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Determination of hinokitiol in skin lotion by high-performance liquid chromatography–ultraviolet detection after precolumn derivatization with 4-fluoro-7-nitro-2,1,3-benzoxadiazole

YASUHIKO HIGASHI and YOUICHI FUJII, *Department of Analytical Chemistry, Faculty of Pharmaceutical Sciences, Hokuriku University, Kanazawa 920-1181, Japan.*

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Synopsis

Hinokitiol, a potent, broad-spectrum antibacterial agent, is a component of various personal care products. In this study, the concentration of hinokitiol in skin lotion was analyzed by means of high-performance liquid chromatography–ultraviolet detection (380 nm) after precolumn derivatization with 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F). A standard curve was obtained after derivatization of the authentic compound with NBD-F in borate buffer (pH 9.0) at 60°C for 10 min. The retention time of NBD-hinokitiol was 7.2 min. The calibration plot was linear in the range of 0.2 to 4 mg/ml with an r^2 value of 0.9985, and the lower limit of detection was 0.05 μg/ml (at a signal-to-noise ratio of 3, absolute amount of 0.33 ng/20 μl injection). The coefficient of variation was less than 9.4%. It was found that the amount of hinokitiol in the tested skin lotion was 194 ± 14 μg/ml (range: 180–212 μg/ml). Recovery in addition-recovery tests was within the range of 84.5% to 98.0%. This system is simple, sensitive, and convenient, and should be suitable for routine quality assessment of personal care products containing hinokitiol.

INTRODUCTION

Hinokitiol (β-thujaplicin, 4-isopropyl-2-hydroxycyclohepta-2,4,6-trien-1-one; Fig. 1) is a naturally occurring toxic compound belonging to the class of tropolones that contain an unsaturated seven-membered carbon ring. The compound is found in heartwood of several cupressaceous plants, such as western red cedar (*Thuja plicata*), eastern white cedar (*T. occidentalis*), hinoki cypress (*Chamaecyparis obtusa*), and hiba (*Thujopsis dolabrata*) (1,2). Since hinokitiol has potent antibacterial activity (minimum inhibitory concentration of 0.2 μg/ml for *Staphylococcus epidermidis* and *Daedalea dickinsii*) (2,3), it is often added to skin lotions, body soaps, and other personal care products.

Direct determination of hinokitiol, by means of gas chromatography (GC) or high-performance liquid chromatography (HPLC) is difficult because of the chelating activity

Address all correspondence to Yasuhiko Higashi at y-higashi@hokuriku-u.ac.jp.

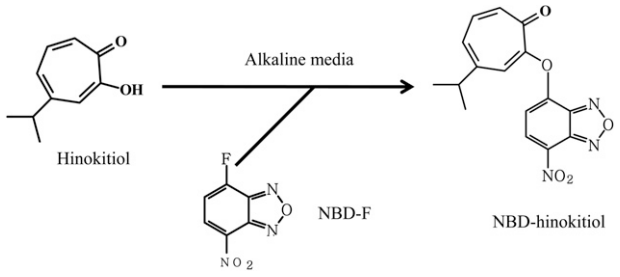

Figure 1. Scheme of hinokitiol derivatization with NBD-F.

of the tropolone ring, its instability to heat, and its adsorption on the stationary phase. Quantitative determinations of hinokitiol by GC and capillary GC have been performed after derivatization with trimethylsilyl chloride and with diazomethane, respectively (1,4). Hanafusa *et al.* (5) presented an HPLC determination of hinokitiol in cosmetics by HPLC with ultraviolet (UV) detection by adding copper (II) to the mobile phase to form the hinokitiol–copper (II) complex. However, the sensitivity of these methods was not discussed. Also, it is difficult and time-consuming to treat waste containing copper (II). Endo *et al.* (6) developed a sensitive HPLC determination of hinokitiol based on formation of the difluoroborane derivative, with the detection limit of 40 pg. Dyrskov *et al.* (7) determined hinokitiol by using a capillary zone electrophoresis–UV method, which provided the detection limit of 0.21 μM. Recently, we established an HPLC-dual UV (240 and 345 nm) method of hinokitiol determination in personal care products by using a reversed-phase C_4 column with detection limits of 0.005 μg/ml (absolute amount of 1 ng) and 0.01 μg/ml (absolute amount of 2 ng) at 240 and 345 nm, respectively (8). Nevertheless, a simple, sensitive, and convenient assay that would be suitable for routine quality control of personal care products containing hinokitiol is still needed.

As a fluorescent labeling agent of primary and secondary amino groups for HPLC-fluorescence detection (9–13), 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) has been used and it has also been used as a UV-labeling reagent reactive with the phenolic hydroxyl group of *N*-acetyltyrosine, chlorophenols, and eugenol (14–16), and the phenol-like hydroxyl group of kojic acid (17). Hinokitiol contains a reactive hydroxyl group (vinyl alcohol) derived from the tropolone structure. In this article, we present a simple HPLC–UV method for the determination of hinokitiol in skin lotion after precolumn derivatization with NBD-F. The derivatization scheme is illustrated in Fig. 1. Our data and validation results indicate that this method will be suitable for routine quality control purposes.

EXPERIMENTAL

APPARATUS

The HPLC system consisted of a model LC-10ATvp pump (Shimadzu, Kyoto, Japan), a Rheodyne injection valve (Cotati, CA) with a 20-μl loop, and a model SPD-10Avp UV detector (Shimadzu) operating at 380 nm. The HPLC column (C18-MS-II, Nacalai Tesque, Kyoto, Japan) was 150 × 3.0 mm i.d., containing 5 μm particles of C18 packing material. The quantification of peaks was performed using a Chromatopac Model C-R8A

integrator (Shimadzu). The mobile phase was prepared by the addition of acetonitrile (500 ml) to 500 ml of Milli-Q water containing trifluoroacetic acid (0.1 v/v%). The samples were eluted from the column at room temperature at a flow rate of 0.40 ml/min.

REAGENTS

Hinokitiol, NBD-F, and seven parabens (methyl 4-hydroxybenzoate, ethyl 4-hydroxybenzoate, propyl 4-hydroxybenzoate, isopropyl 4-hydroxybenzoate, butyl 4-hydroxybenzoate, isobutyl 4-hydroxybenzoate, and benzyl 4-hydroxybenzoate) were obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). General reagents were obtained from Wako Pure Chemical Industries (Osaka, Japan). Skin lotion was purchased from a market in Kanazawa city, Ishikawa Prefecture, Japan.

PROCEDURES

Preparation of standard solutions. A stock solution of hinokitiol (40 μg/ml) in 1% ethanol was prepared in a dark glass bottle and stored at 4°C. It was diluted appropriately with water to prepare several working solutions. The working concentration of hinokitiol was set at 0, 0.2, 0.4, 0.7, 1, 2, and 4 μg/ml.

Derivatization. Ultrapure water was from a Milli-Q water purification system (Simplicity® UV, Millipore Corporation, Bedford, MA). Borate buffer (0.1 M) was adjusted to pH 9.0 by the addition of NaOH. Borate buffer (50 μl) was added to each working standard solution (50 μl); then NBD-F solution in acetonitrile (2 mg/ml, 50 μl) was added. The mixture was vortexed and allowed to react for 10 min at 60°C; then an aliquot (20 μl) was injected into the HPLC system.

Sample preparation and addition-recovery tests. Tested skin lotion contains hinokitiol, paraben, allantoin, perfume, and so on. Concentration of the composition was not expressed. An aliquot of skin lotion (500 μl) was diluted to 100 ml with water, and analyzed after derivatization as described earlier. Addition-recovery tests were carried out to assess the accuracy of the method by spiking skin lotion (500 μl) with hinokitiol (50 or 100 μg) and diluting it in the same manner. An aliquot of 50 μl was analyzed, and the hinokitiol concentration in the sample was determined. Recovery was calculated as follows:

$$\text{Recovery (\%)} = \frac{\left(\text{Total amount after spiking}\right) - \left(\text{Spiked amount}\right)}{\left(\text{Original amount}\right)} \times 100$$

RESULTS AND DISCUSSION

DERIVATIZATION OF HINOKITIOL WITH NBD-F

For the time course study, the reaction time was set at 5, 7.5, 10, 15, 20, or 30 min at 60°C. Hinokitiol (50 μl, 2 μg/ml), borate buffer (50 μl, pH 9.0), and NBD-F (50 μl, 2 mg/ml) were mixed as described in the Experimental section. The derivatization of hinokitiol reached a plateau at 10 min (Fig. 2).

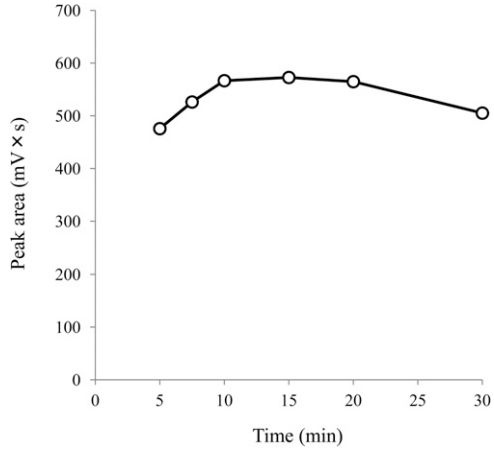

Figure 2. Time course of the formation of the NBD derivative of hinokitiol. The standard sample (2 μg/ml) was reacted with NBD-F in borate buffer, pH 9.0, at 60°C.

Next, the pH dependency (pH 7.5–10.0) was examined at the derivatization time of 10 min at 60°C. The peak area of NBD-hinokitiol was maximal at pH 9.0 (Fig. 3). Thus, the derivatization time of 10 min at pH 9.0 was selected.

CHROMATOGRAMS

Fig. 4 shows typical chromatograms obtained from blank (A) and standard sample (B, 1 μg/ml). The retention time of NBD-hinokitiol was 7.2 min. The running time was 15 min.

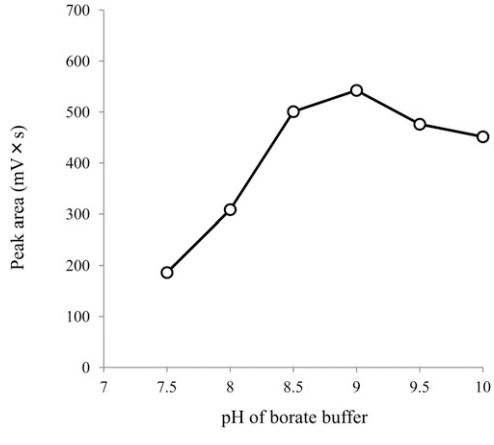

Figure 3. pH dependency of the formation of the NBD derivative of hinokitiol. The standard sample (2 μg/ml) was reacted with NBD-F for 10 min at 60°C in borate buffer at various pH values.

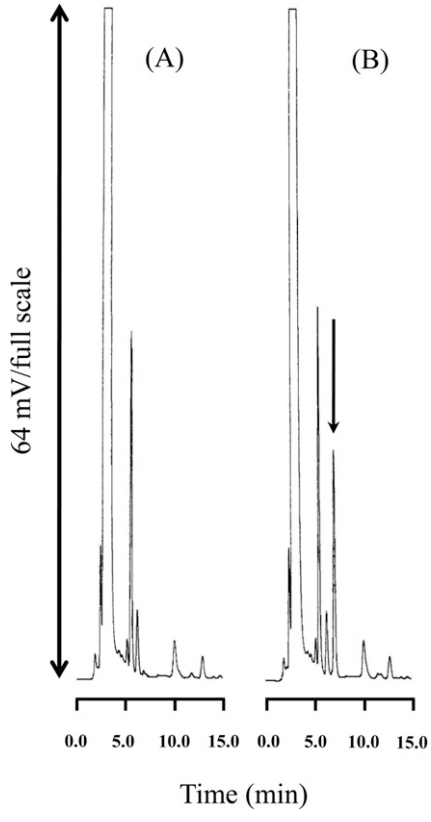

Figure 4. Typical chromatograms of blank (A) and the standard sample (B, 1 μg/ml) after derivatization with NBD-F. Samples were reacted with NBD-F for 10 min in borate buffer, pH 9.0, at 60°C. Retention time of NBD-hinokitiol: 7.2 min (arrowed peak).

METHOD VALIDATION

Linearity. A standard curve was constructed by plotting the integrated peak area vs the concentration of hinokitiol. The plot was linear ($y = 271.8x + 3.252$) in the range of 0.2 to 4 μg/ml, with an r^2 value of 0.9985.

Sensitivity. The values of the lower limits of quantification and detection were 0.17 (absolute amount of 1.1 ng/20 μl injection; signal-to-noise ratio of 10:1) and 0.05 μg/ml (absolute amount of 0.33 ng/20 μl injection; signal-to-noise ratio of 3:1), respectively. As shown in Table I, the sensitivity of the presented method is about 8-fold less than that of the method by Endo *et al.* (6) and can be classed as moderate, compared with other reported methods (6–8), although the sensitivity values of some GC methods were not described (1,4).

PRECISION AND ACCURACY

Precision and accuracy for intra- and interday assays of hinokitiol are shown in Table II. In the intraday assay, the range of standard deviation was within 4.6% to 7.1% of the

Table I
Sensitivity of Various Methods for Determination of Hinokitiol

Method	Limit of detection	Reference
Capillary GC	Not described	1
GC	Not described	4
HPLC	40 pg	6
Capillary zone electrophoresis	0.21 μM	7
HPLC	1 ng (at 240 nm), 2 ng (at 345 nm)	8
HPLC	0.33 ng; 0.05 μg/ml	This article

mean, and recoveries were within the range of 92.0% to 102.8%. In the interday assay, the range of standard deviation was within 5.4% to 9.4% of the mean, and recoveries were within the range of 90.5% to 101.0%.

INTERFERENCE

Skin lotion usually contains paraben, ester possessing phenol-like hydroxyl group, as an effective preservative. Therefore, selectivity was preliminarily investigated on the detection of NBD-hinokitiol peak. All retention times of methyl 4-hydroxybenzoate, ethyl 4-hydroxybenzoate, propyl 4-hydroxybenzoate, isopropyl 4-hydroxybenzoate, butyl 4-hydroxybenzoate, isobutyl 4-hydroxybenzoate, and benzyl 4-hydroxybenzoate derivatives (each paraben concentration; 1 μg/ml) by NBD-F were 6.9 min. Peaks of tested paraben derivatives were found to be slightly separated from NBD-hinokitiol peak (retention time; 7.2 min). One question arises about same retention time of paraben derivatives. Although it is considered that these parabens will be hydrolyzed to generate

Table II
Intra- and Interday Assay Reproducibility for Determination of Hinokitiol

Hinokitiol (μg/ml)	Measured (μg/ml, mean ± S.D., n = 5)	C.V. (%)	Recovery (%)
Intraday assay			
0.2	0.184 ± 0.013	7.1	92
1	0.942 ± 0.058	6.2	94.2
4	4.11 ± 0.19	4.6	102.8
Interday assay			
0.2	0.181 ± 0.017	9.4	90.5
1	0.962 ± 0.081	8.4	96.2
4	4.04 ± 0.22	5.4	101

C.V.: Coefficient of variation.

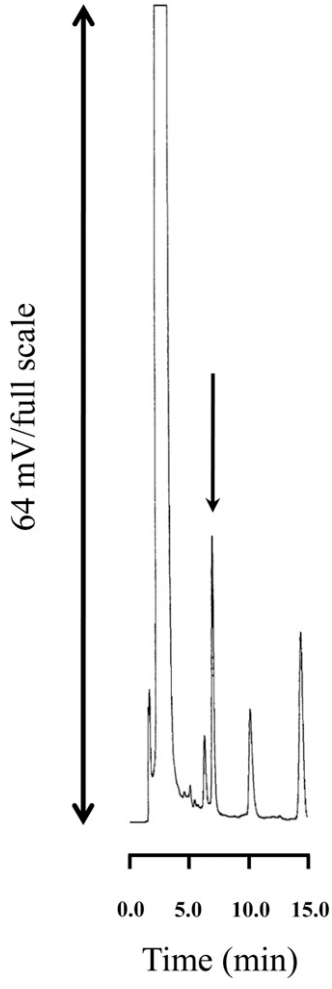

Figure 5. Typical chromatogram of a skin lotion sample (500 µl/100 ml, 200-fold diluted) after derivatization with NBD-F. The sample was reacted with NBD-F for 10 min in borate buffer, pH 9.0, at 60°C. Retention time of NBD-hinokitiol: 7.2 min (arrowed peak).

4-hydroxybenzoate under derivatization (60°C, pH 9.0, 10 min), further studies are needed to solute the point.

APPLICATION TO SKIN LOTION

Figure 5 shows a typical chromatogram obtained from a skin lotion sample. The peak of NBD-hinokitiol was detected at 7.2 min, and the concentration of hinokitiol in the cosmetic was determined as follows.

The described method was used to determine hinokitiol in the skin lotion and in samples spiked with standards. As shown in Table III, the concentration of hinokitiol in the skin

Table III
Level of Hinokitiol in a Skin Lotion and Recovery of Spiked Hinokitiol

		Recovery (%)	
Assay	Amount (μg/ml)	Added (50.0 μg)	Added (100 μg)
Day 1	204	88.8	93.2
Day 2	180	94.1	98
Day 3	212	91.3	91.9
Day 4	192	92.2	94.4
Day 5	183	84.5	89.2
Average ± S.D. (R.S.D.)	194 ± 14 (7.2%, n = 5)	91.8 ± 3.7 (4.0%, n = 10)	

lotion was found to be 194 ± 14 μg/ml (range: 180–212 μg/ml). Recovery of spiked hinokitiol from the lotion was 91.8 ± 3.7% (range: 84.5–98.0%).

CONCLUSION

We have developed a simple HPLC–UV method for the determination of hinokitiol after precolumn derivatization with NBD-F. Although the sensitivity of our method can be classed as only moderate compared with other reported methods, it is superior to that of our previous method. The newly developed HPLC method is simple, convenient, and sufficiently sensitive for routine assay of hinokitiol, for example in quality control for personal care products.

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