonitrile to the mark in 10 mL measuring flask.

2.6. Preparation serum samples spiked with FCA, PGS, TST and Vit. D₃

A 1.0 mL of samples of blood collected from healthy volunteers was centrifuged for 15 min at 4000 rpm to remove proteins. 0.1 mL of the serum sample was added to 1.0 mL of each drug working solution of concentration 1.0 × 10⁻⁶ mol L⁻¹ and the volume was complete to 10 mL by acetonitrile to obtain 1.0 × 10⁻⁷ mol L⁻¹ for each drug in four separate 10 mL measuring flasks.

2.7. Preparation of Tb-SIM biosensor embedded in PEG

Tb-SIM complex was prepared in the solid state by mixing an equal volume of 1.0 × 10⁻⁴ mol L⁻¹ Tb ion and 3.0 × 10⁻⁴ mol L⁻¹

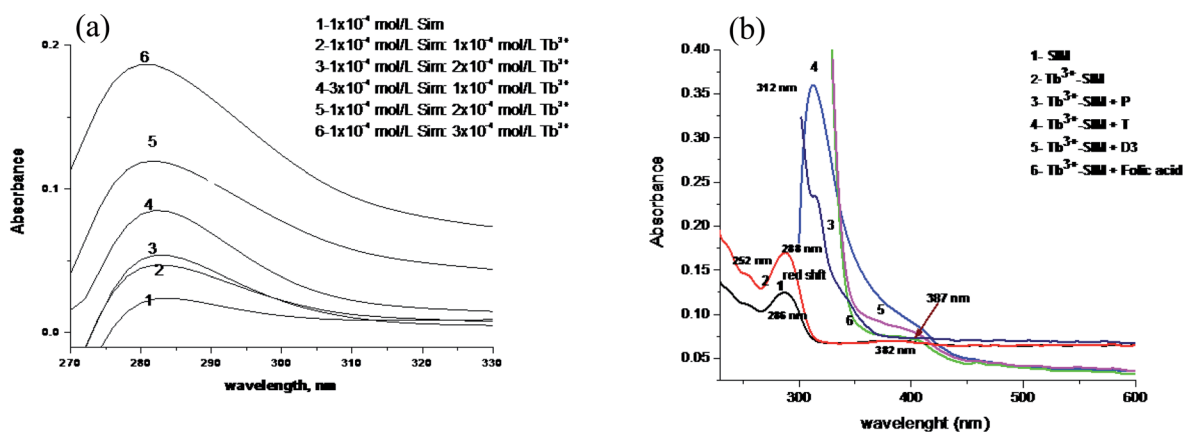


Fig. 2 (a) The absorption spectra of different molar ratios of 1×10^{-4} M Tb(NO₃)₃·6H₂O with 1×10^{-4} M of simvastatin in acetonitrile. (b) The absorption spectrum of (1) simvastatin, (2) Tb³⁺-simvastatin complex (Tb³⁺-SIM), (3) Tb³⁺-simvastatin-progesterone (P), (4) Tb³⁺-simvastatin-testosterone (T), (5) Tb³⁺-simvastatin-vitamin D₃ (Vit D₃), (6) Tb³⁺-simvastatin-folic acid.

simvastatin in ethanol, then evaporation near the dryness of the solution, a pale pink solid was obtained after cooling in air. The thin film was prepared by dissolving 0.1 g of the solidified and seamless complex in 3 mL ethanol and then adding 10 mL of viscose freshly prepared PEG with stirring for about one hour until a homogenous solution was obtained. A thin film was fabricated by spin-coating on a small quartz slide (width 8.5 mm, height 25 mm) to quick fit in the cuvette of the spectrofluorometer.

2.8. Recommended procedure

An appropriate volume (100 μL) of various standard concentrations of folic acid, progesterone, testosterone and vitamin D₃ should be diluted to 3 mL with acetonitrile. The dilute solution was mixed with a thin film of biosensing Tb-SIM doped in PEG matrix in the quartz cell of a spectrofluorometer. The luminescence spectra were recorded at the excitation wavelength $\lambda_{\text{ex}} = 340$ nm. After each measurement, the optical sensor was washed with acetonitrile, and the calibration curve was built by applying the Stern's Volmer equation by plotting (F/F_0) the at $\lambda_{\text{em}} = 545$ nm on the y-axis versus the folic acid, progesterone,

testosterone and vitamin D₃ concentration in mol L^{-1} on the x-axis.

2.9. Determination of FCA in tablet dosage form

The tablet dosage form solutions previously prepared under (2.4) were analyzed using the following procedures: in the spectrofluorometer cell, 1.0 mL of the tablet solutions was separately added followed by the 1.5 mL of acetonitrile in presence of the biosensor film. After mixing, the obtained solutions were scanned, and luminescence spectra were recorded at $\lambda_{\text{ex}}/\lambda_{\text{em}} = 340/545$ nm. The concentrations of the real samples were calculated using corresponding regression equation.

2.10. Determination of FCA in spiked urine samples

The luminescence spectra of the previously prepared spiked urine samples as detailed under (2.5) were scanned at $\lambda_{\text{ex}}/\lambda_{\text{em}} = 340/545$ nm and the concentration of spiked FCA was determined using the corresponding regression equation adopting the standard addition technique.

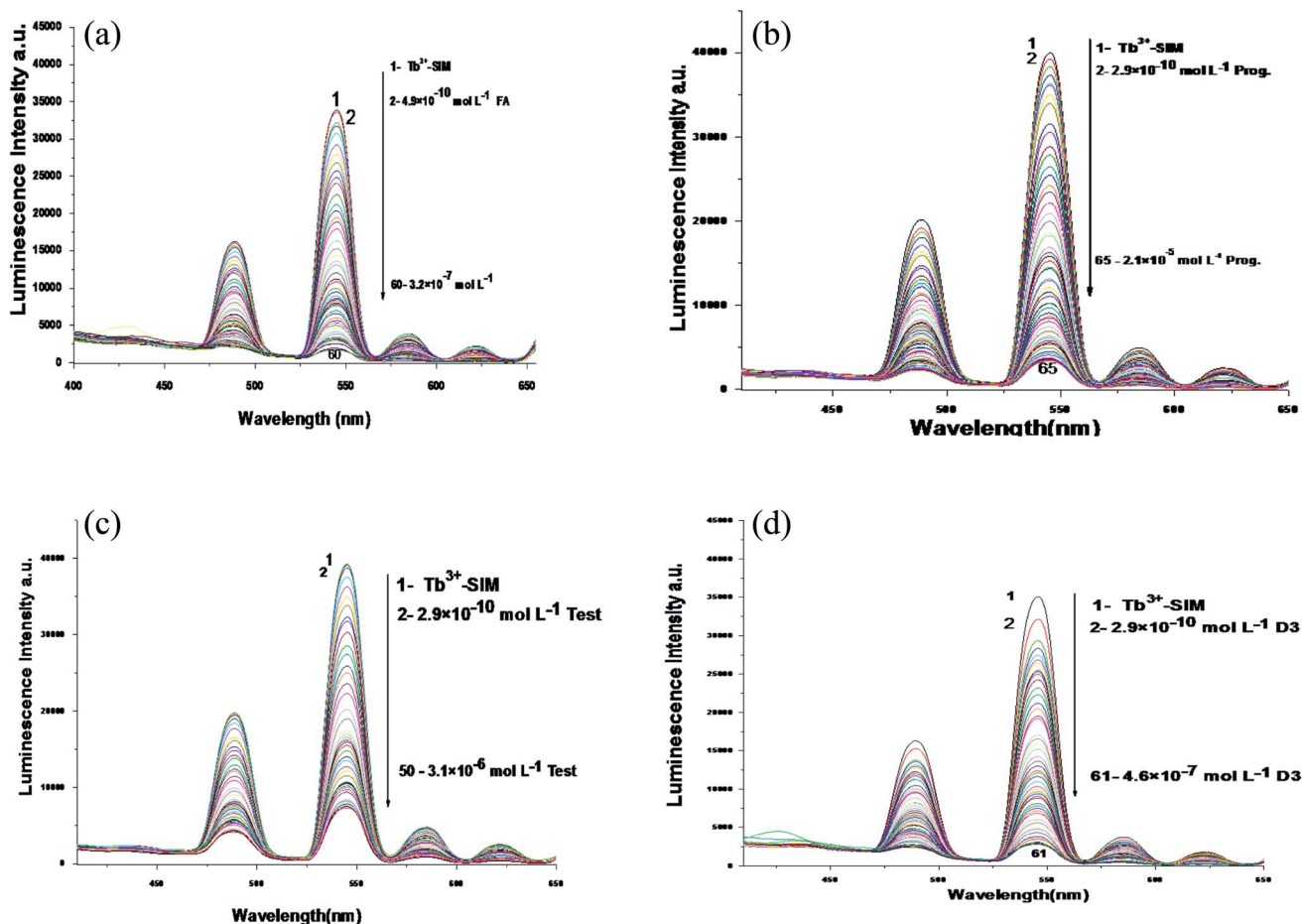


Fig. 3 (a) The emission spectra of Tb³⁺-SIM complex at $\lambda_{\text{ex}} = 340$ nm and pH 5.0 in presence of different folic acid concentrations using acetonitrile as a solvent. (b) The emission spectra of Tb³⁺-SIM complex at $\lambda_{\text{ex}} = 340$ nm and pH 6.2 in presence of different progesterone concentrations using acetonitrile as a solvent. (c) The emission spectra of Tb³⁺-SIM complex at $\lambda_{\text{ex}} = 340$ nm and pH 7.5 in presence of different testosterone concentrations using acetonitrile as a solvent. (d) The emission spectra of Tb³⁺-SIM complex at $\lambda_{\text{ex}} = 340$ nm and pH 9.0 in presence of different vitamin D₃ concentrations using acetonitrile as a solvent.

2.11. Determination of FCA, progesterone, testosterone and vitamin D₃ in serum samples

The luminescence spectra of the serum samples previously prepared as described under (2.6) were measured adopting the same procedures followed under (3.2). The concentrations of each real sample were calculated using corresponding regression equation.

3. Result and discussion

3.1. General features of absorption and emission spectra of Tb–SIM complex

Owing to the f–f transition forbiddance of trivalent ion (Tb³⁺), there is a restriction to directly absorb light which could be overcome through the antenna effect *via* the coupling between

Tb³⁺ and a prominently absorbing organic ligand leading to efficient energy transfer and light absorption processes. Regarding the proposed photo probe, Tb³⁺ is surrounded covalently by 3 molecules of simvastatin ligand responsible for efficient absorption of light and transfer of energy to populate ⁵D₄ state of Tb³⁺.⁴³

The emission of the formed complex Tb–SIM exhibited four specific and intense bands because of the ⁵D₄–⁷F_J transitions (*J* = 6, 5, 4 and 3).⁴⁴

3.2. Absorption and emission spectra

The absorption spectra of Tb(NO₃)₃, simvastatin and Tb³⁺–simvastatin complex are shown in Fig. 2a. A red shift by 7 nm and the absorbance value is enhanced denoting that simvastatin could form a stable complex with Tb³⁺. The absorption

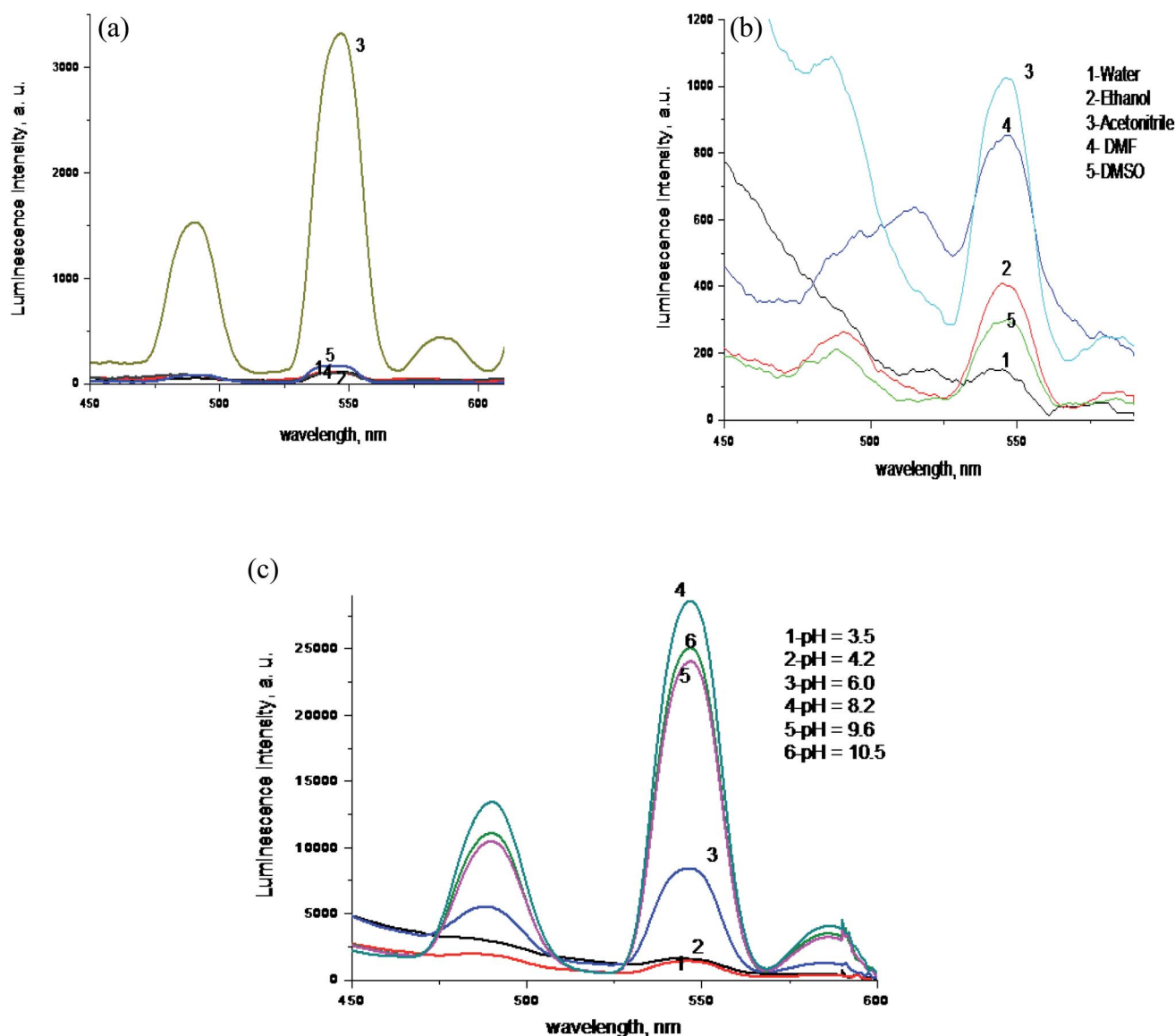


Fig. 4 (a) Luminescence spectra of $1.0 \times 10^{-4} \text{ mol L}^{-1} \text{ Tb}^{3+}$ with $1.0 \times 10^{-4} \text{ mol L}^{-1}$ of simvastatin in different molar ratio; 1 – (1Tb : SIM), 2 – (1Tb : 2SIM), 3 – (1Tb : 3SIM), 4 – (2Tb : 1SIM), 5 – (3Tb : 1SIM) in acetonitrile at $\lambda_{\text{ex}} = 340 \text{ nm}$. (b) Emission spectra of Tb³⁺–SIM optical sensor in different solvents at $\lambda_{\text{ex}} = 340 \text{ nm}$ and pH 8.2. (c) Emission spectra of Tb³⁺–SIM optical sensor in acetonitrile at $\lambda_{\text{ex}} = 340 \text{ nm}$ and different pHs.

spectra of FCA, PGS, TST and Vit. D₃ were scanned alone and in the presence of the optical sensor are shown in Fig. 2b.

The emission spectra of Tb³⁺-SIM complex after adding different concentrations of FCA, PGS, TST and Vit. D₃ using acetonitrile as solvent are shown in Fig. 3a-d, respectively. The characteristic electrical emission band of Tb³⁺ exhibited at λ_{em} 545 nm was quenched due to energy transfer from the optical sensor to FCA, PGS, TST and Vit. D₃.

3.3. Experimental variables

3.3.1. Tb³⁺ and simvastatin amounts. The Tb³⁺-simvastatin complex was formed in ratio 1 M: 3 L indicating that the metal coordinates to the ligand at different sites of coordination not *via* oxygen only, Fig. 4a.

3.3.2. Solvent effect. The intensity of luminescence of solutions containing Tb³⁺ (1.0×10^{-4} mol L⁻¹) and simvastatin (3.0×10^{-4} mol L⁻¹) was investigated in different solvents and the results revealed that maximum enhancement was noticed in acetonitrile as presented in Fig. 4b. Solvents with hydroxyl group as ethanol diminishes the luminescence intensity due to transfer of vibrational energy to molecules of solvents.⁴⁵⁻⁵²

3.3.3. pH effect. The medium pH has a significant influence on the luminescence intensity of the formed Tb³⁺-simvastatin complex. Solutions of NH₄OH and HCl, both 0.1 mol L⁻¹ were used for pH adjustment. The highest luminescent intensity at λ_{em} 545 nm was observed at pH = 8.2 as shown in Fig. 4c.⁵³⁻⁶⁰

3.4. Mechanism of emission quenching

Upon adding different concentrations of FCA, PGS, TST and Vit. D₃ to the Tb-SIM photo probe a notified quenching in its luminescent intensity occurs owing to the approach of the analytes under study and formation of H-bond between the hydroxyl group in both of TST and Vit. D₃, carboxylic group in FCA and enol group in PGS with the SIM. The formation of H-

bonding lead to the depression or decrease in the transfer of energy to the Tb³⁺ ion and consequently the luminescence intensity is significantly quenched.

The pH effect on the luminescence intensity after the addition of the studied analytes to the proposed photoprobe was studied and the luminescence quenching was observed at pH 5.0, 6.2, 7.5 and 9.0 for FCA, PGS, TST and Vit. D₃ respectively.

4. Analytical performance⁶¹

4.1. Linearity

Correlations between the luminescence of emission intensity of optical sensor at λ_{em} 545 nm and FCA, PGS, TST and Vit. D₃ within concentration ranges of (2.4×10^{-9} to 1.28×10^{-6}), (5×10^{-9} to 1.9×10^{-6}), (5×10^{-9} to 2.8×10^{-6}) and (5×10^{-9} to 4.2×10^{-6}) mol L⁻¹ respectively were found to be linear as presented in respective calibration graphs, Fig. 5a and b obtained by applying the Stern-Völmer plot.

The critical concentration of FCA, PGS, TST and Vit. D₃ values are (3.31, 3.1, 2.2, 1.3) and (0.005×10^{-7} to 3.18×10^{-7} , 2.49×10^{-10} to 2.12×10^{-5} , 2.4×10^{-10} to 3.18×10^{-6} , 4.9×10^{-10} to 4.8×10^{-7}) mol L⁻¹ respectively. The distance between the cited compounds and the ionophore is 3.36 Å indicating the electron transfer mechanism of quenching.

The regression equations were computed and the regression parameters in addition the LOD and LOQ were calculated, and results were presented in Table 1.

4.2. Accuracy and precision

The accuracy of the developed method was further investigated *via* applying the standard addition technique and calculating the recovery%. Assessing the obtained recovery was performed through determination of agreement extent between the measured and actual added standard concentration of analyte. All assays were repeated 3 times within the same day and different days to assess the repeatability and intermediate

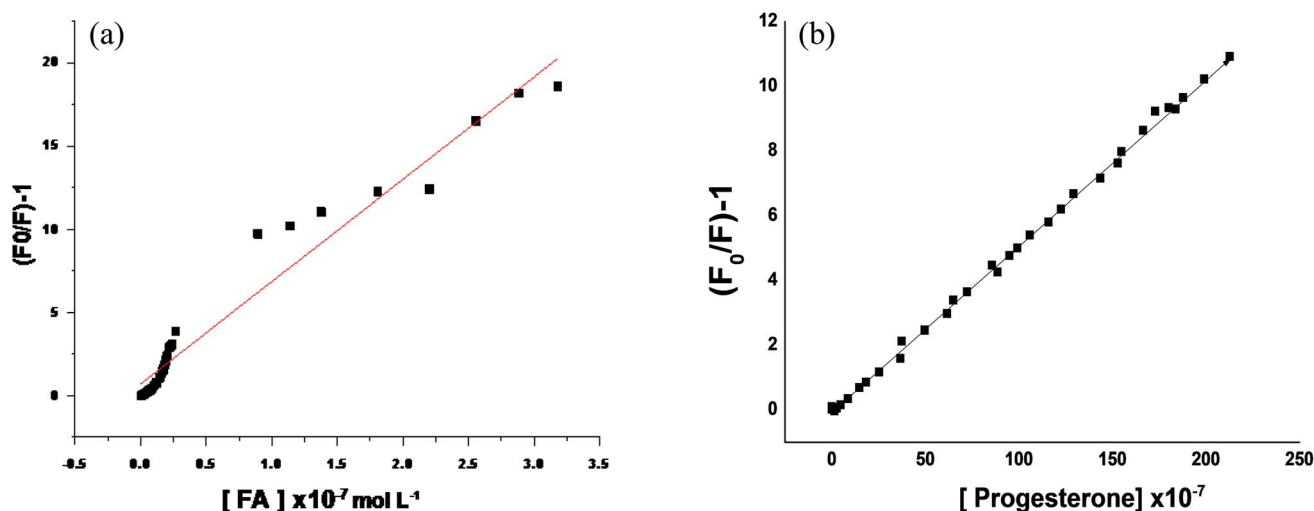


Fig. 5 (a) Stern-Volmer plot $(F_0/F) - 1$ against corresponding concentrations of folic acid. (b) Stern-Volmer plot $(F_0/F) - 1$ against corresponding concentrations of progesterone.

Table 1 Validation sheet and parameters of the regression equations of the proposed optical sensor

Parameter	Folic acid	Progesterone	Testosterone	Vitamin D ₃
λ_{em} (nm)	545			
Linearity (mol L ⁻¹)	1.28×10^{-6} to 2.49×10^{-9}	1.9×10^{-6} to 5×10^{-9}	2.8×10^{-6} to 5×10^{-9}	4.2×10^{-6} to 5×10^{-9}
LOD (mol L ⁻¹)	1.99×10^{-9}	1.5×10^{-9}	3.02×10^{-9}	1.59×10^{-9}
LOQ (mol L ⁻¹)	5.94×10^{-9}	4.5×10^{-9}	9.0×10^{-9}	2.8×10^{-9}
Regression equation	$(Y = a + bX)^a$			
Intercept (<i>a</i>)	0.32	47.2	105	84.5
Slope (<i>b</i>)	3.31	3.1	2.2	1.30
Standard deviation	0.04	15.5	20.5	6.40
Variance (<i>S</i> ²)	0.0016	240.25	420.25	4.90
Regression coefficient (<i>r</i>)	0.99	0.99	0.99	0.99

^a Where *Y*: intensity of luminescence, *X*: analyte concentration (mol L⁻¹), *a*: intercept and *b*: slope.

precision, respectively. Three different levels of the analyte concentrations were used in the assays and the results were summarized and presented in (Table 2).

4.3. Selectivity

The selectivity of the proposed method was investigated through analyzing placebo blank and synthetically prepared mixtures. All possible interfering inactive compounds were used to prepare a placebo containing; 50 mg calcium carbonate, 20 mg calcium dihydrogen orthophosphate, 30 mg lactose, 100 mg magnesium stearate, 40 methyl cellulose, 70 mg sodium alginate, 300 mg starch and 250 mg Talc. Extraction was performed using water and the solution was manipulated as detailed under 2.4. A suitable aliquot of the obtained solution was analyzed after the addition of the optical sensor Tb³⁺-

simvastatin, and the luminescence spectra were recorded at $\lambda_{ex}/\lambda_{em} = 340/545$ nm following the optimized conditions.

The validity and selectivity were further assessed in presence of some proteins and hormones that may interfere as cortisol, Thyroid stimulating hormone, norepinephrine, dopamine and albumin within concentration range of 0.08 g L⁻¹. The interference of 0.006 g L⁻¹ urea, 0.08 g L⁻¹ glucose, uric acid and folic acid was also studied, and the resulting data revealed that there was no significant effect on the observed luminescence activity of the proposed photo probe under optimized conditions.

In addition, the proposed optical probe was successfully applied for selective determination of FCA, PGS, TST and Vit. D₃ either as single or in combination in synthetically prepared mixtures. Four synthetic mixtures were prepared by adding different concentrations of FCA, PGS, TST and Vit. D₃ within

Table 2 Evaluation of repeatability and intermediate precision of the proposed optical method^a

Sample	Concentration taken ($\times 10^{-7}$ mol L ⁻¹)	Repeatability			Intermediate precision		
		Average found \pm CL ^b	% RE ^c	% RSD ^d	Drug average found \pm CL	% RE	% RSD
Progesterone in serum	1.0	1.03 \pm 0.13	3.0	3.39	1.06 \pm 0.11	6.0	2.13
	2.0	1.95 \pm 0.18	2.5	2.33	2.05 \pm 0.17	2.5	3.12
	4.0	4.19 \pm 0.24	4.75	2.99	4.20 \pm 0.23	5.0	2.11
Testosterone in serum	1.0	1.11 \pm 0.13	11.0	3.46	0.99 \pm 0.11	1.00	3.11
	2.0	2.02 \pm 0.18	1.00	2.41	2.04 \pm 0.16	2.00	2.06
	4.0	3.89 \pm 0.26	2.75	2.95	4.13 \pm 0.21	3.25	3.02
Vitamin D ₃ in serum	1.0	1.06 \pm 0.23	6.00	2.22	1.09 \pm 0.21	9.00	2.01
	2.0	2.05 \pm 0.28	2.50	2.26	2.14 \pm 0.36	7.00	4.35
	4.0	4.19 \pm 0.48	4.75	2.25	4.23 \pm 0.31	5.75	2.51
Tablet, 500 μ g of folic acid MEPACO	3.0	3.04 \pm 0.024	1.33	0.33	3.07 \pm 0.052	2.33	0.68
	6.0	5.99 \pm 0.050	0.16	0.35	6.08 \pm 0.070	1.33	0.47
	9.0	8.96 \pm 0.025	0.33	0.11	9.09 \pm 0.062	1.00	0.28
Folic acid in serum	4.0	3.98 \pm 0.20	0.50	0.38	4.08 \pm 0.038	2.00	0.37
	6.0	5.98 \pm 0.15	0.33	0.61	6.09 \pm 0.080	1.50	0.53
	9.0	9.01 \pm 0.22	0.22	0.33	9.06 \pm 0.062	0.67	0.28
Folic acid in urine	4.0	3.99 \pm 0.20	0.50	0.10	4.06 \pm 0.043	2.03	0.32
	6.0	5.99 \pm 0.15	0.33	0.66	6.07 \pm 0.070	1.54	0.51
	9.0	8.99 \pm 0.22	0.22	0.44	9.04 \pm 0.066	0.74	0.38

^a *n* = 3. ^b CL: confidence limits (supplementary material). ^c % RE: percent relative error. ^d RSD: relative standard deviation.

their linearity range in 4 similar sets of 10 mL volumetric flasks containing 1.0 mL of the serum sample as mentioned under 2.6.

The pH of the first set was adjusted to 5.0 for selective determination of FCA in presence of PGS, TST and Vit. D₃, the pH of the second set was adjusted to 6.2 for the determination of PGS in presence of FCA, TST and Vit. D₃, the pH of the third set was adjusted to 7.5 for determination of TST in presence of

FCA, PGS, and Vit D₃, finally the pH of the fourth set was adjusted to 9 for determination of Vit D₃ in presence of FCA, PGS, TST and the volume was completed with acetonitrile for the four sets. Thus, each mixture was prepared 4 times but at different pH (5.0, 6.2, 7.5 and 9.0) for selective estimation of FCA, PGS, TST and Vit. D₃, respectively. Each solution was in triplicates and yielded recovery% of $99.60 \pm 0.47, 100.8 \pm 2.10,$

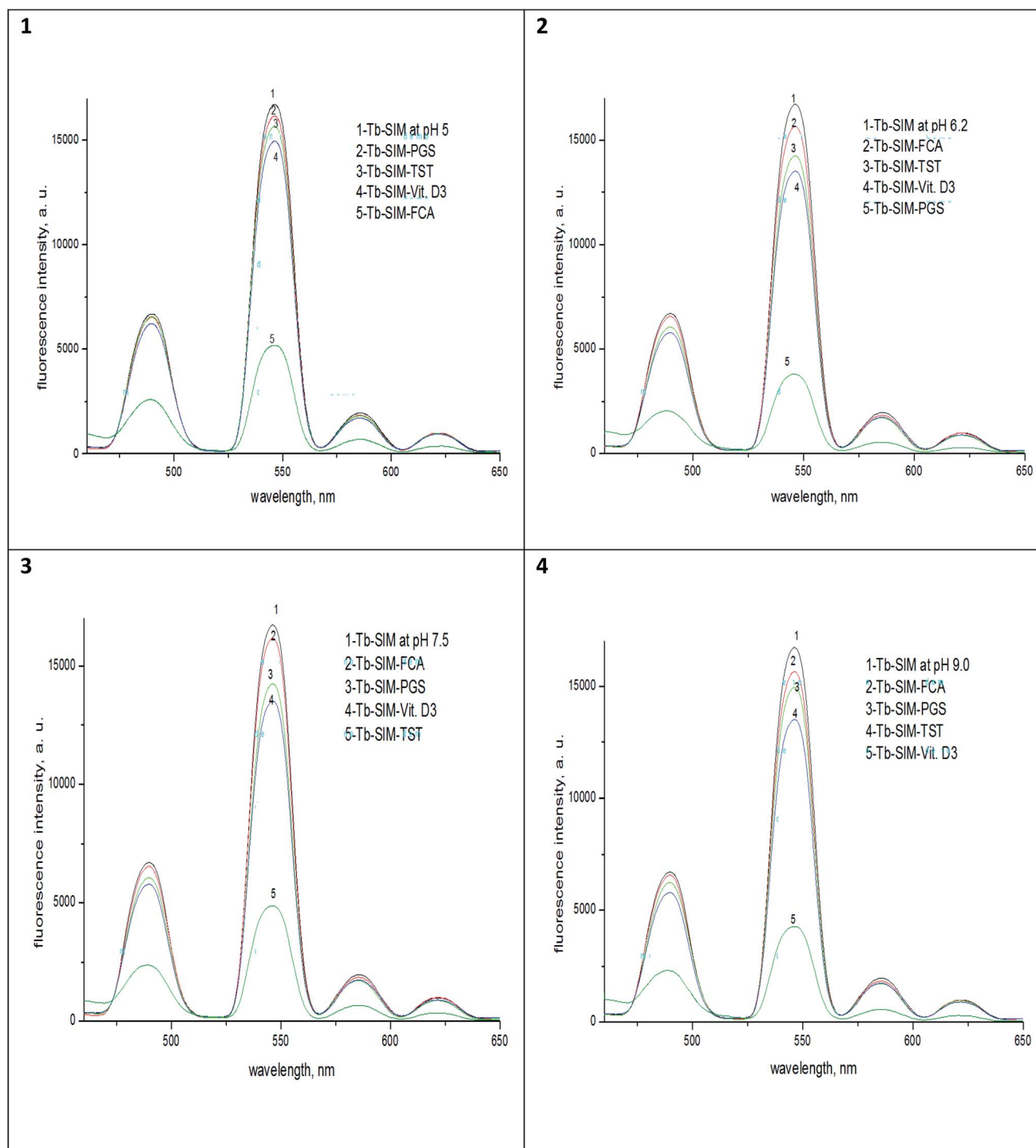


Fig. 6 The luminescence spectra of the complexes Tb-SIM, Tb-SIM-FCA, Tb-SIM-PGS, Tb-SIM-TST and Tb-SIM-Vit-D₃ at $\lambda_{\text{ex}} = 340$ nm and different pHs; (1) 5.0, (2) 6.2, (3) 7.5 and (4) 9.0.

Table 3 Determination of folic acid, progesterone, testosterone and vitamin D₃ samples using Tb-SIM optical sensor

Sample	Added ($\times 10^{-7}$ mol L ⁻¹)	Found ($\times 10^{-7}$ mol L ⁻¹)	Average ^a ($\times 10^{-7}$ mol L ⁻¹)	Average recovery \pm % R.S.D	B.P. (LC)
Progesterone serum sample	3.5	3.52, 3.48, 3.57	3.52	100.2 \pm 2.1	98.6 \pm 0.5
	7.0	6.97, 7.05, 7.03	7.01		
	9.5	9.55, 9.46, 9.46	9.49		
Testosterone serum sample	3.5	3.49, 3.53, 3.52	3.51	99.6 \pm 2.5	99.2 \pm 0.6
	7.0	6.95, 6.99, 6.98	6.97		
	9.5	9.51, 9.45, 9.48	9.48		
Vitamin D ₃ serum sample	3.5	3.39, 3.43, 3.62	3.48	103.1 \pm 2.9	99.4 \pm 0.5
	7.0	6.85, 6.89, 6.88	6.87		
	9.5	9.41, 9.55, 9.58	9.51		
Tablet, 500 μ g of folic acid MEPACO-MEDIFOOD	3.0	3.04, 3.05, 3.03	3.04	101.33 \pm 0.33	99.8 \pm 0.055
	6.0	6.02, 5.98, 5.99	5.99		
	9.0	8.97, 8.96, 8.95	8.96		
Folic acid serum sample	4.0	3.98, 3.97, 4.00	3.98	99.5 \pm 0.38	99.6 \pm 0.050
	6.0	6.01, 5.97, 5.95	5.98		
	9.0	8.98, 8.95, 9.01	9.01		
Folic acid urine sample	4.0	3.99, 3.97, 4.01	3.99	99.75 \pm 0.10	99.5 \pm 0.050
	6.0	6.02, 5.98, 5.99	5.99		
	9.0	8.99, 8.97, 9.00	8.99		

^a Average of nine measurements.

99.4 \pm 2.60 and 101.9 \pm 2.20 for FCA, PGS, TST and Vit. D₃, respectively.

Results in Fig. 6 show that the luminescence of Tb³⁺-SIM complex in its second coordination sphere in which the quaternary mixture of FCA, PGS, TST and Vit. D₃ is quite sensitive to four variant sets of pHs. For Tb³⁺-SIM-FCA, λ_{ex} = 340 and pH 5.0, give the more quenching of luminescence intensity of Tb³⁺-SIM while that for Tb³⁺-SIM-PGS was of λ_{ex} = 340 and pH 6.2 and that for Tb³⁺-SIM-TST was of λ_{ex} = 340 and pH 7.5, and that for Tb³⁺-SIM-Vit-D₃ was of λ_{ex} = 340 and pH

9.0. Thus, a dual-controlled luminescence of smoothly dynamic reversibility is achieved and a reversible on/off switchable Tb³⁺ emission of one system was observed by tuning its optimal values of pH to the optimal ones of the second and so on for the third and fourth. By this dual controlled luminescence, the quaternary mixture of FCA, PGS, TST and Vit. D₃ was simultaneously resolved with average error <3.5%.

Also, the data obtained upon assaying single PGS, TST and Vit. D₃ separately in serum sample and FCA in serum, urine and dosage form, without any interference from inactive excipients,

Table 4 Comparison of proposed optical luminescent technique versus some previously reported methods for estimation of progesterone, testosterone, vitamin D₃ and folic acid

Analyte	Methods	Linearity	Limit of detection	References
Progesterone	HPLC-MS-MS	0.2–50 ng mL ⁻¹	0.2 ng mL ⁻¹	18
	Microfluidic immunosensor system	0.5–12.5 ng mL ⁻¹	0.2 ng mL ⁻¹	21
	Enzyme-linked fluorescence assay	3–40.0 ng mL ⁻¹	—	22
	Spectrofluorometric using Tb ³⁺ -SIM	1.9×10^{-6} to 5×10^{-9} mol L ⁻¹	1.49×10^{-9} mol L ⁻¹	
Testosterone	HPLC in plasma	1.6–400 ng mL ⁻¹	1.6 ng mL ⁻¹	32
	HPLC in serum	1–20 ng mL ⁻¹	0.4	33
	HPLC in urine	10–500 ng mL ⁻¹	1 ng mL ⁻¹	30
	HPLC in dosage form	50–200 μ g mL ⁻¹	5 μ g mL ⁻¹	31
	HPLC in urine	2–300 ng mL ⁻¹	2 ng mL ⁻¹	29
	Spectrofluorometric using Tb ³⁺ -SIM	2.8×10^{-6} to 5×10^{-9} mol L ⁻¹	3.1×10^{-9} mol L ⁻¹	
Vitamin D ₃	HPLC	15–200 nmol L ⁻¹	3 nmol L ⁻¹	62
	LC-MS/MS	3.5 to 75 ng mL ⁻¹	14 ng mL ⁻¹	63
	HPLC-APCI-MS	5–400 nmol L ⁻¹	1–4 nmol L ⁻¹	64
	Spectrofluorometric using Tb ³⁺ -SIM	4.2×10^{-6} to 5×10^{-9} mol L ⁻¹	1.6×10^{-9} mol L ⁻¹	
Folic acid	LC-MS/MS	4.5×10^{-8} to 5×10^{-10} mol L ⁻¹	5×10^{-10} mol L ⁻¹	15
	Chemiluminometric and fluorimetric determination	114–6.0 μ g mL ⁻¹ , 1.10–0.022 μ g mL ⁻¹	2.0 μ g mL ⁻¹ , 0.002 μ g mL ⁻¹	8
	Chemiluminescence	8×10^{-7} to 6×10^{-9} mol L ⁻¹	6×10^{-103} mol L ⁻¹	9
	HPLC method	2500 to 50 μ g mL ⁻¹	1.3 ng mL ⁻¹	14
	Spectrofluorometric method: using Tb ³⁺ -SIM	1.28×10^{-6} to 2.49×10^{-9} mol L ⁻¹	1.99×10^{-9} mol L ⁻¹	

were processed and results were tabulated as shown in Table 3. The results of the proposed method were comparable to that obtained from the reference chromatographic methods mentioned in the British pharmacopeia.⁶² The limitations of the proposed method in real samples in which a hormones and proteins are existed. These biological molecules contain OH, NH and SH groups may make an interference with the analytes at different pHs.

4.4. Comparison with previously reported methods

The results obtained from the proposed spectrofluorometric technique was compared with obtained from other previously reported methods assuring the applicability, accuracy, and precision of the proposed method as presented in Table 4,^{8,9,14,15,17,20,21,29–33,63,64}

5. Conclusion

The proposed analytical method based on the use of Tb³⁺-simvastatin complex is simple and economic and can be successfully applied for sensitive and accurate determination of folic acid, progesterone, testosterone and vitamin D₃ in different matrices including dosage forms, urine and serum. The analysis of the FCA, PGS, TST and Vit. D in biological samples can contribute to early diagnosis of some chronic diseases associated with their abnormal levels.

Conflicts 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.

Acknowledgements

Authors thank Taif University Researchers Supporting Project (TURSP-2020/03), Taif University, Taif, Saudi Arabia, for supporting this work.

References

- 1 U. Binesh, Y.-L. Yang and S.-M. Chen, Amperometric Determination of Folic Acid at Multi-Walled Carbon Nanotube-Polyvinyl Sulfonic Acid Composite Film Modified Glassy Carbon Electrode, *Int. J. Electrochem. Sci.*, 2011, **6**, 3224–3227.
- 2 D. B. Kirsten, C. G. David, J. C. Susan, W. D. Karina, W. E. John, A. R. G. Francisco, R. K. Alex, H. K. Alex, E. K. Ann, C. Landefeld, M. M. Carol, R. P. William, G. P. Maureen, P. P. Michael, S. Michael and T. Chien-Wen, Folic Acid Supplementation for the Prevention of Neural Tube Defects: US Preventive Services Task Force Recommendation Statement, *JAMA, J. Am. Med. Assoc.*, 2017, **317**, 183–189.
- 3 Y. Feng, S. Wang, R. Chen, X. Tong, Z. Wu and X. Mo, Maternal folic acid supplementation and the risk of congenital heart defects in offspring: a meta-analysis of epidemiological observational studies, *Sci. Rep.*, 2015, **5**, 8506.
- 4 I. M. Ebisch, C. M. Thomas, W. H. Peters, D. D. Braat and R. P. Steegers-Theunissen, The importance of folate, zinc and antioxidants in the pathogenesis and prevention of subfertility, *Hum. Reprod. Update*, 2007, **13**, 163–174.
- 5 A. M. Treon, M. Shea-Budgell, B. Shukitt-Hale, D. E. Smith, J. Selhub and I. H. Rosenberg, B-vitamin deficiency causes hyperhomocysteinemia and vascular cognitive impairment in mice, *Proc. Natl. Acad. Sci. U. S. A.*, 2008, **105**, 12474–12479.
- 6 M. Jägerstad, Folic acid fortification prevents neural tube defects and may also reduce cancer risks, *Acta Paediatr.*, 2012, **101**, 1007–1012.
- 7 M. V. Ribeiro, I. S. Melo, F. C. Lopes and G. C. Moita, Development and validation of a method for the determination of folic acid in different pharmaceutical formulations using derivative spectrophotometry, *Braz. J. Pharm. Sci.*, 2016, **52**, 741–750, DOI: 10.1590/S1984-82502016000400019.
- 8 T. Meropi, A. Panagiotis, M. Triantafillos, S. Magdalini and T. Thalia, Chemiluminometric and Fluorimetric Determination of Folic Acid, *Anal. Lett.*, 2007, **40**, 2203–2216.
- 9 B. T. Zhang, L. Zhao and J. M. Lin, Determination of folic acid by chemiluminescence based on peroxomonosulfate-cobalt (II) system, *Talanta*, 2008, **74**, 1154–1159; E. P. Azevedo, E. M. Alves, S. Khan, L. Silva, J. R. de Souza and B. S. Santos, Folic acid retention evaluation in preparations with wheat flour and corn submitted to different cooking methods by HPLC/DAD, *PLoS One*, 2020, **15**, e0230583, DOI: 10.1371/journal.pone.0230583.
- 10 A. Mahato, S. Vyas and N. S. Chatterjee, HPLC-UV Estimation of Folic Acid in Fortified Rice and Wheat Flour using Enzymatic Extraction and Immunoaffinity Chromatography Enrichment: An Interlaboratory Validation Study, *J. AOAC Int.*, 2020, **103**, 73–77.
- 11 E. Mine Öncü-Kaya, Determination of Folic Acid by Ultra-High Performance Liquid Chromatography in Certain Malt-based Beverages after Solid-Phase Extraction, *CBU J. Sci.*, 2017, **13**, 623–630.
- 12 K. Sasaki, H. Hatate and R. Tanaka, Determination of 13 Vitamin B and the Related Compounds Using HPLC with UV Detection and Application to Food Supplements, *Chromatographia*, 2020, **83**, 839–851.
- 13 R. M. Kok, D. E. C. Smith, J. R. Dainty, J. T. V. Akker, P. M. Finglas, Y. M. Smulders, C. Jakobs and K. D. Meera, 5-Methyltetrahydrofolic acid and folic acid measured in plasma with liquid chromatography tandem mass spectrometry: applications to folate absorption and metabolism, *Anal. Biochem.*, 2004, **326**, 129–138.
- 14 E. Dokur, Ö. Gördük and Y. Şahin, Differential Pulse Voltammetric Determination of Folic Acid Using a Poly(Cystine) Modified Pencil Graphite Electrode, *Anal. Lett.*, 2020, **53**, 2060–2078, DOI: 10.1080/00032719.2020.1728540.
- 15 M. C. Brucker and T. L. King, *Pharmacology for Women's Health*, 2nd edn, Jones & Bartlett Publishers. 2010, p. 372.

- 16 D. Maliwal, P. Jain, A. Jain and V. Patidar, Determination of progesterone in capsules by high-performance liquid chromatography and UV- spectrophotometry, *J. Young Pharm.*, 2009, **1**, 371–374.
- 17 P. Naderi and F. Jalali, Poly-L-serine/AuNPs/MWCNTs as a Platform for Sensitive Voltammetric Determination of Progesterone, *J. Electrochem. Soc.*, 2020, **167**, 027524, DOI: 10.1149/1945-7111/ab6a7f.
- 18 A. F. Javier, M. G. Alejandro, M. P. Gabriela, Z. M. Alicia, R. Julio and F. Héctor, Determination of progesterone (P4) from bovine serum samples using a microfluidic immunosensor system, *Talanta*, 2010, **80**, 1986–1992.
- 19 N. Brugger, C. Otzdorff, B. Walter, B. Hoffmann and J. Braun, Quantitative Determination of Progesterone (P4) in Canine Blood Serum Using an Enzyme-linked Fluorescence Assay, *Reprod. Domest. Anim.*, 2011, **46**, 870–873.
- 20 C. Yu, Y. Mehrdad, R. H. Barry, P. D. Eleftherios and W. Pui-Yuen, Rapid determination of serum testosterone by liquid chromatography-isotope dilution tandem mass spectrometry and a split sample comparison with three automated immunoassays, *Clin. Biochem.*, 2009, **42**, 484–490.
- 21 J. Bain, The many faces of testosterone, *Clin. Interventions Aging*, 2007, **2**, 567–576.
- 22 D. French, Development and validation of a serum total testosterone liquid chromatography-tandem mass spectrometry (LC-MS/MS) assay calibrated to NIST SRM 971, *Clin. Chim. Acta*, 2013, **415**, 109–117.
- 23 R. S. Tan and S. J. Pu, A pilot study on the effects of testosterone in hypogonadal aging male patients with Alzheimer's disease, *Aging Male*, 2003, **6**, 13–17.
- 24 B. A. Andre, M. D. Julia, A. S. Elizabeth, M. M. Hassan, T. G. Lin and A. W. Gary, Endogenous Testosterone and Mortality in Men: A Systematic Review and Meta-Analysis, *J. Clin. Endocrinol. Metab.*, 2011, **96**, 3007–3019.
- 25 S. P. Tuck and R. M. Francis, Testosterone, bone and osteoporosis, *Front. Horm. Res.*, 2009, **37**, 123–132.
- 26 A. J. Kadhem, S. Xiang, S. Nagel, C.-H. Lin and M. Fidalgo de Cortalezzi, Photonic Molecularly Imprinted Polymer Film for the Detection of Testosterone in Aqueous Samples, *Polymers*, 2018, **10**, 349–352.
- 27 M. M. Abd-Elzaher, M. A. Ahmed, A. B. Farag, M. S. Attia, A. O. Youssef and S. M. Sheta, A Fast and Simple Method for Determination of Testosterone Hormone in Biological Fluids Based on a New Eu(III) Complex Optical Sensor, *Sens. Lett.*, 2017, **15**, 977–981.
- 28 L. Konieczna, A. Plenis, I. Ołędzka, P. Kowalski and T. Bączek, Optimization of LC method for the determination of testosterone and epitestosterone in urine samples in view of biomedical studies and anti-doping research studies, *Talanta*, 2011, **83**, 804–814.
- 29 C. He, S. Li, H. Liu, K. Li and F. Liu, Extraction of testosterone and epitestosterone in human urine using aqueous two-phase systems of ionic liquid and salt, *J. Chromatogr. A*, 2005, **1082**, 143–149.
- 30 R. K. Gupta, R. K. Roy, Anurag and A. N. Jha, HPLC Method Development for Testosterone Cipionate in Bulk Drug and Oil-Based Injectables, *Biosci., Biotechnol. Res. Asia*, 2010, **7**, 505–511.
- 31 K. H. Yuen and B. H. Ng, Determination of plasma testosterone using a simple liquid chromatographic method, *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.*, 2003, **793**, 421–426.
- 32 V. Loi, M. Vertzoni, A. Vryonidou and C. Phenekos, Development and validation of a simple reversed-phase high-performance liquid chromatography method for the determination of testosterone in serum of males, *J. Pharm. Biomed. Anal.*, 2006, **41**, 527–532.
- 33 J. Albrethsen, M. L. Ljubicic and A. Juul, Longitudinal Increases in Serum Insulin-like Factor 3 and Testosterone Determined by LC-MS/MS in Pubertal Danish Boys, *J. Clin. Endocrinol. Metab.*, 2020, **105**, 3173–3178, DOI: 10.1210/clinem/dgaa496.
- 34 S. Alvi and M. Hammami, An improved method for measurement of testosterone in human plasma and saliva by ultra-performance liquid chromatography-tandem mass spectrometry, *J. Adv. Pharm. Technol. Res.*, 2020, **11**, 64, DOI: 10.4103/japtr.JAPTR_162_19.
- 35 W. C. Chang, D. A. Cowan, C. J. Walker, N. Wojek and A. D. Brailsford, Determination of anabolic steroids in dried blood using microsampling and gas chromatography-tandem mass spectrometry: application to a testosterone gel administration study, *J. Chromatogr. A*, 2020, **1628**, 461445, DOI: 10.1016/j.chroma.2020.461445.
- 36 X. Li, T. Yuan, T. Zhao, X. Wu and Y. Yang, An Effective Acid-Base-Induced Liquid-Liquid Microextraction Based on Deep Eutectic Solvents for Determination of Testosterone and Methyltestosterone in Milk, *J. Chromatogr. Sci.*, 2020, bmaa051, DOI: 10.1093/chromsci/bmaa051.
- 37 W. Xu, H. Li, Q. Guan, Y. Shen and L. Cheng, A rapid and simple liquid chromatography-tandem mass spectrometry method for the measurement of testosterone, androstenedione, and dehydroepiandrosterone in human serum, *J. Clin. Lab. Anal.*, 2017, **31**, e22102, DOI: 10.1002/jcla.22102.
- 38 R. Heidarimoghadam, O. Akhavan, E. Ghaderibi, E. Hashemi, S. S. Mortazavi and A. Farmany, Graphene oxide for rapid determination of testosterone in the presence of cetyltrimethylammonium bromide in urine and blood plasma of athletes, *Mater. Sci. Eng., C*, 2016, **61**, 246–250.
- 39 K. H. Liu, D. O'Hare, J. L. Thomas, H. Z. Guo, C. H. Yang and M. H. Lee, Self-assembly Synthesis of Molecularly Imprinted Polymers for the Ultrasensitive Electrochemical Determination of Testosterone, *Biosensors*, 2020, **10**, 16, DOI: 10.3390/bios10030016.
- 40 B. Du, J. Zhang, Y. Dong, J. Wang, L. Lei and R. Shi, Determination of testosterone/epitestosterone concentration ratio in human urine by capillary electrophoresis, *Steroids*, 2020, **161**, 108691, DOI: 10.1016/j.steroids.2020.108691.
- 41 A. W. Norman, From vitamin D to hormone D: fundamentals of the vitamin D endocrine system essential for good health, *Am. J. Clin. Nutr.*, 2008, **88**, 491–499.

- 42 M. S. Attia, W. H. Mahmoud, A. O. Youssef and M. S. Mostafa, Cilostazol Determination by the Enhancement of the Green Emission of Tb³⁺ Optical Sensor, *J. Fluoresc.*, 2011, **21**, 2229–2235, DOI: 10.1007/s10895-011-0927-y.
- 43 M. S. Attia, M. H. Khalil, M. S. A. Abdel-Mottaleb, M. B. Lukyanova, Yu. A. Alekseenko and B. Lukyanov, Effect of Complexation with Lanthanide Metal Ions on the Photochromism of (1,3,3-Trimethyl-5-Hydroxy-6-Formyl-Indoline-Spiro2,2-[2H] chromene) in Different Media, *Int. J. Photoenergy*, 2006, 1–9, DOI: 10.1155/IJP/2006/42846.
- 44 M. S. Attia, A. A. Essawy, A. O. Youssef and M. S. Mostafa, Determination of Ofloxacin using a Highly Selective Photo Probe Based on the Enhancement of the Luminescence Intensity of Eu³⁺ - Ofloxacin Complex in Pharmaceutical and Serum Samples, *J. Fluoresc.*, 2012, **2**, 557–564, DOI: 10.1007/s10895-011-0989-x.
- 45 M. S. Attia, A. M. Othman, A. O. Youssef and E. El-Raghi, Excited state interaction between Hydrochlorothiazide and Europium ion in PMMA polyme, and its application as optical sensor for Hydrochlorothiazide in tablet and serum samples, *J. Lumin.*, 2012, **132**, 2049–2053, DOI: 10.1016/j.jlumin.2012.03.012.
- 46 M. S. Attia, A. O. Youssef and A. A. Essawy, A novel method for tyrosine assessment in vitro by using fluorescence enhancement of the ion-pair tyrosine- neutral red dye photo probe, *Anal. Methods*, 2012, **4**, 2323–2328, DOI: 10.1039/C2AY25089F.
- 47 M. S. Attia, A. O. Youssef and R. H. El-Sherif, Durable diagnosis of seminal vesicle and sexual gland diseases using the nano optical sensor thin film sm-doxycycline complex, *Anal. Chim. Acta*, 2014, **835**, 56–64, DOI: 10.1016/j.aca.2014.05.016.
- 48 M. S. Attia, M. Diab and M. F. El-Shahat, Diagnosis of some diseases related to the histidine level in human serum by using the nano optical sensor Eu–Norfloxacin complex, *Sens. Actuators, B*, 2015, **207**, 756–763, DOI: 10.1016/j.snb.2014.10.132.
- 49 M. S. Attia, A. O. Youssef, Z. A. Khan and M. N. Abou-Omar, Alpha fetoprotein assessment by using a nano optical sensor thin film binuclear Pt-2-aminobenzimidazole-Bipyridine for early diagnosis of liver cancer, *Talanta*, 2018, **186**, 36–43, DOI: 10.1016/j.talanta.2018.04.043.
- 50 M. S. Attia and N. S. Al-Radadi, Progress of pancreatitis disease biomarker alpha amylase enzyme by new nano optical sensor, *Biosens. Bioelectron.*, 2016, **86**, 413–419, DOI: 10.1016/j.bios.2016.06.079.
- 51 M. S. Attia and N. S. Al-Radadi, Nano optical sensor binuclear Pt-2-pyrazinecarboxylic acid-bipyridine for enhancement of the efficiency of 3-nitrotyrosine biomarker for early diagnosis of liver cirrhosis with minimal hepatic encephalopathy, *Biosens. Bioelectron.*, 2016, **86**, 406–412, DOI: 10.1016/j.bios.2016.06.074.
- 52 M. S. Attia, Nano optical probe samarium tetracycline complex for early diagnosis of histidinemia in newborn children, *Biosens. Bioelectron.*, 2017, **94**, 81–86, DOI: 10.1016/j.bios.2017.02.018.
- 53 M. S. Attia, K. Ali, M. El-Kemary and W. M. Darwish, Phthalocyanine-doped polystyrene fluorescent nanocomposite as a highly selective biosensor for quantitative determination of cancer antigen 125, *Talanta*, 2019, **201**, 185–193, DOI: 10.1016/j.talanta.2019.03.119.
- 54 M. S. Attia, M. N. Ramsis, L. H. Khalil and S. G. Hashem, Spectrofluorimetric assessment of chlorzoxazone and Ibuprofen in pharmaceutical formulations by using Eutetracycline HCl optical sensor doped in sol-gel matrix, *J. Fluoresc.*, 2012, **22**, 779–788, DOI: 10.1007/s10895-011-1013-1.
- 55 A. A. Elabd and M. S. Attia, A new thin film optical sensor for assessment of UO₂²⁺ based on the fluorescence quenching of Trimetazidine doped in sol gel matrix, *J. Lumin.*, 2015, **165**, 179–184, DOI: 10.1016/j.jlumin.2015.04.024.
- 56 A. A. Elabd and M. S. Attia, Spectrofluorimetric assessment of UO₂²⁺ by the quenching of the fluorescence intensity of Clopidogrel embedded in PMMA matrix, *J. Lumin.*, 2016, **169**, 313–318, DOI: 10.1016/j.jlumin.2015.08.007.
- 57 A. A. Essawy and M. S. Attia, Novel application of pyronin Y fluorophore as high sensitive optical sensor of glucose in human serum, *Talanta*, 2013, **107**, 18–24, DOI: 10.1016/j.talanta.2012.12.033.
- 58 E. Hamed, M. S. Attia and K. Bassiony, Synthesis, spectroscopic and thermal characterization of Copper (II) complexes of folic acid and their absorption efficiency in the blood, *Bioinorg. Chem. Appl.*, 2009, 1–7, DOI: 10.1155/2009/979680.
- 59 M. S. Attia, K. Ali, M. El-Kemary and W. M. Darwish, Phthalocyanine-doped polystyrene fluorescent nanocomposite as a highly selective biosensor for quantitative determination of cancer antigen 125, *Talanta*, 2019, **201**, 185–193.
- 60 IHT Guideline, *Validation of analytical procedures: text and methodology, Q2 (R1)*, 2005, vol. 1, pp. 1–15.
- 61 Stationery Office (Great Britain), *British pharmacopoeia 2009*, Stationery Office, London, 2008.
- 62 G. Snellman, H. Melhus, R. Gedeberg, L. Byberg, L. Berglund, L. Wernroth and K. Michaëlsson, Determining vitamin D status: a comparison between commercially available assays, *PLoS One*, 2010, **5**, e11555.
- 63 U. Turpeinen, S. Linko, O. Itkonen and E. Hämäläinen, Determination of testosterone in serum by liquid chromatography-tandem mass spectrometry, *Scand. J. Clin. Lab. Invest.*, 2008, **68**, 50–57.
- 64 M. S. Newman, T. R. Brandon, M. N. Groves, W. L. Gregory, S. Kapur and D. T. Zava, A liquid chromatography/tandem mass spectrometry method for determination of 25-hydroxy vitamin D₂ and 25-hydroxy vitamin D₃ in dried blood spots: a potential adjunct to diabetes and cardiometabolic risk screening, *J. Diabetes Sci. Technol.*, 2009, **3**, 156–162.