[Chem. Pharm. Bull. 32(12)4946—4950(1984)]

Fluorophotometry of Barbaloin in Aloe

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(Received January 26, 1984)

We have developed a new method for the determination of barbaloin, the main laxative component in Aloe. Our method utilizes fluorophotometry based on Schoutelen's reaction which takes place between barbaloin and borax. Barbaloin was heated in 0.2 M borax solution at 60 °C for 5 min, then the reaction mixture was immediately diluted with an equal volume of water and the mixture was cooled. The yellow-green fluorescence intensity of the solution was measured at excitation and emission wavelengths of 383 and 510 nm. Barbaloin contents in Aloe pulv. and pulv. of aloe leaf were determined by using this method. The data were compared with those obtained by the methods of the European pharmacopeia (EP) and the German pharmacopeia (DAB 7). It was concluded that our method is superior to the EP and DAB 7 methods in sensitivity, accuracy and simplicity.

Keywords——Aloe; *Aloe arborescence* var. *natalensis*; Kidachi-aloe; barbaloin; fluorophotometry; Schoutelen's reaction; borax

Aloe species are well-known crude drugs having peptic and laxative activities. Two Aloe species are famous in Japan. One is Cape aloe which is accepted as a colonic laxative in the Japanese pharmacopeia.[1] The other is *Aloe arborescence* MILL. var. *natalensis* BERGER (Kidachi-aloe in Japanese), the leaves of which are used as a folk drug for many purposes[2] and as a component of some cosmetics.[3]

The main laxative component in these Aloe species was proved to be barbaloin, an anthraquinone glycoside, by us.[4] However, the pharmacodynamics of barbaloin have not been investigated. One reason for this is the absence of a suitable determination method for barbaloin in biological samples. The known methods for the determination of barbaloin are colorimetric methods, in alkaline solution after the oxidation of barbaloin with ferric chloride (European pharmacopeia; EP method)[5] or metaperiodate (German pharmacopeia; DAB 7),[6] thin layer chromatography (TLC),[7] high-performance liquid chromatography[8] and gas chromatography.[9] However, these methods are tedious and/or nonspecific. Further, they have not been applied to the determination of barbaloin in biological samples.

In this paper, we present a simple method for the quantitative determination of barbaloin in Cape aloe pulv. (Aloe pulv.) and pulv. of Kidachi-aloe leaf (pulv. of aloe leaf). Our method utilizes fluorophotometry based on Schoutelen's reaction which takes place between barbaloin and borax. Schoutelen's reaction has been employed for the identification test of Aloe in the Japanese pharmacopeia, and was used for the colorimetric determination of barbaloin in Aloe.[10] However, the yellow-green-colored fluorescence of barbaloin in the presence of borax has not been used for the quantitative analysis of barbaloin. Therefore, we made use of this reaction for the fluorophotometry of barbaloin in Aloe.

Experimental

Apparatus——Fluoroscence spectra and intensities were measured with a Hitachi 204 fluorescence spectropho-

tometer equipped with a Hitachi QPD-54 recorder. Absorbance was measured with a Hitachi 124 double-beam spectrophotometer.

Reagents——Aloe pulv. (Japanese pharmacopeia grade) and pulv. of aloe leaf were supplied by Aloe Pharm. Co., Ltd. (Shizuoka, Japan). Barbaloin was isolated from aloin (Merck) and purified by a conventional method. The barbaloin obtained was identical with an authentic sample in terms of infrared (IR), nuclear magnetic resonance (NMR), and ultraviolet (UV) spectra and TLC behavior.

Determination of Barbaloin in Aloe——A) Fluorophotometry: About 0.1 g of Aloe pulv. or pulv. of aloe leaf which had been kept in a silica gel desiccator was weighed out exactly. Fifty ml of water was added and the material was extracted for 5 min at 100 °C (10 min at 60 °C for pulv. of aloe leaf). After filtration of the extract, the filtrate was made up to 100 ml with water. A 0.25 ml aliquot of this extract (5 ml for aloe leaf) was evaporated to dryness *in vacuo*. The residue was dissolved in 50 ml of 0.2 M borax aqueous solution and heated at 60 °C for 5 min. The reaction solution was diluted with water and cooled, then made up to exactly 100 ml with water. The fluorescence intensity of the solution was measured at excitation and emission wavelengths of 383 and 510 nm. The concentration of the standard solution of fluorescein used was 1 μg/ml in 0.1 N NaOH solution.

B) Column Chromatography–Fluorophotometry: Five ml of the extract of Aloe pulv. obtained in A, or methanol extract of pulv. of aloe leaf (0.1 g) obtained by extraction with 25 ml methanol at 60 °C for 10 min, was evaporated to dryness *in vacuo*. The residue was dissolved in $CHCl_3$–methanol mixture (1 : 1, 10 ml). The solution (0.5 ml) was chromatographed on a Sephadex LH-20 column (1.1 × 55 cm) using $CHCl_3$–methanol mixture (1 : 1) as an eluent to obtain the barbaloin-containing fraction (36—56 ml). This fraction was evaporated to dryness *in vacuo*. After the addition of 0.2 M borax aqueous solution (50 ml) to the resulting residue, the barbaloin content was analyzed as described above in A. The mean recovery of added barbaloin (*ca.* 1 mg) on the LH-20 column was 99.4 ± 0.75% (n = 5, ±: S.D.).

C) EP Method: The method described in the European pharmacopeia was used. The amount of sample was 0.2 g for Aloe pulv. and 2 g for pulv. of aloe leaf.

D) DAB 7 Method: Yasuhara's method, which is a modification of the German pharmacopeia's method, was used.[8] However, in the case of aloe leaf, the supernatant of the reaction mixture obtained by centrifugation was employed. The amount of sample was 50 mg for Aloe pulv. and 200 mg for pulv. of aloe leaf.

Results and Discussion

A. Optimum Conditions for the Fluorophotometry of Barbaloin

Fluorospectrum——Figure 1 shows the fluorospectrum of the reaction product of barbaloin with borax in Schoutelen's reaction. The reaction product showed an excitation maximum at 383 nm and an emission maximum at 510 nm. On the other hand, barbaloin or

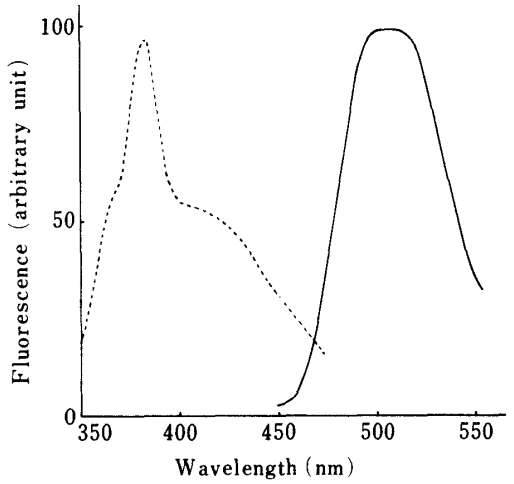

Fig. 1. Excitation and Emission Spectra of the Reaction Product of Barbaloin with Borax

Barbaloin (50 μg) was reacted with 0.2 M borax aqueous solution.
Maximum excitation wavelength = 383 nm.
Maximum emission wavelength = 510 nm.
------, excitation; ——, emission.

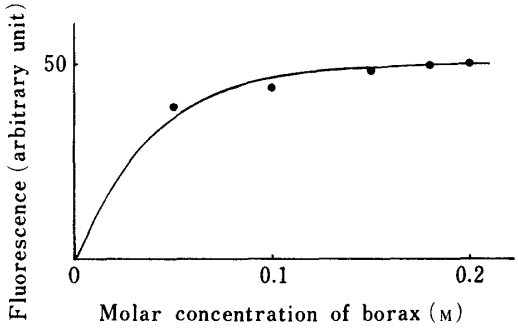

Fig. 2. Effect of Molar Concentration of Borax Aqueous Solution on the Fluorescence Intensity of the Reaction Product of Barbaloin with Borax

The amount of barbaloin used was 50 μg.

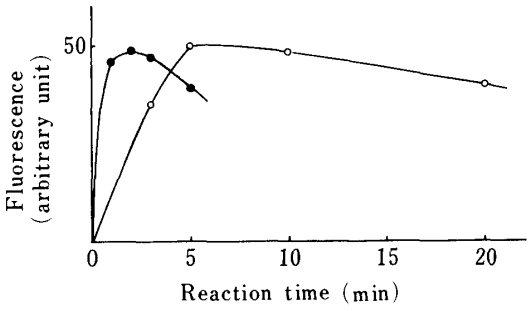

Fig. 3. Effect of Reaction Time at 60 and 100 °C on the Fluorescence Intensity of the Reaction Product of Barbaloin with Borax

The amount of barbaloin used was 50 μg.
●—●, at 100 °C; ○—○, at 60 °C.

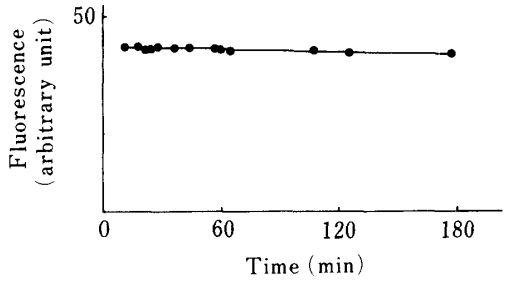

Fig. 4. Stability of the Fluorescence of the Reaction Product of Barbaloin with Borax after Dilution by Water

The amount of barbaloin used was 50 μg.
The reaction solution was diluted with an equal volume of water after the end of the reaction.

borax alone showed no fluorescence at these wavelengths of excitation and emission. Therefore, the excitation wavelength of 383 nm and the emission wavelength of 510 nm were selected for the determination of barbaloin.

Effect of Concentration of Borax Aqueous Solution——The effect of concentration of borax aqueous solution on the reaction was examined in the range of 0.05 to 0.2 M. Constant fluorescence intensity was obtained with over 0.18 M solution when 50 μg of barbaloin was used (Fig. 2). Thus, 0.2 M borax aqueous solution was used in our experiments.

Effect of Reaction Time——The effect of reaction time at 60 and 100 °C (the extraction temperatures for barbaloin in the two Aloe species) was studied. As shown in Fig. 3, the fluorescence intensity decreased rapidly within a reaction time of 5 min at 100 °C. However, a stable fluorescence intensity was obtained at over 5 min reaction time at 60 °C. On the other hand, the temperature of the reaction solution during measurement of the fluorescence was found to affect the intensity. As less effect was observed below 30 °C, we chilled the solution with tap water for 5 min soon after the dilution of the reaction solution. Dilution of the reaction mixture with water is suitable for both lowering the temperature and preventing borax precipitation when the room temperature is below 15 °C.

Stability of the Fluorescence Intensity——As the reaction solution was diluted with an equal volume of water, the stability of the fluorescence intensity of the diluted solution was examined. The result is shown in Fig. 4.

The fluorescence intensity was stable for over 60 min. Therefore, measurement of the fluorescence intensity of the diluted solution was to be completed within 60 min after the end of the reaction.

Calibration Curve——The calibration curve for the determination of barbaloin was obtained under the optimum conditions mentioned above. Thus, about 10 mg of barbaloin, accurately weighed, was dissolved in water and made up to exactly 100 ml. This solution was diluted with water 10—100 times. The diluted solutions corresponding to 10, 20, 30, 40 and 50 μg of barbaloin were each placed in a flask. Each solution was concentrated to dryness *in vacuo*. The residue was dissolved in 50 ml of 0.2 M borax solution and heated for 5 min at 60 °C. The solution was immediately cooled with tap water and diluted with water to exactly 100 ml. The fluorescence intensity of the diluted solution was measured. The limit of detection was 2 μg of barbaloin, which is much better than those in colorimetric methods such as the DAB 7 method (limit = 85 μg; absorbance = 0.02) and the EP method (limit = 50 μg; absorbance = 0.02). The calibration curve was linear up to barbaloin amounts exceeding 1 mg. A calibration curve in the range of 10—50 μg of barbaloin is shown in Fig. 5. The coefficient

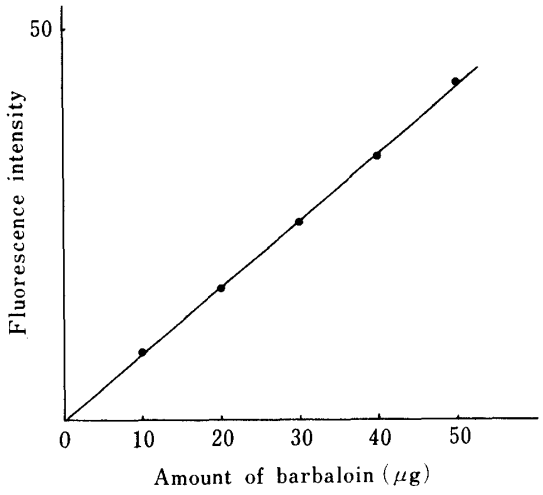

Fig. 5. Calibration Curve for Barbaloin
$r=0.9999$.

TABLE I. Comparison of Determination Methods for Barbaloin in Aloe

Method[a]	Barbaloin content (%)[b]	
	Aloe pulv.	Pulv. of aloe leaf
A	22.87±0.429	1.25±0.018
B	21.59±0.265	1.22±0.016
C	24.26±0.532	1.47±0.022
D	19.88±0.425	1.71±0.058

a) A, fluorophotometry; B, column chromatography–fluorophotometry; C, European pharmacopeia method; D, German pharmacopeia method.
b) Each value represents the mean±S.D. of 5 experiments.

of variation was 0.47% ($n=10$) for 40 μg of barbaloin [0.82% ($n=5$) for 5 μg of barbaloin].

B. Determination of Barbaloin in Aloe

Comparison of Four Methods——As the determination of barbaloin by using fluorophotometry based on Schoutelen's reaction was proved to be effective, the quantitative determination of barbaloin content in Aloe was attempted. Our method described in Experimental (method A) was compared with column chromatography–fluorophotometry as described in Experimental (method B), the EP method (method C) and the DAB 7 method (method D). The reason for including method B was because Aloe might contain some impurity giving a positive response in Schoutelen's reaction. The isolation of barbaloin in Aloe by Sephadex LH-20 column chromatography was done according to Yasuhara's method.[7]

The results are shown in Table I. The mean recovery of barbaloin (5—20 μg) added to 100 mg of Aloe pulv. was 99.3±2.46% ($n=5$, ±: S.D.). The results obtained by methods C and D differed greatly from those by methods A and B in spite of using the same sample. The result obtained by method A for Aloe pulv. was higher ($p<0.05$) than that by method B, which should be accurate in theory. The difference seems to be due to the presence of some fluorescent substance or substance which reacts with borax.[10] However, aloe-emodin, an anthraquinone, was found not to react with borax. Terefore, further work to identify the other substances in Aloe that is positive in Schoutelen's reaction is necessary. On the other hand, the result obtained by method A for pulv. of aloe leaf did not differ significantly from that by method B.

TABLE II. Effect of Filtration Methods on the Determination of Barbaloin in Aloe Pulv. by using Method D

Filtration method	Barbaloin content (%)
No filtration	18.65
Filter paper No. 2 (Toyo)	19.51
Filter paper No. 5c (Toyo)	20.60
Sephadex LH-20 column	21.43

The amount of Aloe pulv. used was 50 mg.

Method C has several disadvantages such as a longer experimental time (4 h for the oxidation of barbaloin with $FeCl_3$–HCl), and a positive error due to anthraquinone derivatives closely related to barbaloin. In fact, it detected components which showed a color change to red upon addition of an alkaline solution after the oxidation of fractions containing no barbaloin in column chromatography of Aloe (Aloe pulv. and pulv. of aloe leaf). Thus, the result obtained by method C for Aloe pulv. was the highest.

Method D is influenced by the filtration method used. That is, a smaller pore size gives a higher value as a rule. We investigated the effect of pore size in filtration on the determination of barbaloin in Aloe pulv. As shown in Table II, our result was consistent with that of Yasuhara *et al.*[8]

On the other hand, when we used method D for the determination of barbaloin in pulv. of aloe leaf, we found turbidity after the addition of sodium periodate and ammonium solution. Therefore, we centrifuged the reaction solution before measurement of the absorbancy. Nevertheless, quantitative determination of barbaloin by method D succeeded only at sample sizes below 300 mg of pulv. of aloe leaf. However, the determination value after column chromatography agreed with that by method B. It seems that pulv. of aloe leaf contains substances interfering with the determination of barbaloin.

From these results, it was concluded that fluorophotometry based on Schoutelen's reaction is superior to the EP and DAB 7 methods in sensitivity, accuracy and simplicity. This fluorophotometric seems to be the most suitable for the micro-determination of barbaloin, because the final volume of 100 ml used in this paper can be easily scaled down to below 10 ml. We are now planning to apply this method to biological samples from animals.

Acknowledgement We are indebted to Mrs. M. Suzuki, the president of Aloe Pharmaceutical Co., Ltd. for a generous supply of Aloe.

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