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ORIGINAL PAPER

Sensitive and Selective Determination of Orotic Acid in Biological Specimens Using a Novel Fluorogenic Reaction

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Abstract Orotic acid is an intermediate in the synthesis pathway of uridine-5′-monophosphate, and increases in body fluids of patients suffering from hereditary disorders such as orotic aciduria and hyperammonemia. In this study, we developed a spectrofluorometric method with or without high-performance liquid chromatography for the selective and sensitive quantification of orotic acid in human biological specimens, using 4-trifluoromethylbenzamidoxime (4-TFMBAO) as a fluorogenic reagent. This reagent provided intensive fluorescence for only orotic acid amongst 62 compounds including structurally related bio-substances such as nucleic acid bases, nucleosides, nucleotides, amino acids, vitamins, bilirubin, uric acid, urea, creatine, creatinine and sugars. Under optimized reaction conditions, orotic acid was reacted with 4-TFMBAO, $K_3[Fe(CN)_6]$ and K_2CO_3 in an aqueous solution. The fluorescence produced from the orotic acid derivative was measured at an excitation of 340 nm and an emission of 460 nm. A concentration of 1.2 μM orotic acid per 1.0 mM creatinine in normal urine and 0.64 nmol orotic acid per 5.0×10^5 HeLa cells were determined by this method. The present method permitted the facile quantification of orotic acid in healthy human urine and cultured HeLa cells by spectrofluorometry and/or high-performance liquid chromatography.

Keywords Orotic acid · Fluorogenic reaction · Quantitative determination · Human urine · HeLa cells

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Introduction

Orotic acid is an intermediate in the de novo biosynthesis of uridine-5′-monophosphate (UMP). This pathway starts from the formation of carbamoyl phosphate, which is used for UMP synthesis and the urea cycle [1–3] as shown in Fig. 1. Deficiencies in urea cycle enzymes such as ornithine carbamoyltransferase and argininosuccinate synthetase, results in an overload of carbamoyl phosphate into the UMP pathway [4, 5]. In turn, an increase in the concentration of orotic acid in body fluids is observed. Additionally, a deficiency of UMP synthase causes accumulation of orotic acid in biological specimens, and thus orotic acid is intensively excreted in urines of patients suffering from hereditary disorders such as orotic aciduria and hyperammonemia [6–9].

Conventional methods for the quantification of orotic acid in biologically complex samples generally require sophisticated instrumentation to completely separate and detect biogenic orotic acid from many other biogenic substances. Commonly, column-switching high-performance liquid chromatography (HPLC) with ultraviolet (UV) absorption detection [10–12], and mass spectrometry (MS) coupled with HPLC [13] or gas chromatography [14] using stable isotope-labeled orotic acid as internal standard for quantification, are employed. Thus, a facile and inexpensive analytical technique has been required for the quantification of orotic acid in biological specimens.

Fluorescence (FL) is one of the most widely used methodologies for the sensitive and selective detection of biological specimens. Herein, we describe the development of a novel FL-derived reaction specific towards orotic acid. The reaction was performed by heating orotic acid with 4-TFMBAO, $K_3[Fe(CN)_6]$, and K_2CO_3 in an aqueous solution at 80 °C for 4 min, followed by either spectrofluorometric or chromatographic quantification of orotic acid in human urine and cultured cells. The usefulness of the method in terms of facility,

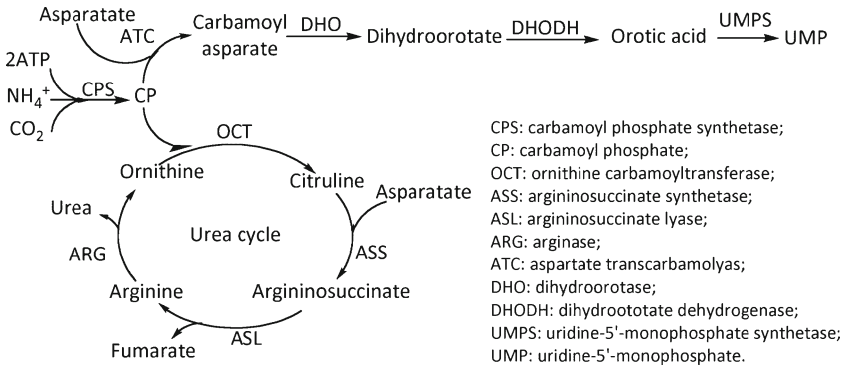

Fig. 1 Synthetic pathway of orotic acid

selectivity and sensitivity for the determination of biogenic orotic acid is also discussed.

Materials and Methods

Reagents

Chemicals obtained from commercial supplies were used without further purification. Orotic acid was obtained from TCI (Kyoto, Japan). 4-TFMBAO and other benzamidoxime (BAO) analogs were purchased from Sigma–Aldrich (St. Louis, MO, USA). All other chemicals were of analytical or guaranteed reagent grade. H_2O was purified by a Milli-Q system and used for the experiments. Orotic acid and its related bio-substances, 4-TFMBAO and its analogs, $K_3[Fe(CN)_6]$ and K_2CO_3, were weighed in each flask or polypropylene tube (Japan Eppendorf, Tokyo, Japan) and then dissolved in water.

Preparation of Urine and Lysate of HeLa Cells

A urine sample was collected from one healthy donor (male, 25 years old) and centrifuged at 3500×*g* for 15 min. The supernatant was stored at −40 °C. Then, the urine specimen was diluted in water to 5 % (*v*/*v*) before analysis. Creatinine concentration (8.85 mM) in the undiluted urine was measured to normalize the rate of kidney filtration using a colorimetric assay kit (AUTION Sticks) purchased from ARKRAY Factory Inc. (Shiga, Japan).

HeLa cells (1.0×10^7) were cultured, collected and then suspended in 4.0 ml of H_2O. The cells were sonicated in an ice bath for 30 min followed by centrifugation at 3500×g for 5 min. The supernatant was collected for analysis.

Typical Procedure for the FL Reaction of Orotic Acid in Urine or Cells

A portion (200 μl) of the biological specimen of 5 % urine or cell lysate (5.0×10^5 cells) was mixed with 50 μl of 0, 10 or 20 μM standard orotic acid. The sample solution (250 μl) was mixed with 250 μl of 4.0 mM of 4-TFMBAO, 250 μl of 8.0 mM $K_3[Fe(CN)_6]$ and 250 μl of 80 mM K_2CO_3. The mixture (1.0 ml) was then heated at 80 °C for 4.0 min, followed by cooling in an ice bath for ca. 2.0 min to stop the reaction.

FL Measurement by Spectrofluorometry

The relative FL intensity produced by the reaction with 4-TFMBAO was measured with an FP-6300 spectrofluorometer (Jasco, Tokyo, Japan) at maximum excitation and emission wavelengths of 340 and 460 nm, respectively. For the reactions with other BAO analogs, the wavelengths at each maximum of excitation and emission were set at 310–340 and 410–460 nm, respectively. Bandwidths were set at 5 nm for both the excitation and emission.

HPLC Measurement

A reversed-phase HPLC system was employed, consisting of a quaternary gradient pump (PU-2089 Plus; Jasco), a reversed-phase C_{18} column (COSMOSIL 5C18-AR-II, 4.6 mm i.d. × 150 mm; Nacalai Tesque, Kyoto, Japan), a UV absorption spectrometer (UV-2070 Plus; Jasco), and a spectrofluorometer (FP-2020 Plus, Jasco). The separation was carried out using 40 % (*v*/*v*) methanol in H_2O as the mobile phase. The flow rate was 1.0 ml/min. The column eluate was monitored at 460 nm (emission) and 340 nm (excitation) for FL detection, and 280 nm (absorption) for UV detection.

Analysis of FL Product of Orotic Acid by MS

Orotic acid (1.0 mM) was reacted according to the above-described procedure for the FL reaction with 4-TFMBAO. The FL product in the reaction mixture was separated and collected by reversed-phase HPLC using a mobile phase (50 % methanol in H_2O) at a flow rate of 1.0 ml/min. The FL product was observed at 5.4 min as a single peak. The separation (100 μl each per injection) was repeated 24 times. The collected fractions were evaporated to remove methanol,

and then lyophilized. A pale blue powder of the product was obtained and dissolved in 150 μl of methanol. The product in the solution was analyzed by a negative electrospray ionization mass spectrometer (ESI-MS; JEOL Ltd., Tokyo, Japan).

Results and Discussion

FL Reaction Conditions

Recently, we have developed two FL reactions specific for uracil [15] and cytosine [16] using BAO and 4-TFMBAO, respectively. These reactions were performed by heating in a strongly alkaline KOH solution. In this study, however, the orotic acid reaction was performed by heating with 4-TFMBAO in a weakly alkaline K_2CO_3 solution in the presence of an oxidant. To specifically yield a FL product from orotic acid, different concentrations of K_2CO_3, 4-TFMBAO and $K_3[Fe(CN)_6]$ were first investigated using uracil and cytosine as additional model compounds and varying the reaction time and temperature (Fig. 2).

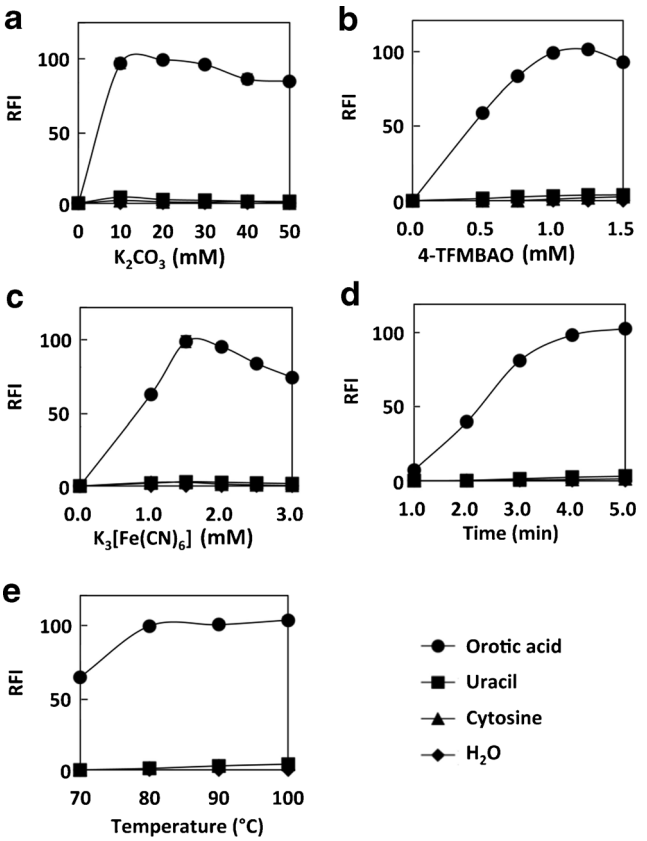

Fig. 2 Effects of concentrations of K_2CO_3 (**a**), 4-TFMBAO (**b**), $K_3[Fe(CN)_6]$ (**c**), reaction time (**d**) and temperature (**e**) on the production of the FL derivative from the analytes at 100 μM in the reaction mixture. Analytes: orotic acid (●), uracil (■), cytosine (▲) and H_2O (♦) as the reagent blank. Each plot is the mean value of three experiments

The maximum and selective FL production from orotic acid was obtained with the final concentrations of 20–30 mM K_2CO_3 in the reaction mixture (Fig. 2a) while a portion (250 μl) of 80 mM K_2CO_3 was employed for the typical procedure. By using 20 mM K_2CO_3 (Fig. 2a), 1.0 mM 4-TFMBAO (Fig. 2b) and 2.0 mM $K_3[Fe(CN)_6]$ (Fig. 2c), the maximum FL intensity were obtained from orotic acid. A reaction time between 1.0 and 5.0 min (Fig. 2d) at 80 °C (Fig. 2e) provided an increasing FL intensity for orotic acid while a 4.0 min reaction time provided better selectivity for orotic acid.

Figure 3 shows FL excitation and emission spectra of the FL product obtained from orotic acid at different concentrations between 1.0 and 10 μM in the FL reaction mixture. The maximum wavelengths of excitation and emission were 340 and 460 nm, respectively. The FL intensities were proportional to the concentrations of orotic acid.

Various analogs of BAO were studied for the production of FLs from orotic acid, uracil and cytosine under the optimized reaction conditions for 4-TFMBAO (Fig. 4). 2-MethylBAO, 3-hydroxyBAO, 4-hydroxyBAO, 2-aminoBAO, 3,5-bis(trifluoromethyl)BAO, 3-nitroBAO and 4-nitroBAO gave negligible FLs for the tested analytes. 4-TFMBAO was found to be the most selective and intensive fluorogenic reagent toward orotic acid, although BAO, 4-trifluoromethoxyBAO, 3-methylBAO, 4-methylBAO, 3-aminoBAO and 4-aminoBAO provided FLs for orotic acid.

Specificity for Orotic Acid

Specificity of the proposed FL reaction for orotic acid was investigated by using 62 different bio-substances that might be present in biological specimens (Fig. 5). Under the optimized reaction conditions, the most intensive FL was obtained from orotic acid. Negligible FL intensities compared with the reagent blank (H_2O) were observed with uracil, cytosine, folic acid, thiamine and pyridoxine. Other compounds such as analogues of orotic acid (dihydroorotic acid, 1-methyluracil, 5-fluorouracil, 6-methyluracil, 5,6-dihydrouracil, 5-methylcytosine,

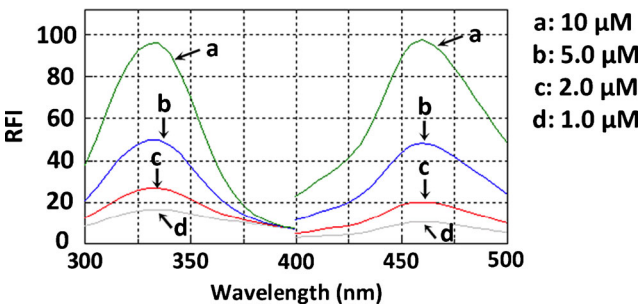

Fig. 3 FL excitation and emission spectra from orotic acid at 10 μM (**a**), 5.0 μM (**b**), 2.0 μM (**c**) and 1.0 μM (**d**) in the FL reaction mixture

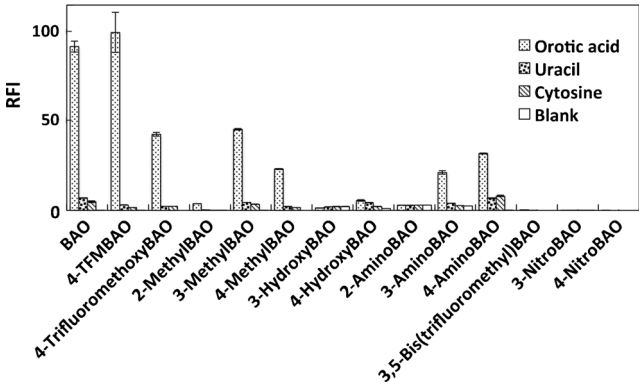

Fig. 4 FL intensity obtained from orotic acid, uracil and cytosine at 100 μM each in the reaction mixtures with various BAO analogs. H_2O was used instead of the analytes for the reagent blank. Each *bar* is the mean value of three experiments (n=3)

thymine, adenine and guanine), nucleosides (uridine, pseudouridine, cytidine, thymidine, adenosine and guanosine), nucleotides (5′-UMP, 5′-CMP, 5′-TMP, 5′-AMP and 5′-GMP), 20 kinds of amino acids, vitamins (D-pantothenic acid, L-ascorbic acid, biotin, riboflavin, nicotinic acid and nicotinamide), other bio-substances (bilirubin, uric acid, creatine, creatinine and urea) and sugars (sucrose, glucose, fructose, lactose and ribose) did not exhibit any FL. Based on these results, it was suggested that the developed FL reaction can be used for the specific determination of orotic acid in biological specimens.

Figure 6 shows a plausible mechanism of the proposed reaction for the production of a FL compound from orotic acid. Our previously reported FL reaction with BAO under strong alkaline conditions using KOH [15] was selective for uracil, but not for orotic acid. Therefore, under weakly alkaline conditions with oxidative heating, it was possible that the carboxyl group of orotic acid was first eliminated and then its carbanion reacted with the amidoxime moiety of 4-TFMBAO. On the basis of the results shown in Fig. 5, substitution at either the *N*1 or *C*5 position inhibited the formation of the FL product. Thus, other related nucleosides and nucleotides having no carboxyl group did not yield any FL. The FL product was separated by reversed-phase HPLC and analyzed by ESI-MS. The MS spectrum in Fig. 6 shows a molecular ion peak at m/z=295 that likely corresponds to the [M-oxygen-H]-ion, because *N*-oxide compounds were reported to show a strong [M-oxygen] ion peak in MS spectra [17, 18]. Therefore, the FL product was suggested to be a phenyl-substituted purine derivative as shown in Fig. 6.

Quantification of Orotic Acid in Urine and Cell Lysate

Concentrations of orotic acid in healthy human urine and the lysate of cultured HeLa cells was determined by HPLC (Fig. 7a, b and c) or spectrofluorometry (Fig. 7d) after the FL reaction with 4-TFMBAO. A standard addition assay was performed for the quantification of orotic acid in these biological specimens (Fig. 7c and d). As shown in Fig. 7a and b, use of reversed-phase HPLC coupled with FL and UV detectors provided separation and detection of orotic acid in the biological specimens as its FL product. The retention time of the FL product of orotic acid was 9.6 min.

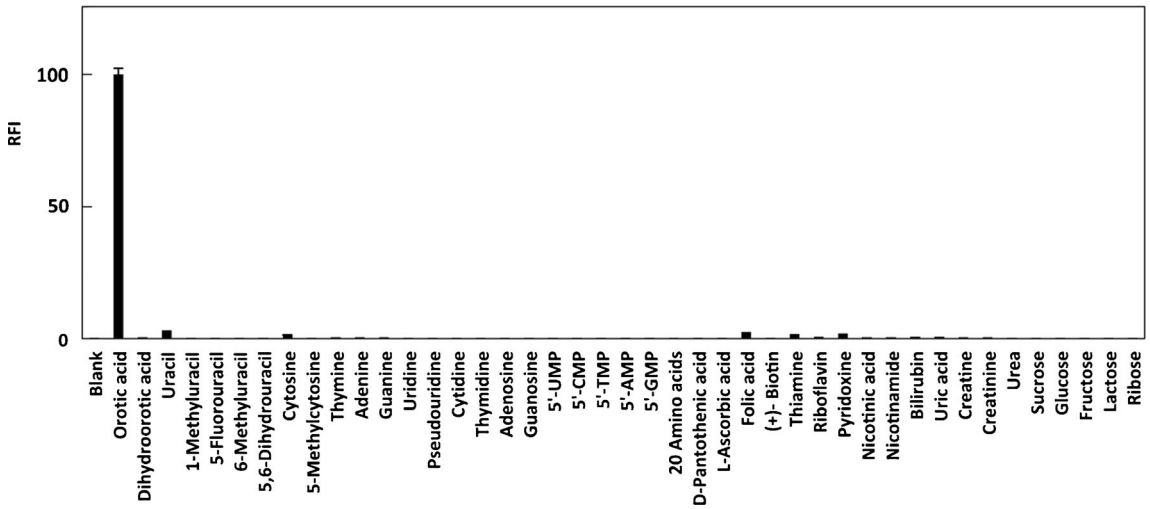

Fig. 5 FL production from orotic acid and other related bio-substances under the optimized reaction conditions for 4-TFMBAO. The analytes were examined at 100 μM in the reaction mixtures, expect for the amino acids. Twenty kinds of amino acids were mixed in the aqueous solution at 10 mM each. Each *bar* is the mean value of three experiments (n=3)

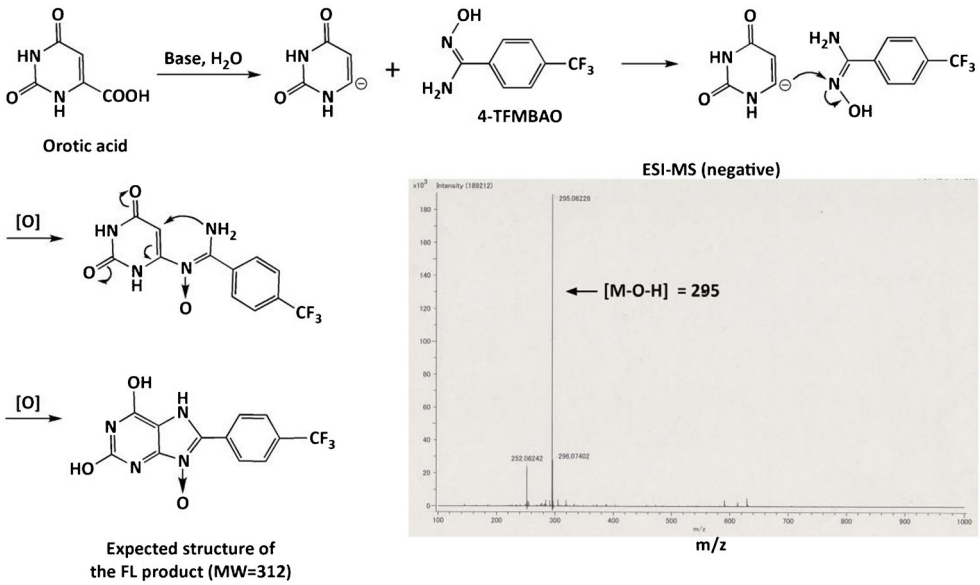

Fig. 6 Plausible reaction mechanism for the FL reaction of orotic acid with 4-TFMBAO, $K_3[Fe(CN)_6]$ and K_2CO_3 in aqueous solution, and the MS spectrum of the FL product. The identification of the FL product from orotic acid was performed by reversed-phase HPLC. The fraction of the FL peak of the product was used for ESI-MS

However, the UV (280 nm) absorption peak of the FL product could not be detected, because the sensitivity of the UV detection was much lower than that of the FL detection. We also tried to detect native orotic acid in the specimens before the FL reaction by HPLC. However, native orotic acid co-eluted with several biogenic other compounds at 3.5 min corresponding to the dead volume of the column.

The endogenous concentrations of orotic acid in human urine and HeLa cells were quantified by addition of standard orotic acid (2.5 and 5.0 μM) to each specimen. Using the HPLC method with FL detection (Fig. 7c), the endogenous concentrations of orotic acid in 5 % urine and the lysate of HeLa cells (5.0×10^5 cells in 200 μl) were 0.56±0.02 μM and 3.35± 0.20 μM, respectively. The linear equations of the standard addition curves (Y=FL intensity, X=mM concentration of added orotic acid, R^2=linear regression) were Y=8.6X+4.8333 (R^2=0.99124) for the urine, and Y= 3.58X+11.983 (R^2=0.9901) for the HeLa lysate.

For the spectrofluorometric determination (Fig. 7d), the FL values obtained from the full reaction for the urine and cell lysate specimens should be first corrected for the background FL obtained from the reaction without 4-TFMBAO in the presence of $K_3[Fe(CN)_6]$ and K_2CO_3. This background correction can discriminate the presence of endogenous orotic acid in the specimens from any FL materials such as vitamins and/or oxidized by-products. By the spectrofluorometric determination, the concentration of orotic acid was 0.55±0.01 μM in the 5 % urine sample and 3.26±0.14 μM in the cell lysate (5.0×10^5 cells in 200 μl). The linear equations of the standard addition curves were Y=43X+23.833 (R^2=0.9974) for the urine and Y=18.8X+61.333 (R^2= 0.99985) for the cell lysate.

The concentration of the endogenous orotic acid obtained by the HPLC and spectrofluorometric methods were consistent with each other, and corresponded to 1.2 μM per 1.0 mM creatinine in the urine, which agreed well with a previous report [9]. A total of 0.64 nmol of endogenous orotic acid could be quantified in 5.0×10^5 HeLa cells. The precision of both the methods was evaluated by the repeated determination (n=5) of endogenous orotic acid over the course of a day. The relative standard deviation of each value for the concentrations of orotic acid was 4.37 % for the urine and 5.89 % for the cell lysate by the HPLC method, and 2.22 % for the urine and 4.43 % for the cell lysate by spectrofluorometry. The lower detection limits of standard orotic acid in the FL reaction mixture were approximately 1.0 nM by the HPLC method and 0.1 μM by the spectrofluorometric method. Their fluorescent signals were at least three fold higher than those of the base-line noise for the HPLC or the reagent blank for the spectrofluorometry.

The recovery of the endogenous orotic acid was 75 % for the urine, and 35 % for the cell lysate (5.0×10^5 cells in 200 μl) compared with the calibration standards in the absence of the biological specimens. The FL reaction of endogenous orotic acid with 4-TFMBAO might be influenced by the large amount of other bio-substances, because the FL production from orotic acid was inhibited by the bio-matrices in the cell lysate. However, the FL signals were proportionally increased with increasing concentrations of orotic acid spiked in the cell lysate, providing a good linear

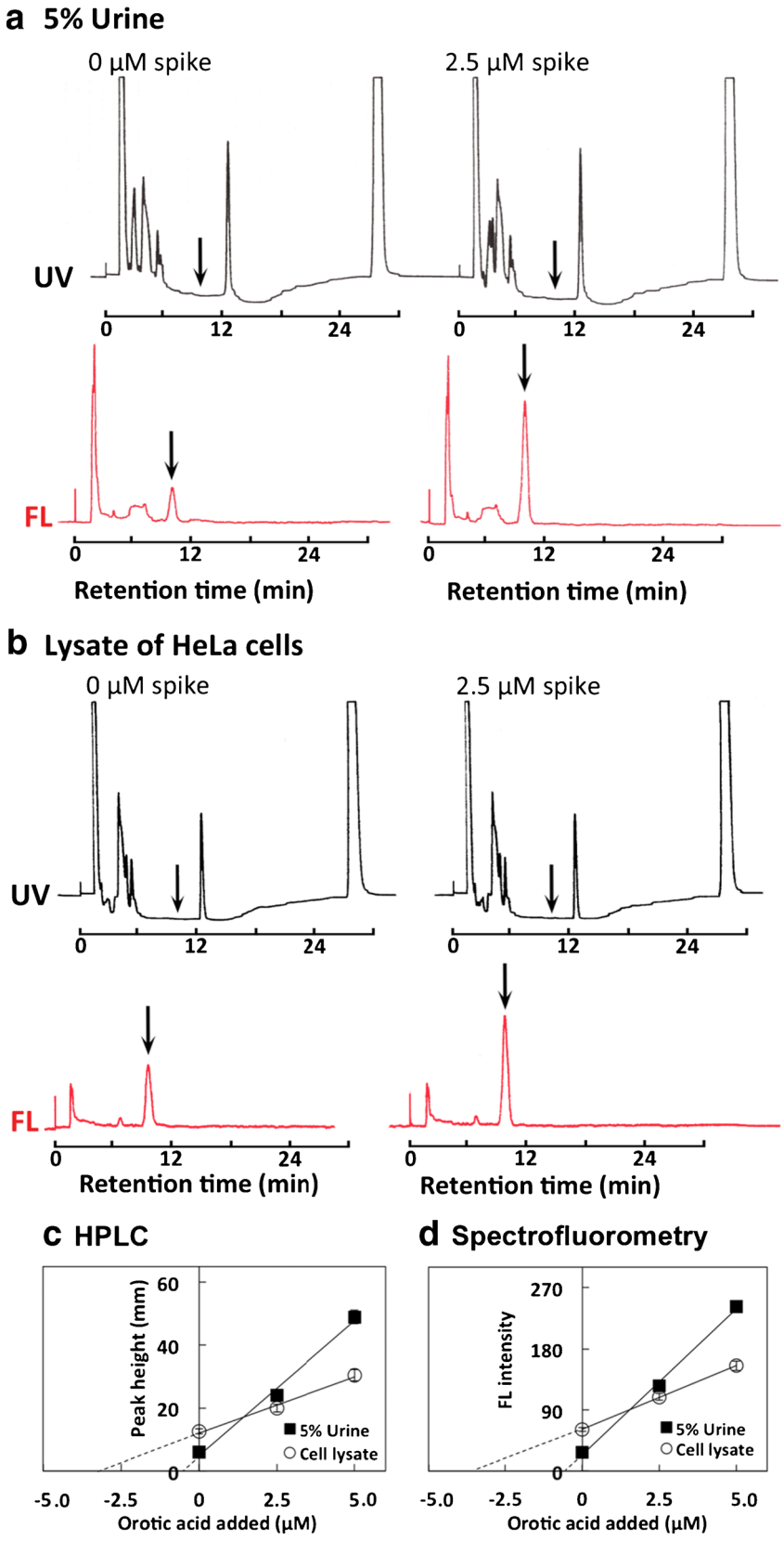

Fig. 7 Quantitative determination of orotic acid in urine and HeLa cell lysate by HPLC with FL and UV detection (**a**–**c**) and spectrofluorometry alone (**d**) after the FL reaction with 4-TFMBAO. Portions (200 μl each) of 5 % urine or cell lysate (5.0×10^5 cells) from HeLa cells were spiked with orotic acid, and then reacted with 4-TFMBAO. The FL product of orotic acid in the biological specimens was separated and detected by reversed-phase HPLC (**a**–**c**), or by a spectrofluorometer (**d**). The calibration curves of orotic acid using the standard addition assay for the urine and cell lysate (n=5 each) were obtained by chromatographic (**c**) and spectrofluorometric (**d**) measurements. See the Materials and Methods section for the full protocols

regression of R^2 of 0.9901–0.99985 as shown in Fig. 7c and d. To avoid the inhibition of the FL reaction, the specimens should be diluted with water as possible. When the cell lysate was twice diluted to 2.5×10^5 cells in 200 μl

of H_2O, this treatment increased the reaction recovery by approximately two folds.

Conclusion

In this study, we developed for the first time a novel fluorescent reaction method that provides a rapid fluorescent derivatization of orotic acid using 4-TFMBAO as a fluorogenic reagent. This reaction was specific toward orotic acid among 62 kinds of bio-substances tested (Fig. 5). Thus, this reaction could be readily applied to FL-based HPLC method for the sensitive determination of biogenic orotic acid in healthy human urine and cultured HeLa cells. In the presence of high concentrations of uracil and cytosine, however the 4-TFMBAO reaction gave an increased FL intensity for orotic acid since the 4-TFMBAO reagent slightly reacted with uracil and cytosine, and their FL products eluted at almost the same retention time as that for the FL product of orotic acid in the FL-based HPLC. When 1.0 μM orotic acid in the presence of 10 μM uracil or 10 μM cytosine was reacted with 4-TFMBAO, the FL-peak height of orotic acid was increased by 25 and 10 %, respectively. The co-elution of those FL products might result in a large value of the endogenous concentration of orotic acid in patient's urines especially that contain intensively higher concentrations of uracil and/or cytosine than that of orotic acid [7]. Therefore, the present FL-based HPLC method permits the determination for the endogenous orotic acid in biological specimens containing low concentrations of uracil and cytosine.

However, the methods described herein provide advantages in terms of facility and sensitivity. For example, the entire assay time including the sample preparation of the endogenous orotic acid was approximately 20 min by the spectrofluorometric method and 40 min by the HPLC method. These assay times are much shorter than those of conventional methods (50–120 min) such as column-switching HPLC with UV detection [10–12] and chromatographic MS [13, 14] using stable isotope-labeled orotic acid as an internal standard. Although the HPLC methods coupling UV detection [10] can afford a wide linear range for the calibration of orotic acid from approximately 1.0 to 1000 μM in the sample, this sensitivity is approximately one tenth lower than that of the present FL-based HPLC method. Our FL-based HPLC method provided a determinable range from 0.1 to 100 μM orotic acid in the reaction mixture. Concentration of orotic acid higher than 100 μM caused saturation for the FL production. In this case, samples should be diluted for the FL reaction. Consequently, the present FL-based method is readily operable and fairly rapid because no pre-treatment was required for the biological specimens.

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