Feature Article

Flow Injection Analysis of Allopurinol by Enzymeless Approach at Glassy Carbon Electrodes

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Abstract
Allopurinol is a pharmaceutical drug mainly used for the treatment of chronic gout. It is an antioxidant as well as a powerful inhibitor of xanthine oxidase. Very few electroanalytical assays were reported for allopurinol detection so far. No faradic process of allopurinol was observed by cyclic voltammetry at a glassy carbon electrode. A systematic decrease in current signal of ascorbic acid was observed upon the addition of allopurinol due to the inhibitory action. By taking ascorbic acid as the mobile phase, the above property was utilized to develop a sensitive detection scheme for allopurinol by flow injection analysis. Possible inhibition pathway is derived in terms of radical coupling mechanism. Under optimized conditions, the calibration plot showed a linear range up to 150 μM with a detection limit ($S/N = 3$) of 0.5 μM (i.e., 1.3 ng in 20 μL sample loop). The precision for 18 successive injection of 0.1 mM allopurinol showed a relative standard deviation of less than 2%. The proposed method is used to detect the amount of allopurinol in a commercially available pharmaceutical drug to show its potential use in real applications.

Keywords: Allopurinol, Ascorbic acid, Flow injection analysis

1. Introduction

In physiological media, free radicals are continuously generated in almost all aerobic cells. If free radicals are not scavenged by the integrated comprehensive array of endogenous antioxidant mechanisms, tissue damage occurs and may cause a large number of medical disorders including cancer, inflammation, atherosclerosis, coronary heart disease, and diabetes [1]. Allopurinol (4-hydroxypyrazolo[3,4-*d*] pyrimidine, AP) is a radical scavenging clinical drug used for the treatment of chronic gout and hyperuricemia resulting from uric acid. It has been reported as a potential inhibitor of xanthine oxidase (XOD), the enzyme of which catalyzes the conversion of hypoxanthine and xanthine to uric acid [2, 3]. The recommended AP dosage is 100 mg/day and 600 – 800 mg/day, respectively, for adults in the treatment of chronic gout and cancer therapy without any combination drugs [4].

Conventional methods for AP detection often depend on UV absorption measurements through derivation with mercurochrome to form colored chromogens [5 – 7]. Electrochemical techniques, on the other hand, offer an attractive and easy route without any derivatization procedure [8 – 11]. For example, Cataldi et al. [8] reported flow injection analysis (FIA) of AP in pharmaceutical formulations by polarography through the formation of sparingly soluble Hg-AP complex in alkaline solution with a sample throughput of 90 samples/h. Wang and coworkers [11] reported determination of AP and its active metabolite oxypurinol by capillary electrophoresis (CE) with amperometric detection on a carbon fiber electrode at 1.2 V in alkaline solution. Nevertheless, future regulations and occupational health considerations of Hg as an electrode material (first case) and a high operation potential at nonphysiological working pH (second case) restrict their real sample applications. An alternative method is electrochemical determination of AP based on its interaction with XOD. Martin and Rechnitz [10] reported an XOD immobilized-carbon paste electrode for the determination of AP with a linear range of 0.2 – 50 μM. A general problem for an enzyme-based sensor, however, is the stability and long-term workability. Hence, there is no ideal analytical procedure reported for AP quantification up to now. Any assay method allowing rapid and reliable quantitation of AP in pharmaceutical formulations is still desirable.

We report here an enzymeless FIA method for AP determination using a glassy carbon electrode (GCE). The drawbacks of electrochemical approaches mentioned above are totally overcome by this method. In the present technique, XOD is replaced by ascorbic acid (AA) and AP is quantified from its inhibition action by FIA with a low applied potential of 0.4 V (vs. Ag/AgCl) at physiological pH. To the best of our knowledge, such an inhibitory quantitative approach has never been reported before. There are two main facts to support the idea mentioned above. First, AP and its metabolites were known to inhibit basal oxidation of ascorbate [12]. Second, AA is also a radical scavenger in living media and is easy to detect on a bare GCE [1, 13 – 15]. Thus, the purpose of the present work is to construct a high precision analytical method in aim with an inexpensive and easy route. Necessary instrumental and solution parameters were systematically optimized in this study.

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2. Experimental

Allopurinol (M.W. 136.11) was obtained from Sigma (St. Louis, MO, USA) and used as received. All the other compounds (ACS-certified reagent grade) were used without further purification. Aqueous solutions were prepared with doubly distilled deionized water. Stock solution of 20 mM AA was prepared in 0.1 M, pH 8 phosphate buffer solution (PBS) and stored in dark. An aliquot was diluted to the appropriate concentrations with pH 8 PBS before actual analysis.

Voltammetric measurements were carried out with a BAS 50W workstation (BAS, West Lafayette, IN, USA). A BAS VC-2 type electrochemical cell was employed for electrochemical experiments. The three-electrode system consisted of a GCE working electrode, a Ag/AgCl reference electrode (Model RE-5, BAS), and a platinum wire auxiliary electrode. UV absorption measurements were carried out using a Hitachi U-3000 spectrophotometer.

The FIA system consisted of a carrier reservoir, a Cole-Parmer Masterflex microprocessor pump drive, a Rehodyne Model 7125 sample injection valve (20 μL loop), interconnecting Teflon tubing, and a BAS Model CC-5 thin layer electrochemical detector with a BAS MF-1000 dual GCE. The dual GCE (5 mm dia.) used in FIA was first polished with the BAS polishing kit and rinsed with deionized water and further cleaned ultrasonically in 1:1 nitric acid and deionized water successively. The mobile phase in FIA consists of 20 mM AA in pH 8 PBS. The working electrode was equilibrated at an applied potential of 0.4 V until the current response becomes constant. It usually took 10 to 15 min. The AP quantification was achieved by measuring the inhibitory current (i_{inh}) of AP on the AA signals. The flow rate was 1.5 mL/min.

Commercial pharmaceutical tablet, Zyloric-100 (The Wellcome Foundation Ltd. London, UK), with an average content of 100 mg AP was used as the dosage form. Each tablet was weighed and finely ground and 30 mg of each sample was then transferred into a 100 mL calibrated flask to which 1 mM NaOH was added to the mark. After 5 min, in an ultrasonic cleaning bath, each suspension was filtered and aliquot of this was diluted 100 times.

3. Results and Discussion

3.1. The Inhibitory Action of AP on AA Oxidation

Figure 1 shows cyclic voltammetric (CV) response of 20 mM AA in the presence of 0–0.5 mM AP at a GCE. An irreversible anodic peak (E_{pa}) at 0.4 V corresponding to the oxidation of AA to dehydroascorbic acid (DHA) was observed [16, 17]. The latter species subsequently undergoes a hydration reaction at the carbonyl group to form the final electro-inactive product [17–19]. The current function was calculated as 34.74 $AV^{1/2}s^{-1/2}mol^{-1}cm^3$. The AA voltammetric signals regularly decreased upon addition of 0.1–0.5 mM AP. About 50% decrease in the inhibitory signal of 20 mM

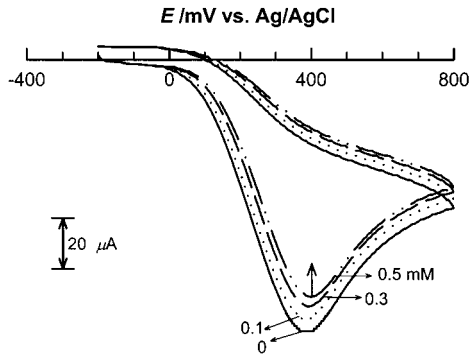
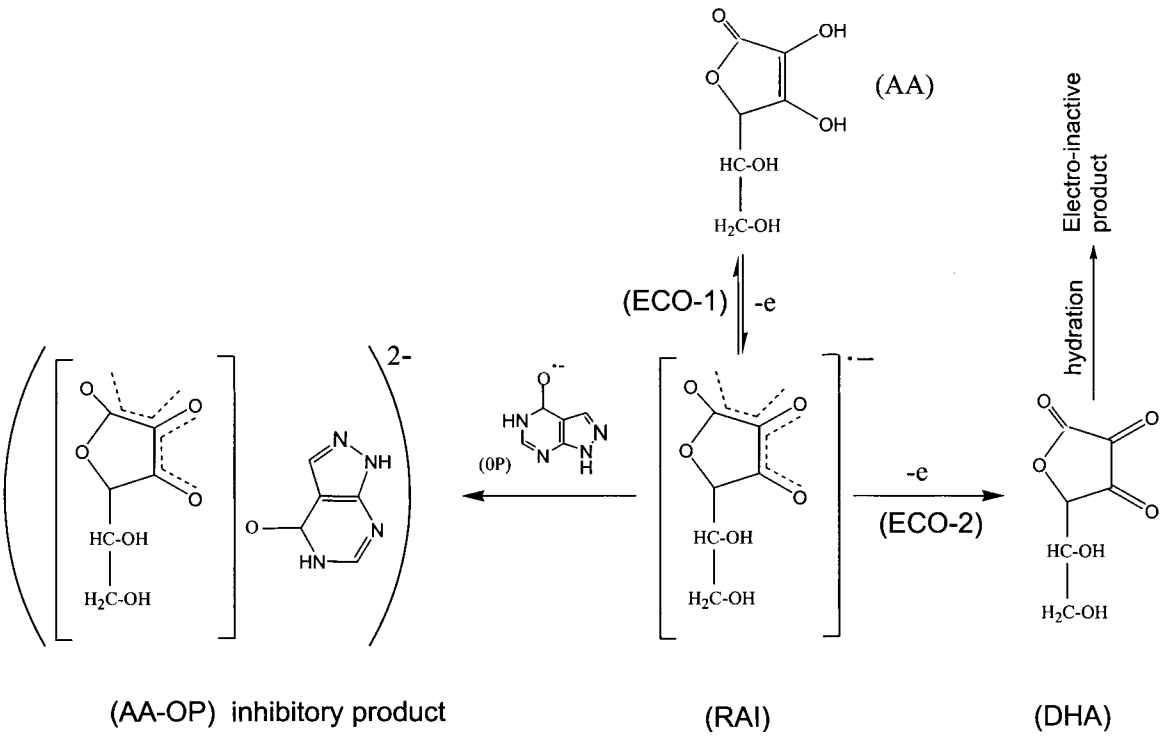


Fig. 1. CV responses for 20 mM AA at various AP concentrations of 0.1–0.5 mM in pH 8 PBS. Scan rate = 50 mV/s.

AA was observed at a high concentration of 19 mM AP. The fact that AP did not show any faradic process on GCE in the potential window of −0.2 to 0.8 V is inconsistent with the electrochemical behavior of AP on carbon fiber electrode reported earlier [11]. It has been established that the electrochemical oxidation (ECO) of AA to DHA proceeds via two consecutive one-electron processes (namely ECO-1 and ECO-2) with the participation of a radical anion intermediate (RAI) as illustrated in Scheme 1 [17, 18]. Radical pathway of AA oxidation was confirmed by Aldaz et al. using paramagnetic resonance spectroscopy [18]. The decrease in voltammetric signals in this study is believed to be due to the inhibitory effect of AP on AA oxidation by arresting of the ECO-2. More specifically, it is the oxypurinol (OP) radical anion (formed from AP) reacts with the RAI that leads to the expulsion of the ECO-2 to DHA step. Consequently, the chemical reaction between RAI and OP is the essential step for the inhibitory action and hence for the decrease in the current signal of AA.

Systematic UV-visible absorption analysis after bulk electrolysis of AA, AP, and (AA + AP) further confirmed the proposed mechanism. Figure 2A shows the typical UV-visible absorption spectra for AA, AP, and (AA + AP) in pH 8 PBS before electrolysis. As can be seen, the interaction between AA and AP before electrolysis can retain their respective characteristics of spectrum. The absorption band of AA at about 260–280 nm, however, completely disappeared after bulk electrolysis at an applied potential of 0.4 V as shown in Figure 2B. The absence of 265 nm band of AA after electrochemical oxidation on GCE at 0.4 V is reasonable due to the disappearance of double bond to form the electro-inactive product. The oxidation of AA obviously follows the reaction pathway of first to DHA and finally to the electroinactive product. However, in the presence of AP, the AA absorption peak only decreased by 30% in intensity after 4 h of bulk electrolysis. Meanwhile, no appreciable change in the absorption spectrum was observed for AP after electrochemical oxidation. These observations confirm that the pathway of ECO-2 on AA oxidation is less favorable after the addition of AP. In other words, there is indeed an inhibitory action on the oxidation of AA in the presence of AP. Scheme 1 represents the tentative reaction pathway and mechanism of the inhibitory action of AA by AP.



Scheme 1. Possible reaction pathways for the formation of dehydro-ascorbic acid (DHA) and (AA-OP) products from ascorbic acid (AA) by electrochemical oxidation (ECO) of oxypurinol (OP) and the anion intermediate radical (RAI) of AA.

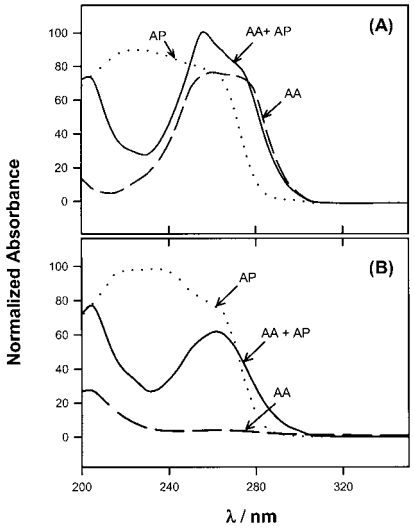


Fig. 2. UV-absorption spectra before (A) and after (B) 4 h electrochemical oxidation of AA, AP and (AA + AP) at 0.4 V in pH 8 PBS. [AA] = 1 mM; [AP] = 1 mM.

3.2. Analytical Application

The inhibitory current, i_{inh} (i.e., $i_{AA} - i_{AA+AP}$), was measured as the difference in current signal of AA before (i_{AA}) and after addition of AP (i_{AA+AP}). The detection scheme can be simplified in FIA by using AA as the mobile phase at its oxidation potential. In that case, the i_{inh} represents the decrease in the baseline of AA after the addition of AP. Necessary solution phase and instrumental parameters were taken into consideration to amplify the detecting signals in FIA. The effect of solution pH to the AP signals in FIA was first studied with 20 mM AA in mobile phase (Fig. 3A). Since very low i_{inh} value was obtained below pH 5 and the baseline showed fluctuation above pH 9, we thus restricted our working window to moderate pH regions. The i_{inh} values increased steadily with pH as ~300 nA/pH in the pH range of 5 – 9. The AA species involved in the oxidation process is the one with a proton released since the working pH lies in-between the pK_{a1} and pK_{a2} ($4.17 < pH < 11.5$) [19]. The increase in i_{inh} values in the pH window of 5 – 9 may be due to the conversion of AP → OP and in turn to the high inhibitory action. For convenience, the working pH is chosen as 8 for further experiments. Similarly, the increase in AA concentration also leads to a marked increase in i_{inh} up to 20 mM (Fig. 3B). Around 2.5× of current magnification is noticed for 20 mM AA compared to that at 5 mM AA. Thus, a mobile phase having relatively higher concentration of AA (20 mM) in pH 8 PBS is used in all the subsequent experiments.

Figure 4 shows the effects of applied potential and flow rate to i_{inh} for 0.1 mM AP. The hydrodynamic voltammograms indicate almost no response up to 0.3 V and start to increase rapidly after that (Fig. 4A). This trend showed the effective AA oxidation on GCE and hence an increase in i_{inh}. Although a higher i_{inh} can be obtained with an applied potential of 0.5 V, the baseline also showed fluctuation at this condition. Hence, the optimized applied potential was chosen as 0.4 V. The i_{inh} increased as the flow rate increased

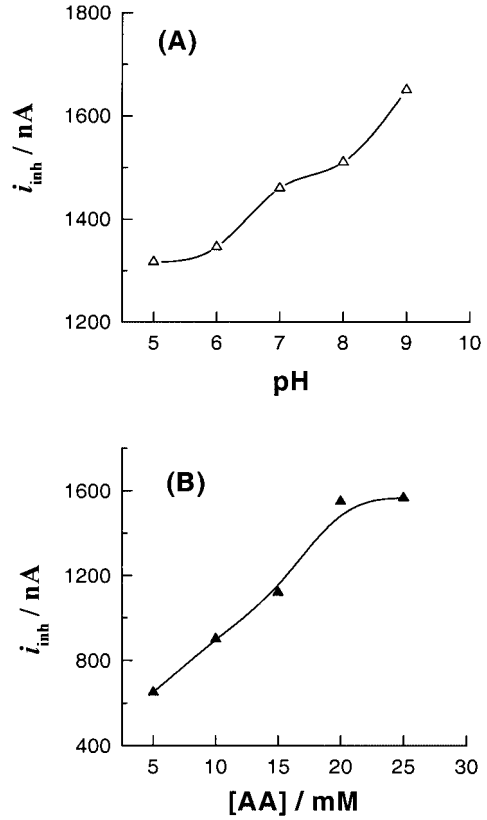


Fig. 3. Effect of solution pH (A) and AA concentration (B) on the detection of 0.1 mM AP by FIA. Applied potential was 0.4 V at a flow rate of 1.5 mL/min.

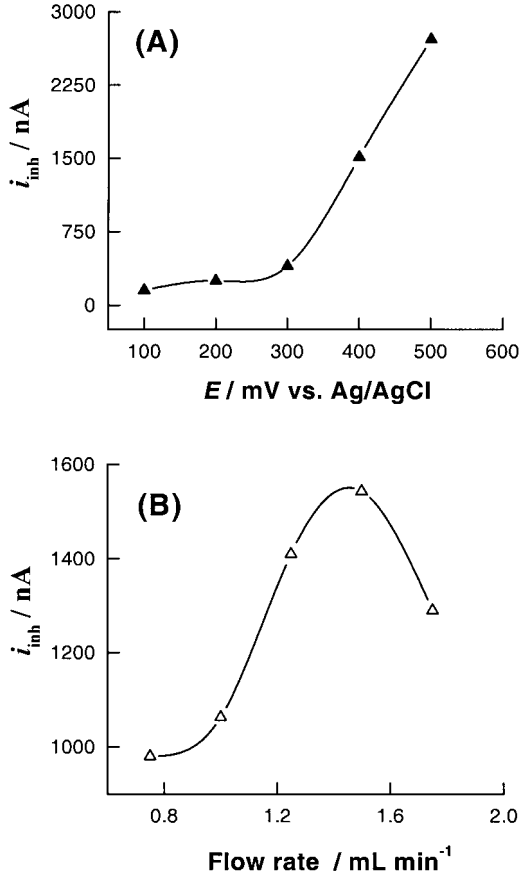


Fig. 4. Hydrodynamic voltammograms with respect to applied potential (A) and flow rate (B) on the detection of 0.1 mM AP by FIA. Other conditions are as in Figure 3.

initially and reached a maximum at 1.5 mL/min (Fig. 4B). An applied potential of 0.4 V together with 1.5 mL/min of flow rate is therefore used in FIA.

Under the optimized conditions, Figure 5 shows the calibration plot and typical FIA responses for the AP detection. A sharp and negative increasing current signal confirms the proper function of the AP inhibitory action on AA oxidation. The obtained linear range is up to 150 μM with slope, intercept, and correlation coefficient of 13.58 nA/μM, 40.56 nA, and 0.9998, respectively. Meanwhile, the baseline of AA in the absence of AP was also checked under optimized FIA conditions and no obvious fluctuation and alteration was observed. Successive injections of 0.1 mM AP ($n = 18$) results in a RSD < 2% indicating the good repeatability of the working system. The detection limit ($S/N = 3$) is 0.5 μM (i.e., 1.3 ng in 20 μL sample loop), which is about 4 times more sensitive than the Hg-based system reported earlier [8]. The matrix and interference effect was checked as shown in Figure 6. Both anionic and cationic surfactant gave almost no interference effect even at 10 times excess in concentration to 40 μM AP. Similarly, the organic matrixes like glucose, oxalic acid, and gelatin showed no appreciable change in the FIA signals. These results indicate the practical applicability of the present system.

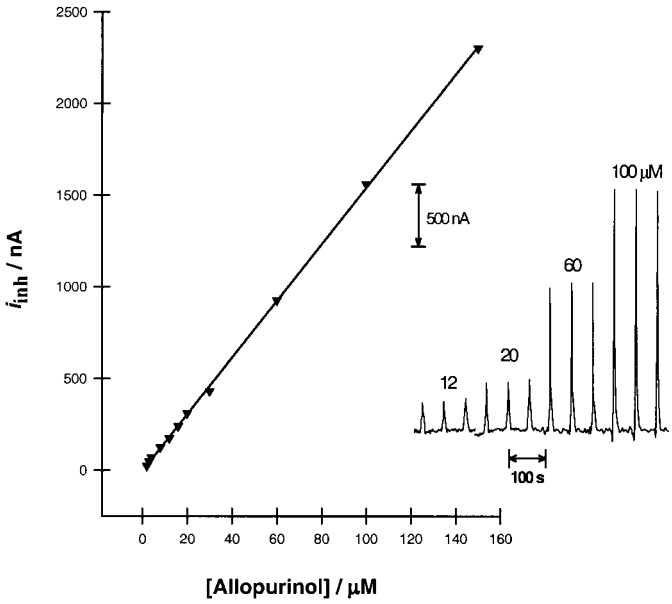


Fig. 5. Calibration curve for AP in terms of i_{inh}. Adjacent figure is the typical FIA response for various concentrations of AP. Other conditions are as in Figure 3.

Table 1. Results for the assay of AP in commercial pharmaceutical drug by FIA.

Sample No.	*Weighed mass (mg)*	*Amount found (mg)*	*Difference from labeled value claimed*
1	317.1	101.9	+1.90
2	311.1	99.8	−0.02
3	303.4	98.9	−1.1
4	310.3	98.8	−1.2
5	307.8	99.8	−0.02

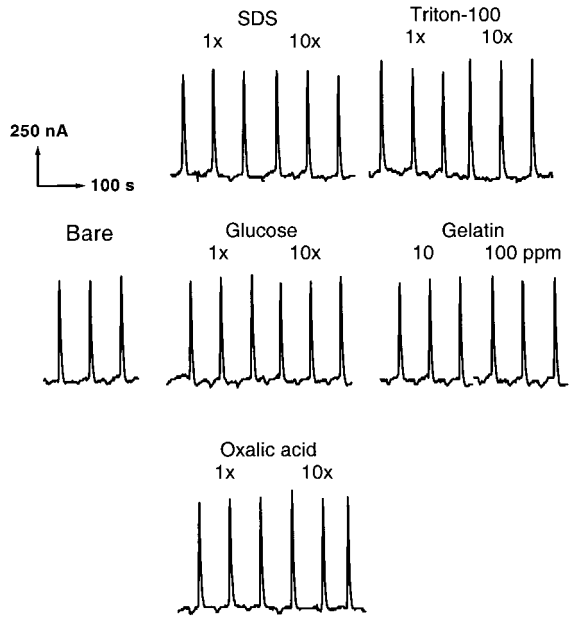


Fig. 6. Effect of interfering agents on the detection of 0.1 mM AP by FIA. Other conditions are as in Figure 3

Finally, a commercial pharmaceutical drug (Zyloric-100) containing 100 mg AP was used for test by FIA in pH 8 PBS. Under optimized solution phase and FIA conditions, standard addition method yielded an AP amount of 99.84 ± 1.25 mg with a recovery of ca 100% ($n=5$) for the pharmaceutical tablet (Table 1). These results again provided the sufficient evidence about the practical utility of the present system. Overall, the inhibitory property of AP on AA worked very well for the quantification assay of AP with good sensitivity by FIA. Real sample assays were in good agreement with standard values. The simplicity, reliability, and high precision prove the ideality of the present system to AP detection over the earlier methods.

4. Acknowledgement

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