Spectrophotometric determination of thrombin in pure samples and biological fluids using π-acceptors

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Determinación espectrofotométrica de trombina en muestras puras y fluidos biológicos utilizando aceptores π.

Determinació espectrofotomètrica de trombina en mostres pures i fluids biològics utilitzant aceptors π.

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RESUMEN

La trombina es la enzima central de la coagulación. Está implicada en funciones opuestas en la sangre. Como factor procoagulante, convierte el fibrinógeno en un coágulo de fibrina insoluble y activa las plaquetas, como anticoagulante, cuándo activa la Proteína C. Este hecho se utiliza para el control farmacológico de la coagulación de la sangre, así que el seguimiento de su actividad es un indicador fiable del índice y extensión de la coagulación. Se propone un método espectrofotométrico sencillo, rápido, sensible y exacto para la determinación de trombina en forma pura y en fluidos biológicos. Se describe la utilidad de algunos aceptores π como 2,3-dicloro-5,6-dicianobenzoquinona (DDQ), 7,7,8,8-tetracianoquinodimetano (TCNQ) y tetracianoetileno (TCNE) para la determinación de trombina (como donante de electrones). Estos aceptores π proporcionan especies complejas altamente coloreadas que han sido estudiadas espectrofotométricamente. Las condiciones experimentales óptimas para estas reacciones CT han sido estudiadas cuidadosamente. La ley de Beer se cumple en los rangos de concentraciones de 10-130, de 50-150 y de 10-100 µg ml-1 de trombina utilizando DDQ, TCNQ y TCNE como reactivos respectivamente. Los porcentajes de recuperación son entre 99.33 y 100.1% (SD = 0.032-0.075), entre 99.50 y 102.5% (SD = 0.016-0.076) y entre 99.5 y 101.4% (SD = 0.034-0.088) para cuatro a seis experimentos. Los reactivos se han utilizado en la determinación de trombina en plasma pobre en plaquetas de pacientes de diálisis con un porcentaje de recuperación de 98.76-103.3% (para n = 5). No se encontró ninguna interferencia de compuestos endógenos. Los resultados obtenidos utilizando reactivos aceptores-π son comparables con aquellos obtenidos por el método oficial.

Palabras clave: Trombina, DDQ, TCNQ, TCNE, espectrofotometría, complejos de transferencia de carga.

SUMMARY

Thrombin is the central enzyme of coagulation. It is engaged in opposing functions in blood. As a procoagulant factor, it converts fibrinogen into an insoluble fibrin clot and activate platelet, as anticoagulant when it activates Protein C. This knowledge is used for the pharmacologic control of blood coagulation, so monitoring its activity is reliable indicator of the rate and extent of coagulation. A simple, rapid, sensitive and accurate spectrophotometric method is suggested for the determination of thrombin in pure form and in biological fluids. The utility of some π-acceptors as 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ), 7,7,8,8-tetracyanoquinodimethane (TCNQ) and tetracyanoethylene (TCNE) for thrombin (as electron donor) determination is described. These π-acceptors give highly coloured complex species that have been spectrophotometrically studied. The optimum experimental conditions for these CT reactions have been studied carefully. Beer's law is obeyed over the concentration ranges of 10-130, 50-150 and 10-100 µg ml^{-1} thrombin using DDQ, TCNQ and TCNE reagents, respectively. The percentage recovery amounts to 99.33-100.1% (SD = 0.032-0.075), 99.50-102.5% (SD = 0.016-0.076) and 99.5-101.4% (SD = 0.034-0.088) for four to six experiments. The reagents are utilized for the determination of thrombin in poor platelet plasma of dialysis patients with a percentage recovery amount to 98.76-103.3% (for n = 5). No endogenous compounds were found to interfere. The results obtained applying the π-acceptors reagents are comparable with those obtained by the official method.

Keywords: Thrombin, DDQ, TCNQ, TCNE, Spectrophotometry, Charge transfer complexes.

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RESUM

La trombina és l'enzim central de la coagulació. Està implicat en funcions oposades en la sang. Com a factor procoagulant, converteix el fibrinògen en un coàgul de fibrina insoluble i activa les plaquetes, com anticoagulant, quan activa la Proteïna C. Aquest fet s'utilitza per al control farmacològic de la coagulació de la sang, així que el seguiment de la seva activitat és un indicador fiable de l'índex i extensió de la coagulació. Es proposa un mètode espectrofotomètric senzill, ràpid, sensible i exacte per a la determinació de trombina en forma pura i en fluids biològics. Es descriu la utilitat d'alguns acceptors-π com 2,3-dicloro-5,6-dicianobenzoquinona (DDQ), 7,7,8,8-tetracianoquinodimetà (TCNQ) i tetracianoetilé (TCNE) per a la determinació de trombina (com a donant d'electrons). Aquests acceptors π proporcionen espècies complexes altament acolorides que han estat estudiades espectrofotomètricament. Les condicions experimentals òptimes per a aquestes reaccions CT han estat estudiades acuradament. La llei de Beer es compleix en els rangs de concentracions de 10-130, de 50-150 i de 10-100 µg ml-1 de trombina utilitzant DDQ, TCNQ i TCNE com a reactius respectivament. Els percentatges de recuperació estan entre 99.33 i 100.1% (SD = 0.032-0.075), entre 99.50 i 102.5% (SD = 0.016-0.076) i entre 99.5 i 101.4% (SD = 0.034-0.088) per entre quatre i sis experiments. Els reactius s'han utilitzat en la determinació de trombina en plasma pobra en plaquetes de pacients de diàlisis amb un percentatge de recuperació de 98.76-103.3% (per a n = 5). No es va trobar cap interferència de compostos endògens. Els resultats obtinguts utilitzant reactius acceptors π són comparables amb els obtinguts pel mètode oficial.

Paraules clau: Trombina, DDQ, TCNQ, TCNE, espectrofotometria, complexos de transferència de càrrega.

1. INTRODUCTION:

Thrombin, a multifunctional serine protease, recognizes multiple macro-molecular substrates and plays a key role in both procoagulant and anticoagulant functions (Tasset et al., 1997). So the detection of thrombin is very important for clinical diagnosis and biological research. Aptamers are short oligonucleotides selected by an in vitro selection process (Ellington et al., 1990) that can bind to a broad range of molecular targets with high-affinity, such as amino acids, drugs, proteins and other molecules, and are considered as promising recognition elements for biosensor applications. The discovery of aptamers offers a new way for the detection of thrombin. A series of detection techniques using aptamers to determine thrombin have been reported, such as fluorescence (Fang et al., 2003; Myoyong et al., 2000; Jiangwei et al., 2002; Hamaguchi et al., 2001; Stojanovic et al., 2001), chemiluminescence (Yaxin et al., 2004), colourimetric (Stojanovic et al., 2002; Huang et al., 2005; Pavlov et al., 2002), electrochemistry (Ikebukuroet al., 2005; Giusto et al., 2005), and AFM (Jiang et al., 2003). Among them, fluorescence detection is most widely used. Unfortunately, most of these measurement techniques need fluorescence labeling or some other functional changes of the aptamer before designing the aptamer probes, which may result in high cost, complicated designing and operating. In recent years, great attention has been focused on the use of nanoparticle labels on the aptamer in order to overcome the problems associated with fluorescent labels and other functional. For example, Pavlov et al. designed a colorimetric method based on gold nanoparticles to detect thrombin in homogeneous and heterogeneous forms (Pavlov et al., 2002). Since Pasternack (Pasternack et al. 1993) applied the resonance light scattering (RLS) technique to study the aggregation of porphyrin and chlorophyll, this technique gradually drew many researchers' attention because of high sensitivity and operational simplicity. As a new spectral analysis technique, the RLS technique has been widely used to quantitatively determine proteins (Huang et al., 2001; Jia et al., 2004) in aqueous solution in view of the enhanced RLS signals. The present research aims chiefly to study the reaction of DDQ, TCNQ and TCNE reagents (electron acceptors) with thrombin (electron donor) and use these reagents in spectrophotometric determination of the given drug in pure form and in some biological fluids.

2. EXPERIMENTAL

2.1. Reagents

All chemicals and reagents were of analytical reagent grade and used without any further purification. The water was always de-ionized. Thrombin was provided by Diagnostica Stago, 2,3-dichloro-5,6-dicyano-p-benzoquinone (DDQ) was supplied from Arcos-USA, while 7,7,8,8-tetracyanoquinodimethane (TCNQ) and tetracyanoethylene (TCNE) were supplied from Aldrich company, USA. Fresh solutions of DDQ (0.01% w/v) and TCNQ or TCNE (0.02%, w/v) in acetonitrile were freshly prepared. Standard solution of the drug containing 1 mg ml^{-1} in acetonitrile is freshly prepared for studying reactions with DDQ, TCNQ and TCNE reagents.

2.2 Apparatus

The spectrophotometric measurements were carried out using the manual Unico 1200 (United Products and Instruments, Inc.) in the wavelength range from 325 - 1000 nm. Small volumes were taken using micropipette.

2.3. General Procedure

2.3.1. Bulk sample

In calibrated 10 ml volumetric flask, different aliquots containing 1.0 ml of $1x10^{-3}$ mol/l of thrombin were added to 1 ml of 0.1% (w/v) DDQ or 0.02% (w/v) TCNQ or TCNE solutions. The volumes were completed to the mark with acetonitrile. The solutions were left to stand for few minutes at room temperature before the absorbance was measured at λ = 470, 842 and 394 nm for DDQ, TCNQ, TCNE reagents, respectively, against a blank solution prepared in the same manner without drugs. The thrombin drug concentrations were calculated from the standard calibration graph prepared under the same identical condition.

2.3.2. Determination of thrombin in biological fluids:

Freshly blood was collected by taking it into 0.11 mol\l trisodium citrate in the ratio of 9 parts of blood to 1 part of anticoagulant. The sample was centrifuged immediately for 10 minutes at 3000 rpm and plasma was separated into a clean test tube. The plasma was stored at -4 °C until analysis. An aliquot was used for the determination of thrombin drug according to the procedure mentioned above.

3. RESULTS AND DISCUSSION

3.1. Absorption spectra

The amino group of thrombin reacting being n-electron donors, reacts with π-acceptors giving characteristic colour reaction products due to the formation of charge transfer complexes (Abdel-Hamid et al 1988). Addition of the different π-acceptors (namely, DDQ, TCNQ and TCNE) to the drug solutions cause changes in the absorption spectra due to the formation of charge transfer complexes with new characteristic bands at maximum absorption depending on the type of the π-acceptors (Fig.1a-c). The CT complexes are formed by the interaction of the investigated drug as n-electron donor and DDQ, TCNQ and TCNE reagents as π–acceptors. The spectrophotometric properties of the coloured CT complexes as well as the different parameters affecting the colour development between thrombin and DDQ, TCNQ and TCNE are extensively studied to determine the optimal conditions for the assay procedure. The interaction of thrombin with DDQ in acetonitrile at room temperature gives red coloured chromogen with a strong absorption maximum at 470 nm (Abdel-Hamid et al, 1985 and Abdel-Salam et al, 1985).

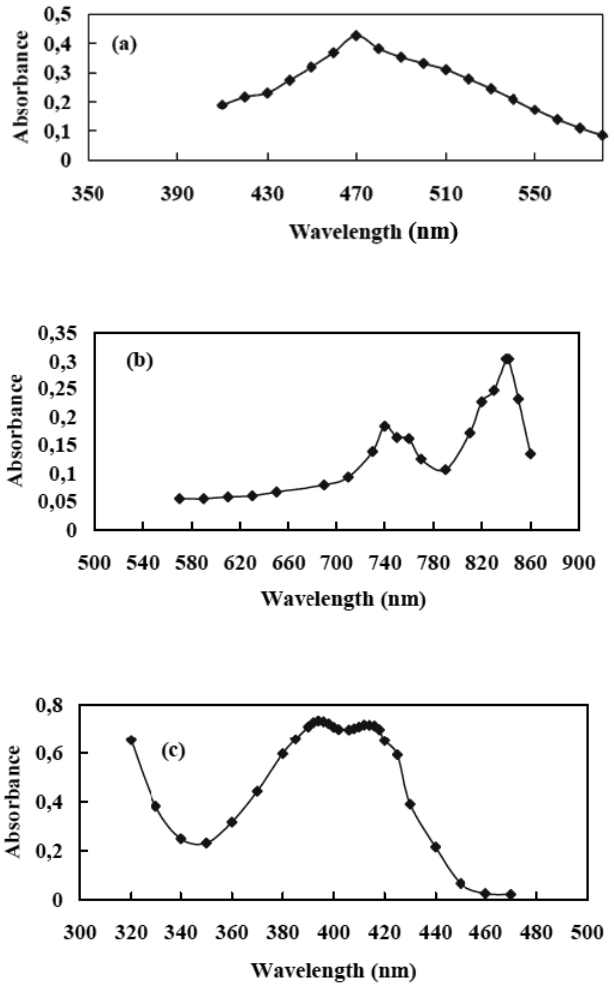


Fig (1). *Absorption spectra of (a) Thrombin-DDQ, (b) Thrombin-TCNQ and (c) Thrombin-TCNE CT complexes.*

The interaction of thrombin with TCNQ gives a green chromogen, which exhibits strong absorption maxima at 842 and 740 nm. The wavelength 842 nm is selected as it gives reproducible results and higher molar absorptivity. These bands may be attributed to the formation of the radical anion TCNQ (Nour El-Dien et al, 2006; 2009), which was probably formed by the dissociation of an original donor–acceptor (D–A) complex which is promoted by the high ionizing power of the acetonitrile solvent (Figure 2).

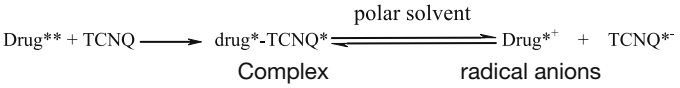


Figure 2. *Charge transfer complex formation.*

The interaction of thrombin with TCNE in acetonitrile at room temperature give intense yellow coloured chromogen with a strong absorption maximum at 394 nm.
Optimization of reaction conditions

3.1. Effect of solvents

In order to select the suitable solvent for CT complex formation, the reaction of DDQ, TCNQ and TCNE reagents with thrombin is made in different solvents. These solvents included acetonitrile, chloroform, n-propanol, methanol, acetone, tetrahydrofuran, 1,4-dioxane, carbon tetrachloride, butanol, 1,2-dichloromethane and dimethyl formamide. It is found that acetonitrile is considered to be an ideal solvent for the colour reaction because it offered an excellent solvating power for DDQ and TCNE reagents. While for TCNQ reagent, it is found that, although acetonitrile has low absorbance values than acetone and ethanol, the absorbance readings are more stable and reproducible. Moreover, being the acetonitrile a polar solvent, it facilitates the complete transfer of charge from donor to acceptor with the formation of radical anion as the predominant chromogen indicated by high ε values, which is attributed to its high dielectric constant (Vogel's Textbook of Practical Organic Chemistry, 1989).

3.2. Effect of time

The optimum reaction time is determined by following the colour development spectrophotometrically at temperature of 25ºC - 40°C for DDQ, TCNQ and TCNE reagents. It is found that, complete colour development is attained after 25, 25 and 30 minutes for DDQ, TCNQ and TCNE reagents, respectively. Also the colour remains stable for one day (Fig: 3 a).

3.3. Effect of temperature

The effect of temperature on the CT complexes formed as the results of the reaction of thrombin drug with DDQ, TCNQ and TCNE reagents in acetonitrile is shown in Fig (3b). It is found that colour development occurs at ambient temperature 30 ± 3ºC, 20 ± 5ºC or 20 ± 1ºC for DDQ, TCNQ and TCNE reagents, respectively.

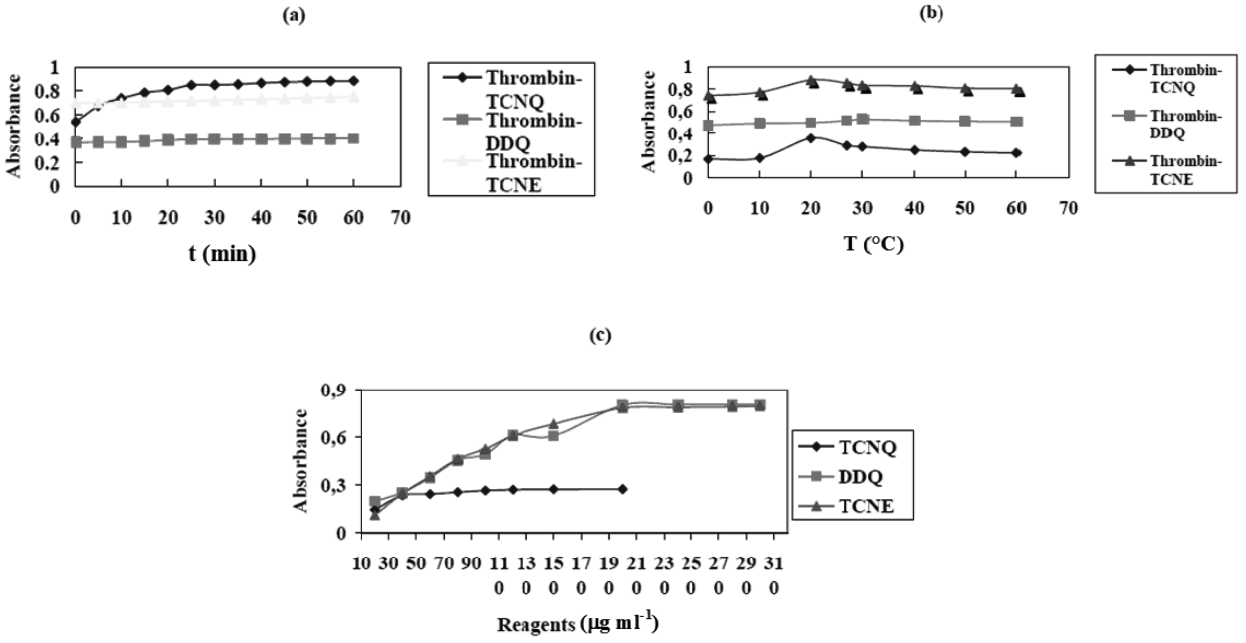
3.4. Effect of reagents concentrations

Various concentrations of DDQ, TCNQ and TCNE reagents are added to fixed concentration of thrombin in acetonitrile (Fig 3c). It is obvious from the figure that 2.0, 1.2 or 2.0 ml of DDQ, TCNQ or TCNE solutions, respectively, are found to be sufficient for the production of maximum and reproducible colour intensity. Higher concentrations of

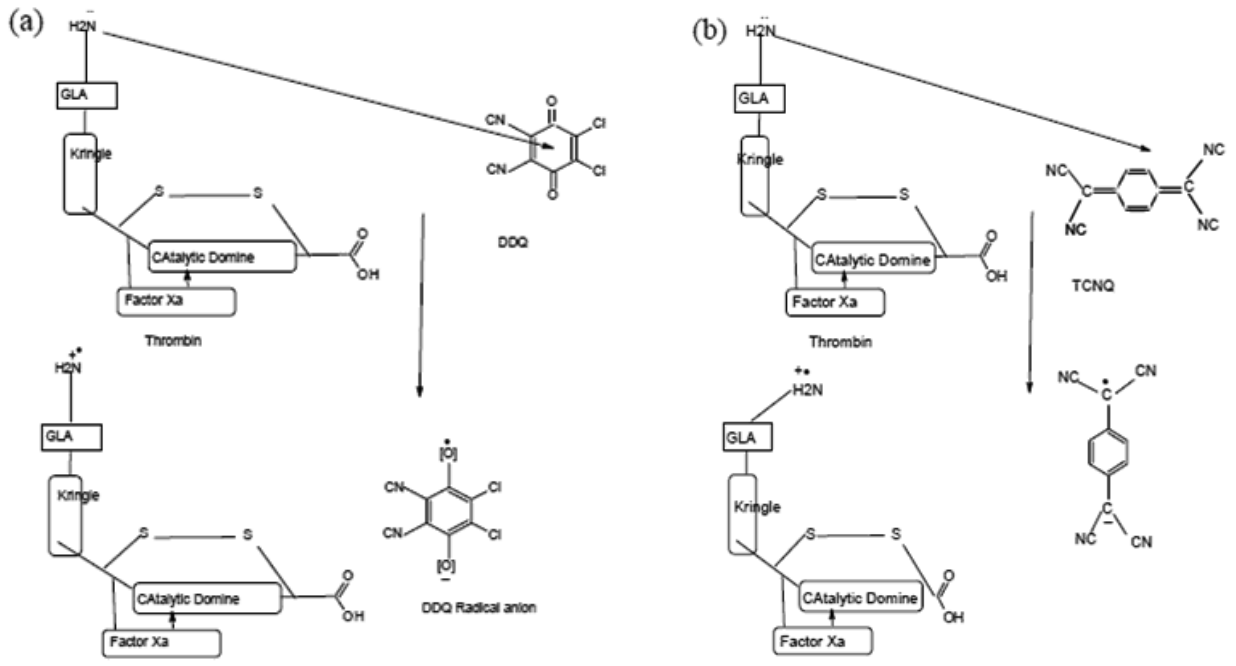
the reagents have no effect, but may be useful for rapidly reaching equilibrium, thus minimizing the time required for attaining maximum absorbance readings at the corresponding wavelengths.
The proposed structures of the CT complexes are given in Figure (4 a-c)

3.6. Spectrophotometric determination of thrombin in pure form using DDQ, TCNQ and TCNE reagents

Under the optimum conditions described above, the calibration graphs were constructed for the drug. Table (1) shows the different analytical parameters obtained. It is obvious from Table (1) that Beer's law is obeyed over the concentration ranges from 10-130, 50-150 and 10-100 $\mu g \cdot ml^{-1}$ of thrombin using DDQ, TCNQ and TCNE reagents, respectively. The molar absorptivity, Sandell sensitivity (S) and regression equation for each drug is tabulated in Table (1). The correlation coefficients of the data obtained are 0.9948, 0.9924 and 0.9926 with DDQ, TCNQ and TCNE reagents, respectively. The SD values are found to be 0.032 – 0.075, 0.016 - 0.076 and 0.034 – 0.088 and the relative standard deviations (RSD) are found to be 0.23 – 1.35, 0.22 - 1.30 and 0.49 – 1.70 with DDQ, TCNQ and TCNE,



Fig(3). *Effect of (a) Time, (b) Temperature and (c) Reagents concentrations on the CT complexes of thrombin with DDQ, TCNQ and TCNE reagents.*



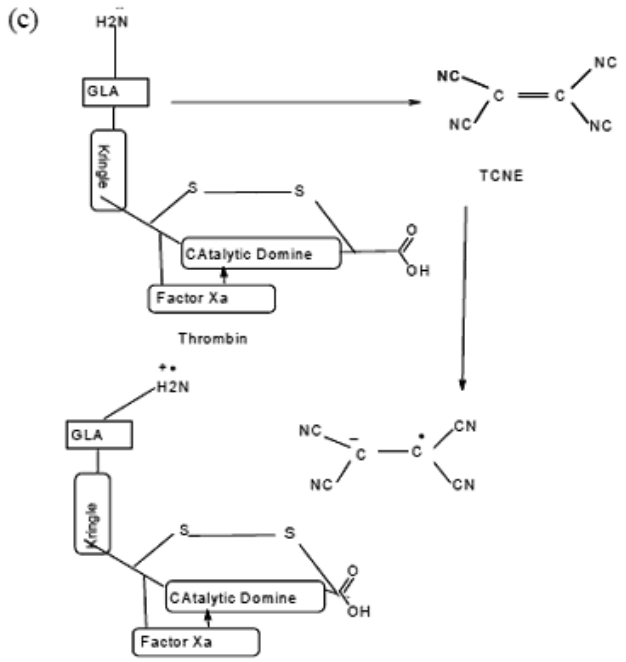


Figure (4): *Proposed structures of CT complexes of thrombin with (a) DDQ, (b) TCNQ and (c) TCNE reagents.*

reagents, respectively. The small values of relative standard deviations indicate the high accuracy and high precision of the proposed spectrophotometric method. The limits of detection and quantification are also calculated and tabulated in Table (1).

Table (1). *Analytical parameters for the determination of thrombin colored reaction products and the characteristics (accuracy and precision) of these reactions.*

Parameters	Results		
	DDQ	TCNQ	TCNE
λ_{max}, nm	470	842	394
Molar absorptivity, l. mol^{-1}. cm^{-1}	11.7×10^4	10.1× 10^4	24.3× 10^4
Sandell Sensitivity, µg·cm^{-2}	2.5×10^{-4}	2.98×10^{-4}	1.24×10^{-4}
Beer's law limit, , µg·ml^{-1}	10-130	50-150	10-100
Percentage recovery, %	99.33-100.1	99.50-102.5	99.50-101.4
Range of Error, %	0-0.83	0-0.66	0-1.4
Standard Deviation (SD)	0.032-0.075	0.016-0.076	0.034-0.088
Relative Standard Deviation, (RSD) %	0.23- 1.35	0.22- 1.30	0.49- 1.70
Regression equation *, slope (b)	0.0025	0.0038	0.0034
Intercept (a)	0.0591	0.0175	0.1589
Correlation coefficient (r)	0.9948	0.9924	0.9926
LOD , µg ml^{-1}	0.95	0.90	0.92
LOQ , µg ml^{-1}	3.17	3.00	3.07

**A = a + bC; where C is the concentration in µg ml^{-1}*

3.7. Between-day measurement

In order to prove the validity and applicability of the proposed method and reproducibility of the results obtained, five replicate experiments at different concentrations of thrombin were carried out. Table (2) shows the values of the between-day relative standard deviations for different concentrations of the thrombin drug, obtained from the experiments carried out over a period of four days. It was found that, the within day relative standard deviations are less than 2%, which indicates that the proposed method is highly reproducible and DDQ, TCNQ and TCNE reagents are successfully applied to determine thrombin via the charge transfer reaction.

3.10. Spectrophotometric determination of thrombin in poor platelet plasma of dialysis patients using DDQ, TCNQ and TCNE reagents.

The specificity of the method is confirmed by the analysis of a variety of different blank plasma samples from healthy volunteers (n = 5), none of which yielded any endogenous interference. The proposed method is successfully applied to the determination of thrombin in poor platelet plasma for dialysis patient before and after dialysis and the results obtained are given in Tables (3-5). There is no official reported method for thrombin determination, therefore, a similar work is done and the results obtained are taken as the standard comparison method. From the calculated t- and F- values, it is clear that, the results obtained by the proposed method are in good agreement with those obtained by applying other - -acceptor reagent namely p-chloranilic acid and subsequently spectrophotometric measurement (Amin et al. 1995). The proposed method is found to be accurate, with high recoveries amounting to 96.70 – 100.9, 98.70 – 101.3 and 99.5 – 103.3% for DDQ, TCNQ and TCNE reagents, respectively.

4. CONCLUSION

A simple, rapid and accurate spectrophotometer method for the determination of thrombin drug in raw material and plasma was developed in this study. The method is based on charge transfer formation using π-acceptor reagents such as DDQ, TCNQ and TCNE reagent. Different experi-

mental factors have been extensively studied in order to obtain the optimal analysis conditions. The concentration limits, the limit of detection and the limit of quantification were calculated. According to the results thrombin drug can be determined in a wide concentration range using charge transfer method complex formation. The smaller values of SD and RSD indicated the reliability, accuracy and precision of the suggested procedure.

Table (2). *Between-day precision of the determination of thrombin drug using DDQ, TCNQ and TCNE reagents.*

Reagents	[Drug] Taken, μg ml^{-1}	[Drug]* Found, μg ml^{-1}	Percentage Recovery (%)	SD	RSD (%)
DDQ	30.00	29.75	99.16	0.056	0.28
	50.00	50.20	100.4	0.023	0.68
	70.00	69.90	99.85	0.043	1.50
	90.00	90.10	100.1	0.064	0.23
	100.0	100.0	100.0	0.011	0.63
TCNQ	40.00	40.02	100.1	0.015	1.30
	50.00	49.90	99.80	0.024	0.61
	60.00	60.10	100.2	0.049	0.43
	80.00	80.00	100.0	0.027	0.81
	100.0	100.4	100.4	0.076	1.60
TCNE	10.00	10.10	101.0	0.036	0.29
	30.00	29.80	99.33	0.030	1.05
	60.00	59.96	99.93	0.072	0.79
	70.00	71.00	101.4	0.072	0.64
	100.0	101.0	101.0	0.073	1.20

Table (3). *Spectrophotometric determination of thrombin in poor platelet plasma of dialysis patients using DDQ and official methods.*

Samples	[Drug] Taken μg ml^{-1}	[Drug] μg ml^{-1} DDQ Method	[Drug] μg ml^{-1} Official Method	SD*	SD**	F-test	t-test
Thrombin before Dialysis Processes	25.00	24.91	25.05	0.018	0.015	1.44	1.55
	30.00	29.60	30.01	0.056	0.011	0.19	1.46
	40.00	40.00	40.00	0.015	0.023	0.43	0.00
	45.00	44.75	45.03	0.021	0.018	1.36	2.60
	50.00	50.10	49.96	0.032	0.040	0.64	0.87
	60.00	60.30	59.85	0.034	0.021	2.60	2.40
	85.00	85.50	85.00	0.013	0.018	0.52	2.70
Thrombin after Dialysis Processes	30.00	29.00	29.02	0.058	0.013	0.050	0.69
	55.00	55.00	55.01	0.041	0.040	1.050	0.48
	60.00	60.00	60.00	0.035	0.016	0.220	0.00
	65.00	65.10	65.03	0.081	0.035	0.190	1.75
	70.00	70.00	70.00	0.043	0.020	0.220	0.00
	82.00	82.80	82.04	0.090	0.019	0.044	1.67
	100.00	99.80	100.01	0.011	0.044	0.063	1.90
Percent recovery of DDQ method				96.70-100.9%			
Percent recovery of official method				96.70-100.2%			

No. of replicates (n) = 5.
Tabulated F-value at 95 % confidence level = 5.05Tabulated t-value at 95 % confidence level = 2.571
** Standard deviation values using proposed method.*
*** Standard deviation values using official method.*

Table (4). *Spectrophotometric determination of thrombin in poor platelet plasma of dialysis patients using TCNQ and official methods.*

Samples	[Drug] Taken μg ml^{-1}	[Drug] μg ml^{-1} TCNQ Method	[Drug] μg ml^{-1} Official Method	SD*	SD**	F-test	t-test
Thrombin before Dialysis Processes	25.00	25.30	25.05	0.010	0.015	0.44	0.45
	30.00	29.90	30.01	0.063	0.011	0.03	0.19
	40.00	40.50	40.00	0.015	0.023	0.43	0.67
	45.00	44.60	45.03	0.018	0.018	1.00	2.70
	50.00	49.90	49.96	0.024	0.040	0.36	1.20
	60.00	60.00	59.85	0.049	0.021	0.18	0.61
	85.00	84.60	85.00	0.035	0.018	0.26	2.20
Thrombin after Dialysis Processes	30.00	29.90	29.02	0.063	0.013	0.04	2.70
	55.00	54.30	55.01	0.071	0.040	0.32	2.25
	60.00	60.00	60.00	0.049	0.016	0.11	0.00
	65.00	64.90	65.03	0.052	0.035	2.21	0.50
	70.00	69.80	70.00	0.013	0.020	0.42	2.30
	82.00	83.10	82.04	0.027	0.019	2.01	1.48
	100.00	100.1	100.01	0.076	0.044	0.28	0.24
Percent recovery of TCNQ method				98.70-101.3%			
Percent recovery of official method				96.78-100.5%			

No. of replicates (n) = 5.
Tabulated F-value at 95 % confidence level = 5.05
Tabulated t-value at 95 % confidence level = 2.571
** Standard deviation values using proposed method.*
*** Standard deviation values using official method.*

Table (5). *Spectrophotometric determination of thrombin in poor platelet plasma of dialysis patients using TCNE and official methods.*

Samples	[Drug] Taken μg ml^{-1}	[Drug] μg ml^{-1} TCNE Method	[Drug] μg ml^{-1} Official Method	SD*	SD**	F-test	t-test
Thrombin before Dialysis Processes	25.00	25.15	25.05	0.033	0.015	0.21	0.62
	30.00	31.10	30.01	0.030	0.011	0.14	2.70
	40.00	39.80	40.00	0.019	0.023	0.68	2.10
	45.00	44.80	45.03	0.021	0.018	1.36	2.20
	50.00	49.60	49.96	0.011	0.040	0.08	2.90
	60.00	60.30	59.85	0.027	0.021	1.65	0.85
	85.00	85.50	85.00	0.072	0.018	0.06	1.38
Thrombin after Dialysis Processes	30.00	31.00	29.02	0.035	0.013	0.14	1.13
	55.00	54.90	55.01	0.041	0.040	1.05	1.17
	60.00	60.00	60.00	0.027	0.016	2.85	0.00
	65.00	64.80	65.03	0.039	0.035	1.24	1.30
	70.00	69.80	70.00	0.072	0.020	0.09	0.65
	82.00	83.20	82.04	0.016	0.019	0.71	1.45
	100.00	99.85	100.0	0.073	0.044	2.80	0.68
Percent recovery of TCNE method				99.50-103.3%			
Percent recovery of official method				96.70-100.2%			

No. of replicates (n) = 5.
Tabulated F-value at 95 % confidence level = 5.05
Tabulated t-value at 95 % confidence level = 2.571
** Standard deviation values using proposed method.*
*** Standard deviation values using official method.*

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