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Technical note

Interference of a methotrexate derivative with urinary oncopterin [N^2-(3-aminopropyl)biopterin] measurement by high-performance liquid chromatography with fluorimetric detection

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Abstract

We previously reported a HPLC assay method using fluorimetric detection for the simultaneous determination of urinary N^2-(3-aminopropyl)biopterin (oncopterin, a natural pteridine newly found in urine from cancer patients), biopterin and neopterin. We now have observed that an unknown substance, which may be derived from methotrexate, in urine from a patient with stomach cancer interfered with the assay of oncopterin and demonstrated that oncopterin could be completely separated from the unidentified substance by HPLC using a Nucleosil 100-5SA strong cation-exchange column. Furthermore, oncopterin was not detectable by this HPLC–fluorimetric method in urine samples from patients with stomach cancer who were not treated with methotrexate. The content of urinary oncopterin from cancer patients is supposed to be very low, with less than 1 μmol/mol creatinine. The present results indicate that the peak found with elution from the C_{18} column was a methotrexate-derived compound and co-eluted with the analyte oncopterin.

Keywords: Oncopterin; N^2-(3-Aminopropyl)biopterin; Methotrexate

1. Introduction

We have previously described a high-performance liquid chromatography (HPLC)–fluorimetry procedure [1] for the simultaneous determination of N^2-(3-aminoprophyl)biopterin (oncopterin, a natural pteridine newly found in urine from cancer patients) [2,3], biopterin and neopterin in urine. By use of 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 were completely separated and measured simultaneously by fluorescence detection. The Develosil ODS-K-5 reversed-phase column gave the most satisfactory resolution. The lower detection limit was approximately 1 pmol for each pteridine derivative. Our previous results indicated that the oncopterin concentration was markedly increased in urine samples from cancer patients and could be a very sensitive marker for cancer.

However, in the present study, we discovered that an unknown substance, especially in urine from cancer patients treated with methotrexate, co-eluted with oncopterin, thus interfering with the assay of

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oncopterin under the HPLC conditions described in our previous report [1]. The interfering substance could be separated from oncopterin under different HPLC conditions using a Nucleosil 100-5SA strong cation-exchange column.

2. Experimental

2.1. Chemicals

N^2-(3-Aminopropyl)biopterin (oncopterin) was synthesized as described by Sawada et al. [4]. Develosil ODS-K-5 and Develosil ODS-HG-5 reversed-phase C_{18} columns were purchased from Nomura Chemicals (Seto, Aichi, Japan) and the Nucleosil 100-5SA strong cation-exchange column came 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 employs a silica surface coated with a polymeric layer. All reagents were of analytical or HPLC grade.

2.2. Chromatographic conditions

HPLC analysis was carried out either on a Develosil ODS-K-5 (150×4.6 mm I.D.; 5 μm) column, a Develosil ODS-HG-5 (150×4.6 mm I.D.; 5 μm) column or a Nucleosil 100-5SA (150×4.6 mm I.D.; 5 μm) column. Isocratic elution was performed at a flow-rate of 1.0 ml/min with the following solvents: for a reversed-phase column, 50 m*M* ammonium phosphate buffer, pH 3.0, at 23°C for Develosil ODS-K-5 or at 22°C for Develosil ODS-HG-5; for the Nucleosil 100-5SA strong cation-exchange col-

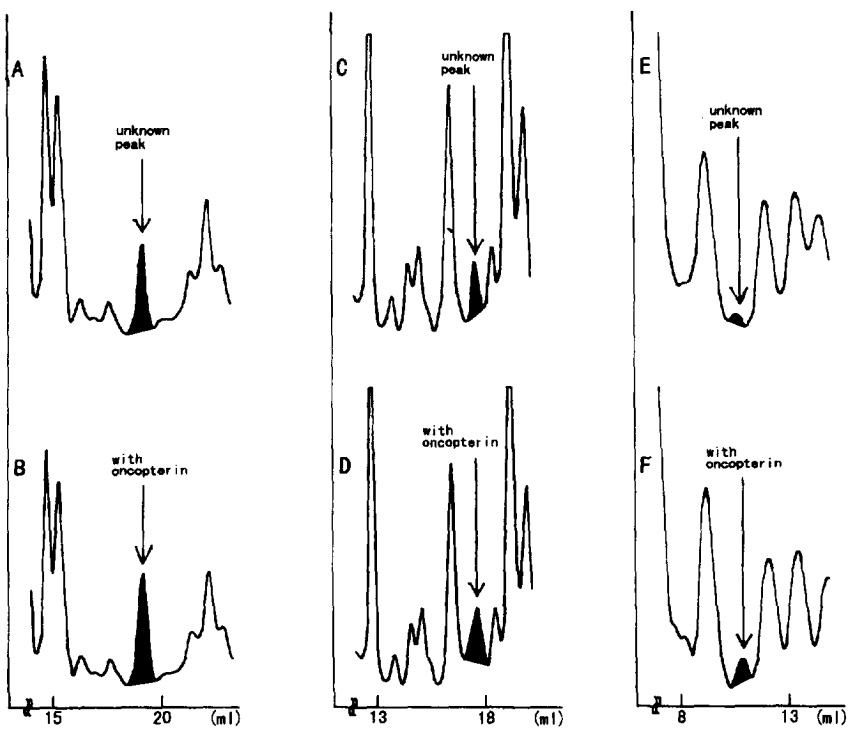

Fig. 1. Chromatograms of urine samples (5 μl) from a patient with stomach cancer under methotrexate chemotherapy. The chromatograms were obtained from a Develosil ODS-K-5 reversed-phase column eluted with 50 m*M* ammonium phosphate buffer, pH 3.0, without (A) or with (B) oncopterin (2 pmol) spiking; a Develosil ODS-HG-5 reversed-phase column eluted with 50 m*M* ammonium phosphate buffer, pH 3.0, without (C) or with (D) oncopterin (2 pmol) spiking; and a Nucleosil 100-5SA strong cation-exchange column eluted with 100 m*M* ammonium phosphate buffer, pH 3.0, without (E) or with (F) oncopterin (2 pmol) spiking.

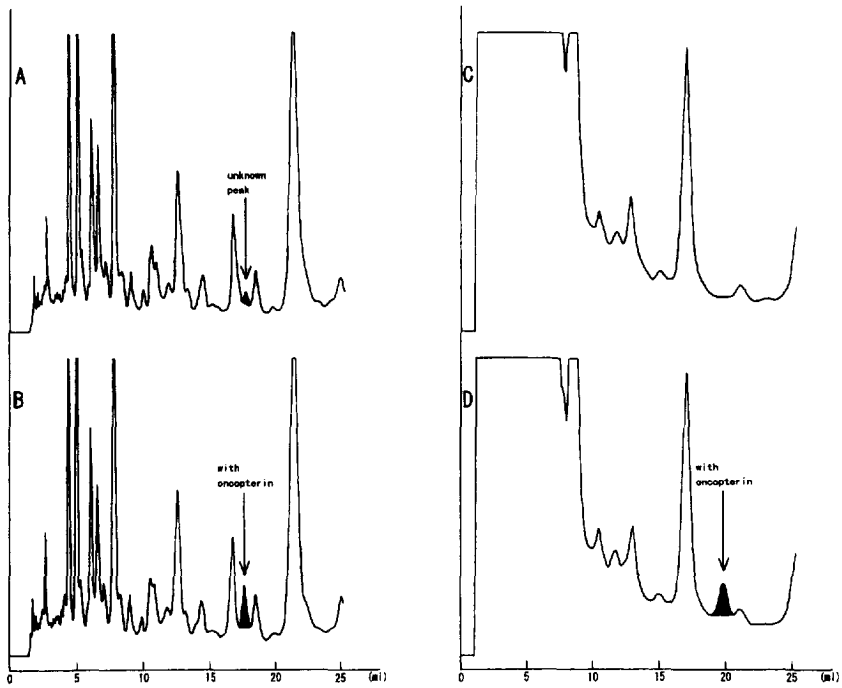

Fig. 2. Chromatograms of urine samples (5 μl) from a patient with stomach cancer under methotrexate chemotherapy. The chromatograms were obtained from a Develosil ODS-K-5 reversed-phase column eluted with 50 m*M* ammonium phosphate buffer, pH 3.0, without (A) or with (B) oncopterin (2 pmol) spiking and Nucleosil 100-5SA strong cation-exchange column eluted with 25 m*M* ammonium acetate buffer containing 10% methanol, pH 6, without (C) or with (D) oncopterin (4 pmol) spiking.

umn, 100 m*M* ammonium phosphate buffer, pH 3.0, at 26°C, or 25 m*M* ammonium acetate buffer containing 10% methanol, pH 6.0, at 25°C. A fluorescence detector (Jasco, Tokyo, Japan) was used with 355 nm excitation and 450 nm emission.

2.3. Preparation of urine samples

Samples from the first urination in the morning were frozen immediately after collection and kept at −80°C in the dark until analyzed. A mixture of urine (120 μl) and 6 *M* hydrochloric acid (60 μl) was heated in a glass tube, sealed with a rubber stopper, to 100°C for 2 h and then lyophilized. The residue was mixed well with the HPLC solvents 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 was measured by a photometric method [5].

3. Results

HPLC chromatograms of oncopterin in urine from a patient with stomach cancer, without or with oncopterin (2 pmol) spiking and obtained from reversed-phase Develosil ODS-K-5, Develosil ODS-HG-5, or strong cation-exchange Nucleosil 100-5SA column under the conditions described previously [1], are shown in Fig. 1. The unknown substance at the position of the oncopterin had the same retention time as the added oncopterin on each of the three columns.

In contrast to the Develosil ODS-K-5 column eluted with 50 m*M* ammonium phosphate buffer (pH 3.0), which gave a single peak without or with oncopterin spiking (Fig. 2A and Fig. 2B), the Nucleosil 100-5SA column eluted with 25 m*M* ammonium acetate buffer containing 10% methanol (pH 6.0) separated the unknown substance from the oncopterin (Fig. 2C and Fig. 2D).

We found that the cancer patient was being treated with methotrexate, so we analyzed a urine sample from a patient with stomach cancer without methotrexate treatment. As shown in Fig. 3, no oncopterin peak was identified. When a methotrexate aqueous solution was subjected to the same acid hydrolysis procedure as the urine sample, an unidentified substance was co-eluted with oncopterin from the Develosil ODS-K-5 (Fig. 4B and Fig. 4C). This peak was completely separated from the oncopterin peak on a Nucleosil 100-5SA strong cation-exchange column eluted with 25 m*M* ammonium acetate buffer containing 10% methanol (pH 6.0) (Fig. 4E and Fig. 4F). The unknown peak that co-eluted with oncopterin on the C_{18}-stationary phase appeared only when the methotrexate solution was subjected to the acid hydrolysis procedure. When methotrexate was directly injected into the columns, no interfering peak appeared at the retention time of oncopterin. The results indicate that an unknown substance derived from methotrexate co-eluted with the analyte oncopterin on a C_{18}-stationary phase, but was resolved on cation-exchange material.

The within-run and between-run coefficients of variation ($n=5$) of oncopterin on cation-exchange material was 4.9 and 4.1%, respectively. The recovery of spiked 4 pmol oncopterin was 90.9±4.5 (S.D.) % ($n=5$). The limit of detection was 1 pmol at a signal-to-noise ratio of 5.

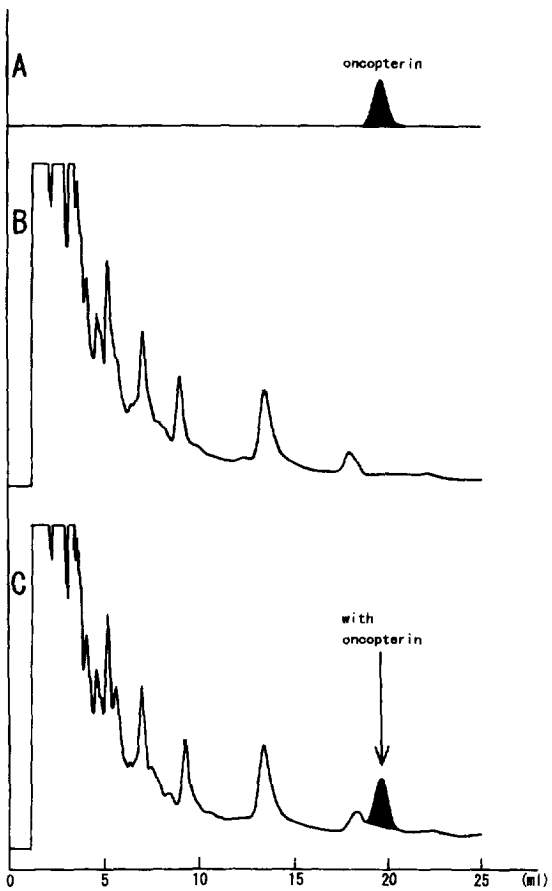

Fig. 3. Chromatograms from a Nucleosil 100-5SA strong cation-exchange column eluted with 25 m*M* ammonium acetate buffer containing 10% methanol, pH 6.0: (A) 4 pmol oncopterin; (B) a urine sample (5 μl) from a patient with stomach cancer before administration of methotrexate; (C) the same sample as in (B) but spiked with oncopterin (4 pmol).

4. Discussion

We also previously reported that oncopterin, a new pteridine derivative isolated from urine of cancer patients, could be measured simultaneously with biopterin and neopterin by HPLC–fluorimetry [1] and that urinary oncopterin was greatly elevated in various types of cancer. In the present study, however, an oncopterin-like substance in urine from cancer patients was separated from oncopterin by HPLC using a Nucleosil 100-5SA strong cation-exchange column. The patients under treatment with methotrexate showed a compound that co-eluted with oncopterin on a Develosil ODS-K-5 reversed-phase column (Figs. 1 and 2). No oncopterin appeared in urine from cancer patients without methotrexate treatment (Fig. 3). These results suggest that the fluorescent substance that co-eluted with oncopterin on a Develosil ODS-K-5 reversed-phase column is not oncopterin and may be a compound derived from methotrexate.

As we also reported previously, a very low but significant oncopterin-like peak appeared in normal subjects [1]. However, we did not observe this peak on a HPLC chart in normal urine with a Nucleosil 100-5SA strong cation-exchange column. This substance in normal subjects may not be oncopterin, but could be some unknown pteridine-like substance(s), possibly derived from food.

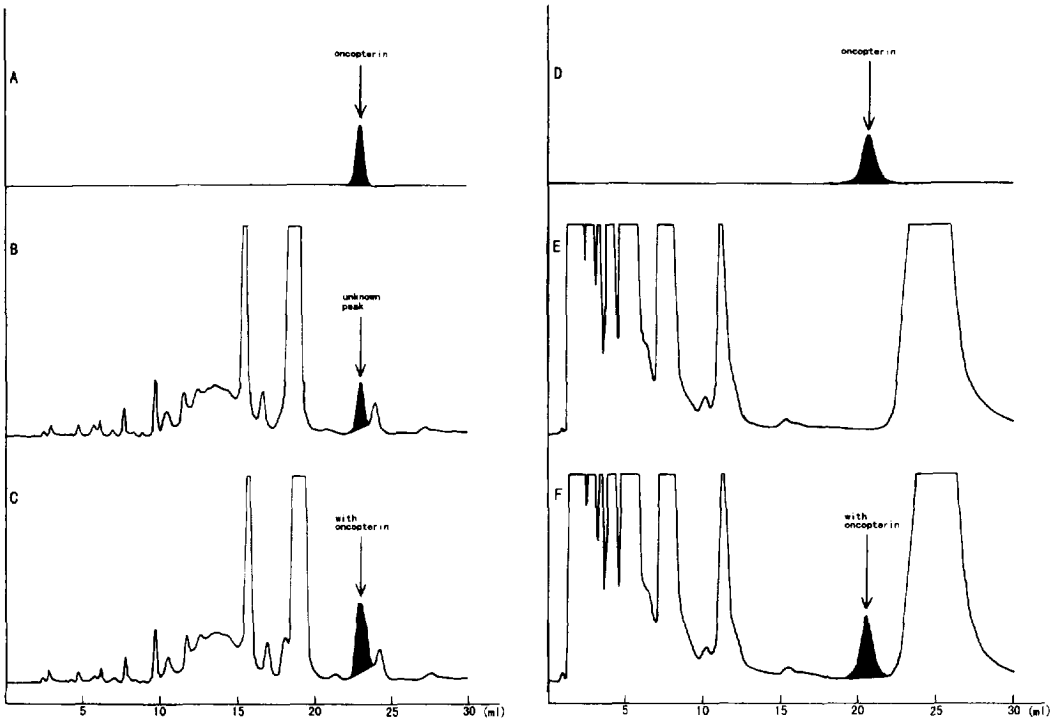

Fig. 4. Chromatograms of a methotrexate aqueous solution subjected to the same acid hydrolysis procedure as the urine sample: (A)–(C) the solution on a Develosil ODS-K-5 reversed-phase column eluted with 50 m*M* ammonium phosphate, pH 3.0; (D)–(F) on a Nucleosil 100 5-SA strong cation-exchange column eluted with 25 m*M* ammonium acetate buffer containing 10% methanol, pH 6.0. (A) and (D), 4 pmol oncopterin; (B) and (E), a methotrexate aqueous solution; (C) and (F), a methotrexate aqueous solution spiked with 4 pmol oncopterin.

Oncopterin was originally isolated and identified in urine from cancer patients treated with methotrexate, but not in urine from normal subjects [2]. However, it is difficult to speculate from the chemical structure that oncopterin is a metabolite of methotrexate. Therefore, we speculate that oncopterin is a new endogenous pteridine and that the amount in cancer patients or in normal subjects may be less than the lower limit of detection (1 pmol) of our HPLC method.

In conclusion, the substance we eluted from the C_{18} column is a methotrexate-derived compound that co-elutes with the analyte oncopterin. A specific assay method with increased sensitivity for oncopterin in urine from normal subjects and cancer patients is currently under investigation.

Acknowledgments

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