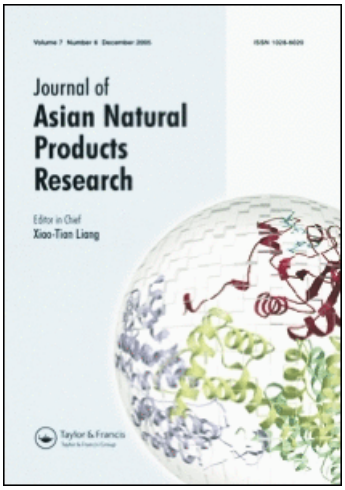

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Biotransformation of nodakenin and simultaneous quantification of nodakenin and its aglycone in incubated system of human intestinal bacteria by HPLC method

Peng Zhang[a]; Xiu-Wei Yang[a]
[a] State Key Laboratory of Natural and Biomimetic Drugs (Peking University), Department of Natural Medicines, School of Pharmaceutical Sciences, Peking University, Beijing, China

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Biotransformation of nodakenin and simultaneous quantification of nodakenin and its aglycone in incubated system of human intestinal bacteria by HPLC method

Peng Zhang and Xiu-Wei Yang*

State Key Laboratory of Natural and Biomimetic Drugs (Peking University), Department of Natural Medicines, School of Pharmaceutical Sciences, Peking University, Beijing 100191, China

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When nodakenin (**1**) was anaerobically incubated with human intestinal bacteria, nodakenetin (**2**) was found as a main biotransformed product. We developed a simple and selective reversed-phase high-performance liquid chromatographic method for simultaneous quantification of **1** and **2** in incubated system of human intestinal bacteria with **1**. Chromatographic separation of **1** and **2** was performed on an analytical C_{18} column, with a mobile phase of MeOH–H_2O (4:6, v/v) at a flow rate of 1.0 ml/min and the UV detection was at 330 nm. The calibration curves were linear over the range of 0.15–24.0 μg/ml for **1** and 0.7–13.2 μg/ml for **2**. The lower limits of detection and quantification were 0.01 and 0.1 μg/ml for **1**, and 0.005 and 0.05 μg/ml for **2**. The recoveries were (87.66 ± 1.66), (79.89 ± 2.53), and (82.96 ± 5.61)% at 1.0, 2.0, and 8.0 μg/ml, respectively, for **1** and (88.32 ± 4.12), (78.15 ± 4.39), and (76.22 ± 3.29)% at 1.0, 4.0, and 16.0 μg/ml, respectively, for **2**. The intra- and interday precision and accuracy were validated by relative standard deviation, which were in the ranges of 1.25–4.16 and 2.16–6.12% for **1**, and 1.98–6.45 and 2.56–4.57% for **2**, respectively. This method has been applied to the simultaneous quantitation of **1** and **2** in incubated system of human intestinal bacteria with **1**.

Keywords: nodakenin; nodakenetin; biotransformation; human intestinal bacteria; column liquid chromatography

1. Introduction

Studies on bacterial flora in alimentary canals of mammalia have proved that 99% of the bacteria are anaerobic. The classification and distribution of anaerobes in the digestive trace have also been brought to light, and the metabolizing abilities of intestinal bacteria and biochemical interactions between a host and its intestinal flora have become the object of research. On the other hand, the intestinal flora plays an important role in the metabolism of compounds administered orally [1]. Besides endogenous compounds (biliary components, etc.), xenobiotic compounds (drugs, food components, etc.) undergo metabolic transformation by intestinal microorganisms. Some of the transformations provide insights into the mechanism of the therapeutic benefits or adverse effects of drugs. Therefore, we have studied the transformation of the components from traditional Chinese medicines, which are taken orally in general, by human intestinal bacteria [2–6].

Qiang-huo is a well-known traditional Chinese medicine [7] and well used in China, Japan, and Korea as a sedative and anodyne. The crude drug produced in China is made of the roots and rhizomes of two species of

*Corresponding author. Email: xwyang@bjmu.edu.cn; xwyang@hsc.pku.edu.cn

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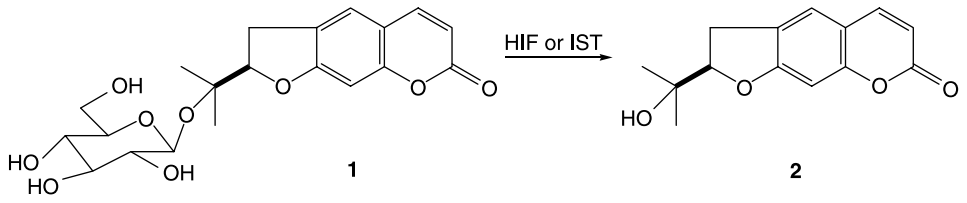

Figure 1. Structures of nodakenin (**1**) and nodakenetin (**2**), and mechanism of biotransformation of nodakenin by human intestinal flora (HIF) or its isolated strains (IST).

Notopterygium incisum Ting ex H.T. Chang and *Notopterygium forbesii* Boiss. It has been reported that nodakenin (**1**) (chemical structure shown in Figure 1) is one of the main coumarin constituents in the roots and rhizomes of *N. incisum* [8,9] and *N. forbesii* [8,10], and has anti-platelet aggregation [11] and anodyne [12] activities. It is interesting to note that **1** exhibits the ability to improve or ameliorate spatial long-term and working memory dysfunction after oral administration in mice, in part, by enhancing the cholinergic nervous system, which suggest that **1** may represent a starting point for the development of novel anti-amnesics [13]. Despite its varied biological activities, the biotransformation of **1** in human intestinal bacteria has not been reported. In addition, our preliminary studies suggest that the oral bioavailability of **1** is extremely low in rats, which is shown by its poor gastrointestinal absorption, and its active metabolites produced in the intestine by gut microflora may be involved in the pharmacological actions. The aim of the present study was focused on developing a sensitive, simple, and accurate method for the simultaneous determination of **1** and its biotransformed product, nodakenetin (**2**), in human intestinal bacteria or isolated strains.

2. Results and discussion

2.1 Separation and specificity

Because the universal C_{18} columns are widely used, have excellent selections, and are cheap, a Diamonsil™ ODS C_{18} analytical column was chosen as the stationary phase. Mobile phase compositions were also screened. When the stationary phase was fixed, the composition of the mobile phase was a critical factor for separating **1** and **2** from interferential substances in the biotransformation system and retention time. Initially, phosphate buffer was used as a component of mobile phase and mixed with methanol (MeOH) or acetonitrile in different proportions, but no significant improvement of peak shape and resolution could be achieved. Finally, a mixture solution of MeOH–H_2O (4:6, v/v) was selected as a mobile phase to obtain significant improvement of peak shape and resolution with interferential substances. Under the condition described in Section 4, the reversed-phase high-performance liquid chromatographic (HPLC) profiles of blank medium solution, heat-inactivated medium solution spiked with standard **1** and **2**, and the medium solution obtained 1 h after biotransformation are shown in Figure 2. A suitable retention time with no interferential substances in the medium of **1** and **2** were 17.12 and 34.37 min, respectively. In the more short retention time, **1** had not been separated with interferential substances.

2.2 Linearity and quantification

The calibration curves for the heat-inactivated general anaerobic medium (GAM) solution assay developed with peak area (y) versus the concentrations (x) of **1** and **2** were found to be linear over the concentration range of 0.15–24.0 μg/ml for **1** and 0.7–13.2 μg/ml for **2**. The mean linear regression equation of the calibration curves were $y = 2.4320 \times 10^{-7}x - 0.8666$ with $r = 0.9997$ ($n = 3$)

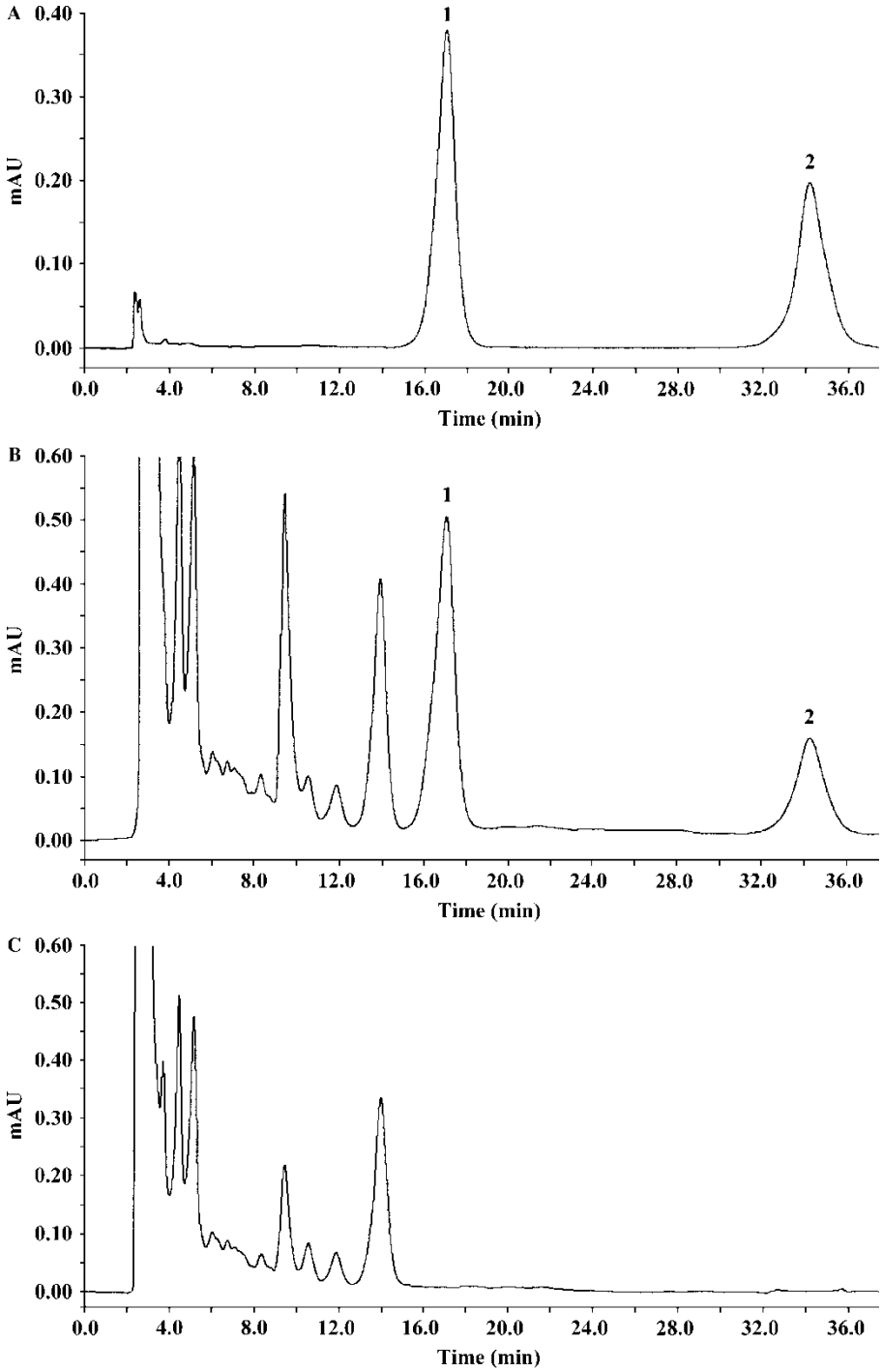

Figure 2. Typical chromatograms of nodakenin (**1**) and nodakenetin (**2**): (A) standard nodakenin (3 μg/ml) and nodakenetin (2.8 μg/ml); (B) the medium solution obtained 1 h after biotransformation of nodakenin by human intestinal flora; (C) blank the medium solution. The retention time of nodakenin and nodakenetin were 17.12 and 34.37 min, respectively.

Table 1. Precision and accuracy of nodakenin and nodakenetin determination in the heat-inactivated GAM solution ($n = 3$).

	Concentration (μg/ml)			
	Added	Found (mean ± SD)	Precision (RSD, %)	Accuracy (%)
Nodakenin	Intra-day			
	1	0.968 ± 0.042	1.25	96.8
	2	1.879 ± 0.127	2.65	93.9
	8	7.761 ± 0.671	4.16	97.0
	Inter-day			
	1	1.012 ± 0.040	2.16	101.2
	2	2.074 ± 0.113	4.38	103.7
	8	7.881 ± 0.579	6.12	98.5
Nodakenetin	Intra-day			
	1	0.963 ± 0.029	1.98	96.3
	4	4.056 ± 0.127	2.56	101.4
	16	15.608 ± 0.375	6.45	97.6
	Inter-day			
	1	0.981 ± 0.051	2.56	98.1
	4	3.872 ± 0.268	3.69	96.8
	16	15.533 ± 1.026	4.57	97.1

for **1**, and $y = 4.8295 \times 10^{-7}x - 0.1417$ with $r = 0.9994$ ($n = 3$) for **2**. The lower limits of detection (LLOD) of **1** and **2** were 0.01 and 0.005 μg/ml, and their lower limits of quantification (LLOQ) were 0.1 and 0.05 μg/ml, respectively.

2.3 Extraction recovery

The extraction recoveries of samples in the heat-inactivated GAM solution were also optimized in our preliminary studies by comparing protein precipitation and extract solvent, such as cyclohexane, chloroform, and ethyl acetate (EtOAc). The results were satisfactory when EtOAc was used. The extraction recoveries from the heat-inactivated GAM solution were found to be (87.66 ± 1.66), (79.89 ± 2.53), and (82.96 ± 5.61)% at the concentrations of 1.0, 2.0, and 8.0 μg/ml for **1**, and (88.32 ± 4.12), (78.15 ± 4.39), and (76.22 ± 3.29)% at the concentrations of 1.0, 4.0, and 16.0 μg/ml for **2**. The relative standard deviations (RSDs) of extraction recoveries were 6.53, 5.77, and 3.14% for **1**, and 3.23, 3.69, and 7.58% for **2**, respectively. The study showed that at least equal volume was needed for complete protein denaturization when 10 ml of EtOAc was added into 10 ml of GAM solution sample.

2.4 Precision and accuracy

The data presented in Table 1 show the intra- and interday precision for the heat-inactivated GAM solution quality control (QC) samples. The intra- and inter-day precision and accuracy in the heat-inactivated GAM solution were evaluated at the three concentration levels, respectively. The intra-day accuracies were between 96.8 and 97.0% for **1** and 96.3 and 101.4% for **2**, while inter-day accuracies were between 98.5 and 103.7% for **1** and 96.8 and 98.1% for **2**. The values of precision and accuracy were acceptable and the results indicated that the method was reproducible.

2.5 Analyte stability

The stability of **1** and **2** in the heat-inactivated GAM solution during the sample storage and

processing procedures was fully evaluated by analyzing QC samples at three concentrations. After 12-h storage at ambient temperature, the accuracies of samples were 90.8, 92.8, and 94.3% at the concentrations of 1.0, 2.0, and 8.0 μg/ml for **1**, and 87.9, 93.6, and 96.1% at the concentrations of 1.0, 4.0, and 16.0 μg/ml for **2**. The RSDs of the samples at corresponding concentrations above were 3.47, 4.26, and 4.73% for **1**, and 2.01, 2.56, and 5.74% for **2**. In addition, **1** and **2** showed no degradation over a 24-h period at 4°C, and was found to be stable at −20°C for 2 months.

2.6 Time course and ability of the biotransformation of nodakenin by human intestinal flora or isolated strains

The method developed here was successfully applied to the biotransformation study of **1** by human intestinal flora or isolated strain. As shown in Figure 3, **1** was completely converted to **2** in 16-h period by incubated system of human intestinal flora and not decreased obviously after prolonged incubation, suggesting that it was not further converted to other negative compounds or incorporated into the bacterial cells.

The human intestinal bacterial strains used in this study were listed in Table 2 and were examined for ability to transform **1** to **2**. Although all of the isolated strains transformed 3.47–51.63% of the mole of **1** in 8 h as calculated from the percentages of the mole of the **2**, because 1 mol of **1** molecule theoretically hydrolyze with glycosidase to form 1 mol of **2**, there were a low transformation rate in most isolated strains (Table 2), combined with the above results of **1** converted by human intestinal flora, suggesting that other strains or interactions between strains in human intestinal flora may well also play important roles in the formation of **2** from **1**.

Figure 1 shows processes leading to the formation of **2** from **1** by human intestinal flora or isolated strain, which was a mechanism of deglycosidation of **1**.

3. Conclusion

Determination and quantification of **1** and **2** in the crude drugs by RP-HPLC method have been reported [8]. In the present study, a RP-HPLC method for the simultaneous quantification determination of **1** and **2** in biotransformation system of human intestinal flora or isolated strains using a universal Diamonsil™ C_{18} analytical column and a mobile phase consisting of MeOH–H_2O (4:6, v/v) have been developed for the first time. Although we did not find a suitable internal standard to improve precision and accuracy of analysis, the method used here was simple, rapid, reproducible, precise, and accurate, and can satisfy the requirement of simultaneous quantification determination study of **1** and **2** in biotransformation system of human intestinal flora or isolated strains. The sample preparation procedure is easy to

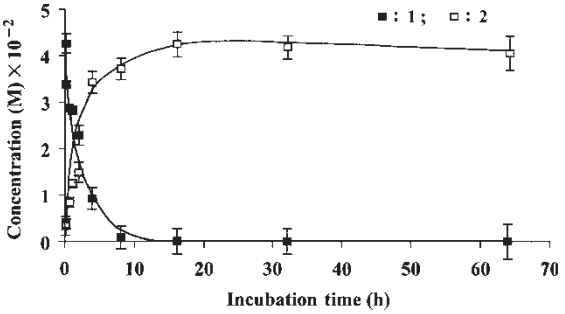

Figure 3. Time course of biotransformation of nodakenin (**1**) to nodakenetin (**2**) by human intestinal flora ($n = 3$, mean ± SD).

Table 2. Ability of bacterial strains from human feces to metabolize nodakenin[a].

	Concentration (M)					
	Nodakenin			Nodakenetin		
Bacterial species	2 h	4 h	8 h	2 h	4 h	8 h
Bacteroides fragilis ss. thetaotus	1.05	1.01	0.54	0.36	0.98	1.34
Bacteroides fragilis ss. vulgatus	1.48	0.75	0.57	0.54	0.65	1.52
Bifidobacterium angulatum	1.37	1.03	0.25	0.66	1.33	2.17
Bifidobacterium breve S-2kz 1287	1.20	0.64	0.15	0.12	0.37	1.87
Bifidobacterium longum IV-55	1.47	0.67	0.53	0.52	0.58	2.36
Clostridium butyricum	1.25	0.91	0.59	0.04	0.15	0.28
Clostridium innocuum ES 24-06	3.38	1.90	0.25	0.11	0.35	2.52
Clostridium perfringens TO-23	2.45	1.36	0.15	0.04	0.09	1.92
Enterococcus faecalis II-136	1.53	1.48	1.28	0.08	0.12	0.51
Escherichia coli O-127	2.42	1.80	0.79	1.48	1.93	2.45
Eubacterium aerofaciens	2.33	1.78	1.21	0.23	0.57	0.61
Fusobacterium nucleatum	1.16	0.75	0.43	0.36	0.38	0.62
Klebsiella pneumoniae ATCC 13833	3.78	3.19	1.48	0.37	0.43	0.49
Lactobacillus brevis II-46	1.52	1.11	0.96	0.21	0.28	0.54
Proteus mirabilis S2	1.17	1.04	0.80	0.47	0.49	0.77
Ruminococcus sp. PO1-3	1.05	0.95	0.84	0.09	0.15	0.17
Veillonella parvula ss. parvula ATCC 10790	2.44	1.21	0.21	0.12	1.21	2.53

[a] A biotransformation system consists of a strain (1 ml), GAM solution (9 ml), and 4.9 M of nodakenin in total volume of 10 ml, and was anaerobically incubated at 37°C. All experiments were carried out in triplicate at each time point.

operate and control. The results provided a firm basis for further studying the biotransformation of **1** by other isolated strains in human intestinal flora and evaluating the clinical efficacy of **1** as a pro-drug and **2**.

4. Experimental

4.1 Chemicals, reagents, and microorganisms

Nodakenin (**1**) was isolated and purified from the MeOH extract of the roots and rhizomes of *N. incisum* and its purity was 99.4% by RP-HPLC analysis described in a previous paper [9]. The chemical structures were shown in Figure 1.

Tryptone, beef extract, beef liver extract powder, digestibility serum powder, soya peptone, and proteose peptone were purchased from Beijing Shuangxuan Microorganism Medium Product Factory (Beijing, China). Sodium thioglycolate, L-cysteine hydrochloride, and glucose were purchased from Sigma Chemical Co. (Deisenhofen, Germany). Yeast extract was obtained from Unipath Ltd (Basingstoke, Hampshire, UK). MeOH of HPLC grade was purchased from Biaoqi Ltd, Co. (Tianjin, China). All other chemicals and solvents used were of analytical grade, and water was milli-Q grade.

Human intestinal bacterial strains (strains were shown in Table 2) kindly provided by Prof. Masao Hattori (Division of Metabolic Engineering, Institute of Natural Medicine, University of Toyama, Japan) had been maintained on EG agar slants in a refrigerator at 4°C prior to use. Fresh feces were kindly provided by Chinese healthy man subjects (State Key Laboratory of Natural and Biomimetic Drugs, School of Pharmaceutical Sciences, Peking University, China).

4.2 Instrumentation

The HPLC system consisted of a SpectraSYSTEM P2000 binary pump, a SpectraSYSTEM UV3000 detector, a SpectraSYSTEM 1000 chromatography data handling system

(Thermo Separation Products, Inc., San Jose, CA, USA), and a 7125 Rheodyne injector (Rheodyne, Cotati, CA, USA) with a loop of 20 μl. An anaerobic incubator YQX-II was purchased from Shanghai Yuejin Medical Instruments Factory (Shanghai, China).

4.3 Chromatographic conditions

The HPLC separation was carried out using an analytical Dikma Diamonsil™ ODS C_{18} column (250 × 4.6 mm i.d., 5 μm; Dikma Co., Beijing, China) equipped with a C_{18} guard column cartridge system (8 × 4 mm i.d., 5 μm; Dikma Co.). The isocratic mobile phase was MeOH–H_2O (4:6, v/v). The mobile phase was filtered through 0.45 μm millipore filters before use. The injection volume was 20 μl and the flow rate was 1.0 ml/min. Peaks of **1** and **2** were detected at 330 nm, which is the maximum absorbance of **1** and **2**. The system was operated at room temperature (25°C).

4.4 Preparation of stock solutions, calibration samples, and QC samples

The stock solutions of **1** (1.5 mg/ml) and **2** (0.7 mg/ml) were prepared in MeOH and kept at 4°C. The working solutions were prepared by appropriately diluting the stock solution. A series of **1** and **2** standard solutions were prepared for working solutions in order to construct the calibration curves. The final concentrations were 0.15, 1.5, 3.0, 6.0, 12.0, and 24.0 μg/ml for **1**, and 0.7, 1.4, 2.8, 5.6, and 13.2 μg/ml for **2** in heat-inactivated nutrient medium of bacteria. The calibration curves were obtained by plotting peak area versus concentrations in the standard samples. QC samples of **1** and **2** in heat-inactivated medium solution were prepared at low, medium, and high concentrations of 1.0, 2.0, and 8.0 μg/ml for **1**, and 1.0, 4.0, and 16.0 μg/ml for **2**. The nutrient medium solution standards were prepared using nodakenin-free nutrient medium solution.

4.5 Sample preparations

The incubation samples (10 ml) of **1** with human intestinal flora or isolated strains were extracted three times with 10 ml of EtOAc. The EtOAc extracts were combined and then evaporated to dryness in vacuum at 40°C. The residue was dissolved in 1.0 ml of MeOH, which also acted as a deproteinization agent. The MeOH solution was mixed in vortexer for 1 min and centrifuged at 16,000 g for 20 min at 4°C. The supernatant was collected and filtered through a 0.45 μm filter. A 20 μl aliquot of the supernatant liquor was submitted to the HPLC system for analysis.

4.6 Validation study

The method was fully validated for its specificity, linearity, LLOD, LLOQ, extraction recovery, accuracy, and precision. Specificity was established by the lack of interference peaks at the retention time for **1** and **2**. Linearity was tested at seven levels of concentrations covering the range of 0.15–24.0 μg/ml ($r = 0.9997$) for **1** and 0.7–13.2 μg/ml ($r = 0.9994$) for **2**.

The LLOD was defined as the concentration that produced a signal-to-noise ratio of 3:1. The LLOQ was determined as the lowest concentration on the calibration curve for which the assay precision (RSD) was lower than 10% and the accuracy was between 80 and 120%.

The intra- and inter-day accuracy and precision were evaluated by assaying the QC samples with low, medium, and high concentrations. The intra-day variation was determined by assaying three replicates on the same day and inter-day variation was assayed for three replicates on 3 days. The precision was expressed as the RSD. The accuracy was determined by comparing the calculated concentration (obtained from the calibration curve) to the theoretical concentration.

The extraction recoveries were determined at three concentrations by comparing peak areas extracted from heat-inactivated medium solution with those of the same amounts of standard solutions in MeOH.

Stability of **1** and **2** in heat-inactivated medium solution was assessed with QC samples ($n = 3$) stored at 4 and -20°C.

4.7 Preparation of GAM

A GAM was prepared as follows: 10.0 g of tryptone, 3.0 g of soya peptone, 10.0 g of proteose peptone, 13.5 g of digestibility serum powder, 5.0 g of yeast extract, 2.2 g of beef extract, 1.2 g of beef liver extract powder, 3.0 g of glucose, 2.5 g of KH_2PO_4, 3.0 g of NaCl, 5.0 g of soluble starch, 0.3 g of L-cysteine hydrochloride, and 0.3 g of sodium thioglycolate in H_2O. Adjust the pH of GAM solution to 7.1–7.2 with 1 M NaOH aq. solution before adjusting the total volume to 1 l. Dispense in tubes and autoclave at 120°C for 30 min, and store at 4°C or immediate use.

4.8 Preparation of a flora of human intestinal bacteria

Fresh feces obtained from the Chinese healthy man were immediately transferred into a vinyl bag filled with oxygen-free nitrogen gas. The bag was then pressed by hand to uniformly mix the contents. The feces (2 g) were suspended in GAM (19 ml) solution. The suspension under anaerobic condition was filtered with gauze and the filtrate was used as a bacterial flora.

4.9 Biotransformation of nodakenin by a bacterial flora from human feces

An activated bacterial flora (10 ml) by established method [1] was added to GAM solution (900 ml) containing **1** (400 mg), and the flora was incubated at 37°C for 8 h in an anaerobic incubator. The reaction mixture was extracted three times with 900 ml of EtOAc. The EtOAc extract was combined and then evaporated to dryness in vacuum at 40°C. The residue was dissolved in EtOAc and subjected to a silica gel column chromatography eluting with cyclohexane–EtOAc mixture solution (10:1, v/v) to yield **2** (260 mg) as a colorless crystal, which was identified by IR, NMR, and MS data, and compared with those of an authentic sample [10].

4.10 Time course of the biotransformation of nodakenin by human intestinal flora or isolated strains

A biotransformation system consists of a human intestinal flora or isolated strains (1 ml) and GAM solution (9 ml) containing 0.5 mM of **1**, and was anaerobically incubated at 37°C. All experiments were carried out in triplicate at each time point. The biotransformation reaction was stopped at 7.5, 15, 30, 60, 120, 240, 480, 960, 1920, and 3840 min after the incubation and mixed with 10 ml of EtOAc. The incubation samples were treated and analyzed as described in Section 4.5.

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