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Analytical method for lipoperoxidation relevant reactive aldehydes in human sera by high-performance liquid chromatography–fluorescence detection

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ABSTRACT

A validated, simple and sensitive HPLC method was developed for the simultaneous determination of lipoperoxidation relevant reactive aldehydes: glyoxal (GO), acrolein (ACR), malondialdehyde (MDA), and 4-hydroxy-2-nonenal (HNE) in human serum. The studied aldehydes were reacted with 2,2′-furil to form fluorescent difurylimidazole derivatives that were separated on a C_{18} column using gradient elution and fluorescence detection at excitation and emission wavelengths of 250 and 355 nm, respectively. The method showed good linearity over the concentration ranges of 0.100–5.00, 0.200–10.0, 0.200–40.0, and 0.400–10.0 nmol/mL for GO, ACR, HNE, and MDA, respectively, with detection limits ranging from 0.030 to 0.11 nmol/mL. The percentage RSD of intraday and interday precision did not exceed 5.0 and 6.2%, respectively, and the accuracy (%found) ranged from 95.5 to 103%. The proposed method was applied for monitoring the four aldehydes in sera of healthy, diabetic, and rheumatic human subjects with simple pretreatment steps and without interference from endogenous components. By virtue of its high sensitivity and accuracy, our method enabled detection of differences between analytes concentrations in sera of human subjects under different clinical conditions.

Oxidative stress has been implicated in a range of diseased conditions, including diabetes [1], hypertension [2], atherosclerosis [3], Alzheimer's disease [4], and rheumatoid arthritis [5]. Oxidants, including reactive oxygen species (ROS),[1] are constantly produced in cells through normal metabolic processes. Oxidative stress occurs when the balance of oxidants within the cell exceeds the levels of antioxidants [6]. The hallmarks of oxidative stress include lipoperoxidation, protein oxidation, and DNA oxidation. The peroxidation of membrane-derived lipid molecules is known to give rise to many products through a series of iterative oxidation and cleavage reactions. The most commonly characterized products are aldehydes. Damage caused by aldehydes can disturb the function of proteins and enzymes, initiating further damage to lipids and leading to the formation of DNA adducts. In addition, some reactive aldehydes such as glyoxal (GO), acrolein (ACR), malondialdehyde (MDA), and 4-hydroxy-2-nonenal (HNE) can reduce intracellular glutathione levels, thereby leading to increased oxidant imbalance within the cell [6,7]. These types of molecular distresses may lead to cell death [8].

Among the carbonyl compounds produced as a result of lipoperoxidation, alkanals are the least reactive. Alkenals containing unsaturated bonds, such as ACR, are usually an order of magnitude more reactive than the alkanals. 4-Hydroxy-2-alkenals, such as HNE, are extremely reactive due to increased reactivity of the α,β-unsaturated bond by the close proximity of the electron-withdrawing hydroxyl at C_4 and the C_1-carbonyl group [6]. The dialdehydes, such as GO and MDA, are also very reactive since the two aldehydic moieties can form Schiff bases with amino acids [6]. Consequently, these four lipoperoxidation relevant reactive aldehydes (LPRRAs), namely GO, ACR, MDA, and HNE, are considered the most reactive and harmful, and could be used as good biomarkers of oxidative damage and disease progression [9]. So there is a strong need for a method that could simultaneously determine LPRRAS to investigate their pattern under healthy and diseased conditions.

Levels of these LPRRAs have been determined in different matrices either individually or in combination with each other or other carbonyl compounds as has been reviewed by Shibamoto [10].

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[1] *Abbreviations used:* ACR, acrolein; FL, fluorescence; GO, glyoxal; HNE, 4-hydroxy-2-nonenal; LPRRAs, lipoperoxidation relevant reactive aldehydes; MDA, malondialdehyde; ROS, reactive oxygen species; TBA, thiobarbituric acid.

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Some methods were recently published for determination of GO such as GC–MS [11], GC–flame ionization detection [12], and HPLC–FL following derivatization with 5,6-diamino-2,4-hydroxypyrimidine sulfate [13] or 4-methoxy-*o*-phenylenediamine [14]. ACR has been determined by HPLC–FL after derivatization with luminarin [15] or 3-aminophenol [16]. MDA has also been determined by LC and GC methods [17], HPLC–CL using a potassium permanganate/formaldehyde system [18], capillary electrophoresis–FL after derivatization with thiobarbituric acid [19], and surface-enhanced Raman spectroscopy after derivatization with thiobarbituric acid also [20]. As for HNE, HPLC–UV detection based on derivatization with 2,4-dinitrophenylhydrazine [21] and GC–electron capture detection [22] have been reported for its determination. Only one GC method with nitrogen phosphorous detection was developed for the simultaneous determination of ACR, MDA, and HNE in lipids following derivatization with *n*-methylhydrazine [23]. Another LC–MS method has been developed for simultaneous determination of several classes of aldehydes including ACR, MDA, and HNE in exhaled breath condensate after derivatization with 2,4-dinitrophenylhydrazine [24]. These methods involved the use of expensive sophisticated instrumentation that is not available in many laboratories and are applied to either lipids [23] or exhaled breath condensate only [24]. This initiates the present study to develop and validate a new analytical method for the simultaneous determination of these LPRRAs in human serum and estimation of their pattern under diseased conditions.

In the present study, a sensitive and selective derivatizing reagent, 2,2′-furil, was used in the presence of ammonium acetate for precolumn derivatizaion of LPRRAs to convert them to highly fluorescent difurylimidazole derivatives. A reaction scheme illustrating the mechanism of fluorogenic derivatization reaction of the targeted aliphatic aldehydes with 2,2′-furil is presented in Fig. 1. 2,2′-Furil has been applied in our laboratory as a new fluorogenic derivatizing agent for the determination of medium-chain-length aliphatic aldehydes in human serum [25]. This reagent has been proven to be selective for aldehydes; also it is stable and safe compared with other fluorescence derivatization reagents such as hydrazine-based reagents [25]. In this study, we adopted the gradient elution to separate the peaks of LPRRAs derivatives from blank peaks within a short time. Hence, the proposed method was applied for the determination of LPRRAs in sera of diabetic and rheumatoid arthritis patients with simple pretreatment steps without interference from biological components. The patterns of these oxidative stress biomarkers in sera of healthy, diabetic, and rheumatic patients were investigated.

Experimental

Materials and reagents

All reagents were of analytical grade and used as received. Acrolein monomer (90%, w/v) and 2,2′-furil were obtained from Tokyo Chemical Industries (Tokyo, Japan). Glyoxal (40%, w/v) and malondialdehyde tetrabutylammonium salt (96%, w/w) were supplied from Sigma Aldrich (St. Louis, MO, USA). 4-Hydroxy-2-nonenal (1%, w/v) was purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Ammonium acetate, citric acid monohydrate, and phosphate buffer saline (PBS) powder were purchased from Wako Pure Chemical Industries (Osaka, Japan). Methanol (HPLC grade) was obtained from Kanto Chemical Company (Tokyo, Japan). Glacial acetic acid and disodium hydrogen phosphate dodecahydrate were purchased from Nacalai Tesque (Kyoto, Japan). The water used was purified by a Simpli Lab UV (Millipore, Bedford, MA, USA). Stock solutions of GO, ACR, MDA, and HNE (5.0 mM) were prepared in methanol. To obtain reproducible results, a mixed standard solution containing the four aldehydes (200.0 μM of each aldehyde) was daily prepared by diluting the stock solutions with methanol and then diluted with the same solvent as needed to obtain the required concentrations. A 8.0 mM 2,2′-furil and 3.0 M ammonium acetate were prepared in methanol and glacial acetic acid, respectively. A 0.01 M PBS was prepared in water. All solutions were kept in the refrigerator at 4 °C except for the HNE stock solution and the mixed standard solution containing the four aldehydes were kept at −80 °C.

Instruments

The HPLC system consisted of two Shimadzu LC-20AD pumps (Kyoto, Japan), a Rheodyne injector (Cotati, CA, USA) with a 20 μL sample loop, a Shimadzu RF-20AXS fluorescence detector, and an

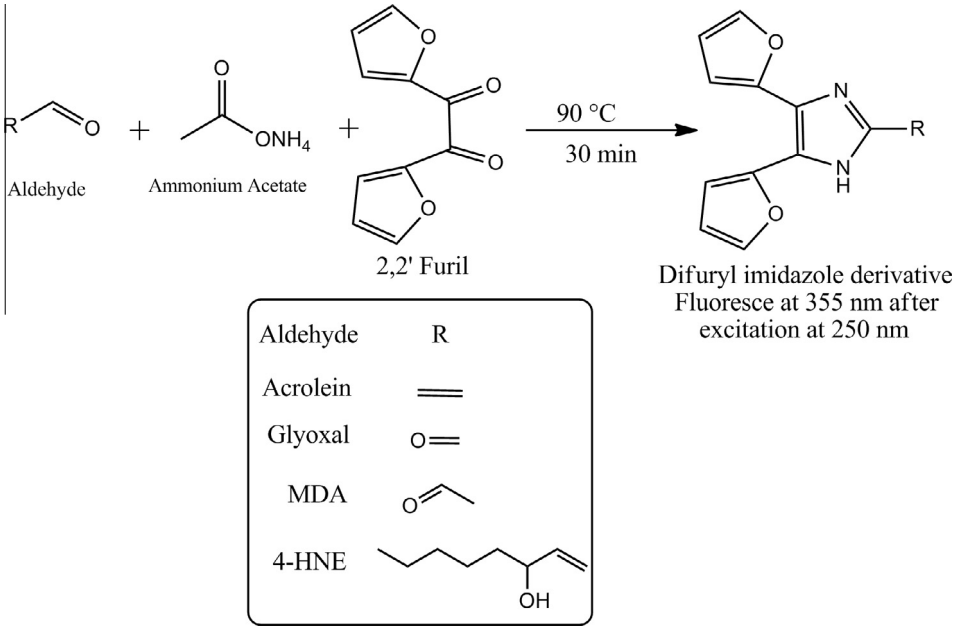

Fig.1. The reaction scheme for fluorogenic derivatization of target aldehydes with 2,2′-furil.