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Note

Highly sensitive assay for xanthine oxidase activity by high-performance liquid chromatography with fluorescence detection

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Xanthine oxidase (xanthine:oxygen oxidoreductase, EC 1.2.3.2) (XO) oxidizes purine, pyrimidine, aldehydes and NADH and is widely distributed among species and within various mammalian organs and bacteria [1]. Bovine milk XO has been intensively studied. This enzyme is a complex flavoenzyme and contains molybdenum, iron and FAD. XO exists in vivo predominantly as an NAD^+-dependent dehydrogenase, which can be transformed into an oxygen-dependent oxidase under various conditions. XO is the rate-limiting enzyme in nucleic acid degradation, but its physiological role is not clearly understood. The enzyme is related to gout and xanthinuria. Gout is characterized by excess uric acid deposition in synovial fluid and tissues, whereas xanthinuria is a hereditary disease lacking XO with a high concentration of extracellular xanthine [2]. XO probably plays an important role in the absorption of iron in small intestine and mobilization of iron in the liver [3, 4], and is assumed to produce oxygen radicals in various tissues, including polymorphonuclear leucocytes [5]. It was also reported that free radicals are formed by XO in organs damaged by ischaemia [6].

The enzymatic activity of XO has been measured by several methods, including manometry [7], colorimetry [8], fluorimetry [9, 10], spectrophotometry [11, 12], radiometry [13, 14] and electrometry [15].

This paper describes a highly sensitive and simple assay method using high-performance liquid chromatography with fluorescence detection (HPLC–FD).

EXPERIMENTAL

Materials

2-Amino-4-hydroxypteridine (AHP), isoxanthopterin (IXP) and lumazine (LZ) were obtained from Sigma (St. Louis, MO, U.S.A.). A Unisil ODS (3 μm)

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packed column was obtained from Gasukuro Industrial (Osaka, Japan). All other chemicals used were of analytical-reagent grade. Glass-distilled water was further purified by using a Milli-Q II system (Millipore, Bedford, MA, U.S.A.).

Enzyme samples were obtained from rat liver, kidney and brain. Each organ was homogenized in four volumes of 100 m*M* sodium phosphate buffer (pH 7.0) per gram wet weight, using a Teflon homogenizer, and centrifuged at 15 000 *g* for 30 min. The supernatant was desalted with Sephadex G-25 and served as an enzyme sample.

Assay of XO activity

The standard reaction mixture consisted of 50 μl of 0.5 *M* sodium phosphate buffer (pH 7.0), 25 μl of 200 μM AHP solution, 25 μl of 10 μM 2,6-dichlorophenolindophenol sodium (DCPIP), 10 μl of an enzyme sample and 10 μl of 10 mg/ml bovine serum albumin, diluted to 250 μl with distilled water. This mixture was incubated at 37°C for 10 min, and the reaction was stopped by adding 25 μl of 60% perchloric acid and cooling for 10 min in an ice-bath. The mixture was centrifuged at 1000 *g* for 10 min and a 10-μl aliquot of the supernatant was injected into the HPLC system. For the controls, the enzyme was denatured by adding perchloric acid before the substrate solution was added.

The concentration of protein was determined using a Bio-Rad Protein Assay Kit (Bio-Rad Labs., Richmond, CA, U.S.A.) using bovine γ-globulin as a standard.

Chromatographic conditions

The HPLC system consisted of a Gilson Type 305 pump (Gilson, Villiers Le Bel, France), an RF-305 fluorescence detector (Shimadzu, Kyoto, Japan), a Rheodyne 7125 injector with a 100-μl sample loop (Rheodyne, Berkeley, CA, U.S.A.) and a Unisil ODS analytical column (50×4.6 mm I.D.).

The mobile phase was 0.1 *M* sodium phosphate buffer (pH 2.0), which had been filtered through a 0.45-μm membrane filter (Toyo Roshi, Tokyo, Japan) and degassed with a water aspirator for a few minutes prior to use. The flow-rate was 1.0 ml/min. Under these conditions the retention times were AHP 2.3 min and IXP 5.3 min. The fluorescence was detected at 410 nm with excitation at 340 nm.

RESULTS

The excitation and emission spectra of AHP, IXP and LZ are shown in Fig. 1. The maximum wavelengths of excitation and emission of IXP, AHP and LZ were 343 and 410 nm, 323 and 470 nm and 328 and 493 nm, respectively. Fig. 2 shows the elution pattern of AHP, IXP and LZ. They were completely separated within 9 min. Only IXP was highly fluorescent, both AHP and LZ being less fluorescent under the conditions used.

IXP was measured with very high sensitivity and with ease by using this HPLC–FD system. The peak-height response of the fluorescence detector was linear for amounts of IXP injected in the range 30 fmol–50 pmol. The rate of IXP formation using the soluble fraction of a rat liver homogenate as an enzyme sam-

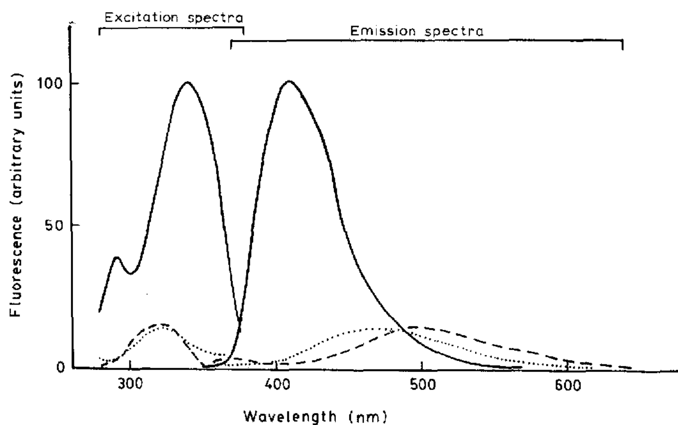


Fig. 1. Fluorescence excitation and emission spectra of isoxanthopterin (IXP), 2-amino-4-hydroxypteridine (AHP) and lumazine (LZ) at the maximum emission and excitation wavelengths. The maximum fluorescence intensity was obtained at the following excitation and emission wavelengths: IXP, 343 and 410; AHP, 323 and 470; LZ, 328 and 493 nm. —, IXP (10 nmol/ml); ·····AHP (200 nmol/ml); - - - -, LZ (200 nmol/ml).

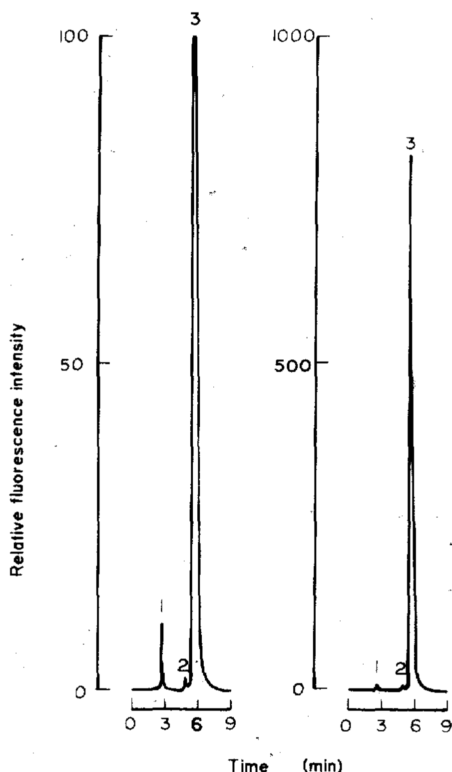
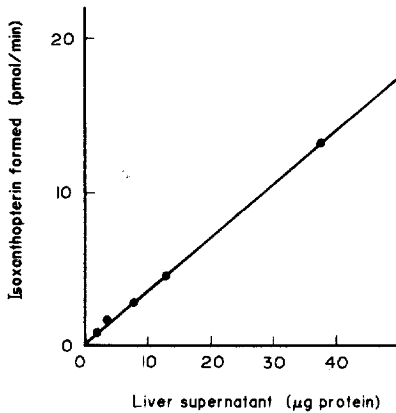


Fig. 2. HPLC elution pattern of the standard compounds (10 pmol each). Peaks: 1 = 2-amino-4-hydroxypteridine (AHP); 2 = lumazine (LZ); 3 = isoxanthopterin (IXP).



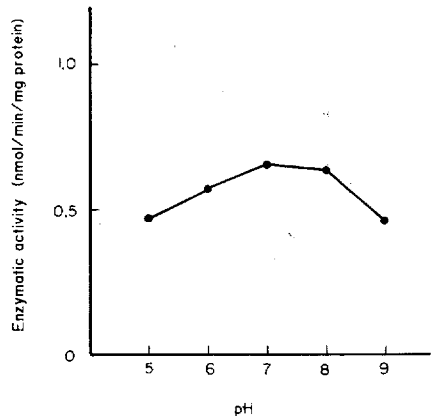


Fig. 3. Xanthine oxidase activity in the soluble fraction of rat liver as a function of enzyme concentration. The standard incubation system was used and incubation was carried out for 10 min at 37°C.

Fig. 4. Effect of pH on the formation of isoxanthopterin by xanthine oxidase in the soluble fraction of rat liver homogenate. Standard incubation mixture containing 10 μl (3 μg of protein) of the enzyme solution and 0.1 *M* sodium phosphate buffer of various pH was used under the conditions described under Experimental.

ple proceeded linearly up to 30 min at 37°C. Accordingly, we incubated the mixture for 10 min in a standard assay. XO activity as a function of enzyme concentration is shown in Fig. 3. Linearity was observed between 0.5 and 40 μg of protein in the soluble fraction. The XO activity in rat liver showed a broad pH optimum in the neutral region with maximum activity over the pH range 7–8 (Fig. 4); we adopted pH 7.0 for the standard assay. Fig. 5 shows the Lineweaver–Burk plot of XO from a rat liver for AHP as a substrate. XO obeyed simple Michaelis–Menten-type kinetics; the Michaelis constant (K_M) was 2 μM

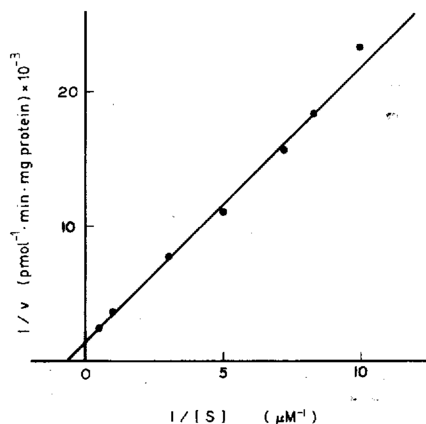


Fig. 5. Lineweaver-Burk plot of xanthine oxidase from rat liver homogenate. Standard incubation mixture containing 10 μl (3 μg of protein) of soluble fraction was used and incubation was carried out for 20 min at 37°C.

for AHP, and the maximum velocity (V_{max}) was approximately 1 nmol/min per mg protein. The method is reproducible. A coefficient of variation of about 10% for the peak height of IXP was obtained.

We applied the method to the measurement of the XO activity in different organs. XO activities in rat kidney and brain in the presence and absence of DCPIP were 152 and 120 pmol/min per mg protein for kidney and 1.06 and 1.05 pmol/min per mg protein for brain, respectively.

DISCUSSION

The sensitivity of the proposed method is approximately 100 times higher than that of ordinary fluorescence assay, because the blank value decreased after HPLC separation of IXP. The limit of sensitivity is approximately 90 fmol/min of IXP enzymatically produced. The sensitivity was found to be comparable to that of the most sensitive radiometric assays, in which the limit of detection is approximately 10 fmol/min of IXP [14].

The procedure is simple. We employed perchloric acid to stop the enzyme reaction and to precipitate proteins. In this reagent the product (IXP) was stable for several hours. The deproteinized reaction mixture can be directly analysed by HPLC, so there is no need to isolate IXP from the reaction mixture as in radiometric assays. Pterin deaminase (EC 3.5.4.11) is known to deaminate AHP as a substrate to form LZ. The HPLC conditions described in this paper permitted the complete separation of IXP, LZ and AHP within 9 min. Therefore, the XO activity could be measured exactly in the presence of pterin deaminase.

The method makes it possible to measure the sum of NAD^+-dependent dehydrogenase and oxygen-dependent oxidase activities by using DCPIP as an electron acceptor, because electrons are transferred directly to DCPIP instead of FAD, an inherent electron acceptor. Oxidase activity alone can be assayed by removing DCPIP from the reaction mixture.

The Michaelis constants for AHP with XO for various preparations and under different conditions have been reported to be 1.5–2.0 μM [16]. In this study, a similar K_M value was observed (0.571 μM with DCPIP and 1.96 μM without DCPIP).

As the method is sensitive, simple and rapid it may be useful for physiological studies with small amounts of enzyme preparations.

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