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Short communication

Simultaneous determination of N^2-(3-aminopropyl)biopterin (oncopterin), biopterin and neopterin by high-performance liquid chromatography with fluorescence detection

M. Hibiya[a], R. Teradaira[a], T. Sugimoto[b], K. Fujita[a], T. Nagatsu[a,*]

[a]Institute for Comprehensive Medical Science, School of Medicine, Fujita Health University, Toyoake, Aichi 470-11, Japan
[b]Department of Natural Science Informatics, School of Informatics and Sciences, Nagoya University, Nagoya 464-01, Japan

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Abstract

A high-performance liquid chromatographic method is described for the simultaneous determination of N^2-(3-aminopropyl)biopterin (oncopterin, a newly found natural pteridine in urine from cancer patients), biopterin, and neopterin in urine. For the detection and quantification of the compounds, fluorometry was used. Using Develosil ODS K-5 and Develosil ODS HG-5 reversed-phase columns and a Nucleosil 100-5SA strong cation-exchange column, oncopterin, biopterin, and neopterin in urine were completely separated and assayed simultaneously by fluorescence detection. Similar values of oncopterin were obtained using each of the three columns, and the Develosil ODS K-5 reversed-phase column gave the most satisfactory separation. The sensitivity was high enough to measure 1 pmol of each pteridine. The HPLC method was highly reproducible. Our preliminary results indicate that oncopterin could be a most sensitive marker for cancer.

1. Introduction

Various types of cancers are associated with increasing levels of unconjugated pteridines, like neopterin and biopterin (Fig. 1) in urine and serum [1,2], of which neopterin is considered particularly important [3,4]. On the other hand, the biogenic polyamines, such as putrescine (1,4-aminobutane), spermidine [N-(3-aminopropyl)-1,4-diaminobutane], and spermine [N,N'-bis(3-aminopropyl)-1,4-diaminobutane] (Fig. 1) are also thought to be of considerable clinical significance as biochemical markers of various solid and blood cancers [5–8]. In our study correlating pteridine metabolism and cancers, we have found a hitherto unknown natural pteridine of a strong base character, tentatively named *oncopterin*, from urine of cancer patients. Oncopterin has been isolated from urine from patients with cancer, and the structure was determined to be N^2-(3-aminopropyl)biopterin [9] (Fig. 1). Thus, oncopterin is composed of the elements of the two biochemical markers for cancers, pteridine and polyamine, and is expected to be one of the most promising diagnostic markers. Attempts to isolate oncopterin from urine of healthy controls have not been successful, suggesting that the compound can only be present at a far lower concentration. In our preliminary study, we tried to measure oncopterin in urine from various

* Corresponding author.

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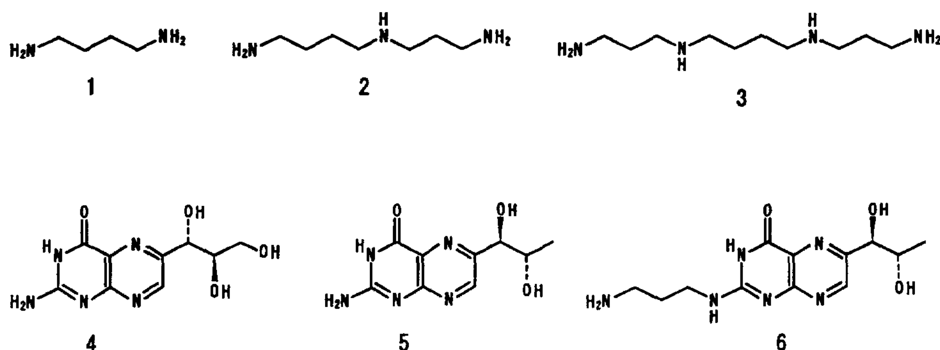

Fig. 1. Structure of putrescine (1), spermidine (2), spermine (3), neopterin (4), biopterin (5), and N^2(3-aminopropyl)biopterin (oncopterin) (6).

cancer patients and from healthy controls by high-performance liquid chromatography (HPLC) with fluorescence detection [10]. The concentration of oncopterin increased after acid hydrolysis, indicating that it exists as an amide in urine. The oncopterin concentrations were very low in the urine from healthy controls and increased markedly in urine from patients with various solid and blood cancers, especially solid cancers. During our study, we frequently encountered difficulties in identifying the oncopterin peak.

In the present study, we have examined the optimum conditions for HPLC to follow the clinical evolution of urinary oncopterin. We have also succeeded in measuring neopterin and biopterin simultaneously with oncopterin by this HPLC method.

2. Experimental

2.1. Chemicals

N^2-(3-Aminopropyl)biopterin (oncopterin) was synthesized as described by Sawada et al. [11]. Develosil ODS K-5 and Develosil ODS HG-5 reversed-phase columns were purchased from Nomura Chemicals (Seto, Japan), and Nucleosil 100-5SA strong cation-exchange column from Macherey-Nagel (Düren, Germany). Develosil ODS K-5 has a silica surface bonded with a monolayer of octadecyl groups, whereas Develosil ODS HG-5 has a silica surface bonded with a polymeric layer.

2.2. Instrumentation

Fluorescence spectra were recorded by a Hitachi 650-40 fluorescence spectrophotometer (Tokyo, Japan). HPLC analysis was carried out with an HPLC system composed of an autosampler AS-8010 (Tosoh, Tokyo, Japan), a CCPM pump (Tosoh), a column oven 860-CO (JASCO, Tokyo, Japan), an FP-777 spectrofluorometric detector (JASCO), and a Chromatopac C-R6A recorder (Shimadzu, Kyoto, Japan).

2.3. Chromatographic conditions

HPLC analyses were carried out either on a Develosil ODS K-5 (150 × 4.6 mm I.D.; 5 μm), a Develosil ODS HG-5 (150 × 4.6 mm I.D.; 5 μm) or a Nucleosil 100-5SA (150 × 4.6 mm I.D.; 5 μm) column. Isocratic elution was performed at a flow-rate of 1 ml/min with the following solvents: for a reversed-phase column, 50 m*M* ammonium phosphate buffer, pH 3.0; for a strong cation-exchange column, 100 m*M* ammonium phosphate buffer, pH 3.0. Column temperature was kept at 23°C for Develosil ODS K-5, 22°C for Develosil ODS HG-5, and 26°C for Nucleosil 100-5SA columns. The fluorescence

detection was used with 355 nm excitation and 450 nm emission to selectively detect pteridines.

2.4. Collection and handling of urine

The first morning urine specimens from 12 cancer patients and 10 healthy controls were frozen immediately after collection and kept at −80°C in the dark until use. A mixture of urine (120 μl) and 6 *M* hydrochloric acid (60 μl) was heated in a glass tube sealed with a rubber stopper at 100°C for 2 h and then lyophilized. The residue was mixed well with 50 m*M* ammonium phosphate buffer, pH 3.0 (120 μl) and then centrifuged at 700 *g* for 10 min. A 5-μl aliquot of the supernatant (equivalent to 5 μl urine) was used for HPLC analysis.

Creatinine concentration in urine was measured according to the photometric method [12].

3. Results

3.1. Fluorescence spectrum of neopterin, biopterin and oncopterin

The fluorescence spectra of neopterin, biopterin, and oncopterin at 1 μ*M* in 50 m*M* ammonium phosphate buffer, pH 3.0, are similar. Maximum excitation wavelengths are: neopterin, 277 and 356 nm; biopterin, 275 and 358 nm; oncopterin, 283 and 360 nm. Maximum emission wavelengths are: neopterin, 443 nm; biopterin, 441 nm; oncopterin, 446 nm. Since excitation at longer wavelengths gives higher selectivity, excitation at 355 nm and emission at 450 nm were used in the present study for simultaneous analysis of the three pteridines.

3.2. Comparison of the measurement of oncopterin with three HPLC columns

HPLC chromatograms of oncopterin in urine from a patient with stomach cancer were compared with each other using the three columns, Develosil ODS K-5, Develosil ODS HG-5 reversed-phase columns and Nucleosil 100 5SA strong cation-exchange column. The values of urinary oncopterin obtained by all three were similar: 45.0, 40.2, and 44.7 μmol/mol of creatinine, respectively. The Develosil ODS K-5 column gave the most satisfactory separation of oncopterin and was thus used in the following experiments.

3.3. Simultaneous determination of neopterin, biopterin, and oncopterin using a Develosil ODS K-5 column

As shown in Figs. 2 and 3, neopterin, biopterin, and oncopterin in urine samples from a patient with kidney cancer and from a healthy subject can be completely separated by HPLC using a Develosil ODS K-5 column. The concentrations of neopterin, biopterin, and oncopterin were calculated from the increased areas of spiked neopterin, biopterin, and oncopterin as internal standards on chromatogram C, and also from the external standards. The results concerning reproducibility of the assay from a cancer patient and a healthy subject are shown in Table 1. Five analyses were made with each sample on a single day. C.V. values were 1–7%, and recovery rates were 92–98%.

3.4. Standard curve of oncopterin

Fluorescence intensity in terms of peak area is completely linear up to 1000 pmol oncopterin. Satisfactory accuracy was obtained down to 1 pmol. The equation of linear regression of the analytical data was: $y = 1.56 \cdot 10^{-4} x$, where y is pmol of oncopterin and x is area (μV·s).

Since the recovery rates of neopterin, biopterin, and oncopterin were over 90%, the assays were generally carried out based on the external standard.

3.5. Assays of neopterin, biopterin, and oncopterin in cancer patients and healthy controls

We applied the present method to the assay of neopterin, biopterin, and oncopterin in urine from patients with various solid cancers and from healthy controls.

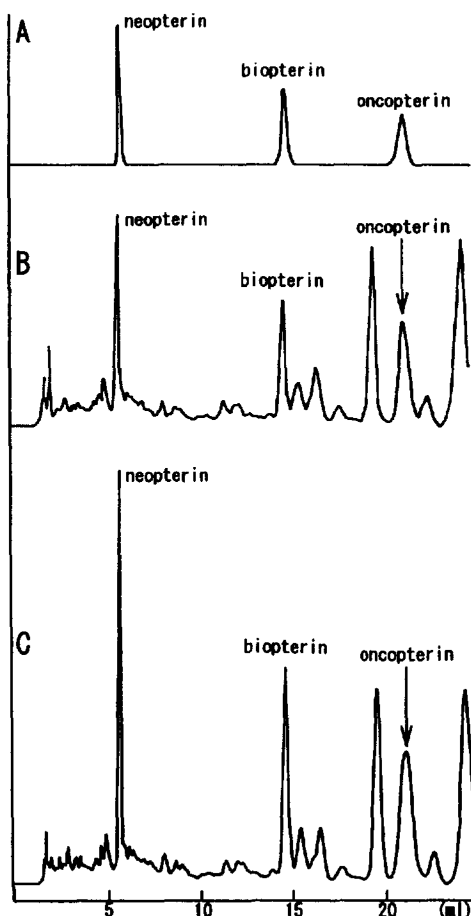

Fig. 2. Chromatograms of simultaneous separation of neopterin, biopterin, and oncopterin by HPLC on a Develosil ODS K-5 reversed-phase column: (A) 3 pmol each of neopterin, biopterin, and oncopterin; (B) a urine sample (5 μl) from a patient with kidney cancer; (C) a urine sample (5 μl) spiked with 3 pmol each of neopterin, biopterin, and oncopterin. Neopterin, biopterin, and oncopterin concentrations were calculated from the increased values in peak areas of internal standards and from the external standard, as shown in Table 1. Creatinine concentration was 54.4 mg/dl urine.

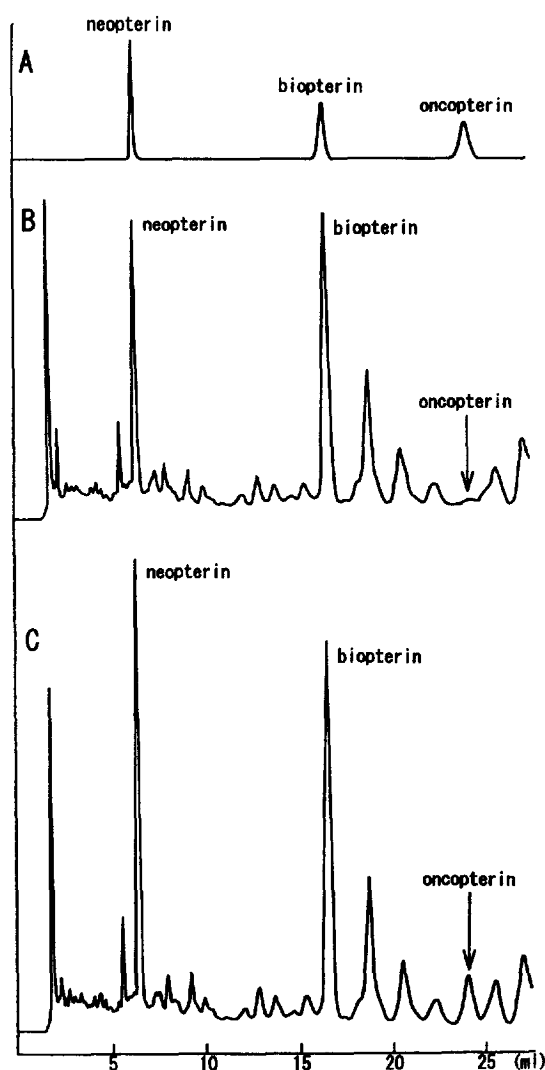

Fig. 3. Chromatograms of simultaneous separation of neopterin, biopterin, and oncopterin by HPLC on a Develosil ODS K-5 reversed-phase column: (A) 3 pmol each of neopterin, biopterin, and oncopterin; (B) a urine sample (5 μl) from a healthy subject; (C) a urine sample (5 μl) spiked with 3 pmol each of neopterin, biopterin and oncopterin. Neopterin, biopterin and oncopterin concentrations were calculated from the increased values in peak areas of internal standards and from the external standard, as shown in Table 1. Creatinine concentration was 126.0 mg/dl urine.

As shown in Table 2, healthy controls showed about 300 and 500 μmol/mol creatinine of neopterin and biopterin, but only about 1 μmol/mol creatinine of oncopterin. Neopterin and biopterin concentrations in urine from cancer patients were about two- to three-fold higher than those in urine from healthy controls, but only the increase in neopterin was statistically significant. In contrast, a marked, about 70-fold elevation in urinary oncopterin was observed in cancer patients, which was statistically significant.

Table 1
Intra-day variability of the simultanous assay of neopterin, biopterin, and oncopterin in a urine sample from a patient with kidney cancer and from a healthy control ($n = 5$)

Compound	Calculated from internal standard		Calculated from external standard		Recovery rate (%)
	Mean ± S.D. (μmol/mol creatinine)	C.V. (%)	Mean ± S.D. (μmol/mol creatinine)	C.V. (%)	
Cancer patient					
Neopterin	235 ± 5	2.1	216 ± 5	2.1	92.5
Biopterin	274 ± 18	6.6	269 ± 17	6.5	97.0
Oncopterin	272 ± 7	2.6	247 ± 6	2.5	94.3
Healthy control					
Neopterin	381 ± 23	6.0	368 ± 13	3.5	93.0
Biopterin	674 ± 21	3.2	544 ± 5	1.0	98.1
Oncopterin	6.9 ± 0.1	1.9	7.1 ± 0.3	4.6	92.6

4. Discussion

We have established an HPLC method to measure simultaneously three pteridines (neopterin, biopterin, and oncopterin) in urine as cancer markers. We had preliminarily described an HPLC method for the assay of urinary oncopterin [10] and reported an increase in various cancer patients. However, there are difficulties with the reproducibility and accuracy of the HPLC assay.

In the present study we have examined the

Table 2
Assay of neopterin, biopterin, and oncopterin in urine samples from healthy controls and from cancer patients

	Concentration (μmol/mol creatinine)[a]		
	Neopterin	Biopterin	Oncopterin
Healthy controls ($n = 10$)	254 ± 111	508 ± 186	1.1 ± 2.4
Cancer patients ($n = 11$)	659 ± 368[b]	1360 ± 1900	77.8 ± 65.1[b]
Stomach cancer	1250	6190	128
	1100	3900	68
Liver cancer	995	951	69
	260	532	59
	910	645	27
Testis cancer	273	518	24
	423	487	99
Kidney cancer	216	269	247
	734	477	25
	697	670	33
Bladder cancer	394	361	77

[a] Mean ± S.D.
[b] Significantly different from controls, $p < 0.01$.

conditions for reproducible and accurate determination of urinary oncopterin in patients with cancer and in healthy controls. The sensitivity of 1 pmol was high enough to measure oncopterin even in healthy controls. It was confirmed that patients with various solid cancers excreted high amounts of oncopterin in urine.

Oncopterin is composed of the elements of the two promising biochemical markers for cancers, pteridine and polyamine. Therefore, oncopterin is expected to be one of the most sensitive diagnostic markers of cancer.

The biosynthetic pathway of oncopterin is under investigation. The enzymatic reactions of putrescine with S-adenosylmethylthiopropylamine introduce one or two 3-aminopropyl groups to putrescine to yield spermidine or spermine, respectively. It is speculated that the 3-aminopropyl substituent in oncopterin also arises from S-adenosylmethylthiopropylamine. In this presumed biosynthetic pathway biopterin might be the precursor of oncopterin.

The present HPLC assay of neopterin, biopterin, and oncopterin should be useful both in the clinical study of pteridines as cancer markers and in the basic study on biochemistry of oncopterin, a newly described natural pteridine derivative.

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