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ORIGINAL ARTICLE

A Novel Method for the Assessment of Cortisol Hormone in Different Body Fluids Using A New Photo Probe Thiazole Derivative

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Abstract A low cost and accurate method for the detection and analytical determination of the cortisol in pharmaceutical preparation, blood serum and urine was developed. The method was based upon the enhancement of fluorescence intensity of the band at 424 nm of the photo probe by different cortisol concentrations in acetonitrile at (pH 5.7, λ_{ex}=320 nm). The influence of the different parameters, e.g. pH, solvent, cortisol concentration and foreign ions concentrations that control the enhancement process of fluorescence intensity of the band of photo probe was critically investigated. The remarkable enhancement of the fluorescence intensity at 424 nm in acetonitrile by various concentrations of cortisol was successfully used as a photo- probe for the assessment of cortisol concentration. The calibration plot was achieved over the concentration range 8.0×10^{-6}–5.5×10^{-9} mol L^{-1} cortisol with a correlation coefficient of 0.998 and a detection limit of 4.7×10^{-9} mol L^{-1}. The developed method is simple and proceeds without practical artifacts compared to the other determination methods.

Keywords Cortisol · Fluorescence enhancement · Photo probe · Selectivity

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Introduction

The hormone Cortisol,(11β)-11,17,21-trihydroxypregn-4-ene-3,20-dione Fig. 1 is the most potent glucocorticoid produced by human adrenals, playing an important role in human physiology. It is a necessary glucocorticoid hormone to maintain life and plays a number of important functions in humans because it affects glucose production, fat metabolism, inflammatory responses, vascular responsiveness, central nervous system and immune functioning [1]. The concentration of blood cortisol generally indicates the hyperirritability level of the body. The concentrations of free cortisol in serum and urine were mostly measured by radioimmunoassay (RIA) in clinical diagnosis. The RIA method was sensitive, but had cross-reactivity with other steroids, giving rise to higher concentrations than reality [2]. Some other methods for the measurements of plasma cortisol were also described, including high performance liquid chromatography (HPLC) [3], LC with tandem mass spectrometry (LC/MS/MS) [4], and chemiluminescence immunoassay techniques [5, 6]. The HPLC method using column switching showed interference by coexisting material [7]. Although the LC/MS/MS method was highly selective and sensitive, it added to the cost and complexity of the assay and decreased its throughput. So far, there seems to be only one report on the application of chemiluminescence for determination of blood cortisol, which utilized the cerium(IV)–sulfite reaction system [8]. Another study related to determination of serum cortisol [9] was by HPLC assisted by chemiluminescence detection.

The present work is focused on the determination of cortisol by the enhancement of the fluorescence intensity of the band at 424 nm of new synthesized photo probe Fig. 1 (5-(p-ethoxy)

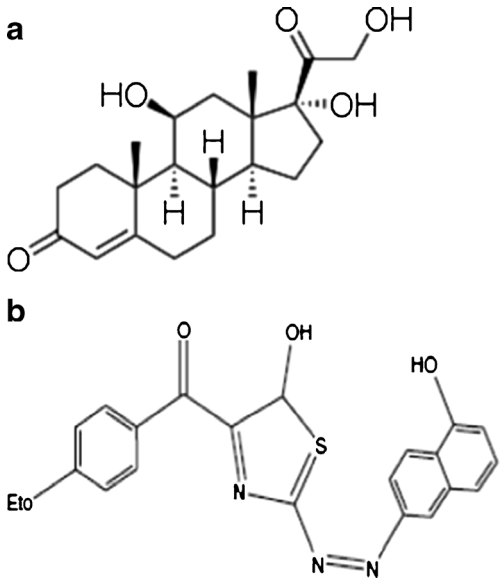


Fig. 1 Chemical structure of Cortisol(a) and 5-(p-ethoxy) benzoyl methyl-2-diazo- α -naphthol thiazol(b)

benzoyl methyl-2-diazo- α -naphthol thiazol) in acetonitrile at pH 5.7.

Experimental

Materials

Pure standard cortisol supplied by the National organization for Drug control and Research (Giza, Egypt). Pharmaceutical preparation of sigmacortin (injection vial) produced by The Nile Co. for Pharmaceuticals and chemical Industries; Egypt is purchased from local market.

Chemicals and Reagents

All chemicals used are analytical-reagent of higher grade. Pure grade solvents from (Sigma- Aldrich) are used for the preparation of all solutions and during the all determinations. β-(p-ethoxy)benzoylacrylic acid and thiourea were purchased from (Sigma- Aldrich). A stock solution of cortisol (1×10^{-3} mol L^{-1}) is freshly prepared and dissolved in ethanol and stored at 4 °C when not in use. The working standard solutions of (3×10^{-5}–1×10^{-9} mol L^{-1}) are freshly prepared by appropriate dilution with acetonitrile. Azothiazole (photo probe) stock solution (1×10^{-4} mol L^{-1}) is prepared by dissolving Azothizole with a small amount of ethanol in 25 ml measuring flask, then diluting to the mark with ethanol. The fluorescence intensity was measured at $\lambda_{ex}/\lambda_{em}$=320/424 nm. Stock and working solutions were stored at 0–4 °C when not in use. In all experiments, clean and sterilized volumetric flasks (10 mL) were used.

Apparatus

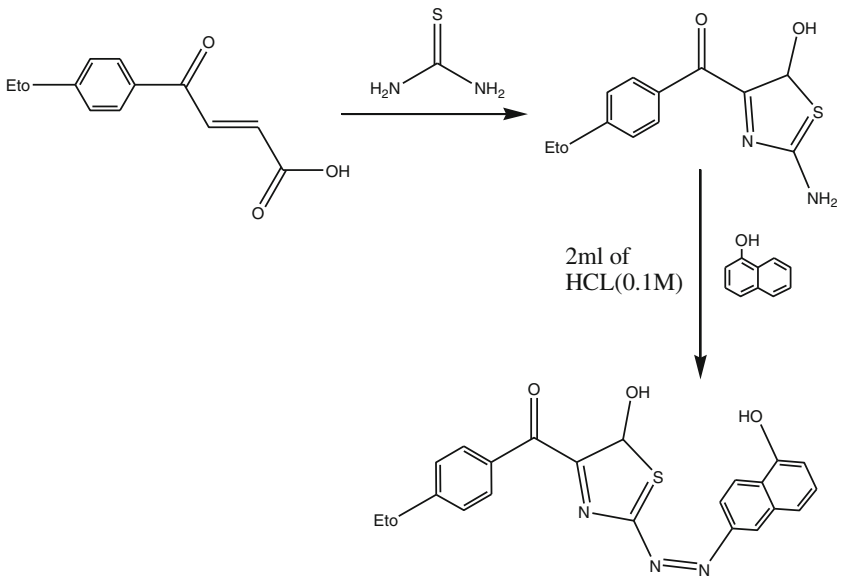
IR Spectroscopy

IR spectra were obtained on a Pye-Unicam SP3100 Spectrophotometer KBr technique (υ_{max} in cm^{-1}).

^{1}HNMR Spectroscopy

^{1}HNMR spectra were recorded on JNMFT 300-Lambda series,chemical shifts were given in ppm (δ-scale) in DMSO using TMS as internal standard.

Scheme 1 synthesis of the photo probe



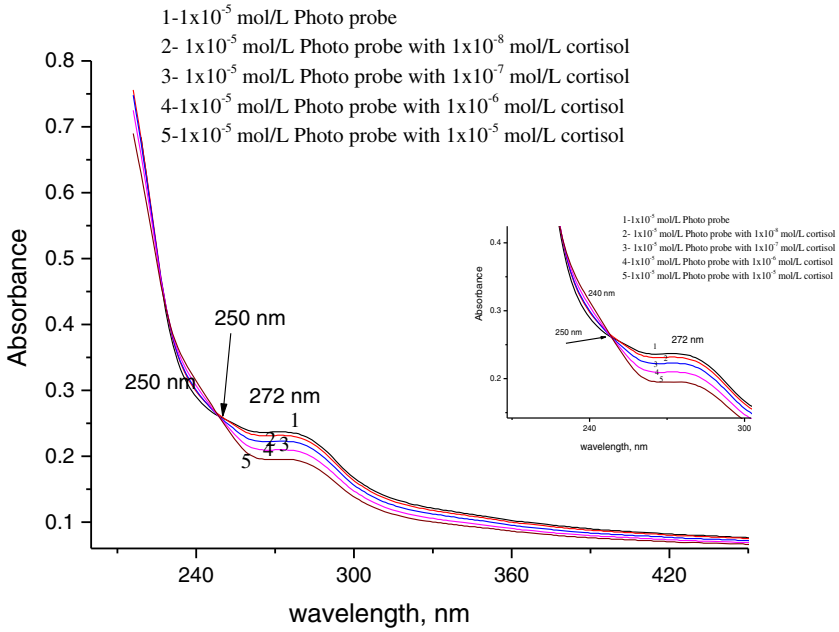


Fig. 2 Absorption spectra of 1×10^{-5} mol/L of photo probe (1) and photo probe in different concentrations of cortisol (2–5)

Mass Spectroscopy

MS spectra were measured on Jeol, JMS 600 Spectrophotometer.

Spectrofluormeter

All fluorescence measurements are carried out on Prekin Almer LS 45 Spectrofluorophotometer in the range (290–750 nm with attenuator 30 %).

Spectrophotometer

The absorption spectra are recorded with Thermo UV-Visible double-beam Spectrophotometer.

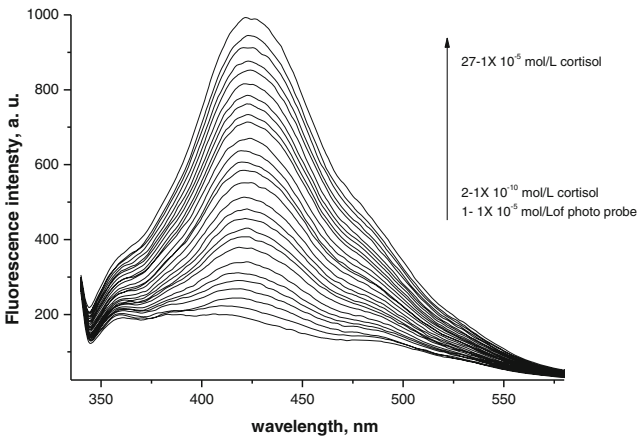


Fig. 3 The fluorescence intensity of 1×10^{-5} mol/L photo probe in the presence of different concentration of cortisol in acetonitrile at pH 5.7 and λ_{ex}=320 nm

pH Meter

All pH measurements are made with Crison Instruments S.A.E.08328 ALELLA-Barcelona (EU).

General Procedure

Preparation of Cortisol Solutions

To 10 ml clean and sterilized measuring flasks, the standard solutions of cortisol were prepared by different additions of (1×10^{-3} mol L^{-1}) cortisol solution. The solutions were diluted to the mark with acetonitrile at room temperature. The above method was used for the subsequent measurements of absorption, emission spectra, effect of pH and effect of solvents. The fluorescence intensity was measured at $\lambda_{ex}/\lambda_{em}$=320/424 nm.

Preparation of 5-(p-ethoxy) benzoyl methyl-2-diazo-α –naphthol-4-hydroxy thiazol

The reaction of β-(p-ethoxy)benzoylacrylic acid(1) with thiourea gives 2-amino-4-hydroxy-5-(p-ethoxy) benzoyl methyl thiazole. Diazotization of 2-amino-4-hydroxy-5-(p-ethoxy) benzoyl methyl thiazole and coupling with naphthols (0.1 mole) of 2-amino-4-hydroxy-5-(p-ethoxy) benzoyl methyl thiazole was dissolving in (0.2 mL HCl), cool at 5 °C then add 0.2 mL of HCl, filter the formed precipitate .add nitrous acid to the filtrate until the brown gases evolved then add this mixture to sod (α-napthoate) crystallized from ethanol in dark brown crystals .m.p. 280 °C,yield 40 %. [10](photo probe) Scheme 1.

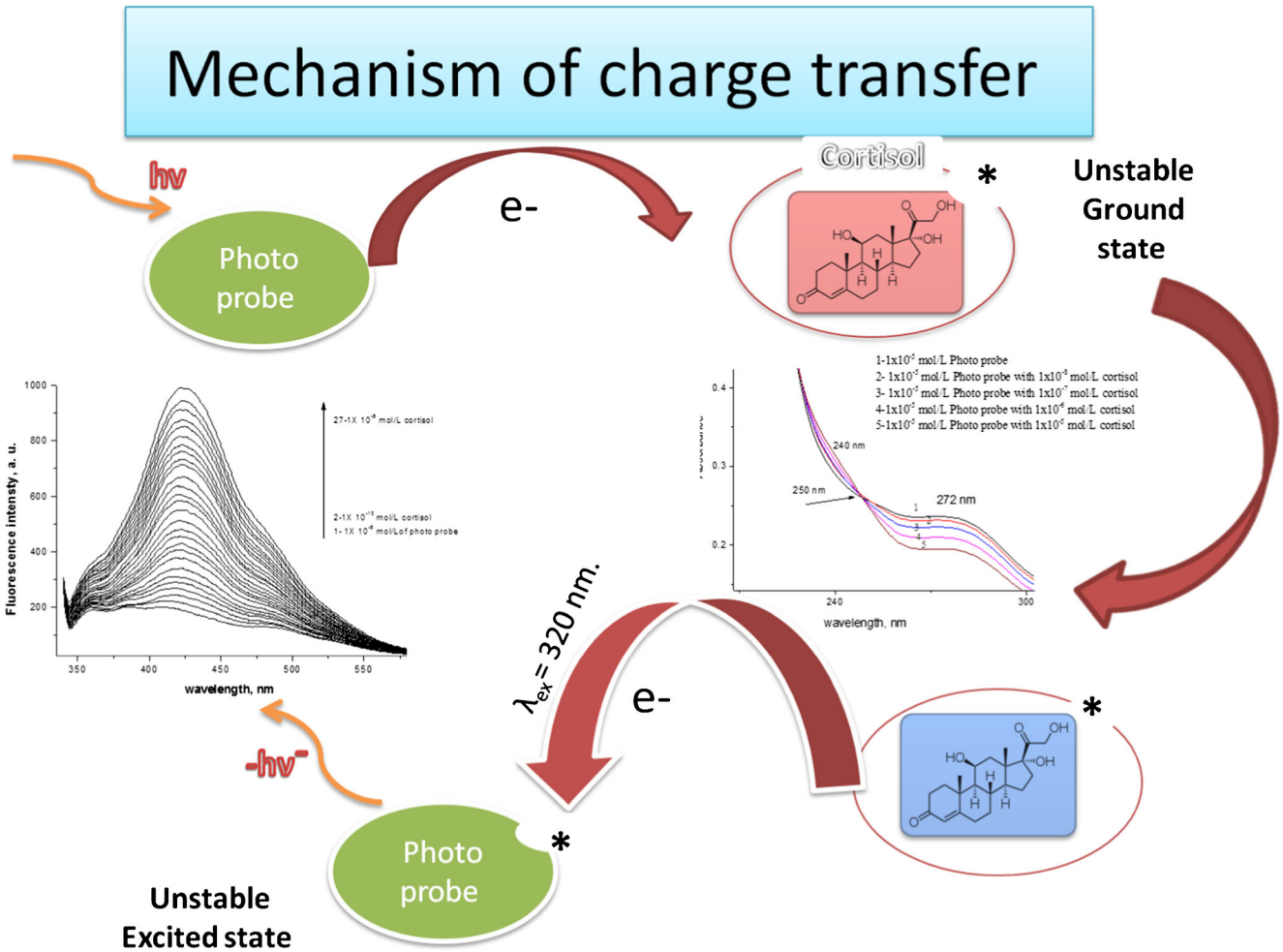


Fig. 4 Mechanism of the charge transfer between cortisol and the photo probe

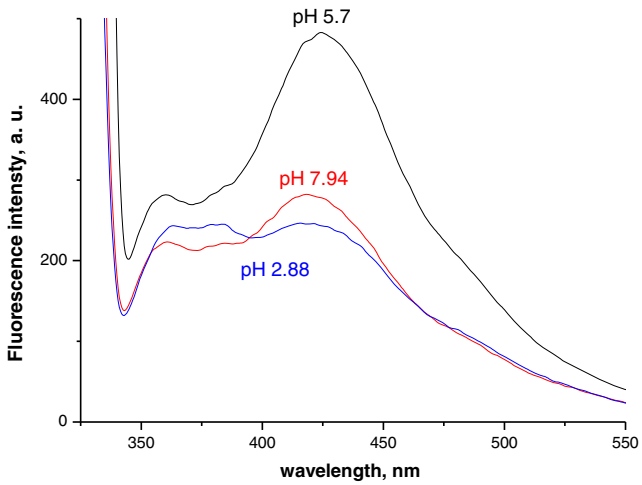


Fig. 5 Emission spectra of 1×10^{-5} mol/L of photo probe at different pHs at λ_{ex}=320 nm

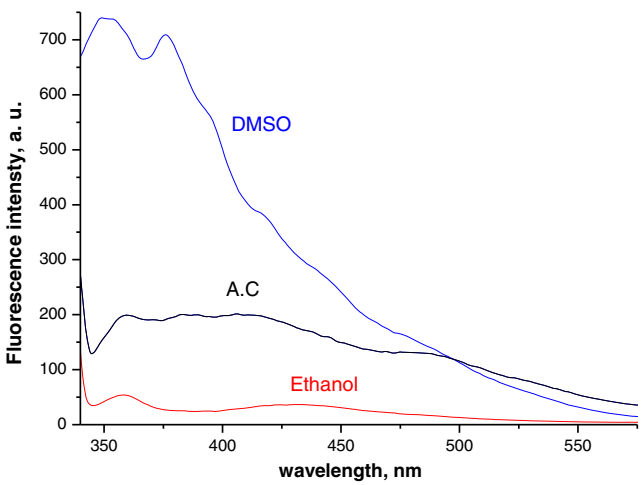


Fig. 6 Emission spectra of 1×10^{-5} mol/L of photo probe in different solvents at λ_{ex}=320 nm

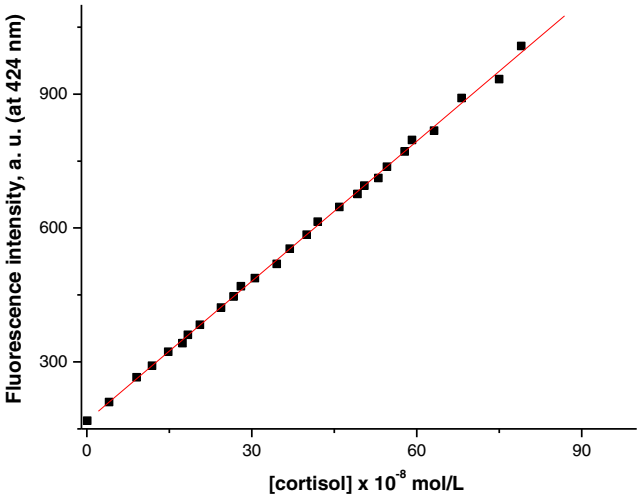


Fig. 7 Linear relationship between the fluorescence intensity of 1×10^{-5} mol/L of photo probe and [cortisol] in acetonitrile at λ_{ex}=320 nm

Characterization of 5-(p-ethoxy) benzoyl methyl-2-diazo-α -naphthol thiazol

IR, υ **(cm^{-1})** υ_{OH} (3422), υ_{CH2}(2853), $\upsilon_{C=O}$(1652), $\upsilon_{C=N}$(1619), $\upsilon_{N=N}$(1477).

^{1}H-NMR (DMSO-d_6, ppm): **9** (s, 1H,OH), **8.8** (s,1H,OH), 8–8.2 (s,2H,NH_2).

3.6) s, 2H, CH_2) (4.2 (q, 2H, CH_2O, J=11.2), 1.3 (t,3H, CH_3, J=13.6).

EI-M m/z(%) 313 ($C_{15}H_{11}N_3O_3S$, 100 %), 285 ($C_{14}H_{11}N_3O_2S$,37 %),263 ($C_{13}H_{12}NO_3S$, 7 %), 157 ($C_{10}H_7NO$,14 %),149 ($C_9H_9O_2$, 32 %), 121 (C_8H_9O,28 %),95 (C_6H_7O, 28 %),66 (C_5H_6,31 %)**, 120** (**C_8H_8O**, 28 %).

Elemental analysis CHNS, Anal.Calcd.for **$C_{23}H_{19}N_3O_4S$ (433): C, 63.7; H, 4.38; N, 9.7; S, 7.** Found C,63.3; H,4.2; N,10; S,7.2.

Table 1 Sensitivity and regression parameters for Photo probe

Parameter	Values
λ_{em}, nm	424
Linear range, mol L^{-1}	8.0×10^{-6}–5.5×10^{-9}
Limit of detection (LOD), mol L^{-1}	4.7×10^{-9}
Limit of quantification (LOQ), mol L^{-1}	1.4×10^{-8}
Regression equation, Y*	
Intercept (a)	167.05
Slope (b)	1.4×10^{9}
Standard deviation	2.03
Variance (Sa^2)	4.1
Regression coefficient (r)	0.993

*Y = a + bX, Where Y is luminescence intensity, X is concentration in n mol L^{-1} , a is intercept, b is slope

Determination of Cortisol in Pharmaceutical Formulation

Appropriate volume was taken from sigmacortin (injection vial) (3×10^{-2} mol L^{-1}) to obtain 3×10^{-4} mol L^{-1} in 50 mL acetonitrile then further dilution to the linear range concentration. Solutions were standing for about 10–15 min. The concentration of the drug was determined by using 3 concentrations for each sample from the corresponding calibration graph.

Determination of Serum 11-Hydroxy Corticosteroid

To 0.5 ml of serum in a glass stopper tube, 0.5 ml of distilled water was added followed by addition of 7.5 ml of methylene chloride (MC). The tube was shaken slowly for 20 min to extract the steroid. The two phases were allowed to settle and supernatant, then the aqueous layer was pipette out and discarded [11]. 1 mL of the extracted MC was transferred into a calibrated 10 mL measuring flask containing 1 mL of (1×10^{-4} mol/L) photo probe then diluted to the mark with acetonitrile at pH 5.7. The fluorescence intensity of the test solution is measured before and after addition of 1 mL of the extracted MC at 424 nm. The change in the fluorescence intensity is used for determination of cortisol in serum sample.

Determination of Urinary 17-Oxogenic Steroids

To 4 mL of urine sample in a screw capped tube 20 mg of potassium borohydride was added which was left open overnight at room temperature. Then 4 mL of glacial acetic acid was added followed by 1 g of sodium bismuthate. The tube was shaken for 30 min in dark and centrifuged for 10 min at 2,000 rpm. The supernatant was collected and 5 mL of it transferred to a clean glass stoppered tube. Then 0.1 mL of 20 % sodium metabisulphite was added to decolourise the sample and the contents were diluted with 5 mL of water. This was followed by addition of 1 mLof conc. hydrochloric acid. The tube was then heated in a boiling water bath for 20 min, cooled and 10 mL of chloroform added. The steroid was extracted into the chloroform by vigorous shaking. The chloroform extract was washed with 3 mL of 1 mol/L sodium hydroxide solution and washed twice with 3 mL of water. 6 mL of this washed chloroform extract was taken in a clean dry test tube. The chloroform was evaporated to dryness by warming the tube in a hot water bath. The dried residue contained steroid from 4 ml of urine [11]. The extracted cortisol was dissolved in 5 mL of acetonitrile after vigorous shaking for 20 min. the dissolved extracted cortisol was transferred into a calibrated 10 mL measuring flask containing 1 mL of (1×10^{-4} mol/L) photo probe then diluted to the mark with acetonitrile at pH 5.7. The fluorescence intensity of the test solution is measured before and after addition of the extracted cortisol at 424 nm. The change in the fluorescence intensity is used for determination of cortisol in urine sample.

Table 2 Comparison of different determination methods for the (Cortisol) with the proposed method

Method	Linear range (mol L^{-1})	Detection limit (mol L^{-1})	References
Isotope dilution liquid Chromatography/mass spectrometry	0.6×10^{-8}–8.27×10^{-8}	2.75×10^{-9}	[13]
Fluorometric liquid chromatographic	4.46×10^{-6}–2.75×10^{-7}	2.75×10^{-7}	[14]
Liquid chromatography	0.01–0.97×10^{-6}	0.01×10^{-6}	[15]
Capillary electrophoretic enzyme immunoassay	1.66×10^{-7}–2.76×10^{-8}	3.5×10^{-9}	[16]
Spectrofluorimetric method	5×10^{-9}–5×10^{-6}	4.7×10^{-9}	Present work

Results and Discussion

Spectral Characteristics

The absorption spectrum of (1×10^{-5} mol/L) photo probe in acetonitrile is shown in Fig. 2. The spectrum (1) in Fig. 2 shows a two intense broad bands at (240 and 272 nm) due to the $\pi\rightarrow\pi^*$ transition in the photo probe. Upon addition of different concentrations of the cortisol to the photo probe in acetonitrile (spectra 2–5) the equilibrium maintained (the intensity of the band at 240 increased and the intensity of band at 272 nm decreased Fig. 2 inset) between the cortisol and photo probe with the isosbestic point at 250 nm. The result reveals that the charge transfer complex was formed between cortisol and the photo probe in the ground state and this is observed in the (Fig. 2 inset).

The emission spectra (Fig. 3) of the photo probe in the acetonitrile at pH 5.7 showed the characteristic emission band at 424 nm (Fig. 3). The fluorescence intensity of the photo probe (acetonitrile and pH 5.7, λ_{ex}=320) at 424 nm increased on adding cortisol confirming the charge transfer from cortisol to the photo probe in acetonitrile at λ_{ex}=320 and the mechanism of the charge transfer from cortisol to the photo probe is shown in Fig. 4.

Effect of Solvent

The effect of the solvent on the fluorescence intensities of 1×10^{-5} mol L^{-1} photo probe and 1×10^{-5} mol L^{-1} of cortisol was studied under the conditions optimized above Fig. 5. The high fluorescence intensity of the photo probe was observed in aprotic solvents like acetonitrile and DMSO this may be attributed to the good charge transfer from cortisol to photo probe. The higher intensity of the photo probe was obtained in case of DMSO but in this solvent the cortisol exists as precipitate. Also, the fluorescence intensities for the photo probe in aprotic solvent like DMSO, acetonitrile and DMF are stronger than in protic solvent like water and ethanol. This could be attributed to the protic solvents prevent the charge transfer from the cortisol to photo probe in the excited state. The protic solvents shield the cortisol from the photo probe and the quenching of the fluorescence intensity at 424 nm is occurred.

Effect of pH

Cortisol contains several functional groups that ionize at different values of pH (Fig. 6). The good result was obtained at pH 5.7 due to suitable configuration of cortisol (the mono negatively charged form of cortisol, in which the ionization of OH group to O^-) at this pH for the good charge transfer

Table 3 Evaluation of intra-day and inter-day accuracy and precision

Method	Cortisol taken[a]	Intra-day accuracy and precision (n=3)			Inter-day accuracy and precision (n=3)		
		Cortisol Average Found[a] ± CL	%RE	%RSD	Cortisol average found[a] ± CL	%RE	%RSD
sigmacortin	1.0	1.01±0.07	1.0	0.03	1.02±0.16	2	0.07
Serum sample	1.0	0.98±0.20	2.0	0.08	0.97±0.39	3	0.15
Urine sample	1.0	0.97±0.30	3.0	0.13	0.96±4.59	4	0.24

[a] The values are mulitiplied by 10^{-7} mol L^{-1} for method

%RE. Percent relative error, %RSD. relative standard deviation and CL. Confidence limits were calculated from: CL = ± tS/√n. (The tabulated value of t is 4.303, at the 95 % confidence level; S standard deviation and n number of measurements)

between cortisol and the photo probe which enhances the fluorescence intensity of the photo probe at 424 nm.

Interference in the Detection of Cortisol

We examined the effects of interfering species commonly observed in biological samples. The concentration of cortisol was maintained at 1×10^{-5} mol/L and the pH was adjusted to 5.7. Potential interference was studied by new photo probe with the selected steroids and drugs at a concentration of 1×10^{-5} mol/L. the results reveal that there is no detectable interference in case of the steroids like (dexamethasone, prednisone, prednisolone, deoxycorticosterone, 11-dehydrocorticosterone, testosterone, androsterone, 17a-hydroxyprogesterone, aldosterone, 11-deoxycorticosterone, tetrahydrocorticosterone, a-cortolone, 3-cortol, 11-deoxycortisol, estrone, estradiol, estriol, and progesterone) and drugs like (ethosuximide, primidone, phenobarbital, phenytoin, carbamazepine, theophylline, procainamide, propranolol, quinidmne, diazepam, chlordiazepoxide, acetaminophen, and acetylsalicylic acid). This attributed to the high selectivity of the photo probe for the cortisol at pH 5.7.

Calibration Curve and Detection Limit

The effect of the concentrations of the cortisol on the fluorescence intensity of photo probe is shown in Fig. 3. As can be clearly seen from the figure and under the optimal conditions, the fluorescence intensity is increased linearly with the concentrations of cortisol over the range 5.5×10^{-9} to 8.0×10^{-6} mol L^{-1} with a correlation coefficient of 0.998 Fig. 7. The detection limit (LOD) and quantification detection limits were calculated according to ICH guidelines [12] using the formulae: LOD=3.3 S/b and LOQ=10 S/b, (where S is the standard deviation of blank luminescence intensity values, and b is the slope of the calibration plot) are also presented in Table 1. The comparison of the proposed photo probe for the determination of cortisol with other published methods indicate that the developed method has good stability, lower limit of detection (4.7×10^{-9} mol L^{-1}) and wide linear range of application (5.5×10^{-9}–8.0×10^{-6} mol L^{-1}) as shown in Table 2.

Accuracy and Precision of the Method

To compute the accuracy and precision, the assays described under "general procedures" were repeated three times within the day to determine the repeatability (intra-day precision) and three times on different days to determine the intermediate precision (inter-day precision) of the method.

These assays were performed for pharmaceutical tablet, serum and urine samples. The results of this study are summarized in Table 3. The percentage relative standard deviation (%RSD) values were in range of 0.03–0.13 % (intra-day), 0.07–0.24 % (inter-day), for pharmaceutical tablet, serum and urine samples indicating high precision of the method. Accuracy was evaluated as percentage relative error (RE) between the measured mean concentrations and the taken concentrations of drug. Bias {bias %=[(Concentration found - known concentration)×100/known concentration]} was calculated at each concentration and these results are also presented in Table 4. Percent relative error (%RE) values were in range of 1.0–3.0 % (intra-day) and 2.0–4.0 % (inter-day) for pharmaceutical tablet, serum and urine samples demonstrates the high accuracy of the proposed method.

Analytical Application. Determination of Cortisol in Different Body Fluids

The analytical utility of the proposed spectrofluorimetric method was tested by measuring the concentration of cortisol in pharmaceutical formulation (sigmacortin), serum and urine samples. The results obtained are summarized in Table 4. Good agreement between the average values obtained by the developed procedure (98–105 %, RSD 0.03–0.13 %) and the standard method (98–99.7, RSD 0.025–0.12 %) [17] and no significant differences between two methods. A comparison between the values of mean for the samples using the standard method with that obtained by the developed method revealed no significant differences between the two methods.

Table 4 Determination of (Cortisol) in pharmaceutical preparation, serum and urine using photo probe

Drug	Taken (× 10^{-7} M)	Found (× 10^{-7} M)	Average[a]	Average recovery ± R.S.D. (%)	B.P. (LC)
sigmacortin The Nile Co	1.0	1.02, 1.03, 0.98	1.01	101±0.03	99.7±0.025
Serum sample	1.0	1.07, 0.93, 0.94	0.98	98±0.08	99.2±0.045
Urine sample	1.0	1.1, 0.92, 1.14	1.05	105±0.13	98.2±0.12

[a] Average of three measurements

Conclusion

The developed method provides an excellent selectivity for cortisol at pH 5.7. The method is sensitive and provides a wide linear dynamic range of cortisol concentrations by measuring the fluorescence intensity of the photo probe under the optimal conditions. A detection limit of 4.7×10^{-9} mol $L^{-1}$was achieved. The interference caused by other steroids and drugs analog for cortisol is minimized in the developed method compared to the reported methods [12–16].

References

1. Raul J, Cirimele V, Ludes B, Kintz P (2004) Detection of physiological concentrations of cortisol and cortisone in human hair. Clin Biochem 37:1105–1111
2. Jonsson BAG, McGinley B, Amilon A, Garde AH, Orbak P (2003) Determination of cortisol in human saliva using liquid chromatography–electrospray tandem mass spectrometry. J Chromatogr B 784:63–68
3. AbuRuz S, Millership J, Heaney L, McElnay J (2003) Simple liquid chromatography method for the rapid simultaneous determination of prednisolone and cortisol in plasma and urine using hydrophilic lipophilic balanced solid phase extraction cartridges. J Chromatogr B 798:193–201
4. DiFrancesco R, Frerichs V, Donnelly J, Hagler C, Hochreiter J, Tornatore KM (2007) Simultaneous determination of cortisol, dexamethasone, methylprednisolone, prednisone, prednisolone, mycophenolic acid and mycophenolic acid glucuronide in human plasma utilizing liquid chromatography–tandem mass spectrometry. J Chromatogr B 859:42–51
5. Boever JD, Kohen F, Serreyn R, Vandekerckhove D, Maele GV (1985) Application of chemiluminescence immunoassays for steroid hormones in clinical endocrinological investigations in women. Anal Chim Acta 170:125–131
6. Lindstrom L, Meurling L, Lovgren T (1982) The measurement of serum cortisol by a solid-phase chemiluminescence immunoassay. J Steroid Biochem 16:577–580
7. Okumura T, Nakajima Y, Takamatsu T, Matsuoka M (1995) Column-switching highperformance liquid chromatographic system with a laser-induced Fluorimetric detector for direct, automated assay of salivary cortisol. J Chromatogr B 670:11–20
8. Deftereos NT, Calokerinos AC (1994) Flow-injection chemiluminometric determination of steroids. Anal Chim Acta 290:190–200
9. Zhang Y, Zhang Z, Song Y, Wei Y (2007) Detection of glucocorticoid residues in pig liver by high-performance liquid chromatography with on-line electrogenerated $[Cu(HIO_6)_2]$5-luminol chemiluminescence detection. J Chromatogr A 1154:260–268
10. Debay R, Danton chdorier S (1961) IP Bull Soc/chim Fr 2061:1, Chem. Abstr. 56(1962) 14157
11. Mattingly D (1964) Steroid analysis. In: King EJ (ed) Microanalysis in medical biochemistry, 4th edn. J & A Churchill (P) Ltd, London, pp 179–183
12. International Conference on hormonisation of technical requirements for registration of pharmaceuticals for human use, ICH harmonised tripartite guideline, validation of analytical procedures: text and methodology q2(R1), complementary guideline on methodology dated 06 November 1996, incorporated in November 2005, London
13. Lee H, Shin H, Park S, Park CJ (2010) Measurement of cortisol in human serum by isotope dilution liquid chromatography/mass spectrometry and a comparison through a proficiency testing. Bull Korean Chem Soc 31:5–13
14. Gotelli GR, Wall JH, Kabra PM, Marton LJ (1981) Fluorometric liquid-chromatographic determination of serum cortisol. Clin Chem 27(3):441–443
15. Caldarella AM, Reardon GE, Canalis E (1982) Analysis for cortisol in serum by liquid chromatography. Clin Chem 28(3):538–543
16. Jia M, Jin WR (2002) Electrochemical determination of cortisol by capillary electrophoretic enzyme immunoassay. Chin Chem Lett 13: 867–870
17. British Pharmacopoeia, (1999). Vol. II, Her Majesty's Stationary Office, London, p. 2705