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ORIGINAL PAPER

Determination of Tetracaine Hydrochloride by Fluorescence Quenching Method with Some Aromatic Amino Acids as Probes

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Abstract A novel fluorescence quenching method for the determination of tetracaine hydrochloride (TA·HCl) concentration with some aromatic amino acids as fluorescence probe has been developed. In pH 6.3 acidic medium, tryptophane (Trp), tyrosine (Tyr) or phenylalanine (Phe) can react with tetracaine hydrochloride to form an ion-association complex by electrostatic attraction, aromatic stacking interaction and Van der Waals' force, which lead to fluorescence quenching of above amino acids. The maximum fluorescence excitation and emission wavelengths of them are located at 278, 274, 258 nm and 354, 306, 285 nm, respectively. The relative fluorescence intensity (F_0/F) is proportional to the TA·HCl concentration in certain range. The linear ranges and detection limits are 1.2–5.0 μg/mL and 0.37 μg/mL for Tyr-TA·HCl system, 1.3–6.0 μg/mL and 0.38 μg/mL for Trp-TA·HCl system, and 1.4–6.0 μg/mL and 0.41 μg/mL for Phe-TA·HCl system. The optimum reaction conditions, influencing factors and the effect of coexisting substances are investigated. And the results show the method has a good selectivity. Judging from the effect of temperature, the Stern-Volmer plots and fluorescence lifetime determination, the quenching of fluorescence of amino acids by TA·HCl is a static quenching process.

Keywords Tetracaine hydrochloride · Tryptophane · Tyrosine · Phenylalanine · Fluorescence quenching

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Introduction

Tetracaine hydrochloride [4-(butylamino) benzoic acid 2-(dimethylamino) ethyl ester 2-(dimethylamino) ethyl 4-(butylamino) benzoate hydrochloride, TA·HCl], is an ester-type local anesthetic. This drug is very potent, long-acting agent with a low therapeutic dose, being commonly used to induce spinal anesthesia. Excessive dose and abuse of local anesthetics may cause sudden medical accidents. So it is important to determine the concentrations of local anesthetics in body fluids in the medical and judicial case in order to guide clinical use of local anesthetics. However, the components of body fluids are complex and the analyte is usually present at low concentration in body fluids. Therefore, it is very necessary to develop an accurate and quick examination technique for TA·HCl. According to The Pharmacopoeia of People's Republic of China, only high concentration of TA·HCl can be determined by titration method [1]. However, the method is tedious and time-consuming, for the samples have to be heated. Judging the end point is difficult and the sensitivity of method is low. Literature survey reveals many methods for the determination of TA·HCl concentration in pharmaceutical preparations and biological fluids such as spectrofluorometry [2, 3], chemiluminesence (CL) [4, 5], spectrophotometry [6], electrochemistry method [7–9], high performance liquid chromatography (HPLC) [10–15], gas chromatograph-mass spectrometer(GC-MS) [16–18], resonance Rayleigh scattering (RRS) [19–21], etc. At present, HPLC is used the most, but it needs complicated pretreatment and operation. Some of these methods have high sensitivity, but the apparatus used are always complex and the manipulations are time-consuming. Hence, it is important to establish a simple and accurate method to determine TA·HCl. In this paper, we study the reaction of TA·HCl with some aromatic amino

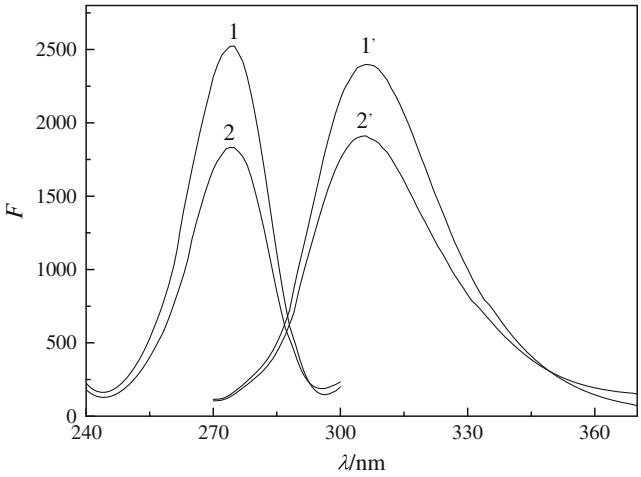


Fig. 1 The excitation and emission spectra of TA·HCl, Tyr and TA·HCl-Tyr system. 1 and 2 are the excitation spectra of Tyr and TA·HCl-Tyr system; 1′ and 2′ are the emission spectra of Tyr and TA·HCl-Tyr system; TA·HCl:2.0 μg·mL^{-1}, Tyr: 4×10^{-5} mol/L, pH 6.3

acids to develop a simple and sensitive spectrofluorimetric method for its determination in biological fluids.

The aromatic amino acids include tryptophan (Trp), tyrosine (Tyr) and phenylalanine (Phe). Trp, Tyr and Phe are well known as three kinds of essential amino acids for human as the precursor of hormones, neurotransmitters and other relevant biomolecules. So it is necessary to study the interaction of above amino acids with medication. This paper develop a new fluorescence quenching method which can apply to the determination of TA·HCl concentration using aboved amino acids as probes.

In this work, the optimum reaction conditons, influencing factors and the effects of foreign substances are investigated. Judging from the effect of temperature, the Stern-Volmer plots and fluorescence lifetime determination, the quenching of fluorescence of amino acids by TA·HCl is a static quenching process. And the method has been applied to the direct determination of TA·HCl concentration in human serum and urine sample.

Experimental

Apparatus and Reagents

A Hitachi F-2500 spectroflurophotometer (Tokyo, Japan) is used to record fluorescence spectra and fluorescence intensity with the slits (EX/EM) of 10.0/10.0 nm for the fluorescence spectra. A FL-TCSPC Fluorolog-3 fluorescence spectrometer (Horiba Jobin Yvon Inc., France) is used to measure fluorescence lifetime of Tyr and Tyr-TA·HCl system at room temperature ($\lambda_{ex}/\lambda_{em}$=280 nm/306 nm). A PH S-3C pH meter (Shanghai Dazhong Analytical Instrument Plant) is used to adjust pH.

Tetracaine hydrochloride (TA·HCl, National Institute for the Control of Pharmaceutical and Biological Products) stock solution concentration is 200.0 μg/mL, the working solution concentration is 20.0 μg/mL.

Tryptophane (Trp) and tyrosine (Tyr) working solution concentration are 4.0×10^{-4} mol/L, and phenylalanine (Phe) is 8.0×10^{-3} mol/L.

Britton–Robinson (BR) buffer solutions with different pH are prepared by mixing the mixed acid (composed of 0.04 mol/L, H_3PO_4, HAc and H_3BO_3) with 0.2 mol/L NaOH in proportion. The pH is adjusted by a pH meter.

All reagents are analytical reagent grade and doubly distilled water is used.

General Procedure

Into a 10.0 mL calibrated flask are added 1.0 mL of pH 6.3 BR, 1.0 mL of amino acids solution and an appropriate TA·HCl standard solution. The mixture are then diluted to the mark and mixed thoroughly. After 10 min, record the fluorescence spectra and measure the fluorescence intensity of reagent blank (F_0) and combining product (F) at $\lambda_{ex}/\lambda_{em}$=278 nm/354 nm for Trp-TA·HCl system, 274 nm/306 nm for Tyr-TA·HCl system, and 258 nm/285 nm for Phe-TA·HCl system.

Results and Discussion

Fluorescence Spectrum

TA·HCl has little fluorescence, its excitation and emission wavelength are located at 308 nm and 375 nm. Trp, Tyr and Phe have different fluorescence intensity, the excitation/

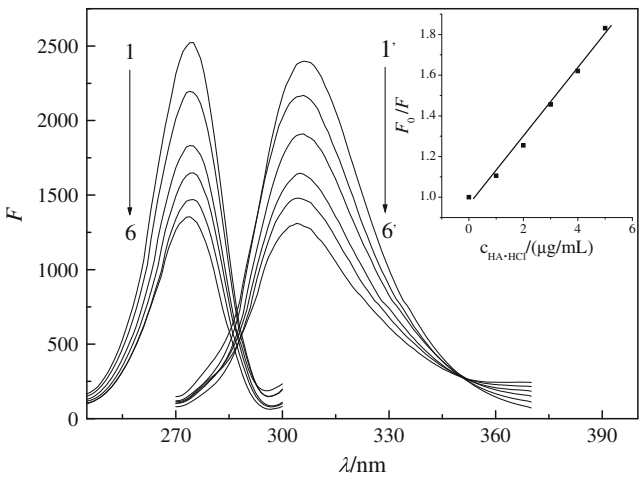


Fig. 2 Fluorescence spectra.1∼6: Excitation spectra; 1′∼6′: Emission spectra; Tyr: 4.0×10^{-5} mol/L, TA·HCl: 0.0, 1.0, 2,0, 3.0, 4.0, 5.0 μg·mL^{-1}, respectively; pH 6.3

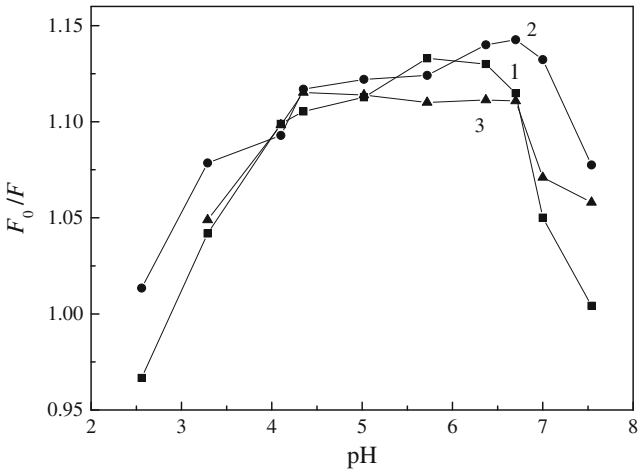


Fig. 3 Effect of pH. 1, TA·HCl-Tyr system; 2, TA·HCl-Trp system; 3, TA·HCl-Phe system; Trp and Tyr: 4.0×10^{-5} mol/L, Phe: 8.0×10^{-4} mol/L, TA·HCl: 1.0 μg·mL^{-1}

emission wavelength are located at 278/354 nm (Trp), 274/306 nm(Tyr) and 258/285 nm(Phe), respectively. Fluorescence intensity of TA·HCl is 2.5% of Trp, 8.0% of Tyr and 22.5% of Phe. Excition spectra and emission spectra of Tyr and TA·HCl-Tyr system are shown in Fig. 1. When Trp, Tyr or Phe with TA·HCl to combine new complexes, their fluorescence spectrum characteristics are almost the same, and fluorescent intensities are significant quenching. Figure 2 shows relative fluorescence intensity (F_0/F) is proportional to the TA·HCl concentration.

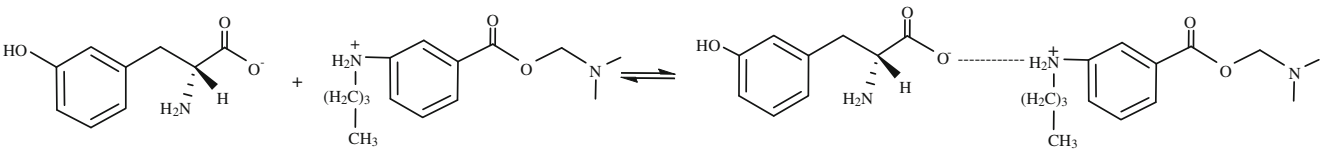
The Optimum Conditions of the Reaction

Effect of Acidity

The influences of different buffer solution on fluorescence intensity of the reaction are tested with BR, HAc-NaAc and

Table 1 Correlative parameters of the calibration graphs and the detection limits for TA·HCl

System	Measurement wavelength ($\lambda_{ex}/\lambda_{em}$/nm)	Linear regression equation (c, μg·mL^{-1})	Correlation coefficient (R)	Linear range (μg·mL^{-1})	Detection limit(3σ) (μg·mL^{-1})
TA·HCl-Tyr	274/306	F_0/F=0.96+0.17 C	0.9951	1.2–5.0	0.37
TA·HCl-Trp	278/354	F_0/F=0.99+0.15 C	0.9989	1.3–6.0	0.38
TA·HCl-Phe	258/285	F_0/F=1.00+0.11 C	0.9977	1.4–6.0	0.41



Tyr TA·HCl TA·HCl-Tyr

Fig. 4 The combined reaction of Tyr with TA·HCl

Table 2 Thermodynamic parameters of system

System	Temperature (K)	*Ksv* (L·mol^{-1})	ΔH^0 (kJ·mol^{-1})	ΔG^0 (kJ·mol^{-1})	ΔS^0 (J·mol^{-1}·K^{-1})
TA·HCl-Tyr	303	6.2×10^4	−9.4	−27.8	60.7
	313	5.5×10^4	−9.4	−28.4	60.7
TA·HCl-Trp	303	2.3×10^4	−15.1	−25.3	33.7
	313	1.9×10^4	−15.1	−25.6	33.5
TA·HCl-Phe	303	4.2×10^4	−14.4	−26.8	40.9
	313	3.5×10^4	−14.4	−27.2	40.9

citrate sodium–HCl. The results show that BR buffer solution is better than other buffer solutions and the optimum pH ranges are (4.1~6.7) for Tyr-TA·HCl system, (4.3~7.0) for Trp-TA·HCl system, and (4.1~6.7) for Phe-TA·HCl system (shown in Fig. 3), respectively. So, pH 6.3 is chosen as reaction acidity for the system and the appropriate amount is 1.0 mL.

Effect of Amino Acids Concentration

The experiment results show that fluorescence intensities reach the maximum and retain stability when the concentration of amino acids are (3.0×10^{-5} ~6.0×10^{-5}) mol/L for Trp and Tyr system, and (7.0×10^{-4} ~9×10^{-4}) mol/L for Phe system. So we choose the experiment concentrations are 4.0×10^{-5} mol/L for Trp and Tyr, and 8.0×10^{-4} mol/L for Phe.

Reaction Speed and the Stability of Fluorescence Intensity

At room temperature, the reactions are finished in 10 min and fluorescence intensity could remain constant for 4 h at least. Experiments are carried out after 10 min.

Sensitivity of the Method

Under the optimum experimental conditions, the different concentrations of TA·HCl react with amino acids are measured at their maximum $\lambda_{ex}/\lambda_{em}$. The calibration graphs are gained by relative fluorescence intensity (F_0/F) being plotted against the concentrations of TA·HCl. The linear ranges, relative coefficients and the detection limits for TA·HCl are listed in Table 1.

It can be seen that fluorescence quenching method for the determination of TA·HCl concentration with Tyr or Trp as fluorescence probes have high sensitivities. The detection limits are 0.37 μg/mL for Tyr-TA·HCl system, 0.38 μg/mL for Trp-TA·HCl system, and 0.41 μg/mL for Phe-TA·HCl system (shown in Table 1). When Trp, Tyr or Phe is as probe, the sensitivities are higher than spectrophotometry [6]. And this method is simple, fast, and more advantageous to the trace determination of TA·HCl concentration.

The Composition Ratio and Fluorescence Quenching of TA·HCl with Amino Acids

The Composition Ratio of TA·HCl with Trp, Tyr and Phe

In pH 6.3 acidic medium, pH>pI, H^+ of amino acids can dissociate to become negatively charged, but TA·HCl is protonized and become positively charged in the acid medium, they can react with each other to form ion-association complex by electrostatic attraction and aromatic stacking interaction.

The composition ratio of the ion-association complex is determined by molar ratio method. The results show that TA·HCl

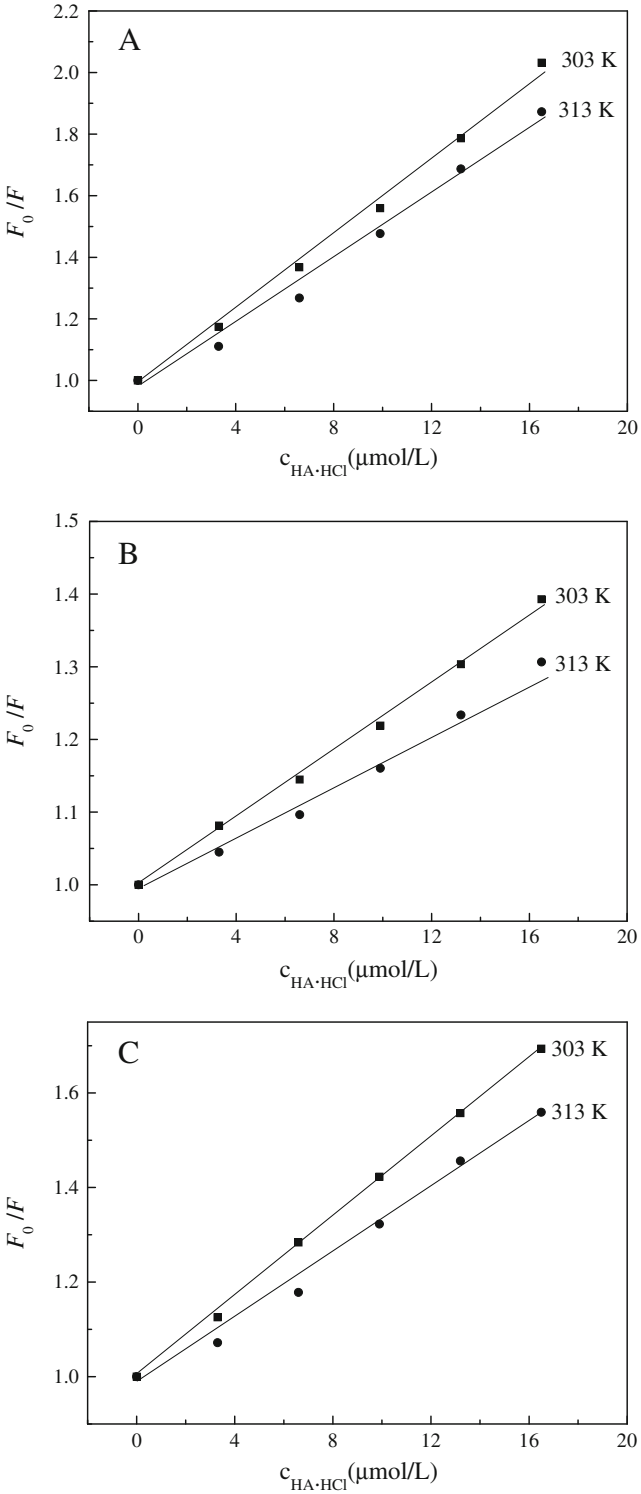


Fig. 5 The Stern-Volmer plots at different temperature. A, TA·HCl-Tyr system, B, TA·HCl-Trp system, C, TA·HCl-Phe system; Tyr and Trp: 4.0×10^{-5} mol/L, Phe: 8.0×10^{-4} mol/L, TA·HCl: 0.0, 3.3, 6.6, 9.9, 13.2, 16.5 μmol·L^{-1}, respectively; pH 6.3

Table 3 Some parameters for Stern-Volmer plots

System	Temperature T/K	Stern-Volmer equation (c, μmol·L^{-1})	Correlation coefficient (R)	Ksv (L·mol^{-1})	K_q (L·mol^{-1}·S^{-1})
TA·HCl-Tyr	303	F_0/F=0.97+6.2×10^4[Q]	0.9980	6.2×10^4	1.7×10^{13}
	313	F_0/F=0.95+5.5×10^4[Q]	0.9951	5.5×10^4	1.5×10^{13}
TA·HCl-Trp	303	F_0/F=1.00+2.3×10^4[Q]	0.9986	2.3×10^4	7.4×10^{12}
	313	F_0/F=0.99+1.9×10^4[Q]	0.9953	1.9×10^4	6.1×10^{12}
TA·HCl-Phe	303	F_0/F=1.00+4.2×10^4[Q]	0.9997	4.2×10^4	6.2×10^{12}
	313	F_0/F=0.97+3.5×10^4[Q]	0.9956	3.5×10^4	5.1×10^{12}

reacts with amino acids to form a 1:1 ion-association complex. The combined reaction of Tyr with TA·HCl is shown in Fig. 4.

In addition, it is important to determine the mode of reactant combination by enthalpy-entropy changes of reaction. The enthalpy change of the reaction (ΔH^0) can be seen as a constant. It is easy to calculate thermodynamic parameters of system by following two formulas,

$$1\mathrm{n}\frac{\mathrm{K}_2}{\mathrm{K}_1} = \frac{\Delta \mathrm{H}^0}{\mathrm{R}}\left(\frac{\mathrm{T}_2 - \mathrm{T}_1}{\mathrm{T}_1\mathrm{T}_2}\right) \quad (1)$$

$$\Delta G^0 = -RT1\mathrm{n}K_A = \Delta H^0 - T\Delta S^0 \quad (2)$$

Thermodynamic parameters of systems of amino acids with TA·HCl are computed by binding constant at different temperatures, conclusions are listed in Table 2. We can see that it is disadvantageous to elevate temperature for the reaction. The reaction of amino acids with TA·HCl are exothermic and spontaneous by $\Delta H^0<0$ and $\Delta G^0<0$. Furthermore, Van der Waals' force may exist between amino acids and TA·HCl by $\Delta H^0<0$ [22].

Quenching Mechanism

Fluorescence quenching include dynamic and static quenching [23]. In this experiment condition, quenching constant K_{SV} reduced as the temperature is rised (Fig. 5 and Table 3), it is a remarkable characteristic of static quenching. According to $K_{SV}=K_q\tau_0$, fluoresence lifetime of Trp, Tyr and Phe are 3.1 ns, 3.6 ns and 6.8 ns [24], we have count the double molecular quenching constant K_q are from 5.1×10^{12} L·mol^{-1}·s^{-1} to 1.7×10^{13} L·mol^{-1}·s^{-1} between 303 K to 313 K (Table 3). As known, the biggest K_q of collisional quenching is (1~2)×10^{10} L·mol^{-1}·s^{-1} [24, 25], K_q value of the experiment has far exceeded 2×10^{10} L·mol^{-1}·s^{-1}, which demonstrate the quenching of fluorescence of amino acids by TA·HCl is a static quenching process.

The measurement of fluorescence lifetime is the most definitive method to distinguish static and dynamic quenching. The lifetime (τ_0) of fluorescence molecule on excited state has no change in the presence of quencher when static quenching takes place. Reversely, τ_0 has to be shorter if dynamic

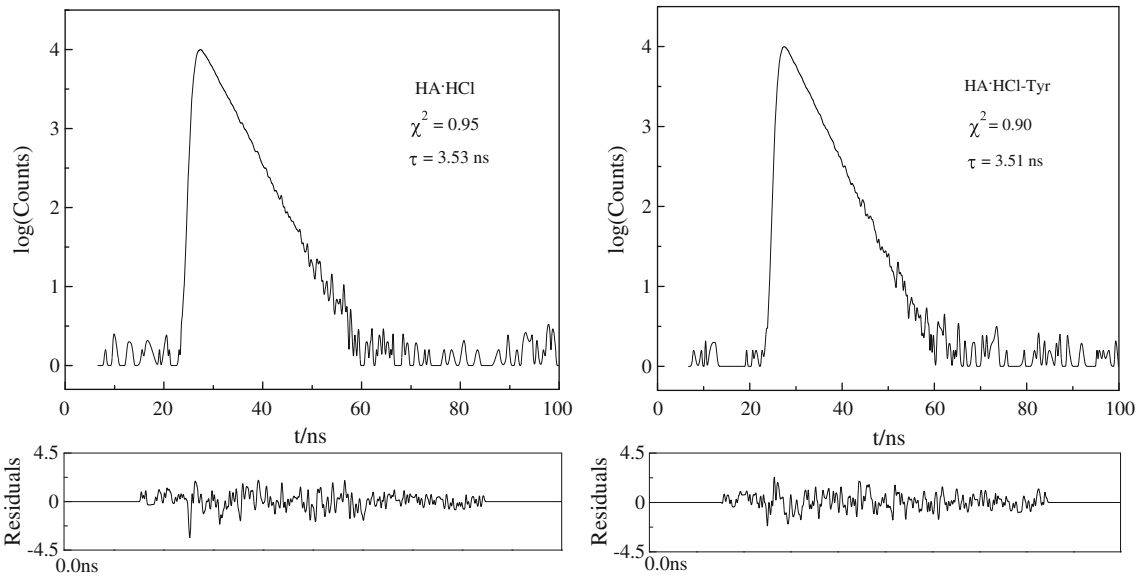


Fig. 6 Fluorescence emission decay curves of Tyr and HA·HCl-Tyr. Tyr: 4.0×10^{-5} mol/L, TA·HCl: 2.0 μg·mL^{-1}, pH 6.3

Table 4 Effects of coexisting substances(solution of TA·HCl:2.0 μg·mL^{-1})

Coexisting substance	Concentration (μg·mL^{-1})	Relative error (%)	Coexisting substance	Concentration (μg·mL^{-1})	Relative error (%)
Maltose	100	4.2	Urea	1000	−4.5
Lactose	200	2.2	VB_4	10	4.8
Glucose	160	1.6	NaAc	30	1.3
Sucrose	100	4.6	KCl	160	−4.9
HSA	34	2.5	NH_4Cl	180	1.4
BSA	30	3.1	$CaCl_2$	220	0.7
L(+)-Arginine	50	1.4	$MgSO_4$	160	−1.7
Lysine	50	5.0	$Al_2(SO_4)_3$	29	−1.6
DL -Aspartic acid	50	3.4	$CuSO_4$	16	4.2
L-Histidine	50	−2.1	$MnSO_4$	100	4.7
Starch	800	3.3	$FeCl_2$	500	2.3
VB_{12}	15	−0.6	$NH_4Fe(SO_4)_2$	66.5	2.6

quenching occurs. That is, $\tau_0/\tau=1$ (τ is the fluorescence lifetimes of fluorescence molecule in the presence of quencher) for static quenching; $\tau_0/\tau=F_0/F$ for dynamic quenching [23, 26]. Take the Tyr system with the highest sensitivity as an example, the measured fluorescence lifetime of Tyr and Tyr-TA·HCl is 3.53 ns and 3.51 ns (Fig. 6), respectively. Fluorescence lifetime have little difference, and $\tau_0/\tau \approx 1$, which demonstrates the addition of TA·HCl don't decrease the lifetime of the excited state and the quenching isn't a dynamic one but from the formation of a non-fluorescent complex in the ground state, which is generally called a static quenching [26]. So we can see that the quenching of fluorescence is a static quenching process.

Selectivity of the Method and Analytical Application

Selectivity of the Method

Take Tyr-TA·HCl system as an example, the effects of some co-existing substances on the determination of TA·HCl concentration are investigated, the results are given in Table 4. It can be seen that when the relative error is lower than ±5% , and the concentration of TA·HCl is 2.0 μg/mL, the common metal ions, acid radical anions, amino acids and saccharides have little interference with the determination of TA·HCl concentration. Therefore, the method has a good selectivity.

Analytical Application

Determination of TA·HCl Concentration in Human Serum

A fresh human serum sample is treated with suitable amounts of trichloroacetic acid and centrifuged 10 min at 4000 rpm. When the proteins in serum are separated thoroughly, into a 10.0 mL volumetric flask are added 0.5 mL supernatant fluid of the sample, 1.0 mL pH 6.3 BR buffer solution and 1.0 mL 4.0×10^{-4} mol/L Tyr solution. The standard addition method is used to determine five parallel samples for each concentration and the results are listed in Table 5.

Determination of TA·HCl Concentration in Human Urine

Into a 10.0 mL volumetric flask is added 1.0 mL filtered human urine, 1.0 mL pH 6.3 BR buffer solution and 1.0 mL 4.0×10^{-4} mol/L Tyr solution. The solution is diluted to the mark and mixed thoroughly. The standard addition method is used to determine five parallel samples for each concentration and the results are listed in Table 5.

It can be seen that fluorescence quenching method has a good repeatability for the determination of TA·HCl concen-

Table 5 Results for the determination of TA·HCl in urine and serum samples

Samples	Found (TA·HCl in samples) /(μg·mL^{-1})	Added TA·HCl/ (μg·mL^{-1})	Total found (n=5)	Recorvery/ %	RSD/ %
Urine 1	0	2.0	2.07 2.03 1.90 1.87 1.97	98.4	4.3
Urine 2	0	2.7	2.70 2.63 2.60 2.77 2.80	100.0	3.2
Urine 3	0	3.3	3.53 3.47 3.27 3.17 3.23	101.0	4.7
Serum 1	0	2.0	2.10 2.00 2.07 1.97 1.93	100.7	3.5
Serum 2	0	2.7	2.73 2.77 2.57 2.53 2.67	98.3	3.9
Serum 3	0	3.3	3.43 3.40 3.47 3.40 3.30	103.0	1.8

tration in serum and urine samples and relative standard deviation are between 1.8% and 4.7%. The method also has a good accuracy and the average recoveries are between 98.3% and 103.0% (Table 5). Therefore, the method can be applied to the rapid monitoring of TA·HCl in serum and urine samples, which will provide valuable evidence for clinical doctors to use TA·HCl reasonably and safely.

Conclusion

In pH 6.3 BR buffer solutions, tryptophane, tyrosine or phenylalanine can react with tetracaine hydrochloride (TA·HCl) to form ion-association complex by electrostatic attraction, aromatic stacking interaction and Van der Waals' force. Compared with other methods, it is obvious advantages not only in the operation but also in the sensitivity. It has been successfully applied to the quick determination of TA·HCl concentration in serum and urine samples.

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