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Resonance Light-Scattering Method for the Determination of Probenecid Based on a Complex with Aniline Blue

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The interaction of probenecid and aniline blue was characterized by resonance light-scattering (RLS) measurement. Thereby, a new method for the determination of probenecid with high sensitivity and selectivity by using resonance light-scattering technique was developed. At pH 0.6-1.2, probenecid reacted with aniline blue to form an ion-association complex which resulted in considerable enhancement of resonance light-scattering intensity and a new spectrum appeared. The maximum scattering peaks of the resonance light-scattering spectrum were at 430 and 463 nm. Under optimal conditions, a linear relationship was obtained between the intensity of resonance light-scattering (I_{RLS}) and the concentration of probenecid ranging from 4.0×10^{-7}-3.2×10^{-5} mol L^{-1} with a detection limit of 4.9×10^{-8} mol L^{-1}. Three synthetic positive urine samples were determined with satisfactory results and the recoveries were in the range of 92.2-106 %.

Key Words: Probenecid, Aniline blue, Resonance light-scattering, Human urine.

INTRODUCTION

Probenecid (PB) is a uricosuric agent with a weak diuretic activity. It is also a classical competitive inhibitor against organic acid and organic anion transport in the kidney and other organs[1,2]. Probenecid has been used clinically to inhibit renal tubular secretion of several drugs such as penicillin and cephalosporin antibiotics[3].

Probenecid (4-[(dipropylamino)sulfonyl] benzoic acid) is a lipid-soluble benzoic acid derivative, whose chemical structure is shown as follows:

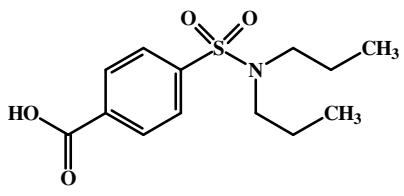

Fig. 1. Constitutional formula of probenecid

Probenecid has been misused in sports in recent years, for two reasons: (1) athletes may wish to reduce their body weight quickly in order to qualify for a

lower class. (2) Athletes may wish to dilute their urine to avoid the positive results of other doping by increasing the flow of urine. Health risks are always involved in abuse because of potential side-effects. The Medical Commission of the International Olympic Committee decided to add probenecid in the list of forbidden substances in 1988.

The methods for the determination of probenecid have been less reported. The classical method is spectrophotometer which has been applied for many years with a lower sensitivity. Gradually, this method has been replaced by high performance liquid chromatography (HPLC)[4,5]. Recently, a micellar electrokinetic capillary chromatographic (MEKC) method and a new HPLC method have been reported for determining probenecid in both pharmaceuticals and biological fluids[6,7]. However, these methods are expensive and have slightly high detection limits. Resonance light-scattering is a technique developed in recent years and has received much attention because of its high sensitivity and simplicity. Resonance light-scattering technique has been successfully utilized for determining several medicines, such as aminoglycosides antibiotics[8], flavonoid[9], tetracycline[10] and labetalol[11]. We focused on the utilization of aniline blue to develop a sensitive and convenient technique for the determination of trace probenecid. This is the first attempt to apply the RLS technique to the determination of probenecid in human urine and the method has needs no time-consuming sample per-treatment.

EXPERIMENTAL

Probenecid was purchased from Sigma Co. and prepared as a 1×10^{-2} mol L^{-1} stock solution by dissolving commercially probenecid in 0.05 mol L^{-1} sodium hydroxide solution. Probenecid solution was then diluted to 1×10^{-3} mol L^{-1} as required. Aniline blue (water soluble) was purchased from Shanghai Specimen Model Plant. The aniline blue stock solution and working solution were aqueous solutions of 1×10^{-2} and 1×10^{-3} mol L^{-1}, respectively. All chemicals used were of analytical reagent grade. All solutions were prepared with deionized water (18.2 MΩ cm).

A 970 CRT spectrofluorometer (Shanghai, China) with a 1 cm quartz cell was utilized for recording RLS spectra. The slit was (EX/EM) 5 nm/5 nm. A TU-1901 double-beam UV-spectrophotometer (Beijing Purkinje General Instrument Co., China) was utilized for recording the absorption spectra. All pH values were measured with a digital pH meter (pHS-25, Shanghai Presition and Scientific Instrument Co., China).

First, 0.3 mL of aniline blue solution and 0.5 mL of acetate-HCl buffer (pH 1) were put in a 10 mL volumetric graduated cuvette. Then a suitable amount of probenecid sample dissolved in 0.05 mol L^{-1} sodium hydroxide solution was added to the cuvette. After that, the mixture was diluted to 5 mL with water and mixed completely. After 15 min mixing, resonance light-scattering spectra were recorded by synchronous scanning at $\lambda_{em} = \lambda_{ex}$ ($\Delta\lambda = 0$ nm). The difference between the I_{RLS} (I) of sample solution and that (I_0) of reagent blank at maximum scattering wavelength was measured, $\Delta I = I - I_0$.

RESULTS AND DISCUSSION

Fig. 2 shows the RLS spectra of probencid, aniline blue and their mixtures. The intensities of RLS for probencid and aniline blue are very weak when they exist separately. When probencid and aniline blue coexisted, however, the RLS intensity was strongly enhanced and a new spectrum appeared because of the formation of the complex of probencid with aniline blue by virtue of electrostatic and hydrophobic interaction forces. Fig. 2 shows also the RLS intensity was enhanced with an increase in concentration of probencid. A maximum intensity of RLS was found at 430 nm and a deuto-intensity signal of RLS was at the wavelength of 463 nm together with other weaker peaks at 352, 383, 405 and 478 nm. Therefore, the wavelength for the light-scattering measurements of probencid was chosen to be at 430 nm in subsequent experiments.

UV-Visible absorption spectra of probencid, aniline blue and their mixtures were also recorded to clarify whether the spectrum in Fig. 2 originates from RLS. As can be seen in Fig. 3, the strong absorption peaks appeared at a wavelength of 314 nm for the aniline blue solution and both mixtures. In general, the RLS effect is observed to have an increasing scattering intensity at or near the wavelength of absorption of an aggregated molecular species[12]. Fig. 2 shows that the RLS intensity of the complex composed of probencid and aniline blue was strongly enhanced in the 350-478 nm wavelength range. Based on the UV-visible absorption spectral feature, the spectrum shown in Fig. 2 should be RLS spectrum originating from the aggregation of probencid-aniline blue complex.

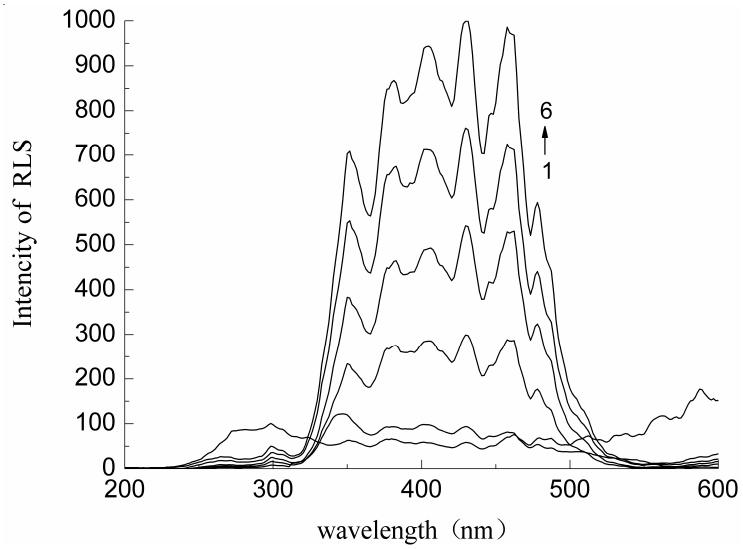

Fig. 2. RLS spectra as a function of probenecid concentration. (1) Aniline blue (6.0×10^{-5} mol L^{-1}); (2) probencid (3.0×10^{-5} mol L^{-1}); (3-6) aniline blue-probencid. Aniline blue concentration: 6.0×10^{-5} mol L^{-1}, probencid concentrations (mol L^{-1}): 8.0×10^{-6}, 1.6×10^{-5}, 2.4×10^{-5}, 3.2×10^{-5}. 1.0 mol L^{-1} acetate-HCl buffer (pH 1) was used

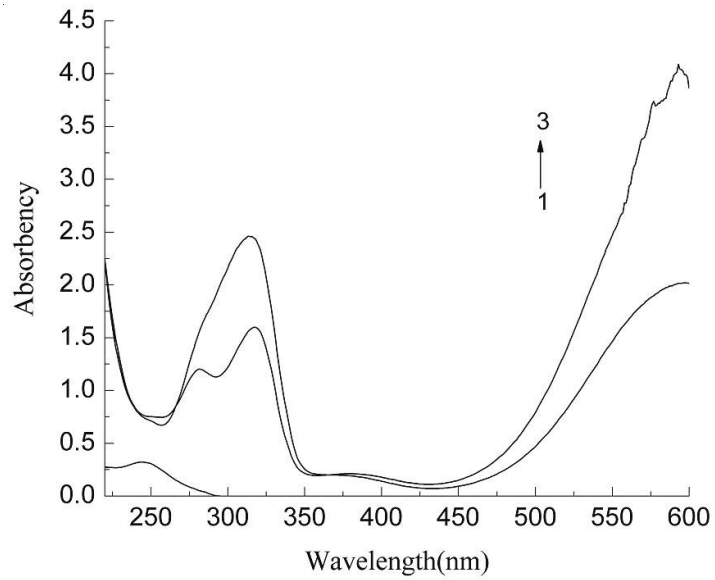

Fig. 3. UV-Visible absorption spectra of PB, AB and their mixtures. (1) PB. (3.0×10^{-5} mol L^{-1}); (2) AB-PB. AB (5.0×10^{-5} mol L^{-1}), PB (1.5×10^{-5} mol L^{-1}); (3) AB. (1.0×10^{-4} mol L^{-1}). 1.0 mol L^{-1} acetate-HCl buffer (pH 1) was used

Based on the RLS spectrum of probencid-aniline blue mixture, a quantitative determination of probenecid was tested. The type of buffers is one of most important conditions for measurements. Three different buffers were investigated: sodium acetate-HCl (pH 0.6-5.0), acetic acid-sodium acetate (pH 6.0-7.0) and NaOH-citric acid (pH 8.0-9.0) buffer systems. It was found that virtually no response or very unstable RLS signal was observed in acetic acid-sodium acetate and NaOH-citric acid buffers, respectively, while significant and stable RLS signal was found in sodium acetate-HCl buffer (pH 0.6-1.2). This indicated that the complex of probencid and aniline blue is stable in stronger acidic solution. Thus, the sodium acetate-HCl buffer at pH 1.0 was employed in this study.

The concentration of aniline blue solution had a significant effect on the RLS intensity of the complex. A well-defined and sensitive RLS signal appeared when the concentration of aniline blue solution was between $4\text{-}8 \times 10^{-5}$ mol L^{-1}. When the concentration of aniline blue solution was higher than 8×10^{-5} mol L^{-1}, the intensity of RLS was reduced. It is likely that a part of aniline blue can self-aggregate when the concentration is too high and the aggregation may disturb the formation of probencid-aniline blue complex. On the other hand, aniline blue solution lower than 4×10^{-5} mol L^{-1} gave weaker RLS signal probably due to incomplete reaction between aniline blue and probencid. Thus, in the present study, 6×10^{-5} mol L^{-1} of aniline blue was used. The intensity of RLS spectrum reached a steady state immediately after mixing and remained constant for at least 6 h at the room temperature (18-32 ºC).

The calibration graph (Fig. 4) for the determination of probenecid was constructed under the optimal procedure. Resonance light-scattering was recorded in the presence of 4.0×10^{-7}-3.2×10^{-5} mol L^{-1} probenecid. Fig. 4 shows that the intensity of RLS increased with an increace in concentration of probenecid. The linear-regression equation is $\Delta I = 284.8C - 6.39$.

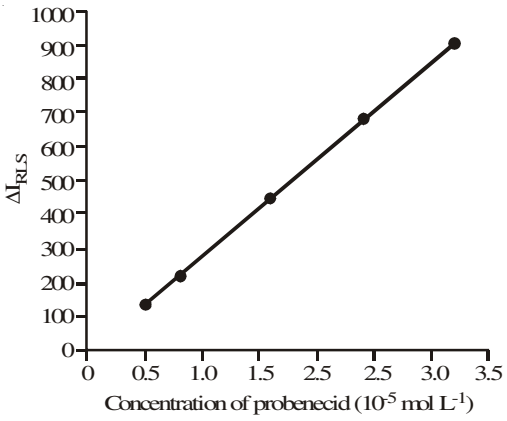

Fig. 4. Calibration graph

The influence of various ions, sugars and amino acids was tested when the concentration of probenecid was 3×10^{-5} mol L^{-1} under the optimal condition. The results indicate that monovalent cations and anions, divalent anions and urea do not interfere with the determination of probenecid. The effects of divalent cations, trivalent cations and sugars on the RLS intensity of the determination system were not significant in the present method. However, the amino acids showed a significant negative effect on the RLS intensity owing to the presence of carboxyl group in amino acids probably (Table-1).

An appropriate amount of probenecid was added in a fresh sample of human urine (5 mL) from a healthy adult to simulate positive urine. Then a small quantity of hydrochloric acid of definite concentration was added to the sample. The sample then was centrifuged at 3000 rpm for 15 min. The assay was completed using 250 μL of this supernatant, according to the procedure reported in experimental section. A higher background value of RLS was found in the urine samples. Some efforts were done for reducing the background and the results of experiment indicate that the determinate amount of hydrochloric acid can decrease the background value in urine samples. The optimal concentration of hydrochloric acid in urine sample was 0.3-0.4 mol L^{-1}. Fig. 5 shows the influence of concentration of hydrochloric acid on the RLS intensity in urine samples.

In the presence of hydrochloric acid, good linearity was obtained between the intensity of RLS and the concentration of probenecid in urine sample. Typical results of recovery test are summarized in Table-2, which shows that the proposed method is acceptable for the determination of probenecid in human urine.

TABLE-1
EFFECTS OF COEXISTING SUBSTANCES ON THE INTENSITY OF RLS ARISING FROM 3×10^{-5} mol L^{-1} PROBENECID

Coexisting substance	Concentration/10^{-5} (mol L^{-1})	Change in I_{RLS}* (%)
Na^+	3000	4.55
K^+	3000	8.65
Cl^-	3000	3.21
NO_3^-	3000	-1.99
SO_4^{2-}	3000	9.65
Urea	3000	-4.80
Al^{3+}	1200	6.09
Fe^{3+}	900	-4.43
Sucrose	600	-5.15
Cu^{2+}	300	-9.87
Pb^{2+}	300	-2.35
Mg^{2+}	180	-9.89
Ca^{2+}	30	-3.15
Glucose	30	6.61
Lactose	30	-2.26
Arginine	6	-2.81
L-Lysine	3	22.28
DL-Cysteine	3	-36.00
Glycine	3	-16.58
Glutamic acid	3	-25.86

*Average values of 3 independent measurements are listed.

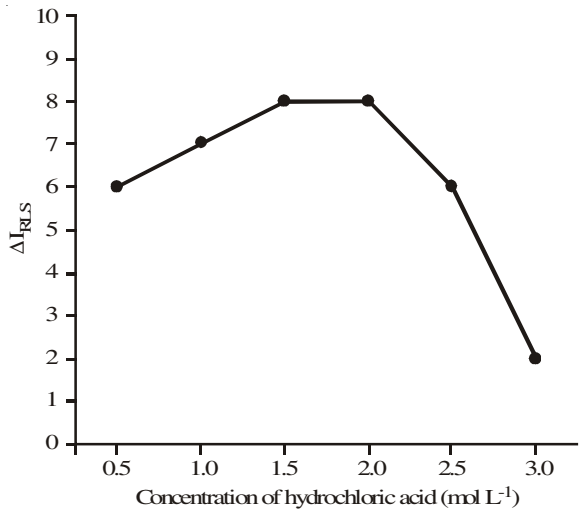

Fig. 5. Influence of concentration of hydrochloric acid on RLS intensity in urine sample AB concentration: 6×10^{-5} mol L^{-1}; PB concentration: 5×10^{-7} mol L^{-1}. The addition of urine was 250 μL. ΔI is the difference of RLS intensity between positive urine sample solution and negative urine sample solution

TABLE-2
DETERMINATION OF PROBENECID IN HUMAN URINE

	Sample added (10^{-7} mol/L)	Found (10^{-7} mol/L)	RSD % (n = 3)	Recovery % (n = 3)
No. 1	5	5.03	5.70	106.00
No. 2	5	4.61	5.50	92.20
No. 3	5	4.92	3.60	98.40

Aniline blue is an anionic triphenylmethane dye with three sulfo-groups. Because the sulfo-groups can completely dissociate under the determined condition (pH 1), the sodium salt of aniline blue existed as AB^{3-} (**Scheme-I**). According as the molecular structure, the maximum positions of negative charge in probenecid molecule should be at the two thiocarbonyl oxygen atoms (Fig. 1) which were easy to associate with H^+. Thus, the probenecid molecule existed as a protonated organic cation $[H_2PB]^{2+}$. In addition, there is a carboxy group in the probenecid molecule and the carboxyl group has interaction with sulfo-groups of aniline blue molecule through hydrogen bond. Accordingly, $[H_2PB]^{2+}$ can react with AB^{3-} to form an ion-association complex through electrostatic attraction, the hydrophobic force and hydrogen bond. These considerations suggest that the ratio of AB^{3-} to $[H_2PB]^{2+}$ in the complex should be 1:1 and the molecular formula should be $[H_2PB]AB$ provided with a ring structure. The molecule free turning was restricted owing to increasing the molecule rigidity on the ring structure and thus, the RLS intensity was enhanced[13]. Because of the distant space position of three sulfo-groups in aniline blue molecule, two OH^+ groups on the thiocarbonyl in probencid do not suit to the binding with aniline blue in synchronization. The possible structures for the complex are suggested as **Scheme-II** A and B.

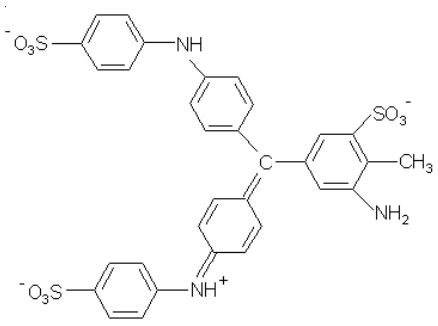

Fig. 6. Structure of aniline blue

Conclusion

In this work, resonance light-scattering method was first applied to study the interaction of probenecid and aniline blue. A sensitive and convenient method for the determination of a trace amount of probenecid has been developed utilizing

RLS technology based on the formation of ion-association complexes $[H_2PB]AB$. The performance of the system indicated that it can be used for determining probenecid in human urine.

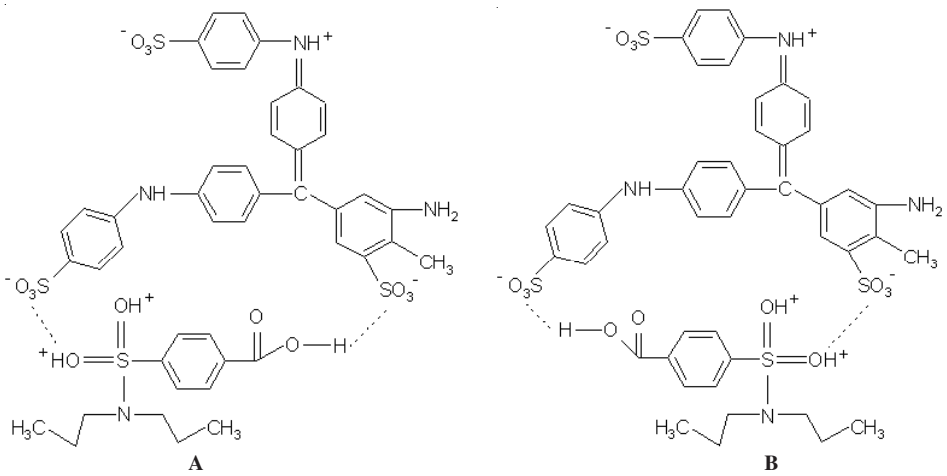

Fig. 7. Structures for the complex composed of aniline blue and probencid

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