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Journal of Pharmaceutical and Biomedical Analysis
35 (2004) 185–191
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Short communication

A selective and sensitive assay of berberine using total internal reflected resonance light scattering technique with fluorescein at the water/1,2-dichloroethane interface☆

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Received 4 August 2003; received in revised form 17 December 2003; accepted 19 December 2003

Abstract

An assay of berberine (BE) was developed with good selectivity and sensitivity based on the total internal reflected resonance light scattering (TIR-RLS) signals from water/1,2-dichloroethane (H_2O/DCE) interface. Under optimal conditions, amphiphilic complex formed by BE and fluorescein (Flu) was adsorbed to H_2O/DCE interface, resulting in good separation of BE from the coexisting foreign substances in aqueous phase and significant enrichment of BE at the interface. This enriched species at the interface was found corresponding to enhanced TIR-RLS signals located at 370.0 nm. Proportional relationships were established between the enhanced TIR-RLS intensity and the BE in the range of 3.2×10^{-9} to 3.2×10^{-6} mol l^{-1} with the limit of detections (3σ) being 1.3 ng ml^{-1}. Favorable sensitivity of TIR-RLS technique was demonstrated superior to that of high-performance liquid-chromatography (HPLC) method. The feasibility of the proposed technique was validated by the satisfactory performance of intra-assay and inter-assay BE in tablets.

Keywords: Total internal reflected resonance light scattering (TIR-RLS); Berberine (BE); Fluorescein (Flu); Water/1,2-dichloroethane (H_2O/DCE) interface

1. Introduction

It has been well recognized that two immiscible liquid/liquid interface plays an essential role in biological systems, particularly in biomedical engineering, pharmacology, and food processing [1]. The biological cells are a practical example of liquid/liquid interfacial systems, in which, an organic wall separates two water phases and various molecular recognitions proceed across the membrane wall [2]. The understanding of the behavior of drugs at biological membranes is an important aspect in pharmacological research, and the water/oil interface can be used to mimic biological membrane since it provides a hydrophobic–hydrophilic medium.

Since resonance light scattering (RLS) technique was first applied for analytical purpose [3,4], it has been widely and successfully used in biomedical

☆ Supplementary data associated with this article can be found at doi: 10.1016/j.jpba.2003.12.013.

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doi:10.1016/j.jpba.2003.12.013

analysis [5–7]. However, with the development of further research, the limitation of RLS technique is clarified gradually. Prominently, RLS technique suffers from serious inference by the coexisting foreign substances in bulk solution, obstructing further improvement of selectivity and sensitivity in biomedical analysis. To overcome these drawbacks, we combine RLS technique with a water/oil interface based on the theory that the amphiphilic species will be repelled from both the water and oil phases, but could be well adsorbed at the interfacial region [8,9]. Thus, analyte can be separated from coexisting foreign substances effectively and enriched at the liquid/liquid interface by forming corresponding amphiphilic species with other reagent. Water/1,2-dichloroethane (H_2O/DCE) interface was selected as the preferred water/oil interface in this experiment since this interface has been commonly applied to total internal reflection spectroscopy [10,11] and to the transfer of small ions and electrons across the liquid/liquid interface [12,13]. In this contribution, the characteristics of berberine (BE) at H_2O/DCE interface were investigated fully by total internal reflected resonance light scattering (TIR-RLS) technique with fluorescein (Flu). The molecular structures of BE and Flu can be obtained in [14,15], respectively (see supplementary material).

BE is the main active component contained in coptis rhizome and phellodendron bark, and it has been proved to be an important anti-inflammatory drug for heart and intestinal disorders [15,16]. More recently, it has been reported to possess anti-tumor promotion and anti-lipase property [17]. In view of its widespread and important use, its quantification is important in pharmacological and clinical analysis [18,19]. In recent years, new techniques have been developed to determine the BE such as HPLC [20–22], self-ordered ring technique [23], voltammetric methods [24], potentionmetric sensor [25], and spectrophotometry [26,27]. However, these methods were limited in either complicated operation or sophisticated instrumentation, or poor selectivity. Therefore, it is necessary to develop a simple and high selective method to analyze BE. Due to the formation of amphiphilic complex through electrical attraction between BE and Flu, the new-formed complex was adsorbed to the water/DCE interfacial region, resulting in strongly enhanced TIR-RLS signals at the water/DCE interface, and the enhanced TIR-RLS intensity from the water/DCE interface was well proportional to the concentration of BE. Therefore, a selective and sensitive assay of BE was developed innovatively based on the introduction of water/DCE interface.

2. Experimental

2.1. Apparatus

TIR-RLS spectra and intensities were measured with a Hitachi F-2500 spectro-fluorometer (Tokyo, Japan). The optical arrangement for the TIR-RLS spectra has been illustrated in our former reports (see supplementary material) [28,29]. The under inside of an optical quartz cuvette (1.0 cm × 1.0 cm) was treated with dichlorodimethylsilane in toluene so as to make the lower inside wall hydrophobic and afford a flat H_2O/DCE interface. Two right-angled prisms (10 mm × 10 mm × 10 mm, Huaguang Optics Co., Chongqing, China) were attached to the cuvette walls facing the excitation light source and the signal detector, respectively. According to the Snell Law, the incidence angle is 72.6°, which is sufficiently greater than the critical angle of 67° for the total internal reflection at H_2O/DCE interface. So the excitation light beam, passing through the quartz prism and organic phase, irradiate the H_2O/DCE interface and undergoes total internal reflection.

HPLC detection was carried out by a Shimadzu LC-6A HPLC system (Kyoto, Japan) composed of LC-6A HPLC pump, SCL-6A HPLC system controller, SPD-6AV UV-Vis spectrophotometric detector (all from Shimadzu, Kyoto, Japan) and Elite YWG-C_{18} (Dalian, China) column (30 cm × 5 mm inner diameter, particle size 5 μm). N-2000 chromatographic workstation (Zhejiang University, China) was used for chromatogram recording and data processing.

An S-10A digital pH meter (Xiaoshan Scientific Instruments Company, Zhejiang, China) was used to measure the pH values of the aqueous solutions.

2.2. Reagent

A 1.00×10^{-3} mol l^{-1} stock solution of BE (E. Merck, Darmstadt, Germany) was prepared by directly dissolving commercially purchased product in doubly distilled water. The working solution

was 1.00×10^{-5} mol l^{-1}. A working Flu solution (1.00×10^{-5} mol l^{-1}) was prepared by dissolving of Flu sodium reagent (Shanghai Chemical Reagent Plant, China) in 500 ml water. Standard samples of palmatine, phellodendrine and magneflorine, which coexist in as mentioned above with BE, were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). 1,2-Dichloroethane was directly used as organic phase. Britton–Robinson buffer solution was employed to control the acidity of the interacting system, and 1.0 mol l^{-1} sodium chloride was used to adjust the ionic strength of the aqueous solution.

All of other reagents are analytical-reagent grade, and used without further purification. Doubly distilled water was used, throughout the experiment.

2.3. Chromatographic conditions

HPLC detection was used to referenced method of our assay. HPLC operation was mainly followed as reference [30]. The mobile phrase was the mixture of methyl-cyanide and water (55:35, v/v) with the addition of 3.4 g potassium dihydrogenphosphate and 1.7 g sodium dodecyl sulfate into 1 l mobile phase. Before delivered into system, the mobile phrase was filtered through 0.45 μm PTFE filter and degassed by vacuum. The chromatogram was measure at 345 nm under condition of a flow rate of 1.2 ml min^{-1}at 30 °C. A 20 μl each sample was injected into system and measured under the above conditions.

2.4. Pretreatment of samples

To demonstrate the present assay feasible, commercial tablets of BE (Kangfulai Pharmaceutical Ltd., Chengdu; and Taiji Pharmaceutical Ltd., Chongqing, China), were directly detected. Pretreatment was made according to reference [31]. Briefly, sugar coat was peeled off carefully at first, and then the residue of the tablets was ground into powder. The appropriate fine powder was directly dissolved in boiling water. After being cooled, it was transferred into a calibrated flask to prepare a 250 ml sample solution.

2.5. General procedure

Into a 10.0 ml volumetric flask were added a typically experimental BE working solution or its sample solution, 1.0 ml Britton–Robinson buffer solution, and 1.0 ml Flu solution of 1.0×10^{-5} mol l^{-1}, successively. The mixture was blended after each addition of the additives. Then the mixture was diluted to the 10 ml scale mark and blended thoroughly. A 1.0 ml 1,2-dichloroethane and 2.0 ml the mixture of BE and Flu were transferred into a dry optical quartz cuvette, respectively, stirred thoroughly, and then allowed to stand for 15 min before the TIR-RLS signal was measured. All the samples were measured against the background of the parallel blanks treated in the same way without the addition of BE.

3. Results and discussion

3.1. Feature of TIR-RLS spectra

As Fig. 1 shows, the TIR-RLS intensity at the interface of H_2O/DCE was very faint in the whole scanning wavelength region without any addition of BE, and it was difficult to survey the variation of TIR-RLS intensity with changing Flu concentration. When a trace amount of BE was added into the aqueous phase, however, a significantly increased TIR-RLS signal in the range of 300–600 nm can be observed with a maximum peak at 370.0 nm, all which indicate that a new chemical species had been formed in the vicinity of the H_2O/DCE interface. It was found that the

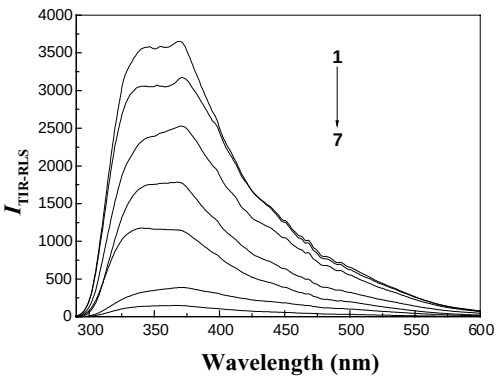

Fig. 1. The TIR-RLS spectra of the complex of BE and Flu from H_2O/DCE interface. Concentrations: Flu, 1.00×10^{-6} mol l^{-1}; BE, 2.00, 1.60, 1.20, 0.80, 0.40, 0.10 and 0×10^{-6} mol l^{-1} from curves 1 to 7, respectively. pH in aqueous media, 8.0; ionic strength of aqueous media, 0.003 mol l^{-1}.

enhanced TIR-RLS intensities were in good proportion to the concentration of BE, indicating that the enhanced TIR-RLS intensities are caused by the addition of BE and also denotes that the determination of BE by TIR-RLS technique is possible in practical application.

3.2. Optimization of the general procedures

It was found that the TIR-RLS intensity varied with the pH of the aqueous phase. In this experiment, the strongest and stable TIR-RLS signal can be found in pH range of 6.1–9.9, and the intensity decreases considerably at other pH values out of this range. This may be attributed to different states of Flu and BE in various pH values. Flu in solution exists in the cation, the zwitterion, the neutral stable lactone, the neutral *p*-quinonoid species, monoanion and dianion ionic species at different pH values [15,32]. When pH of solution is in the range pH 7.5–9.5, Flu exists as dianion ionic species. In acidic medium, pH-sensitive Flu (pK_a 6.31) undergoes protonation at pH 6.1, exists mainly as the cation or the neutral molecule [33] and has no potentiality to interact with BE. The increase of pH from 9.5 will promote Flu molecules to the gradual formation of dimer, obstructing the interaction of Flu and BE [34]. Additionally, since the pK_a of the BE hydroxide is 11.0 [35], the increasing hydroxide concentration would prevent BE hydroxide from the dissociation BE^+ when pH is above 10.0 and obstruct the formation of the BE–Flu complex. Therefore, to obtain stable signals, the pH value of aqueous medium was kept at 8.0 using Britton–Robinson buffer.

It was also found that TIR-RLS intensity decreased sharply with increasing ionic strength from 0.003 to 0.103 mol l^{-1}. The intensity decrease has a strong linear dependence on the salt concentration, indicating that electrostatic interaction plays a dominant role in the binding between BE and Flu. With the ionic strength increasing, the charges of BE and Flu were neutralized gradually by the increasing charge of ionic atmosphere. Thus, it is necessary to control the ionic strength of the medium. No extra NaCl solution was added throughout the experiment.

It was found that the emulsification at interface affected the TIR-RLS signals seriously and even made the intensity unstable. However, if the mixture was allowed to stand motionlessly for 15 min after the two phases in an optical cuvette had been blended thoroughly, a flat interface could be constructed without any emulsified mixture and stable TIR-RLS signals could be obtained.

3.3. Molar ratio of BE and Flu at H_2O/DCE interface

Fig. 2 displays the important role that Flu plays in the system. It can be seen that the enhanced TIR-RLS signals strongly depend on Flu concentration. If Flu concentration was too small in the system, the TIR-RLS intensity would be faint accordingly. The enhanced TIR-RLS signals got stronger gradually when Flu concentration increased, indicating that the concentration of the complex formed by Flu and BE increased with the further addition of Flu. When the concentration of Flu was near that of BE, the enhanced TIR-RLS intensity reached its maximum. Then TIR-RLS intensities remained constant in a concentration range and began to decrease slightly with a further increase of the BE concentration. Therefore, it can be inferred that the molar ratio of Flu to BE in the new-formed complex is 1:1. The molar ratio (R) could also be identified by keeping the total concentration of BE and Flu at 2.0×10^{-6} mol l^{-1}, while the concentrations of two components of the BE–Flu complexes were changed simultaneously. As Fig. 3 shows, when the fraction of BE in complex was about 0.5, the TIR-RLS intensity reached to the maximum.

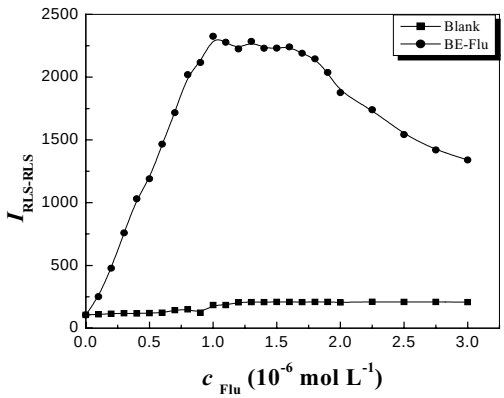

Fig. 2. Effect of Flu concentration on the TIR-RLS intensity. Concentration of BE was kept at 1.0×10^{-6} mol l^{-1}. pH in aqueous media, 8.0; ionic strength of aqueous media, 0.003 mol l^{-1}.

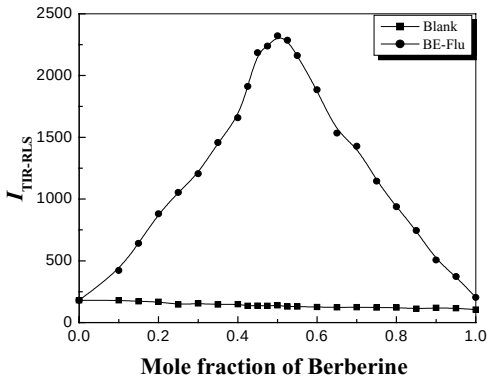

Fig. 3. Binding molar ratio in the BE–Flu complexes. The total concentration of BE and Flu was kept as 2.00×10^{-6} mol l^{-1}. pH in aqueous media, 8.0; ionic strength of aqueous media, 0.003 mol l^{-1}.

The result indicated the molar ratio of BE to Flu was near 1:1. The mole ratio was identical to the result from the Fig. 2. Furthermore, this 1:1 molar ratio could demonstrate that the mechanism of this reaction is the neutralization of equivalent anion and cation. In other parts of this experiment, Flu concentration was kept as 1.00×10^{-6} mol l^{-1}.

3.4. Interferences of coexisting foreign substances

The influence of the analogs of BE, various ions, proteins, sugar, surfactants, amino acids and nucleotides was tested under optimal conditions according to the standard procedure. It was found that common metal ions such as Mg^{2+}, Ca^{2+}, Cd^{2+}, Ba^{2+}, Co^{2+}, Pb^{2+}, could be tolerated at a concentration level above 10^{-3} mol l^{-1}, about 100-fold higher than the tolerant levels reported by other authors [22]. Substances including K^+, Na^+, NH_4^+ and urea seem to have no effect on the TIR-RLS method, even if they coexist at the high concentration about 1.0×10^{-2} mol l^{-1}. Organic compounds, including glucose, lactose, sucrose, maltose, β-cyclodextrin (β-CD), human serum albumin (HAS), bovine serum albumin (BSA), L-lysine, L-phenylalanine, L-glycine, L-tryptophan, L-histidine and starch can also be tolerated at high concentration levels without interference to TIR-RLS signals. The analogs of BE, such as palmatine, phellodendrine and magnoflorine can be tolerated at the concentration of 10^{-7} mol l^{-1} or less. However, some surfactants such as CTAB, Zeph, SDBS, SDS, SLS, and Triton X-100 can be tolerated at a lower concentration. The reason may be that surfactants were agitated and enriched at the H_2O/DCE interface, due to their amphiphilic property, consequently, competing for the assembling site at the interface with the complex. However, their content in tablets is very low. Therefore, this method has good selectivity and can be applied to the direct determination of trace amounts of BE in biological materials without prior separation of interfering species. Thus, TIR-RLS technique is much superior to RLS technique in term of tolerance of foreign substances, since the analyte could be separated from the foreign materials [28].

3.5. Calibration curves and synthetic sample determinations

Under optimal conditions, the linear regression equation is $I = -144.8 + 1755c$ (c, $\times 10^6$ mol l^{-1}; r, 0.995; LOD, 1.3 ng ml^{-1}), in the range from 3.2×10^{-9} to 3.2×10^{-6} mol l^{-1} at Flu concentration of 1.0×10^{-6} mol l^{-1}. When the analysis was carried out by HPLC method, linear regression equation is $I = -33094 + 58432c$ (c, $\times 10^5$ mol l^{-1}; r, 0.998; LOD, 200 ng ml^{-1}), in the range of 2.0×10^{-6} mol l^{-1} to 1.2×10^{-4} mol l^{-1}. In comparison of HPLC method, TIR-RLS technique is great superior in sensitivity. To testify the practical feasibility of this assay, synthetic samples were analyzed and the determination results were listed in Table 1. All of the results indicate that the assay method of BE is feasible in practical application.

3.6. Intra-assay and inter-assay accuracy and precision

The intra-assay accuracy and precision were determined from the analyses of six replicates from two different berberine tablets, which were mentioned above, within a single analytical batch. The accuracies and precisions (R.S.D., %) of the two targets were all satisfactory. The inter-assay accuracy and precision were determined from six separate analytical runs, using the same tablets as in the intra-assay studies. The inter-assay and the precision were also satisfactory. All results were listed in Table 2 with those results from HPLC method.

Table 1
Results for the determination of BE in synthetic samples

BE added (10^{-6} mol l^{-1})	BE found (10^{-6} mol l^{-1}, $n = 5$)	Main additives[a] in samples	R.S.D.[b] (%)	Recovery (%)
0.5	0.55	K(I), Mg(II), Ba(II), BSA, glucose, palmatine	2.4	110.0
1.0	0.96	NH_4(I), Ca(II), Co(II), HAS, sucrose, magnoflorine	3.1	96.0
1.5	1.54	Na(I), Cd(II), urea, β-CD, maltose, phellodendrine	4.5	102.7

[a] Concentrations of additives: K(I), 1.0×10^{-3} mol l^{-1}; NH_4(I), 1.0×10^{-3} mol l^{-1}; Na(I), 1.0×10^{-3} mol l^{-1}; Mg(II), 5.0×10^{-4} mol l^{-1}; Ba(II), 5.0×10^{-5} mol l^{-1}; Ca(II), 5.0×10^{-4} mol l^{-1}; Co(II), 5.0×10^{-5} mol l^{-1}; Cd(II), 5.0×10^{-5} mol l^{-1}; BSA, 0.05 μg ml; HSA, 0.05 μg ml; sugar, 5.0×10^{-5} mol l^{-1}; β-CD, 1.0×10^{-5} mol l^{-1}; urea, 1.0×10^{-3} mol l^{-1}; palmatine, 1.0×10^{-7} mol l^{-1}; phellodendrine, 1.0×10^{-7} mol l^{-1}; magnoflorine, 1.0×10^{-7} mol l^{-1}. Concentration of Flu, 1.00×10^{-6} mol l^{-1}; pH 8.0; ionic strength, 0.003 mol l^{-1}.
[b] Relative standard deviation.

4. Conclusions

Herein, we succeed in using TIR-RLS technique to determine a trace amount of BE at H_2O/DCE interface. The satisfactory results reveal that the TIR-RLS technique is a powerful tool of biomedical analysis with the characters of good selectivity and high sensitivity. Furthermore, water/oil interface provides a mimic of biological membrane to pharmacological and biomedical study. In addition, many medicines have amphiphilic property, and it is favorable to learn their function by TIR-RLS technique. As we know, clinic medicines usually contain some foreign substances such as glucose, starch, NaCl, etc., which lead to serious interference. TIR-RLS technique can be employed satisfactorily without severe interference. Therefore, the TIR-RLS technique is a promising candidate in pharmacological and biomedical analysis of the samples in which the contents of coexisting substances are very high and complicated. TIR-RLS technique also provides a significant insight into biological macromolecules such as proteins, nucleic acids interacting with drug at liquid/liquid interface, which not only helps to learn protein's function and human genetic information but bridges the gap between drug analysis and functional protein.

Table 2
Comparison of inter-assay and intra-assay accuracies and precisions of TIR-RLS technique and HPLC method to determine BE in tablets

Sample	Method	Intra-assay		Inter-assay	
		Recovery (%)	R.S.D. (%)	Recovery (%)	R.S.D. (%)
1	TIR-RLS	95.6	1.8	94.8	4.5
	HPLC	97.5	3.1	95.2	3.4
2	TIR-RLS	110.3	2.4	112.3	3.8
	HPLC	106.4	2.8	108.4	3.6

Reference value of tablet samples was 30 mg of BE per piece supplied by the suppliers. Sample 1 was purchased from Kangfulai Pharmaceutical Ltd. (Chengdu, China), and sample 2 from Taiji Pharmaceutical Ltd. (Chongqing, China). Concentration of Flu, 1.00×10^{-6} mol l^{-1}. pH in aqueous phase, 8.0; ionic strength of aqueous media, 0.003 mol l^{-1}. HPLC chromatographic conditions as mentioned in this paper.

Acknowledgements

This research received that supports from the National Natural Science Foundation of China (NSFC, No. 20275032), the Teaching and Research Award Program for outstanding Young Teachers in Higher Education Institutions of MOE, PRC, and the Municipal Science and Technology Committee of Chongqing, PRC. All authors here express their deep thanks.

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