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ORIGINAL PAPER

Fluorescence Enhancement Effect for the Determination of Adenosine 5′-Monophosphate with 9-Anthracene Carboxylic Acid-Cetyl Trimethyl Ammonium Bromide System

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Abstract A fluorimetric method based on fluorescence enhancement effect was developed for the determination of adenosine 5′-monophosphate (AMP) with 9-anthracene carboxylic acid (9-ANCA)–cetyl trimethyl ammonium bromide (CTAB) system. Fluorescence intensity of 9-ANCA was decreased by the addition of CTAB but addition of AMP again rose the intensity of 9-ANCA gradually. The observed fluorescence enhancement is attributed to the competitive binding reaction of 9-ANCA and adenosine to CTAB. The enhancement in the fluorescence intensity was found proportional to the concentration of AMP over the range 2.0×10^{-4} to 1.2×10^{-3} mol dm^{-3}. The ion pair complex is formed spontaneously between 9-ANCA and CTAB. Since the binding interaction is larger for the adenosine–CTAB pair, the fluorophore 9-ANCA will be released. The quantum yield of free 9-ANCA is higher therefore its fluorescence observed at 417 nm wavelength is enhanced. This mechanism of competitive molecular interaction is further confirmed by conductometric measurements. The method was applied successfully for the determination of AMP from pharmaceutical sample. The method is more selective, sensitive and relatively free from interferences.

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Introduction

The photochemical interaction of chromophores with nucleotides, nucleosides and their bases has been the focus of extensive recent research [1–3]. The studies on interactions between DNA and self-assembled nanostructures, such as micelles and vesicles, have been major concern, due to the possibility to use these complexes as transfection tools [4]. The electrostatic interaction between the nucleotides and fluorophores like coumarine, pyrenes were extremely studied to develop a method for DNA sequencing and possible mechanisms of electron transfer are shown to involve in the interactions [5, 6]. Among the technological applications, the use of micellar systems for the study of synthetic nucleolipids is recently coming up [7]. The electrostatic interactions between the positively charged cationic surfactant molecules and the negatively charged mononucleotides are expected to lead to the formation of complexes. Furthermore by studying these interactions significant information as to the recognition, sensing of anionic analytes and supramolecular chemistry are reported in the literature [8].

The quantitative determination of nucleotides is required in many fields, such as molecular biology, biotechnology and pharmacodynamics studies. The natural fluorescence intensity of nucleotides is very weak to use in direct fluorimetric method [9]. The DNA bases and nucleotides are known to interact with different fluorescent probe in

water and other environment also [6, 10]. The enhancement in fluorescence intensity of ethidium bromide is reported due to direct interaction with DNA bases and nucleotides [11–15]. However, quenching of fluorescence probes by interaction with DNA bases and nucleotides is also seen to be used in analysis [16–18].

In view of a search for new selective and more sensitive fluorescent probes for detection of DNA the electrostatic interactions between the analytes and oppositely charged probe molecule were made of use. The 9-anthracene carboxylic acid (9-ANCA) is widely used as probe molecule because of its high quantum yield for detection of cationic surfactant and trace metal ions [19–21]. Adenosine monophosphate consisting of the phosphate group, the pentose sugar ribose, and the nucleobase adenine is an intermediary substance formed during the body's process of creating energy in the form of adenosine triphosphate (ATP) from food. The body creates AMP within cells during normal metabolic processes. It has medicinal importance and its injection available commercially is used in postherpetic neuralgia treatment. The present work aimed to develop fluorimetric method for analysis of adenosine by using 9-ANCA. The direct molecular interaction between 9-ANCA and AMP is not possible because both are negatively charged species. Hence possible electrostatic interactions between the pairs ANCA-CTAB and CTAB-AMP are used to develop a new fluorimetric method for analysis AMP from aqueous solution and applied successfully for pharmaceutical samples containing AMP. The competitive electrostatic interaction between these two pairs was examined by conductometric studies. The present method is selective and more sensitive to other methods such as spectrophotometry, chemiluminescence, electrochemical and capillary electrophoresis reported in the literature [22–26].

Experimental

Materials

The purity of 9-anthracence carboxylic acid obtained from Merck-Schuchardt was confirmed by noting its melting point, electronic spectra and by production of similar spectra after excitation at different wavelengths. The adenosine 5′-monophosphate, cetyl trimethyl ammonium bromide and Tris (hydroxymethyl) amino methane hydrochloride buffer obtained from s.d. fine-chem. Limited Mumbai were used directly without further purification. Double-distilled water was used in all experiments. All measurements were performed at ambient temperature.

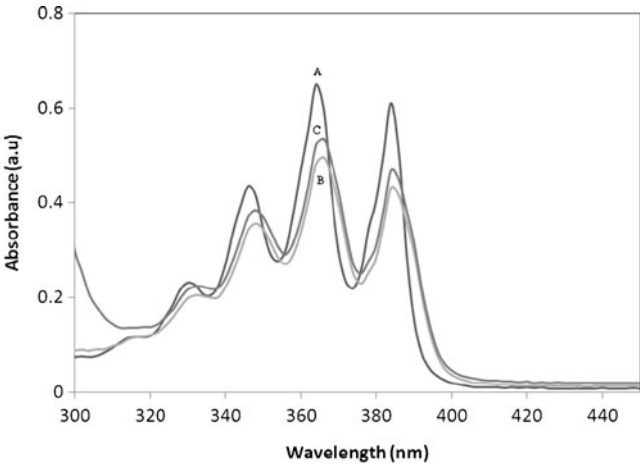


Fig. 1 Absorption spectra of 9-ANCA (*A*), with CTAB (*B*) and with CTAB-AMP mixture (*C*) in aqueous solution

Preparation of Solutions

Stock solution of 9-ANCA of concentration (4.0×10^{-4} mol dm^{-3}) was prepared by dissolving 0.0044 g in 50 mL double distilled water and stirring overnight. Stock solution of AMP (1.0×10^{-2} mol dm^{-3}) was prepared by dissolving 0.0977 g in 25 mL double distillated water. Similarly, stock solution of CTAB (3.0×10^{-3} mol dm^{-3}) was prepared by dissolving 0.1093 g in 100 mL double distillated water. Tris (hydroxymethyl) amino methane hydrochloride buffer (5.0×10^{-2} mol dm^{-3}) was prepared by dissolving 0.7889 in 100 mL deionised water and then the pH was adjusted to 7.5 using reported procedure [27]. The fluorescence intensity of 9-ANCA is maximum in solution of tris (hydroxymethyl) amino methane hydrochloride of pH 7.5 [28]. These solutions were further diluted to the required concentration in the experiments.

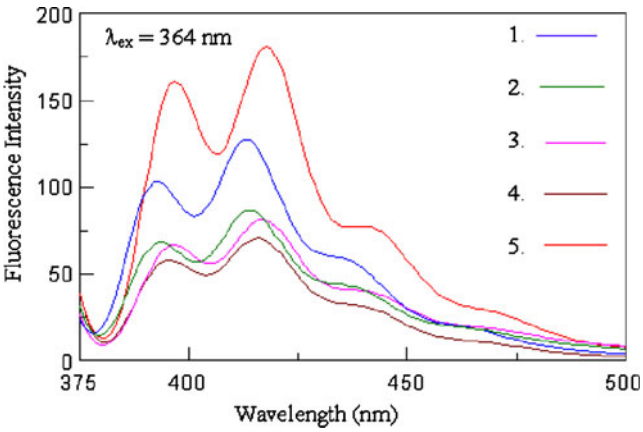
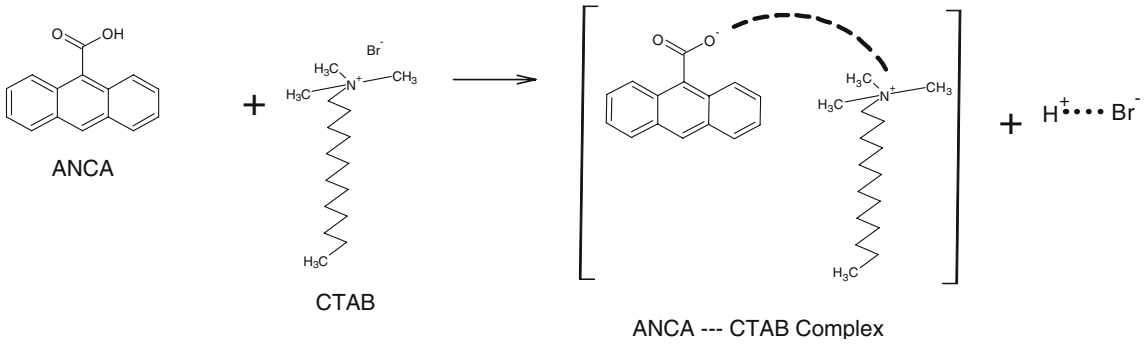


Fig. 2 Steady state fluorescence spectra of 9-ANCA in the absence (*1*) and presence of varying concentrations of CTAB in aqueous solution (*2–5*), 0.7×10^{-4} mol dm^{-3} (*2*), 3.0×10^{-4} mol dm^{-3} (*3*), 6.0×10^{-4} mol dm^{-3} (*4*), 1.0×10^{-3} mol dm^{-3} (*5*)



Scheme 1 Mechanism of interaction of CTAB with 9-ANCA to form complex

Fluorescence and Absorption Measurements

Steady-State fluorescence and excitation spectra of the solutions were recorded on PC based spectrofluorophotometer (JASCO Model-FP-750, Japan) equipped with 150 W Xenon lamp source and 1.00 cm quartz cell. The excitation wavelength 364 nm was obtained from the excitation spectrum and the emission spectrum was monitored at this excitation wavelength. The excitation and emission slit width were kept constant at 10 nm during the measurements. The absorption spectra of solutions were recorded on UV-Visible spectrophotometer (UV-3600 SHIMADZU) using 1.00 cm quartz cell. All solutions were degassed by passing nitrogen gas before subjected to optical measurements. The absorption and excitation spectra were found identical. The conductivity of solution was measured by using conductivity meter (model No.EQ.660A EQUIP-TRONICS) equipped with dip type conductivity cell (cell constant 1.00 cm^{-1}).

Results and Discussion

The interaction between 9-ANCA-CTAB and 9-ANCA-CTAB-AMP were studied by absorption and fluorescence spectrometry. Figure 1 shows, absorption spectra of 9-ANCA alone (A), with CTAB (B) and with CTAB-AMP mixture (C). The absorption spectrum of 9-ANCA is banded with maximum at 364 nm. The addition of dilute solution of CTAB below its critical micelle concentration (CMC) not only decreases the absorbance but also the wavelength of maximum absorbance seems to be shifted slightly from 364 to 368 nm. The observed spectral changes led to believe the formation of ion-pair complex between 9-ANCA and CTAB. However, introduction of AMP in the 9-ANCA-CTAB mixture, again rose the absorbance of 9-ANCA. It is believed that the presence of AMP act to break the ion-association complex. The observed absorption spectral results were used to optimize 9-ANCA-CTAB system as a fluorescent probe for determination of AMP and to develop a fluorimetric method more sensitive to spectrophotometry.

Fluorescence Spectra of 9-ANCA in Presence of CTAB

The aqueous solution of 9-ANCA of concentration of 4.0×10^{-6} mol dm^{-3}, exhibited a strong fluorescence at 413 nm. The cationic surfactant such as CTAB is known to solubilize and stabilize the drugs and DNA molecules of opposite charges and may result in several desirable or undesirable interactions. Hence effect of CTAB concentration on fluorescence of 9-ANCA was studied and given in Fig. 2. From the figure it is observed that the fluorescence intensity of 9-ANCA was quenched by addition of CTAB (spectra 1 to 4) below its CMC (6.3×10^{-4} mol dm^{-3}), due to the formation of non-fluorescent ion-association complex between two molecules [29] .The fluorescence spectrum of 9-ANCA is shifted to red from 413 nm to 417 nm, which is an indication of complex formation as shown in Scheme 1. However, when the concentration of CTAB was higher than critical micelle concentration, CTAB micelles were formed and the fluorescence intensity of the 9-ANCA at 417 nm seen to increased at higher CTAB concentration in Fig. 2 (spectrum 5). This is because of the fact that the 9-ANCA

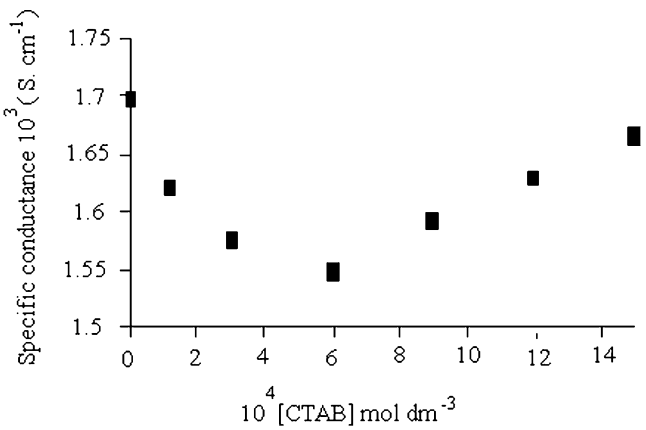


Fig. 3 Conductometric titration curve of 9-ANCA with CTAB system in aqueous solution, [9-ANCA]: 4.0×10^{-6} mol dm^{-3}

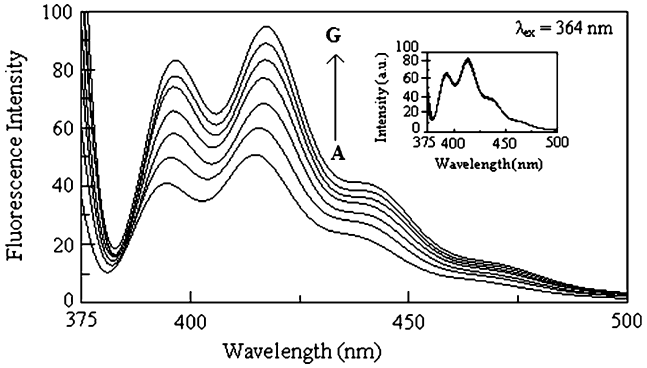


Fig. 4 Steady state fluorescence spectra of the interaction between 9-ANCA-CTAB complexes with varying concentrations of AMP in aqueous solution (*A* to *G*) 0, 2.0, 4.0, 6.0, 8.0, 10.0 and 12.0× 10^{-4} mol dm^{-3} respectively, [9-ANCA]: 4.0×10^{-6} mol dm^{-3}, [CTAB]: 3.0×10^{-4} mol dm^{-3}. Inset shows the fluorescence spectra of 9-ANCA as a function of [AMP] in absence of CTAB

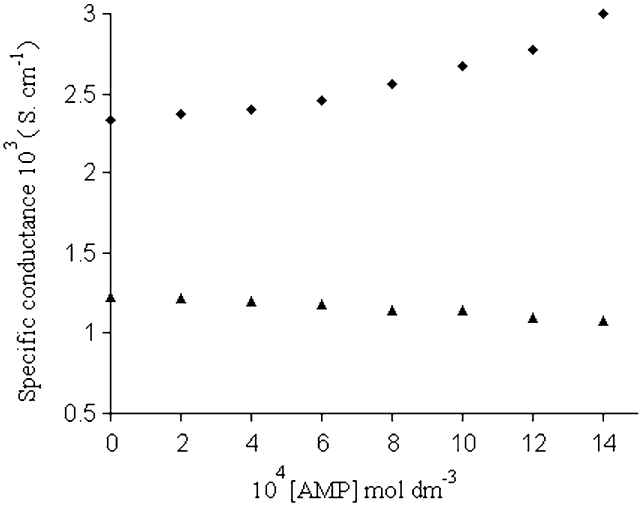
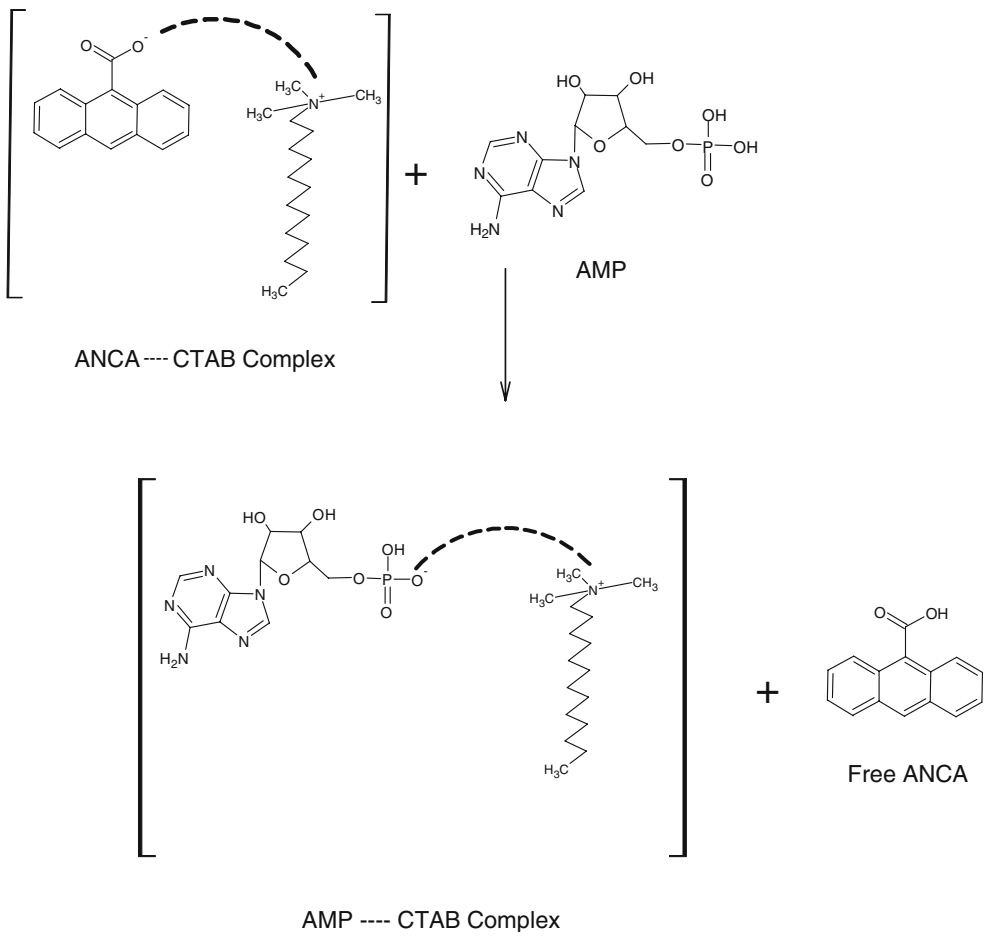


Fig. 5 Conductometric titration curves for 9-ANCA-AMP (▲) and CTAB-AMP (■) system in aqueous solution, [9-ANCA]: 4.0× 10^{-6} mol dm^{-3}, [CTAB]: 3.0×10^{-4} mol dm^{-3}

molecules were bound to the core of CTAB micelles and protected from the ion-association complex formation. The complex formation between 9-ANCA and CTAB was also examined and followed by conductometric titration. A typical conductometry curve for the titration of 9-ANCA against varying concentration of CTAB shown in Fig. 3, exhibits the expected interactions between the two molecules below and above CMC. The decrease in specific



Scheme 2 Mechanism of competitive interaction of CTAB and AMP with 9-ANCA

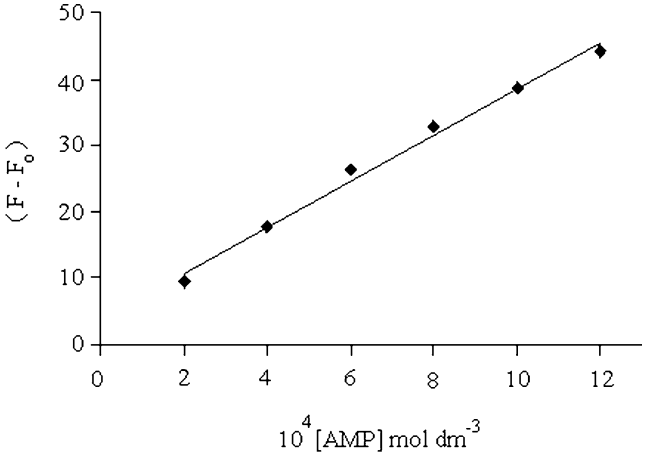


Fig. 6 Plot of the enhanced fluorescence intensity as a function of concentration of AMP in aqueous solution

conductivity is because of reduction in charged species due to complexation before CMC whereas increase in conductivity is due to separation of 9-ANCA ion from complex due to CTAB micelle.

The present work required 9-ANCA-CTAB system having minimum fluorescence intensity. Hence the optimal CTAB concentration was decided from Fig. 2 indicating fluorescence intensity changes of 9-ANCA with CTAB below CMC. The intensity of (4.0×10^{-6} mol dm^{-3}) 9-ANCA is minimum present in (3.0×10^{-4} mol dm^{-3}) CTAB concentration. The complex forming system of these concentrations was used further to study the interaction with AMP solution.

Interaction of the 9-ANCA-CTAB Complex with AMP

The fluorescence spectra of 9-ANCA-CTAB system of optimal concentration as determined above are recorded in presence of varying concentration of AMP and shown in Fig. 4. It is observed that the intensity of fluorescence of emission band at 417 nm increases gradually without any spectral shift. It demonstrated that AMP interacted with CTAB and dissociated the non-fluorescent ion-association complex of the 9-ANCA and CTAB in aqueous solution shown in Scheme 2. These molecular interactions are feasible only in ternary 9-ANCA-CTAB-AMP system because fluorescence spectra of 9-ANCA with AMP concentration shown in inset of Fig. 4, did not show any significant spectral changes. It is concluded that 9-ANCA and AMP did not interact directly because both are negatively charged species. But CTAB-AMP is ion pair of opposite charges and therefore the possible competitive binding interaction between them releases 9-ANCA. The quantum yield of free 9-ANCA is higher therefore its fluorescence observed at 417 nm wavelength is enhanced. The conductometric studies between 9-ANCA-AMP and CTAB-AMP also supports the expected interactions in the CTAB-AMP. A typical conductometry curves for the titration of 9-ANCA and CTAB against varying concentration of AMP are shown in Fig. 5. Figure shows that the specific conductance of CTAB increases appreciably with addition of AMP while that of 9- ANCA does not vary with concentration of AMP. It is concluded that AMP has strong molecular interaction with CTAB rather than with 9-ANCA.

Table 2 Influence of coexisting substances on 9-ANCA-CTAB and AMP system in aqueous solution [9-ANCA]: 4.0×10^{-6} (mol dm^{-3}), [CTAB]: 3.0×10^{-4} (mol dm^{-3}) and [AMP]: 4.0×10^{-4} (mol dm^{-3})

Sr.No.	Foreign substance	Concentration (mg/mL)	Change in intensity (%)
1	Zn^{2+}	1	+2.70
2	Cu^{2+}	1	−4.18
3	Mn^{2+}	1	+1.93
4	La^{3+}	1	−4.44
5	Fe^{3+}	1	−6.8
6	Cr^{3+}	1	−7.82
7	SCN^-	1	+0.32
8	EDTA	100	+12.65
9	Glucose	100	−3.60
10	Citrate	100	+11.08

Calibration Curve for AMP Determination

Under optimal conditions, calibration graph for the determination of AMP was constructed. The enhanced fluorescence intensity was represented as $\Delta F = F - F_o$, where F and F_o were the fluorescence intensities of the systems with and without AMP, respectively. The calibration graph was constructed by plotting the enhanced fluorescence intensity (ΔF) at 417 nm as a function of AMP concentration and presented in Fig. 6. The enhanced fluorescence intensity showed a good linear relationship

Table 1 Analytical characteristics of the AMP determination

Nucleotide	Linear range (mol dm^{-3})	Linear regression equation (mol dm^{-3})	Correlation coefficient (R)	Standard deviation	Relative standard deviation (%)	Detection limit (mol dm^{-3})
AMP	2.0×10^{-4} to 1.2×10^{-3}	$\Delta F = 3.9333 + 3.4679 \times 10^4$	0.9990	0.1209	1.99	1.05×10^{-5}

Table 3 Results of determination of AMP from pharmaceutical sample

Sample	Composition	Amount found (mg)			S.D.	R.S.D. (%)
		I	II	III		
Adenosine injection Sanofi-synthelabo, Glaxo wellcome prod. Bondeville, France	(i)Adenosine Ph. Eur. 6 mg per 2 mL (ii) NaCl solution, 0.9% *w/v*	5.80	6.00	5.80	0.1197	1.97

with the concentration of AMP in the range of 2.0×10^{-4} mol dm^{-3} to 1.2×10^{-3} mol dm^{-3}, and the regression equation was $\Delta F = 3.9333 + 3.4679 \times 10^4\,C$, where C is the concentration, correlation coefficient was 0.9990. The detection limit given by the equation, $C_{lim.} = 3\delta/k$, where δ is the standard deviation of blank determination (n=6) and k is the slope of calibration graph. The relative standard deviation is 1.99% for the determination of 6.0×10^{-4} mol dm^{-3}, shown in Table 1.

Effect of Coexisting Substances and Method of Analysis

Under the optimal conditions, effects of interferences that usually used as the compatibility of coexisting substances on the fluorescence intensity of the system were studied [30]. Initially, coexisting substances to the AMP solution are taken in large excess and the fluorescence intensities are measured. When interference was found to be intensive, the tests were repeated with successive smaller amounts of coexisting substances. According to the proposed system, the most of them have little effect on the determination of AMP. For a relative error of less than ±5%, the results are shown in Table 2.

Analysis of AMP from Pharmaceutical Sample

The observed fluorescence enhancement of 9-ANCA-CTAB system was employed for determination of adenosine 5′ monophosphate in pharmaceutical sample namely Adenosine injection [26]. For the assay of adenosine 5′ monophosphate the sample must be diluted appropriately within the linear range of determination of AMP. The sample solution was analysed by the method developed above. Though adenosine injection contains NaCl, it does not interfere on fluorescence intensity measurements because of use of selective Tris–HCl buffer. The results are in good agreement with certified values performed in triplicate, which affords good precision and accuracy. The values are reported in Table 3.

Conclusion

The absorption and fluorescence studies indicated the molecular interactions between 9-ANCA and CTAB to form ion association pair complex. This system is optimized for fluorescence performance because addition of CTAB below its CMC has quenched the fluorescence of 9-ANCA while the fluorescence is increased when CTAB concentration is above CMC. The optimal 9-ANCA-CTAB system exhibiting minimum fluorescence was used to study the interactions with biologically important analytes AMP. The conductometric studies indicated the competitive electrostatic interactions between 9-ANCA-CTAB and CTAB-AMP. Therefore addition of AMP into optimal 9-ANCA-CTAB enhanced the fluorescence intensity linearly. The calibration curve obtained between fluorescence enhancement and concentration of AMP was used for analysis of adenosine injection. The determination of AMP by this method is reliable, precise and simple. Therefore, this method has the potential of practical applications in the determination of AMP from pharmaceutical sample available commercially.

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