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A high selective and sensitive method for the detection of six psychotropic drugs in human urine by High performance liquid chromatography combined with resonance Rayleigh scattering spectra

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Abstract

A reliable, high selective and sensitive method of high performance liquid chromatography combined with resonance Rayleigh scattering spectra **(** HPLC-RRS) was developed and validated for the synchronous detection of six psychotropic drugs, including Doxepin(DH), Promethazine(PMZ), Imipraine(IMP), Amitriptyline (AH), Chlorpromazine(CPZ) and Clomipramine(CHL). This method was based on the weak intensity of the RRS of six drugs and its significant enhancement after combined with Erythrosine (Ery) in a pH 4.0 - 4.7 BR buffer solution. The linear range was between 0.05 and 20 μg mL^{-1} with correlation coefficient (r) all above 0.9916. The limits of detection(S/N=3) were in the range of 0.42 and 21.05 ng mL^{-1}.Because the RRS detection wavelength was special (The excitation wavelength

was equal to the emission wavelength, λ ex= λ em= 295nm) and some other substances in human urine do not form ion-association complex with the Ery, no interference from the matrix was observed. The proposed method was successfully applied to real human urine samples analysis.

Keywords: high performance liquid chromatography; resonance Rayleigh scattering spectra; erythrosine; psychotropic drugs; urine

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1. Introduction

Doxepin (DH), Imipraine (IMP), Amitriptyline (AH) and Clomipramine (CHL) are the tricyclic antidepressants (TCA). Their main function is to block the reuptake of the central nervous system in noreoinephrine and serotonin for anti-depression[1]. In addition, they can treat other psychotropic diseases such as anxiety, neuropathic pain and infant enuresis[1-2] and so on. Chlorpromazine (CPZ) and Promethazine (PMZ) are the phenothiazine drugs[3-4] and have the similar basic structure, but they showed a big difference for pharmacological effects. CPZ is used as a antihistamine and stabilizer whose main role is sedation and antipsychotic in the clinical. While PMZ is a histamine H1 receptor antagonist[4], it was widely used for antihistaminic, sedative, antipsychotic, analgesic and anticholinergic properties[5]. All the six drugs are commonly used. But they also can cause a series of toxic side effects such as dizziness, headache, sleepiness, seriously may cause arrhythmia，shock even death. Thus, the detection of the concentration of the drugs in human urine is very important. On the one hand, it could provide insight into the relationship between the dosage of the drugs and its clinical effect. On the other hand it could provide some valuable informations for controlling their side effects.

Several methods including electrochemical[6] , HPLC-mass spectrometry/mass spectrometry(HPLC-MS/MS)[7-11] , ultra performance

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liquid chromatography-MS/MS (UPLC-MS/MS)[2,12], gas chromatogram-MS(GC-MS)[13], dispersive liquid–liquid microextraction (DLLME) coupled with GC(DLLME-GC)[14-15],electro-enhanced solid phase microextraction (EME) coupled with GC–MS (EME-GC-MS)[16-17] have been applied for the detection of the concentration of one or more above drugs in different matrices, such as urine, blood, serum, plasma and waste water. Electrochemical detection method is simple, selective and low cost. But the problems of electrode deactivation and frequent pretreatment made it being not as popular as other methods. HPLC-MS/MS, UPLC-MS/MS, GC-MS methods have the advantages of fast speed, efficient, high selective and sensitive with a good precision and accuracy. However, they have not been widely used due to its expensive apparatus and the complex sample pre-treatment. In recent years, more and more studies were focused on the methods of DLLME and EME coupled with HPLC or GC. Through extracted, purified and concentrated the target from the analyzed matrix, it could obtain a high recovery. But the process of sample dispose was still relatively complex and the cost was expensive as well. Although the above methods can obtain good results, high selective and sensitive. But high efficient and low cost method is still needed to develop.

Resonance Rayleigh scattering (RRS) is a new spectral analysis technology. Its wavelength is located or close to the molecular absorption

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band. The scattering can absorb the light energy and to produce a re-scattering process by resonance[18]. As known to all, it has outstanding sensitivity and very fast analysis speed. It has been widely applied in many filed such as biomacromolecules [19-21], inorganics [22-23] and pharmaceuticals [24-25] and so on. But the selectivity of RRS was not meet to synchronous detection of complex mixtures. HPLC was widely applied for its reproducibility, accuracy and selectivity performance. But its sensitivity is low. HPLC- RRS method combined the high sensitivity of HPLC and the high selectivity of RRS. It could provide a new detector type for HPLC and promote the development of the HPLC. At the same time, it could make up the poor selectivity shortcoming of RRS and realize the goal that applied RRS to the detection of complex substances. At present, HPLC-RRS has been successfully appied to the detection of fluoroquinolones[26-27], four tetracycline antibiotics[28], local anesthetics[29], amino acids[30], proteins[31], amino-glycosides[32] and so on.

In this article, a simple, reliable, high selective and sensitive analysis method of HPLC-RRS for the determination of six psychotropic drugs was established. Compared with other derivative methods [33-35], the proposed method did not need tedious sample pretreatment. No interference from the matrix was observed because the RRS detection wavelength was specific and some other substances in human urine do not form ion-association complex with Ery. The experimental conditions

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such as detection wavelength, the length of reaction tube and reaction temperature have been studied. Quantum chemistry calculation, absorption spectra, scanning electron microscope (SEM) have been used to explore the system reaction mechanism, combination mode and binding sites. The proposed method was successfully applied to real human urine samples analysis.

2. Experimental

2.1 reagents and chemicals

Reagents and chemicals and were as follows: DH，IMP, AH, CHL，CPZ and PMZ were purchased from the offices shall of pharmaceutical and biological products (Beijing, China). Ery was purchased from Aladdin reagent net (Shanghai, China). HPLP-grade acetonitrile, Triethylamine and Phosphate were provided from Kermel (Tianjin, China). Britton–Robinson buffer solutions (BR) was used to adjust acidity and prepared by mixing 0.2 mol L^{-1} NaOH and 0.4 mol L^{-1} solutions of HAc, H_3BO_3 and H_3PO_4. Double distilled water was used in the whole experiment process. All solutions was filtered by 0.22 μm Nylon membrane.

2.2 Apparatus

A HPLC (Shimadzu, Japan) including two LC-20AD pumps, a DGU-20A5R degassing unit and a RF-20A fluorescence detector. A PCX-BT post-column derivatization instrument was from Tian Mei Da

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Scientific Instruments Co. Ltd. (Shenyang, China). RRS spectra was measured by a Hitachi F-2500 spectrofluorophotometer (Tokyo, Japan) and the absorption spectra was obtained from a UV-Vis 8500 spectrophotometer (Shanghai, China). The surface images of Ery blank and ion-association complexes were observed by scanning electron microscopy (SEM, S-4800, Hitachi, Tokyo, Japan). The pH was measured by a PHS-FE20 pH meter (Mettler-Toledo Instruments Co. Ltd., Shanghai, China). Double distilled water was prepared using a Millipore SZ-93 system (Shanghai Yarong Biochemical Apparatus Co .).

2.3 Standard solution preparation

DH, PMZ, IMP, AH, CPZ and CHL were dissolved in double distilled water and configured to 1 mg mL^{-1} stock solutions, then preserved in refrigerator at 4 ℃. 6 x 10^{-4} mol L^{-1} Ery working solution was prepared by dissolving pure Ery in double distilled water.

2. 4 Human urine samples preparation

Human urine was got from a health volunteer. 400μL acetonitrile was added to 200 μL urine. Then the urine was vortex for 30 s and centrifuged at 12,000 rpm min^{-1} for 10 min. The supernatant was placed in a new centrifuge tube. Acetonitrile was evaporated in a vacuum drying oven at 37℃for one hour. Finally, all the urine samples were filtered by the 0.22 μm Nylon membrane filters. The analysis was conducted immediately after the samples preparation.

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2.5 HPLC-RRS device and parameters

The HPLC-RRS system was show in Fig.1. A Phenomenex Luna 5 μm100Å C8 column (250 x 4.60 mm) was used for the separation. Mobile phase was acetonitrile: 0.1% triethylamine solution (using phosphoric acid adjusted the pH to 3.1) = 40:60 (V/V) with the flow rate of 0.4 mL min^{-1}. Column temperature was 40℃ and sample quantity was 20 μL. Flow rate for BR buffer solution was 1.0 mL/min and 0.2 mL min^{-1} for Ery. After separated by HPLC, the six drugs would mix with BR buffer solution one by one in the first tee and protonation, then the mixture would mix with Ery in the second tee. Finally the association reaction was completed in the heat tube. The synchronous scanning wavelength of RRS was $\lambda ex = \lambda em = 295$ nm.

3. Results and Discussion

3.1 Optimization of the reaction conditions

3.1.1 Selection of the detection wavelength

All the RRS spectra of Ery, six drugs and the ion－association complex were pre-measured by a Hitachi F-2500 fluorescence spectrophotometer with synchronous scanning. As showed in Fig.2, the maximum wavelength of RRS all around 289 nm. Then a series of RRS wavelengths between 270nm to 310 nm were scanned to obtain the optimal detection wavelength on HPLC-RRS. The results indicated that all the peaks area

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reached the maximum at 295 nm.

3.1.2 Effection of pH

Acidity has a huge impact on the I_{RRS}, a series of BR buffer solutions with pH between 4.0-4.7 were tested. As showed in Fig.3, all the I_{RRS} of the system reached the maximum when the pH between 4.2 - 4.4. When pH was lower than 4.1, Ery would aggregate itself, which would lead to enlarge the noise. when pH was higher than 4.4 , it could not be beneficial to the association reaction, thereby the I_{RRS} was attenuate. Therefore, pH 4.3 was chosen as the optimal condition for the following research.

3.1.3 Effection of HPLC flow rate

The mobile phase flow rate could affect the chromatographic retention time and the I_{RRS} strength. The flow rate of the HPLC mobile phase from 0.3 mL/min to 0.5 ml/min was tested. The results showed that when the flow rate was 0.3 mL/min, the retention time would become longer and the noise increased. When flow rate was 0.5 ml/min, too fast flow rate could result in I_{RRS} attenuate. I_{RRS} would reach the highest when the flow rate was 0.4 mL/min. Therefore 0.4 mL/min was chosen as the optimal condition for the following research.

3.1.4 Effection of BR flow rate

The BR flow rate from 0.6-1.2 mL/min was tested. The results showed

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that when the flow rate less than 1.0ml/min, I_{RRS} increased with the increased of flow rate. When the flow rate was more than 1.0ml/min, the too fast flow rate would cause the impact increased，which was not beneficial to the association reaction, some unstable association substances would disintegrate. Thus the reactions between drugs and Ery were incomplete. Thereby the I_{RRS} attenuate. Therefore, 1.0mL/min was chosen as the optimal condition for the following research.

3.1.5 Effection of Ery flow rate

The Ery flow rate from 0.1-0.3 mL/min was tested in this study. The results showed that the I_{RRS} reached the maximum when the Ery flow rate was 0.2mL/min. When the flow rate was less than 0.2mL/min, the Ery concentration was insufficient and resulted in the I_{RRS} attenuate. When the flow rate was more than 0.2 mL/min, it not only gave rise to the I_{RRS} attenuate but also the noise increased. Because the too fast flow rate would cause the impact increased, which was not beneficial to the association reaction, some unstable association substances would disintegrate. At the same time the Ery concentration increased, which would resulte in the noise increased. So 0.2mL/min was chosen as the optimal condition for the following research.

3.1.6 Effection of Ery concentration

The Ery concentration ranged from 4 x 10^{-4} mol/L to 8 x 10^{-4} mol/L was

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studied to test the effect on the I_{RRS}. The results showed that the I_{RRS} was weak when the Ery concentration less than 6 x 10^{-4} mol/L. When Ery concentration was more than 6 x 10^{-4} mol/L, the I_{RRS} was increased, but the noise was increased as well. Therefore, the concentration of 6 x 10^{-4} mol/L for Ery was selected.

3.1.7 Effection of reaction tube length

The length of the reaction tube affected the reaction time. So the length of the reaction tube from 1m to 6m were tested. The results showed that the I_{RRS} would reached maximum when chose the 3m reaction tube. When the reaction tube was less than 3m, the short reaction time would result in the association reaction incomplete and I_{RRS} attenuate. When the reaction tube was longer than 3m, the dead volume would increased with wider chromatographic peak and lead to the I_{RRS} attenuate, because the column efficiency was reduced. Therefore, 3 m of reaction tube was selected in this study.

3.1.8 Effection of the reaction temperature

Because the reaction temperature also was a significant influence factor of the system, the temperatures ranged from 20℃ to 40℃ were tested, According to the equation of van't Hoff:

$$\frac{d \ln K^{\Theta}}{dT} = \frac{\Delta_r H_m^{\Theta}}{RT^2}$$

When the $\Delta_r H_m^{\Theta} < 0$, the reaction heat would release, K^{Θ} would decrease along with the temperature rose. The chemical reaction which

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have been reached balance which would move into the direction of the reactant. Due to the reaction $\Delta_r H_m^{\Theta} < 0$ (took the reaction of CPZ and Ery as example, $\Delta_r H_m^{\Theta}$=-0.12 a.u), when the temperature rose, the I_{RRS} would decreased. The I_{RRS} would reach maximum at 20℃. Therefore 20℃ was selected as the optimal reaction temperature in the study.

3.2 Reaction mechanism and RRS signal enhancement reason

3.2.1 The reaction mechanism

Due to the chemical structures of the six drugs are similar (Fig.4), they all have a tertiary amine group. In this paper, we took CPZ as an example to study the reaction mechanism.

The composition ratio of Ery and CPZ was determined by molar ratio and Job method (Fig.5). The result showed that CPZ and Ery would formed 1:1 ion－association complex. In this study, all the calculations, the ground state charge distribution of CPZ and full geometry optimizations of the study system were performed by quantum chemistry calculation by using B3LYP method with Lanl2dz as the basis set. Ery is a binary weak acid, according to its acid dissociation constant (pKa1=2.9，pKa2=3.8). Under the experiment conditions, Ery will dissociate to two forms, HL^- and L^{2-}. HL^- is the main existing form because near the hydroxyl oxygen there are two strong electron-with -drawing group (-I) on the xanthene base which will lead to the electron cloud of the hydroxyl oxygen reduce greatly. So it is easier dissociate than -COOH on phenyl. From another

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perspective, the enthalpy changes of -OH and -COOH dissociation systems are -0.109 a.u and -0.063 a.u, respectively. It means that hydroxyl dissociation will release more energy (0.046 a.u) than that of -COOH. Therefore, for Ery system, hydroxyl dissociation way is more stable than carboxyl dissociation. From the approximate calculation to the ground state charge distribution of CPZ by quantum chemistry, the above conclusion was confirmed. As showed in Fig.5, the charge distribution on CPZ branched chain N (1) and N (2) was -0.267 and -0.212, respectively. In weak acidic solution, N (1) was more easily to combine with H^+ to form the quaternary ammonium cationic ($HCPZ^+$). Therefore Ery and CPZ formed 1:1 ion－association complex by the electrostatic force and the hydrophobic force (Fig.7). The result of quantum chemistry calculation and the analysis of perspective of thermodynamics showed that after combination of two molecules, $\triangle G<0$, Therefore the reaction would occur spontaneously.

3.2 .2 RRS signal enhancement reason

3.2.2.1 The influence of absorption spectra

Because the RRS is a scattering-absorption-re-scattering process, its produce is due to the resonance between light scattering and the absorption. When the wavelengh of RRS is located or closed to the light molecular absorption band, the scattering can absorb the light energy and produce a re-scattering process by resonance. As showed in Fig.6，

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Compared with the RRS spectra and the ultraviolet absorption spectra (UV), it can be seen that the RRS spectra peaks are located at 289, 328nm and 565nm respectively. They are very closed to the UV absorption peaks of 258nm, 310nm, 528nm, respectively. Due to the scattering peaks near absorption band, it will produce a resonance enhanced scattering effect which would cause the scattering intensity enhanced significantly.

3.2.2.2 The molecular volume increased

According to the law of Rayleigh scattering, the scattering intensity is in proportion to the square of scattering particles volume (v)[36].

$$I = \frac{24\pi^3 N v^2}{\lambda^4}\left(\frac{n_1^{\,2} - n_0^{\,2}}{n_1^{\,2} + 2n_0^{\,2}}\right)^2 I_0$$

Where I is the intensity of scatting light, I_0 is the intensity of incident light, N is the particle number in per unit volume, v is the volume of a particle, n_1 and n_0 are stand for the refractive index of scattering phase and medium, respectively.

The molecular diameter and shape of Ery and ion-association were observed by SEM (Fig.9). From the SEM images, It can be seen that single Ery molecule (Fig.9A) could only be found at higher magnification. While the ion-association (Fig.9B) have been aggregated to form large spherical particles, it could be seen at a low magnification. It is the most important reason for the I_{RRS} strengthen.

3.2.2.3 Hydrophobicity enhancement

Both $CPZH^+$ and Ery^- have some hydrophilic groups, so they could be

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easier dissolve in water without forming hydrophobic interface. But after the CPZ and Ery formed the ion-association complex, the charges of molecules were neutralized. At the same time, the ion-association complex has a large amount of hydrophobic aryl and alkyl skeleton. Thus it would form a hydrophobic interface between the ion-association complex and water, which would produce a surface enhanced Rayleigh scattering effect and lead to the I_{RRS} enhanced greatly.

3.3 Method validation and application

3.3.1 Method validation

In this study, six drugs could be separated and detected in 27 minutes. To ensure the accuracy and precision of the measurement, each sample was measured with six replicates. Calibration curves were set up for all the analytes and it was constructed by using the peak area as the ordinate(y) and the sample concentration as the abscissa(x, μg mL^{-1}). Satisfactory linearities with correlation coefficient (r) all above 0.9916 were obtained in the range of 0.10-20 μg mL^{-1} for DH, 0.08-20 μgmL^{-1} for PMZ , 0.06-20 μg mL^{-1} for IPM and AH and 0.05-20 μg mL^{-1} for CPZ and CHL, respectively. The limit of detection (LOD) was obtained at a signal-to-noise ratio of 3.0. It was 21.1 ng mL^{-1} for DH, 11.2 ng mL^{-1} for PMZ, 1.68 ng mL^{-1} for IMP, 1.02 ng mL^{-1} for AH, 0.76 ng mL^{-1} for CPZ and 0.42ng mL^{-1} for CHL, respectively. All the analytical parameters were listed in table 1.

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3.3.2 Application of proposed method to real urine samples

In this study, the proposed method was applied to the detection of six drugs in human urine . The prepared urine samples were spiked with 3 μg mL^{-1}, 10 μg mL^{-1} and 20 μg mL^{-1} mix standard solution, respectively. Each sample was measured with six parallel injections. As showed in table 2, the sample recovery was from 93.6% to 102.1%. All standards were within 15% of the expected concentration. Thus it was meet FDA guidelines[37-38]. At the same time the RSDs were lower than 6.4% for all the analytes. Fig.10 showed the chromatograms of blank urine and the urine sample spiked with 3 μg ml^{-1} mixed standard solution. It can be seen that the chromatographic peak were well-separated and no interference from the matrix was observed under the experiment condition. This is because the RRS detection wavelength was specific and some other substances in the human urine do not form the ion-association complex with Ery.

To validate the HPLC-RRS method, the proposed method was applied to the determination in real urine samples. For this purpose, We used the urine samples from a male patient who is suffering from the treatment with the IMP and analyzed them by the proposed method. The patient was taken IMP tablets three times a day and each does was 25mg. The urine samples were collected 6h after he took 25mg IMP tablets. He has not taken the IMP drugs again in this 6h. From the current obtained peak

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of HPLC(Fig.11), the average amounts of IMP was found to be 142.6 ng ml^{-1}, good RSD < 5.6 was obtained (n=6).

IMP has some active metabolins[39]. Comparing the chromatograms of the standard IMP solution at level of 200 ng mL^{-1} (Fig.11,A,red) and the real urine sample (Fig.11,B,black), As seen, the urinary excretion has not change IMP in a way. The result indicated that the metabolins would not interfierence to the detection.

4. Conclusion

The proposed method is the first report using HPLC coupled with RRS to the detection of the above six drugs in human urine. It has the merits of simple operation procedure, reliable and high sensitivity. The results indicated that the proposed method was effective and reliable in the analysis of the human urine with complex matrix. In the end, the proposed method was successfully applied to real human urine samples analysis. The following work should concentrate on developing a suitable flow-through cell to improve the HPLC-RRS sensitivity. In addition, HPLC coupled with RRS also could be used to analyze substances which do not have fluorescence or absorption of UV-Vis light. We believed that HPLC coupled with RRS will have a broad application prospects.

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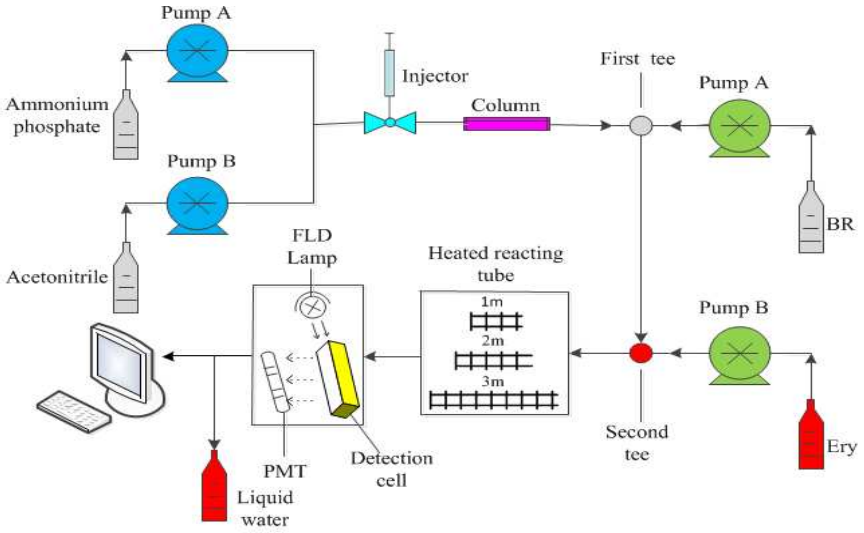

Fig.1. The HPLC-RRS system.

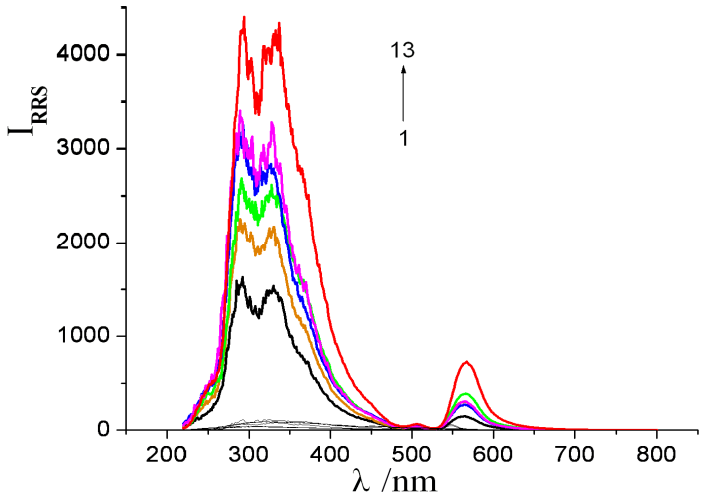

Fig.2. RRS spectra (1) DH（2)PMZ（3)IMP（4)AH（5）CPZ （6）CHL （7）Ery (8) Ery-DH (9) Ery-PMZ (10) Ery-IMP (11) Ery-AH (12) Ery-CPZ (13) Ery-CHL . The concentration of Ery was 2 x 10^{-4} mol L^{-1} , pH was 4.3.

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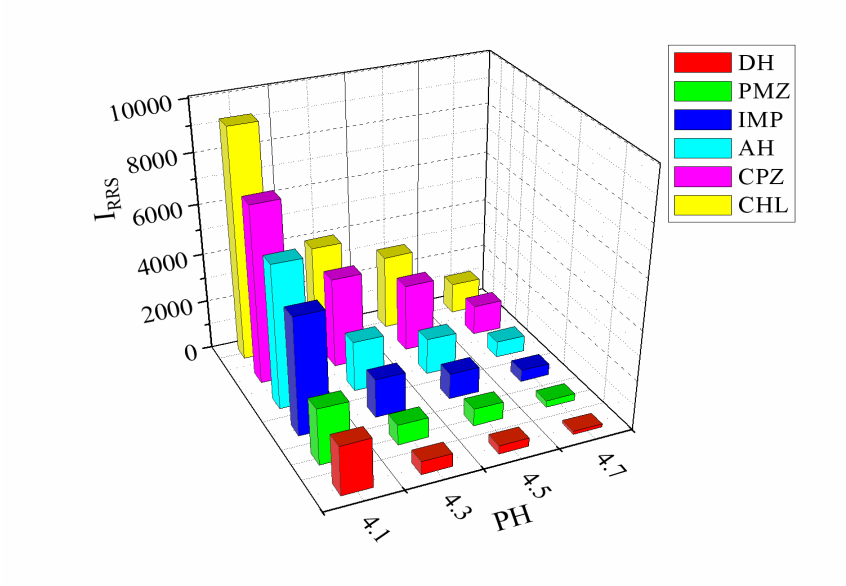

Fig.3. Effection of pH . The concentration of drugs was 20μg mL^{-1}.

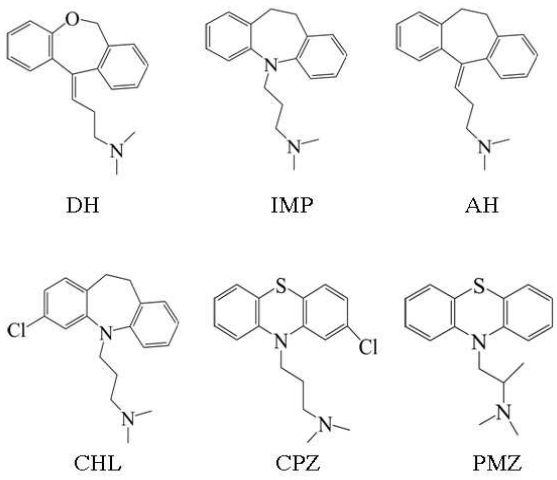

Fig.4. The chemical structures of the six drugs.

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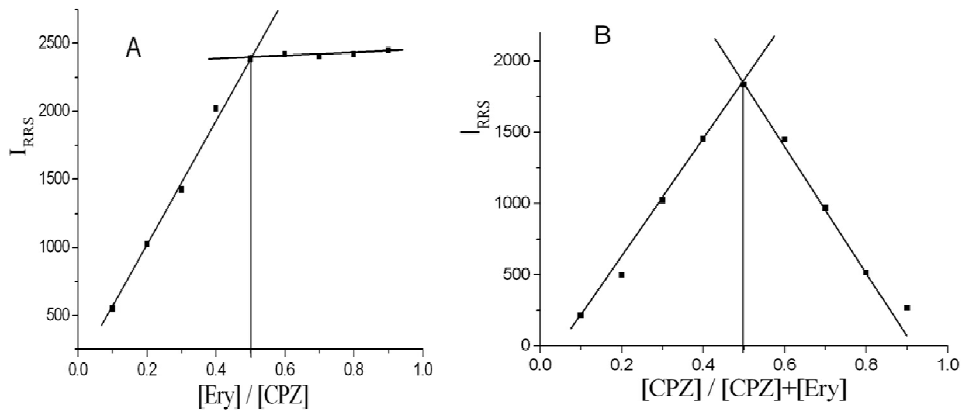

Fig.5. Molar ratio method (A) and Job method (B) for determine the composition ratio of Ery and CPZ . pH was 4.3

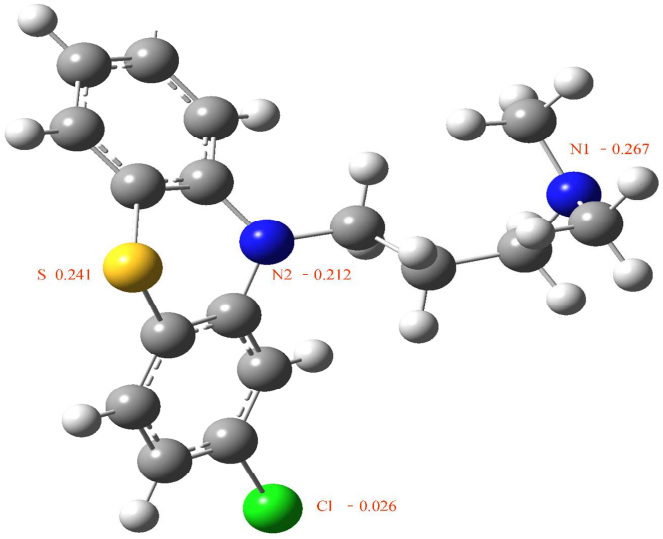

Fig.6. The charge distribution of CPZ (Mulliken atomic charges)

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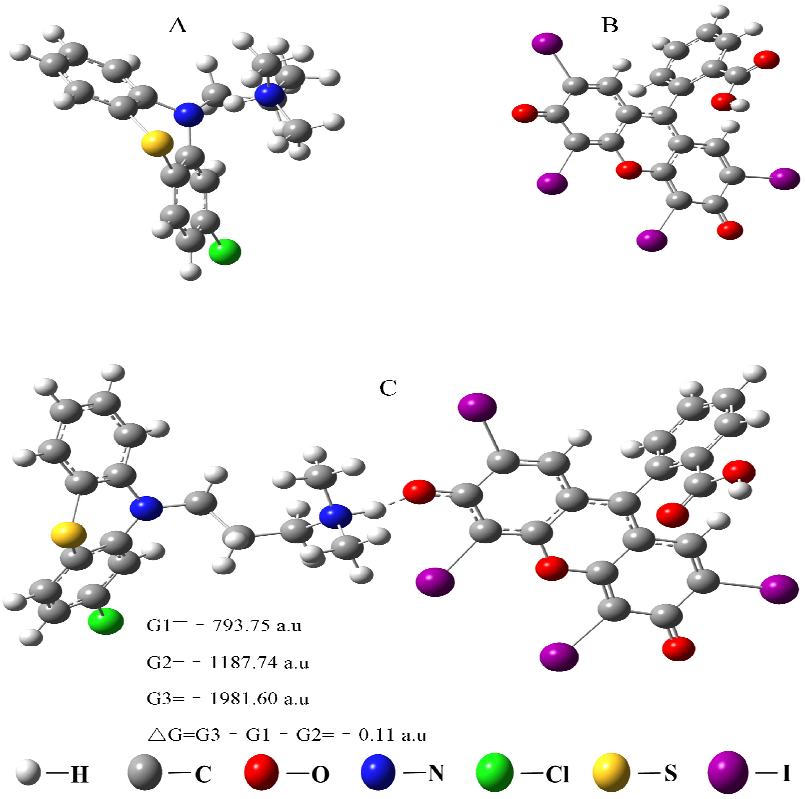

Fig.7. The reaction process and the change of gibbs free energy from B3LYP/lanl2dz method calculation (A) CPZ (B) Ery (C) CPZ-Ery .

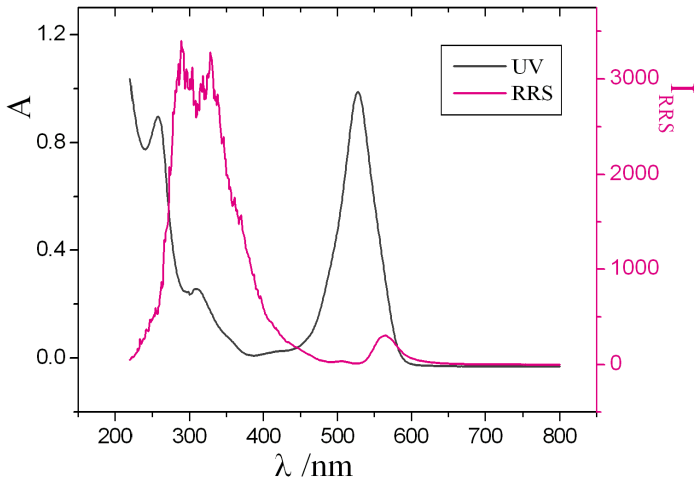

Fig.8. RRS spectra and UV absorption spectra of the Ery-CPZ.

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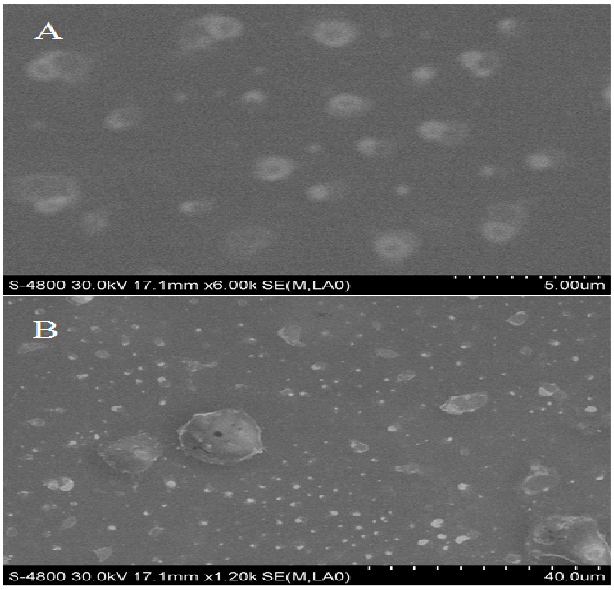

Fig.9.SEM images of Ery (A) and CPZ-Ery(B).

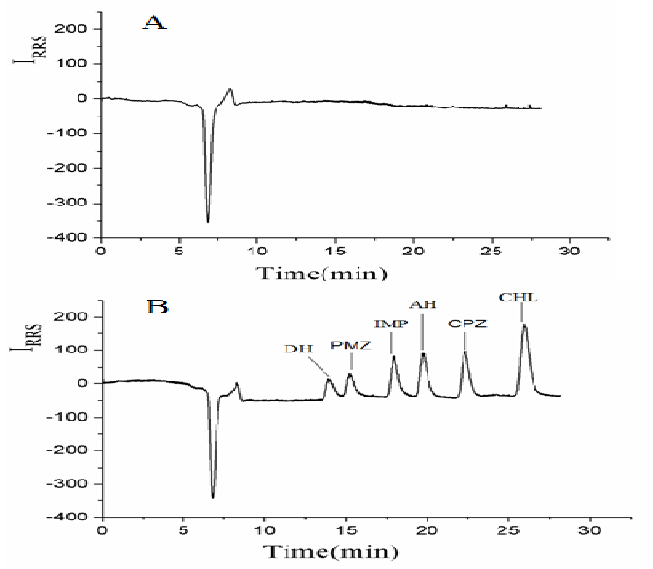

Fig.10.Chromatograms of the blank urine sample(A) and the urine sample spiked with 3μg mL^{-1} mixed standard solution(B).

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Table 1 Calibration linear data for samples by using HPLC-RRS

Samples	Linear regression Equation (c , μg mL^{-1})	Linear range (c,μg mL^{-1})	Correlation Coefficient (r)	LOD (c,ng mL^{-1})
DH	y=0.680x-0.578	0.10-20	0.9952	21.05
PMZ	y=0.656x-0.258	0.09-20	0.9962	11.23
IMP	y=1.619x-0.258	0.06-20	0.9963	1.68
AH	y=2.715x-1.525	0.06-20	0.9976	1.02
CPZ	y=4.233x-4.568	0.05-20	0.9916	0.76
CHL	y=7.493x-4.277	0.05-20	0.9919	0.42

Table 2 Recoveries of samples at different spiked levels in human urine

Samples	Spiked					
	3μg mL^{-1}		10μg mL^{-1}		20μg mL^{-1}	
	Recovery (%)	RSD(%)	Recovery (%)	RSD(%)	Recovery (%)	RSD(%)
DH	98.6	4.3	97.7	3.5	100.2	4.6
PMZ	96.7	2.5	101.4	3.6	97.2	3.9
IMP	101.3	3.9	99.5	5.2	102.1	6.2
AH	97.2	2.7	93.6	4.7	95.2	3.5
CPZ	98.9	2.9	97.1	4.1	97.6	3.9
CHL	95.6	6.4	94.3	5.8	101.5	5.2

Mean value (n=6)

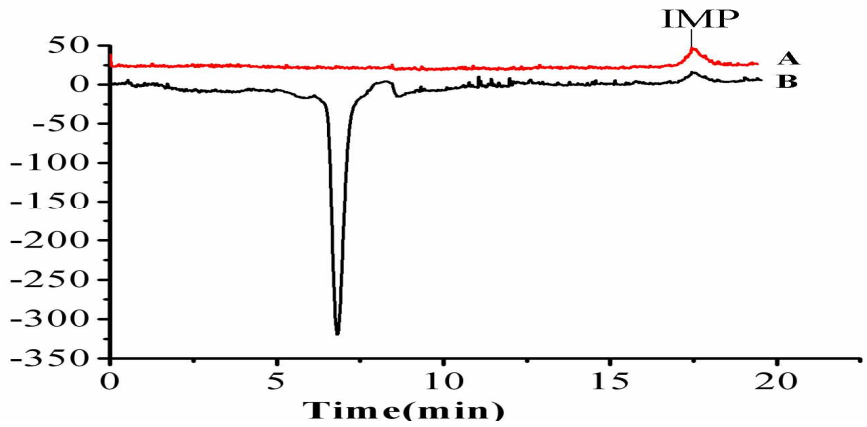

Fig.11. Chromatograms of the standard IMP solution at level of 200 ng mL^{-1} (A,red) and the real urine sample (B,black). The real urine sample was taken 6h after the patient took 25mg IMP tablets.

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