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Determination of human serum semicarbazide-sensitive amine oxidase activity *via* flow injection analysis with fluorescence detection after online derivatization of the enzymatically produced benzaldehyde with 1,2-diaminoanthraquinone

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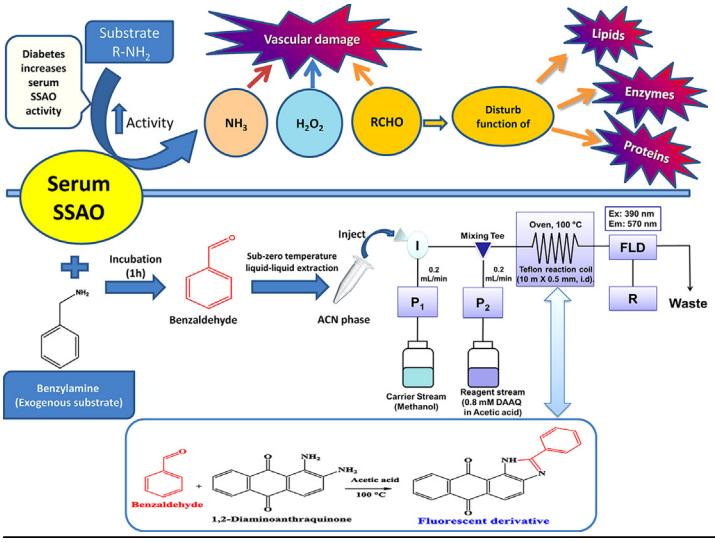
HIGHLIGHTS

- The first FIA-FL method was developed for measurement of serum SSAO activity.
- SSAO causes oxidative deamination to benzylamine, generating benzaldehyde.
- 1,2-Diaminoanthraquinone was used as a novel fluorogenic reagent for benzaldehyde.
- The method is sensitive, selective, and rapid with a sample throughput of 27 h^{-1}.
- The method was applied to compare SSAO activity in different clinical conditions.

GRAPHICAL ABSTRACT



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ABSTRACT

A fast, simple, and sensitive flow injection analysis method was developed for the measurement of semicarbazide-sensitive amine oxidase (SSAO) activity in human serum. Benzaldehyde, generated by the action of SSAO after incubation of serum with benzylamine, was derivatized with a novel aromatic aldehyde-specific reagent (1,2-diaminoanthraquinone) and the fluorescent product was measured by fluorescence detection at excitation and emission wavelengths of 390 and 570 nm, respectively. Serum SSAO activity was defined as benzaldehyde (nmol) formed per milliliter serum per hour. The method was linear over SSAO activity of 0.2–150.0 nmol mL^{-1} h^{-1} with a detection limit of 0.06 nmol mL^{-1} h^{-1}. The % RSD of intra-day and inter-day precision did not exceed 9.4% and the accuracy ranged from −6.5 to −0.6%.

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Benzaldehyde
1,2-Diaminoanthraquinone
Flow injection analysis
Fluorescence detection

The method was applied for the determination of the serum SSAO activity in healthy controls (C, $n = 24$) and diabetes mellitus patients (DM, $n = 18$). It was demonstrated that the activity (mean ± SE) of SSAO in diabetics sera was significantly higher than that in healthy subjects' ones (DM; 73.3 ± 1.8 nmol mL^{-1} h^{-1} *vs* C; 58.9 ± 2.2 nmol mL^{-1} h^{-1}, $P < 0.01$).

1. Introduction

Semicarbazide-sensitive amine oxidase (SSAO) is a family of heterogenous enzymes that can catalyze the deamination of various exogenous and endogenous mono-amines. It is a copper-containing glycoprotein possessing topa-quinone as a cofactor. These enzymes are found in most of the mammalians in two forms: tissue-bound and soluble isoforms (*i.e.*, serum SSAO) [1,2]. The SSAO enzymes are different from the monoamine oxidases (MAO-A and MAO-B) in their inhibition pattern: they have tolerance to MAO inhibitors like clorgiline, pargyline, and deprenyl, but sensitive to semicarbazide and other hydrazines [3]. SSAO causes oxidative deamination to various amine compounds including dopamine, polyamines, tyramine, tryptamine, and benzylamine by converting them to the corresponding aldehydes with the generation of hydrogen peroxide and ammonia. Oxidation of methyl amine, allylamine, and aminoacetone by SSAO gives rise to formaldehyde, acrolein, and methylglyoxal, respectively [3]. These products are all potentially cytotoxic, *e.g.*, formaldehyde is well known to be a major environmental risk factor, acrolein and methylglyoxal may disrupt the functions of proteins, lipids, and enzymes, and hydrogen peroxide is a major generator of oxidative stress which is commonly associated with numerous diseases [4]. The activity of serum SSAO has been reported to be increased in certain clinical conditions such as heart failure [5] and diabetes mellitus [6,7]. Also, when the activity of SSAO is high, the increased enzymatic-mediated deamination is proposed to be involved in the pathogenesis of many vascular disorders [8]. Hence, the determination of SSAO might be a useful marker in the prognostic evaluation of diabetic angiopathy [9].

Several methods have been described for the measurement of SSAO activity, mostly using benzylamine as the preferred substrate. By the action of SSAO, benzylamine is deaminated and oxidized into benzaldehyde, hydrogen peroxide, and ammonia. These methods include HPLC-fluorescence detection after derivatization of benzaldehyde with dimedone [10], HPLC-UV detection [11] and light scattering measurement [12] after derivatization with 2,4-dinitrophenylhydrazine, and radiometric method using [^{14}C]-benzylamine as enzyme substrate [13]. Other methods depend on direct measurement of the enzyme using LC–MS [14], reverse transcription polymerase chain reaction (RT-PCR) [15], and Western blot and immunoassays [2,16]. Although some of these methods are sensitive enough to give valuable data about the SSAO activity, most of them suffer from various drawbacks such as time-consumption, tedious extraction procedures, and poor recoveries of benzaldehyde from biological fluids [11,12], long derivatization reaction times [10–12], use of radioactive compounds [13], harsh condition (*e.g.*, 9 M sulfuric acid) [10], and the use of sophisticated and expensive instruments [2,14–16]. Also, these methods that are based on the direct measurement of the SSAO enzyme [2,14–16] do not reflect its activity inside the human body. In addition, the RT-PCR method [15] was not able to make a precise determination of the expression of SSAO neither *in-vivo* nor *in-vitro*. Also, the Western blot and immunoassays [2,16] need expensive antibodies. So, these methods are not suitable for routine analysis of a large number of samples and there is a need to develop a sensitive, fast, simple, and convenient method for the determination of SSAO activity in serum.

In the present study, we developed a flow injection analysis (FIA) method for the determination of SSAO activity in serum that allows the processing of a large number of samples within a short analysis time. The method is based on incubation of serum with benzylamine, an exogenous SSAO substrate. Then the benzaldehyde generated by the SSAO activity is derivatized online with the novel aromatic aldehyde-specific reagent 1,2-diaminoanthraquinone (DAAQ) and the formed imidazole derivative is determined by fluorescence detection at excitation and emission wavelengths of 390 and 570 nm, respectively. Serum SSAO activity is defined as benzaldehyde (nmol) formed per milliliter serum per hour.

DAAQ is a non-cytotoxic and non-fluorescent probe that was used previously for nitric oxide sensing through the formation of a fluorescent triazole derivative which is detectable by means of fluorescence microscopy [17]. The use of DAAQ as a derivatizing reagent for aldehydes has not been previously reported, which indicates the novelty of the proposed method. Also, the non-fluorescent property of DAAQ allows its use for online derivatization in FIA.

2. Experimental

2.1. Materials and reagents

Benzaldehyde, benzylamine, and glacial acetic acid were purchased from Wako Pure Chemical Industries (Osaka, Japan). Sodium dihydrogenphosphate dihydrate ($NaH_2PO_4{\cdot}2H_2O$) and disodium hydrogen phosphate (Na_2HPO_4) were obtained from Nacalai Tesque (Kyoto, Japan). DAAQ and clorgiline were purchased from Sigma–Aldrich (St. Louis, MO, USA). Acetonitrile and methanol (HPLC grade) were obtained from Kanto Chemical Company (Tokyo, Japan). The water used was purified by a Simpli Lab UV (Millipore, Bedford, MA, USA). Phosphate buffer solution (PBS, 0.1 M, pH 7.8) was prepared by dissolving 0.13 g of Na_2HPO_4 and 0.14 g of $NaH_2PO_4{\cdot}2H_2O$ in 100 mL distilled water and a Horiba F22 pH-meter was used to check the pH of the buffer. Stock solution of benzaldehyde (10 mM) was prepared in acetonitrile and stock solutions of clorgiline (0.2 mM) and benzylamine (5.6 mM) were prepared in PBS. 8.0 mM and 0.8 mM solutions of DAAQ were prepared in glacial acetic acid. Because of the expected photosensitivity of DAAQ, its solution was kept in amber-colored glass bottles. It was found to be stable for at least 2 weeks when kept at 4 °C in the refrigerator.

2.2. Confirmation of the identity of benzaldehyde–DAAQ derivative

The method of Huang et al. [18] was followed for the synthesis of benzaldehyde–DAAQ derivative. The obtained yellowish-brown compound was subjected to electron impact mass spectrometry (EI-MS) to identify the molecular ion and ^{1}H NMR studies to prove the structure. The EI-MS spectra were recorded using JMS DX-303 mass spectrometer (Joel Ltd., Japan) and ^{1}H NMR spectra were recorded using Varian Inova-500 (500 Hz) spectrometer (Varian, CA, USA).

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