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Ultrahigh performance liquid chromatography–triple quadrupole mass spectrometry inhibitors fishing assay: A novel method for simultaneously screening of xanthine oxidase inhibitor and superoxide anion scavenger in a single analysis

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ABSTRACT

Xanthine oxidase (XOD) inhibitors and superoxide anion scavengers play an important role in the treatment of gout and the inhibition of many diseases related to superoxide anion. The respective quantitation of uric acid and superoxide anion by traditional spectroscopic methods is routine in XOD inhibitors and superoxide anion scavengers screening at laboratories worldwide. In the present study, we established an ultrahigh performance liquid chromatography and triple quadrupole mass spectrometry (UHPLC–TQ-MS) method of higher accuracy and speed that combines screening of superoxide anion scavenger and XOD inhibitor in a single analysis by adding WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium sodium salt) to the enzymatic reaction. We applied the established method to determine the XOD inhibitory activities and superoxide scavenging activities of some herbal extracts and compounds from natural products, which could be classified into six groups based on the results of the assay. Our innovative protocol is fast, accurate and robust. Moreover, it can eliminate false positive and false negative results which may occur in the traditional spectroscopic methods.

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1. Introduction

Xanthine oxidase (XOD, EC 1.1.3.22) is an enzyme which has been under investigation for decades. This enzyme plays an important role in the catabolism of purines in some species, including humans. It catalyzes the oxidation of hypoxanthine to xanthine and can further catalyze the oxidation of xanthine to uric acid. These processes are accompanied by the generation of superoxide anion [1–4] and reactions can be written as follows:

$$\text{hypoxanthine} + 2O_2 + H_2O \rightarrow \text{xanthine} + 2O_2^- + 2H^+$$

$$\text{hypoxanthine} + H_2O + O_2 \rightarrow \text{xanthine} + H_2O_2$$

$$\text{xanthine} + 2O_2 + H_2O \rightarrow \text{uricacid} + 2O_2^- + 2H^+$$

$$\text{xanthine} + O_2 + H_2O \rightarrow \text{uricacid} + H_2O_2$$

The overproduction of uric acid can lead to gout, due to the deposition of uric acid in the joints thereby leading to painful inflammation [5]. These superoxide anion and other reactive oxygen species have long been implicated in the oxidative stress on the organism and involved in many pathological processes such as inflammation, atherosclerosis, cancer, and aging [6,7]. Consequently, XOD is considered to be an important enzyme causing gout and many other diseases related to superoxide anion.

The XOD inhibitors can block the synthesis of uric acid and reduce the generation of superoxide anion in vivo. This explains why the use of the XOD inhibitor should be one of the therapeutic approaches for the treatment of hyperuricaemia, gout and other many diseases related to reactive oxygen species [8–14]. Indeed, some effective XOD inhibitors which are being used to treat gout and other disease have been designed, such as allopurinol [9,15,16], Y-700 [17–19] and febuxostat [20–24]. Since XOD inhibitors and superoxide scavengers as an important new class of therapeutic agents are under investigation, there is a need for developing an efficient screening method to evaluate analytes with XOD inhibitory activity and superoxide scavenging potential.

In recent years, several methods for determination of the XOD inhibitory activity and superoxide scavenging activity of compounds have been reported. The XOD inhibitory activity of

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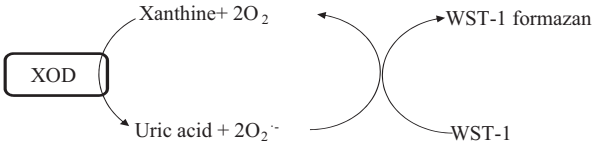


Scheme 1. The principle of the method. The quantification of the enzymatic reaction products (uric acid and superoxide anion) was accomplished by quantifying the reactants (Xanthine and WST-1).

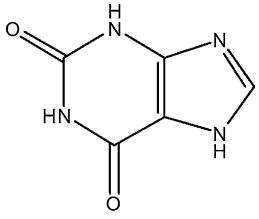
compounds can be evaluated by quantifying products of enzymatic reaction, containing uric acid and superoxide anion. The amount of uric acid produced from xanthine can be quantified by spectrophotometric [25] or HPLC methods [26]. The superoxide anion can be quantified by the chemiluminescence method with the addition of luminol [27], or the spectral method with the addition of nitro blue tetrazolium [28] or cytochrome c [29]. Electron spin resonance method can also be used to detect and quantify superoxide anion [30]. These methods sometimes are also used to evaluate the superoxide anion scavenging activity of compounds. However, the above methods cannot determine the XOD inhibitory activity and superoxide scavenging activity of compounds simultaneously, and some of these methods easily give false positive and negative results. In recent years, mass spectrometry-based methods as a sensitive, rapid and accurate tool have been successfully and widely used to screen enzyme inhibitors [31–33]. In the present study, a new screening method based on Ultrahigh Performance Liquid Chromatography (UHPLC) and Triple Quadrupole Mass Spectrometry (TQ-MS) was developed. It can accurately quantify the amount of analytes by using multiple reaction monitoring (MRM) function which is through monitoring the ratio of mass and charge (m/z) of parent ions and diagnostic fragment ions. This method can simultaneously evaluate the XOD inhibitory activity and superoxide scavenging activity of compounds by indirectly quantifying two products of enzymatic reaction (uric acid and superoxide anion). During this process, WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium sodium salt), which can react with superoxide anion [34], was applied to the enzymatic reaction. The principle of the method is shown in Scheme 1. The structures of the xanthine, WST-1, WST-1 formazan and uric acid are shown in Fig. 1. Compared with the commonly used spectroscopy method, the UHPLC–TQ-MS method is sensitive, accurate and fast, moreover, it can eliminate false positive and false negative results.

In the present study, the experimental conditions including pH value, temperature, reaction time, the concentration of reagent such as WST-1, XOD and xanthine were firstly optimized. The type of buffer solution on the enzymatic reaction, the liquid chromatography method condition and mass spectrometry parameter were also investigated. Subsequently, the validated method was used to evaluate the XOD inhibitory activity and superoxide scavenging activity of some herbal extracts and compounds from natural products. These compounds could be classified into six groups based on the results of the assay: superoxide scavengers without inhibitory activity on XOD (category A), XOD inhibitors without any additional superoxide scavenging activity (category B), XOD inhibitors with an additional superoxide scavenging activity (category C), XOD inhibitors with an additional pro-oxidant effect on the production of superoxide (category D), compounds with a marginal effect on XOD but with a pro-oxidant effect on the production of superoxide (category E), and finally, compounds with no effect on XOD or superoxide (category F). The results obtained by this method were consistent with data reported in the literature [35].

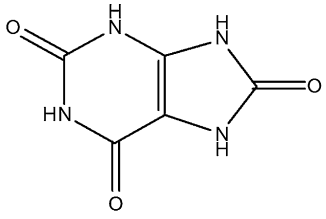
2. Chemicals and methods

2.1. Chemicals

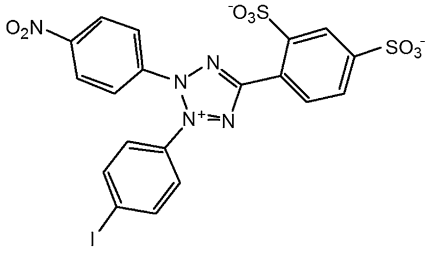
Apigenin, quercetin, catechin, genistein, naringenin, baicalein, berberine and jateorrhizine standards were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China); allopurinol and xanthine were acquired from Alfa Company and used directly; WST-1 was obtained from Nanjing Robiot Co., Ltd. (Nanjing, China); sodium tauroursodeoxycholate (ST) was acquired from Fleton Natural Products Co., Ltd. (Chengdu, China); XOD and Tris were purchased from Sigma (St.



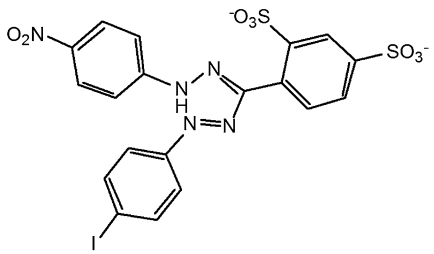
Xanthine Exact Mass:152.03



Uric acid Exact Mass: 168.03



WST-1 Exact Mass: 627.91



WST-1 formazan Exact Mass: 628.91

Fig. 1. The structure and exact mass of xanthine, WST-1, WST-1 formazan and uric acid. The negative ion ESI-MS of xanthine, uric acid, WST-1 and WST-1 formazan produced the abundant deprotonated molecular ions $[M-H]^-$ at m/z 151, 167, 628 and 630 respectively.

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