flow of the incubation medium, continuous observation could be carried out on such cells until the contractile responses were completed. With such procedures, the contraction of isolated cells could be observed continuously and the degrees of contraction were determined photographically with use of phase contrast microscope (Fig. 3). Although cells adhered to the slide glass and remained on it against flow of the perfusate, contraction was not disturbed when silicon-coated slide glass was used. On the other hand, when uncoated slide glass was used, cells adhered so tightly that contraction was not observed even if the experiment was carried out under the best condition for the contraction.

The calcium contraction of the cells was not accelerated by ATP and almost of the cells contracted little or slightly by calcium in normal Tyrode solution. These results suggested that, in contrast to the case of glycerinated muscle cells and skinned fibers, membrane of the isolated cells in this experiment was not damaged.

According to this simple procedure, many isolated smooth muscle cells could be continuously observed before and through contraction with the same preparation. This technique could be applicable to studies on contraction mechanism of smooth muscle.

[Chem. Pharm. Bull.]
[25(9)2451-2455(1977)]

UDC 547.978.1.08 : 543.422.5.061

Extraction-Spectrophotometric Determination of Berberine in Pharmaceutical Preparations with 2,6-Dichloro- phenolindophenol¹⁾

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(Received January 28, 1977)

A new application of monoacidic dye is reported for the determination of berberine. The method is based on solvent extraction into nitrobenzene of an ion-pair compound formed between 2,6-dichlorophenolindophenol and berberine. Berberine is determined by measuring absorbance of the extracts over the range of $(1-5) \times 10^{-6}$ M (0.408–2.04 $\mu\text{g/ml}$) at 650 nm. The molar absorptivity was 3.8×10^4 l, mol⁻¹, cm⁻¹. Continuous variations plots have a maximum at 0.5 mol fraction of DCIP, indicating a 1:1 DCIP-berberine ratio.

Keywords—spectrophotometry; solvent extraction; berberine; 2,6-dichlorophenolindophenol; pharmaceuticals

Introduction

Several reagents such as bromothymol blue³⁾ or bromophenol blue⁴⁾ have been suggested for the spectrophotometric determination of onium compounds. However, in the case of such diprotic acids, the acidity gave a complicated effect on the extraction because stepwise dissociation occurred in the aqueous phase. Therefore, a singly charged tetrabromophenolphthalein

1) A part of this work was presented at 25th Annual Meeting of The Japan Society for Analytical Chemistry, Niigata, October, 1976.

2) Location: a) Takano 1851, Hozumi-cho, Gifu; b) Koyama-cho 1-1, Tottori-shi.

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ethyl ester (TBPE) has been used for the determination of various amines, alkaloids and quaternary ammonium salts as shown in previous papers.^{5,6)} TBPE has some characteristics as follows; (1) the presence of the bulky ester group, (2) wide pH range on extraction and (3) hydrophobic (extractable). As shown in our previous studies,⁶⁾ therefore, TBPE is sensitively extractable as ion-pairs of quaternary ammonium salts or molecular complexes of some amines and alkaloids. In other words, TBPE has no selectivity because of the reaction with many onium compounds. However, 2,6-dichlorophenolindophenol (DCIP), which has one acidic group as well as TBPE, is able to form only a 1:1 complex with R_4N^+ .⁷⁾ And R_4N^+ -DCIP ion-pair complexes are reproducibly extracted at pH 8.5 into only nitrobenzene since DCIP is a smaller molecular dye in comparison with TBPE. Consequently, this DCIP method can supplement TBPE method on interferences of amines. The proposed method with DCIP is, consequently, more suitable for the extraction in high selectivity, no complication and only a little dependence on the pH, as compared with known methods.

Nonaqueous titrimetric,⁸⁾ gravimetric⁹⁾ and spectrophotometric¹⁰⁾ methods were investigated for the determination of berberine. Also DCIP has been used as a reagent for ascorbic acid.^{9,11)}

This paper deals with the determination of berberine as medicines.

Experimental

Apparatus—Hitachi Model 124 spectrophotometer with 10 mm cells, Iwaki Model KM shaker, Hitachi-Horiba pH meter and Kokusan Model H-100B centrifuge (rpm 2000) were used.

Reagents—2,6-Dichlorophenolindophenol (DCIP) Solution: Weighed amounts of 2,6-dichlorophenolindophenol sodium salt (M.W. 209.09) were dissolved in ethyl alcohol. The solution remains stable for at least a week.

Standard Berberine Solution: A proper quantity of berberine hydrochloride was dissolved in the distilled water and diluted to a concentration of 1×10^{-3} M. The working standard solutions were obtained by diluting this solution to the concentration required for the experiments.

Buffer Solution: The borate-phosphate buffer (pH 8.5) was prepared by mixing 0.3 M potassium dihydrogen phosphate with 0.1 M sodium borate, followed by regulation of pH with 1 N sodium hydroxide or 1 N sulfuric acid.

Procedure: Pipette 3 ml of standard berberine solution (5×10^{-5} M), 3 ml of DCIP solution (5×10^{-4} M) and 5 ml of borate-phosphate buffer solution (pH 8.5) into a 100 ml separatory funnel. Dilute the resultant solution to 50 ml with water and shake for 3 min with 10 ml of nitrobenzene. After separation of the two layers, centrifuge the organic layer to remove droplets of water. Measure the absorbance of the extract at 650 nm, using a reagent blank or nitrobenzene as a reference.

Results and Discussion

Absorption Spectra

In the absence of berberine a reddish compound is extracted into nitrobenzene, whilst in the presence a blue ion-pair compound is formed. And the ion-pair compound has the maximum absorbance at 650 nm, apparent molar absorptivity being 3.8×10^4 l·mol⁻¹·cm⁻¹.

Effect of pH

The effect of pH on the extraction was studied by extracting berberine with DCIP from a series of aqueous solution buffered at various pH values. The absorbances of the extracts

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were constant when the pH of the aqueous phase lay within the range of 8.0—9.0. On the other hand, the optimum pH range of TBPE-berberine-1,2-dichloroethane system was found to be 5.5—11. Thus, monoacidic dyes gave a wide suitable pH range. The difference of pH range for these dyes may, however, be attributed to pK_a of each dye (pK_a for TBPE=4.2, DCIP=5.4). Accordingly, the pH range of DCIP is somewhat narrower than that of TBPE. In a more acidic or alkaline solution, the absorbance decreases because of the presence of extracts of DCIP molecule ($\lambda_{\max}$ =530 nm) or decomposition of DCIP.

Effect of Reagents

All other factors being kept constant, the effect of DCIP concentration was studied by adding DCIP solution of various concentrations. It was observed that the extraction of DCIP-berberine was complete and constant from 2 to $4 \times 10^{-5} \text{ M}$ DCIP but not complete at lower concentration of reagent. On the other hand, there was some increase in the absorbance when the concentration of the reagent was increased more than $5 \times 10^{-5} \text{ M}$. When the addition of the buffer solution was less than 2 ml, separation of two layers became to be incomplete.

Effect of Other Parameters

The period of equilibration was varied from 30 sec to 10 min. The extraction was quantitative more than 1 min. It is, therefore, recommended that the mixture is shaken for at least 3 min. The color intensity of nitrobenzene layers remained constant for 30 min. A slight fading was observed after 40 min. Normal room temperature fluctuations caused no measurable effect on the absorbance.

In order to clarify the composition of the extracted species, continuous variation plots were made at 650 nm. The results reveal that a 1:1 compound is formed between DCIP and berberine in the nitrobenzene layer. Similarly, TBPE, which is a monoprotic acid dye, formed a 1:1 compound as shown in our previous paper.⁶⁾

TABLE I. Determination of Best Solvent with DCIP or TBPE for Berberine

Conc. of berberine		$3 \times 10^{-6} \text{ M}$ DCIP				$1 \times 10^{-6} \text{ M}$ TBPE			
Solvent	Dye $\lambda_{\max}$	Ab	As	ΔA	$\lambda_{\max}$	Ab	As	ΔA	
Nitrobenzene	650	0.023	0.592	0.569	610	S.O.	S.O.	0	
Dichloroethane	645	0.012	0.415	0.403	610	0.025	0.475	0.450	
Chloroform	650	0.007	0.184	0.177	612	0.008	0.380	0.372	
MIBK	630	0.150	0.349	0.199	610	S.O.	S.O.	0	
Chlorobenzene	—	0	0	0	610	0.008	0.309	0.301	

Ab, Absorbance of reagent blank; As, Absorbance of sample; S.O., Scale over.

Calibration Curve

Table I lists the absorbances obtained with use of different organic solvents. Of these, nitrobenzene with higher dielectric constant was found to be most suitable for the extraction of berberine-DCIP because DCIP is a smaller molecular dye than TBPE.

The calibration curve obtained at pH 8.5 is linear over the range $(1-5) \times 10^{-6} \text{ M}$ (0.408—2.040 $\mu\text{g/ml}$) of berberine. In tenfold determination of $3 \times 10^{-6} \text{ M}$ (1.224 $\mu\text{g/ml}$) of berberine, the absorbance was 0.569 ± 0.012 , the relative standard deviation being 2%.

Interferences

To a solution containing $2 \times 10^{-6} \text{ M}$ berberine, various amounts of foreign compounds were added and the influences of them were examined by the recommended procedure. The results are summarized in Table II. Glucose, lactose, inorganic and organic ions, except sodium salicylate, do not interfere in the determination of berberine, while other organic

substances, except quaternary ammonium salts, gave a little influence because of their weak interaction with DCIP. Consequently, the proposed method is more selective than TBPE method in which a strong interference of a small amount of alkaloids, amines and quaternary ammonium salts is observed. Therefore, sensitive TBPE method will be able to be supplemented by this method concerning interferences of amines.

TABLE II. Effect of Diverse Substances

Dye Solvent Berberine taken Wavelength	DCIP (pH 8.5) N.B 2×10^{-6} M 650 nm		TBPE (pH 8.5) DCE 1×10^{-6} M 610 nm	
	M ratio	Rec.	M ratio	Rec.
Glucose	1:1000	101	1:1000	100
Lactose	1:1000	101	1:1000	98
Ammonium sulfate	1:1000	100	1:1000	101
Calcium chloride	1:1000	99	1:1000	100
Sodium carbonate	1:1000	103	1:1000	98
Sodium chloride	1:1000	98	1:1000	102
Sodium nitrate	1:1000	96	1:1000	101
Sodium acetate	1:100	100	1:1000	101
Sodium citrate	1:1000	102	1:1000	100
Sodium salicylate	1:1000	75	1:1000	99
0.4% starch	—	103	—	101
Thiamine	1:10	99	1:1	102
Triethanolamine	1:10	101	1:100	100
	1:100	96	1:500	111
Benzethonium	1:0.1	99	1:0.2	100
	1:0.2	110	1:1	169
Caffeine	1:100	101	1:100	101
Papaverine	1:1	101	1:1	103
Diphenhydramine	1:0.1	100	1:0.1	111
	1:1	100	1:1	119
Neostigmine	1:0.1	100	1:0.1	111
	1:1	128	1:1	193
Ephedrine	1:0.2	100	1:0.2	101
	1:1	96	1:1	107
Hydroxylamine	1:5	101	1:1	101
Quinine	1:0.1	101	1:0.1	102
	1:1	105	1:0.2	119
Acrinol	1:0.1	101	1:0.1	106
	1:0.5	139	1:0.5	123

M ratio, Mole ratio; Rec., Recovery (%).

TABLE III. Analysis of Commercial Sample

Sample number	DCIP method Found (mg/l)	TBPE method Found (mg/l)
1	24.79	25.46
2	27.39	27.27
3	29.05	28.75
4	49.92	49.87
5	49.31	49.31

Analysis of Practical Samples

Commercial samples were analyzed according to the TBPE method and the proposed method as follows: Dissolve a sample (tablet) in water. Centrifuge the solution and dilute the supernatant solution with water, and treat the solution according to the "procedure" described above. Table III shows the analytical values obtained by the calibration curve method. The proposed method was, consequently, essentially the same as TBPE method. In pharmaceutical preparations containing amines, such as thiamine or diphenhydramine, however, the proposed method has proved to be applicable to the determination of berberine instead of TBPE method.

Acknowledgement The authors would like to express appreciations to Professor Yuroku Yamamoto of Hiroshima University for his helpful discussion. The authors also wish to thank Dr. Masaya Tanaka of Tottori University for his advice.