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Full Length Research Paper

Development of a new animal model-bioassay procedure for the evaluation of Xanthine oxidase inhibitors

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New Xanthine oxidase inhibitors are in development and these need to be evaluated reliably before being introduced into clinical investigations. The presence of valid, easy and reliable *in vivo* bioassy to evaluate the Xanthine oxidase inhibition is of utmost importance to achieve steady paces in drug discovery attempts to treat gout disease. The objective of the current study was to develop and validate a bioassay procedure that can be employed for *in vivo* evaluation of drugs investigated as xanthine oxidase inhibitors. Exploiting rats as an animal model to carry out the bioassay is proposed to establish a novel approach to screen and evaluate the xanthine oxidase inhibitory activity of potential agents and/or plant extracts. Allopurinol was used as a reference treatment to inhibit Xanthine oxidase while the degree of enzyme inhibition was indirectly measured by determining the blood levels of 6-mercaptopurine (6-MP) as a surrogate chemical marker. In this model, serum obtained from: untreated rats, rats treated with 6-MP alone and rats treated with 6-MP and different doses of allopurinol were analyzed, for levels of 6-mercaptopurine. An elevated plasma 6-mercaptopurine level in the allopurinol treated rats as compared to untreated rats was an indication of an *in vivo* inhibition of the enzyme Xanthine oxidase.

Key words: Xanthine oxidase inhibitors, 6-Mercaptopurine, allopurinol, gout, bioassay, animal model.

INTRODUCTION

Hyperuricemia, characterized by high serum uric acid level, plays a major role in the occurrence of gout. Development of gout occurs as a result of increased uric acid production, impaired renal uric acid excretion, or a combination of these mechanisms. Uric acid, derived from purine catabolism, is sparingly soluble in extracellular body fluids and slight changes in its concentrations may cause supersaturation and precipitation of urate crystals in synovial fluid which leads to attacks of painful acute arthritis (Pennes and Martel, 1986). Gout is a common biochemical abnormality with an estimated prevalence of 8.4 cases per 1,000 persons (Lawrence et al., 1998; Saag Kenneth and Choi, 2006) and is associated with painful arthritis attacks and the development of metabolic syndromes (Choi and Ford, 2007) and nephropathy (Avram and Krishnan, 2008).

Control of hyperurecemia is, most often, achieved by reducing uric acid production with an inhibitor of Xanthine oxidase (XO), the enzyme that catalyzes the production of uric acid, or less frequently, by employing uricosuric agents to increase renal excretion of uric acid. Allopurinol is the most frequently used XO inhibitor (Figure 1) and the only one that is readily available in some countries

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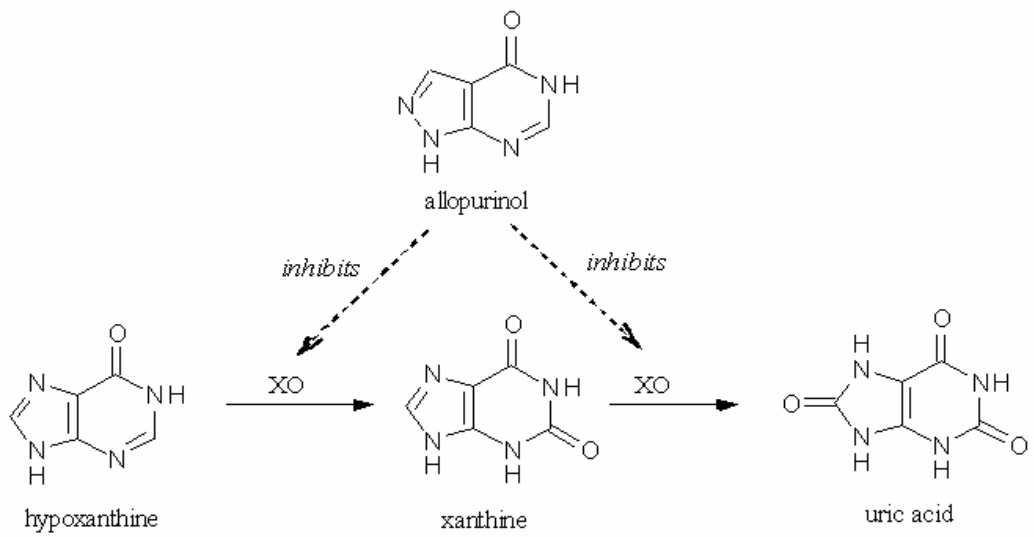

Figure 1. Pharmacological action of allopurinol.

such as France (Bardin, 2004). However, its usage is associated with adverse effects that range from gastrointestinal intolerance and skin rash to the more sever reactions of allopurinol hypersensitivity syndrome that requires permanent discontinuation of allopurinol therapy (Bardin, 2004; Chao and Terkeltaub, 2009; Fagugli et al., 2008; Keith and Gilliland, 2007). Thus, there is a need to provide the market with new urate lowering drugs with high efficacy and low side effects (Chohan and Becker, 2009). It is an active research area that focuses on the exploration of potential XO inhibitory agents, specially, from natural sources that are believed to have relatively lower side effects than synthetic chemicals (Dew et al., 2005; Hsieh et al., 2007; Nguyen et al., 2004; Zhao et al., 2006). Testing the inhibitory potential of these products may lead to the discovery of new medicaments for the management of hyperuricemia and gout.

Several methods are available to screen the effects of potential XO inhibitors. The in vitro XO inhibition assay is one basic step to assess the direct effect of a given XO inhibitor on XO activity. A further screening step prior to clinical trial is the screening in an animal model that is physiologically relevant to human and bridges the gap between the in vitro results and clinical human expectations. However, currently available animal models for the in vivo evaluation of XO inhibitory agents are inadequate. The uricase-inhibited rat model, by using potassium oxonate, is the currently available model (Dan et al., 1994; Stavric and Nera, 1978). Uricase is a purine metabolic enzyme that catalyzes the conversion of uric acid to the more soluble allantoin in most mammals except humans (Wu et al., 1994). As a result, the less soluble uric acid is the end product of purine metabolism in human which requires renal excretion to maintain uric acid level below the supersaturation level that is associated with gouty arthritis. Thus, mammals are unsuitable as experimental models for human hyperuricemia due to the activity of uricase enzyme in converting the uric acid to the more soluble allantoin. Potassium oxonate is used to block the effect of uricase and to produce hyperuricemia in rats to serve as an animal model in the investigation of antihyperuricemic agents. This model is useful in evaluating agents that reduce the overall plasma uric acid level without clarifying the exact antihyperuricemic mechanism. Thus, for XO inhibitor, a further elaborate ex-vivo step, consists of assaying the liver XO activity from the liver of treated rat, to confirm the XO inhibitory activity of the studied antihyperuricemic agents (Dan et al., 1994). In the current study, we developed a new animal model-bioassay procedure that can be employed for direct in vivo evaluation of plant extracts, drugs or any other compounds that possess anti-gout activity, mediated through XO inhibition, employing 6-mercaptopurine (6-MP) as a surrogate marker. 6-MP is a purine analogue used in cancer chemotherapy, primarily in childhood acute lymphoblastic leukemia (Pinkel, 1993), and metabolized mainly by XO (Figure 2).

EXPERIMENTAL

Animals

Experiments were performed on 12-weeks-old male Wistar rats, weighing 180 – 200 g, obtained from Jordan University animal house, Jordan. Animal experiments confirm with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication no. 85-23, revised 1996). Rats were housed in temperature controlled cages (20–22°C) with a 12 h light–dark cycle, and given free access to water and formulated diet

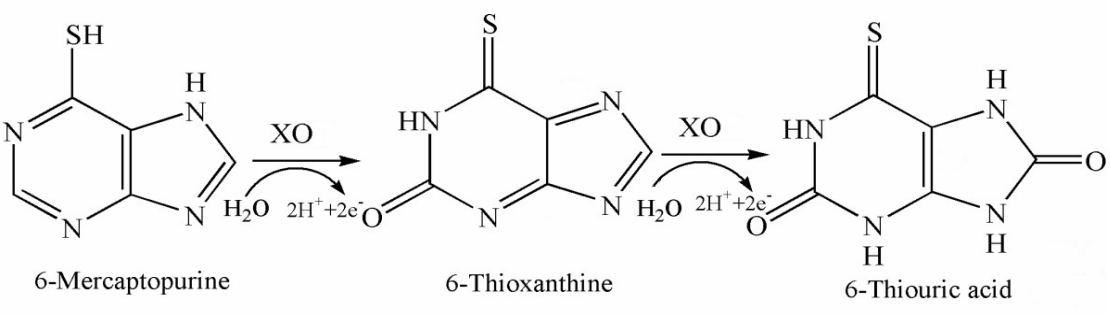

Figure 2. Scheme showing oxidative hydroxylation of 6-mercaptopurine to 6-thioxanthine to 6-thiouric acid by xanthine oxidase (XO).

(Jordan Feed Co. Ltd, Amman, Jordan) for 7 days before experiments began.

Materials

6-Mercaptopurine and allopurinol as well as other reagents used in the experiments were purchased from Sigma, USA. All solvents were of High Performance Liquid Chromatography (HPLC) grade and combined in appropriate proportions and filtered (0.45 mm HA membrane filter, Millipore, Ireland) to prepare the mobile phase. Allopurinol was suspended in distilled water (concentration = dose/0.5 ml water). 6-Mercaptopurine was suspended in 2-pyrrolidone (2-Pyrol) – 0.01M NaOH (0.1: 0.9) solvent (concentration = dose/2 ml solvent). Doses were prepared freshly just before administration.

Animal groups and drug administration

The animals were divided into four experimental groups, 3 rats each, that are matched for body weight: group I was left untreated and served as a blank group, group II was given only water without allopurinol is dissolved and 6-mercaptopurine (50 mg/kg) and served as control group, group III was given allopurinol (25 mg/kg) and 6-mercaptopurine (50 mg/kg), and group IV was given a higher dose of allopurinol (50 mg/kg) and 6-mercaptopurine (50 mg/kg). In all groups; the water or allopurinol solution was given intraperitoneally to rats half an hour before the intraperitoneal administration of 6-mercaptopurine.

Biological sample collection and handling

Blood samples were collected 8 h after allopurinol administration from the retro-orbital plexus of each rat, before being sacrificed. Using sterile heparinized capillary tubes (Vitrex, Herlev, Denmark), 0.5 ml of blood was collected in sterile tubes containing K_2-EDTA as anticoagulant (Minicollect®, Greiner, Germany) so that the blood to anticoagulant ratio was 1:0.075 v/v. Collected blood samples were then centrifuged at 14,000 rpm for 30 min and the harvested plasma portions were transferred to pre-labelled eppendorff tubes and kept under subdued-light and refrigerated conditions during short term handling times and under -20°C for long term storage.

Determination of 6-MP in rat plasma by HPLC.

Determination of 6-MP in rat plasma was carried out using the HPLC method developed by Oliveira et al. (2004) with minor modifications.

Preparation of plasma samples

Concentration of 6-mercaptopurine in each collected rat plasma was determined using RP-HPLC. Plasma samples were prepared as follows: 125 µl of dithiothreitol (DTT) - methanol solvent (10 mg/dl) were mixed with 125 µl of plasma samples and vortexed for 15 s. Samples were then centrifuged for 25 min at 14,000 rpm to remove the formed white precipitate. If supernatant was still turbid, samples were re-centrifuged for additional 10 min. Supernatants were transferred into glass test tubes and 50 µl of the clear supernatants were injected onto the chromatographic system.

Preparation of standard samples

Standards were prepared in the same manner as samples (using blank plasma) and reconstituted with DTT-methanol containing 6-MP at known concentrations. A stock solution (0.1 mg/ml) was prepared by dissolving 10 mg of 6-MP in 100 ml DTT-methanol solvent. Five calibration standards were prepared for 6-MP in plasma, between 0.5 and 10 µg/ml, as follow: 100, 200, 500, 1000 and 2000 µl of the stock solution were diluted up to 10 ml with DTT-methanol and 125 µl of plasma were treated with 125 µl of the above serially diluted solutions to give 0.50, 1.00, 2.50, 5.00 and 10.00 µg/ml 6-MP in plasma. Serially diluted plasma samples were then centrifuged for 25 min at 14,000 rpm and 50 µl of clear supernatant were injected onto the chromatographic system. The calibration curve for HPLC analysis was constructed by plotting the ratio of peak area of drug against the drug concentration in µg/ml.

HPLC system and chromatographic conditions

The 6-MP concentration in plasma samples was determined using a Surveyor Plus HPLC System (THERMO electron corporation, USA) consisting of Finnigan™ Surveyor® Autosampler Plus, Finnigan™ Surveyor® LC Pump Plus and Finnigan™ Surveyor® Photodiode array (PDA) Plus UV Detector. The samples were chromatographed at room temperature on a reverse phase Phenomenex®-C_{18} column (5 mm, 15 cm × 0.5 mm i.d., Thermo electron company, Bellefonte, North America) preceded by a guard column (40 × 4 mm) of the same material. The mobile phase, consisted of Phosphoric acid: Water: Acetonitrile: DTT (2.5 ml : 500 ml : 15 ml : 50 mg), was filtered and degassed under vacuum before use. It was pumped at a flow rate of 0.5 ml/min for the run time of 10 min under these experimental conditions with an injection volume of 50 µl. The

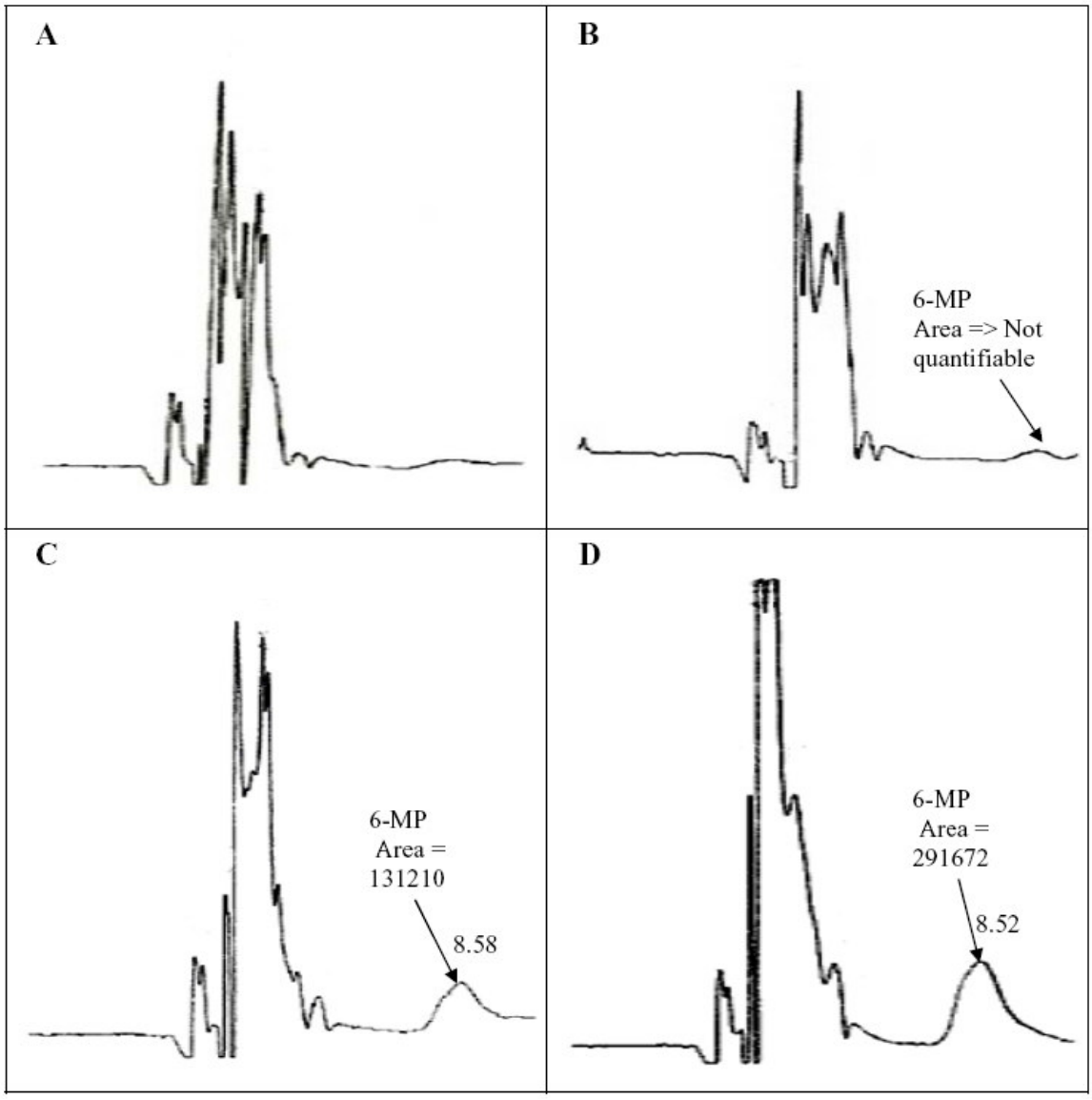

Figure 3. HPLC elution diagrams of serum samples obtained from: (A) untreated control rat (B) rat treated with 0.5 ml water followed by 50 mg/kg 6-MP C) rat treated with 25 mg/kg allopurinol followed by 50 mg/kg 6-MP and (D) rat treated with 50 mg/kg allopurinol followed by 50 mg/kg 6-MP.

column effluent was monitored simultaneously at 335 nm. Data from each chromatographic run were processed using ChromQuest® v 4.1 LC data system (THERMO electron corporation, USA). Concentrations were calculated from the peak area.

RESULTS

The standard curve demonstrated a linear relationship between peak areas and concentrations for the pooled rat plasma spiked with 6-MP standards (Correlation coefficient (mean ± SD) was 0.999 ± 000768 from three consecutive runs). The detection limit of 6-MP was about 0.1 µg/ml.

To rule out any peak overlapping or co-elution between allopurinol and 6-MP, both analytes were injected using the adopted HPLC conditions. The retention time for 6-MP was 8.6 min ± 0.1 min and allopurinol was not detected during the total run time of 10 min.

Figure 3A depicts an HPLC chromatogram of a processed serum samples obtained from an untreated rats. No peaks could be detected at 335 nm after the first 10 min of elution in this chromatogram. Figure 3B represents the HPLC chromatogram of a processed serum samples of rats treated only with 50 mg/kg 6-MP (without allopurinol) showing no peak of 6-MP, not quantifiable, eluting at 8.6 min. Figure 3C shows HPLC chromatogram of a processed serum sample of rat

treated with 50 mg/kg 6-MP and 25 mg/kg allopurinol. In this chromatogram the peak area of 6-MP (retention time = 8.58 min) was almost half the area of 6-MP peak (retention time = 8.52) for the HPLC chromatogram of serum sample obtained from rat treated with 50 mg/kg 6-MP and 50 mg/kg allopurinol (Figure 3D). There were no interfering peaks in the blank plasma in the region of 6-MP elution.

DISCUSSION

Several models have been reported for the evaluation of XO inhibitors. A number of authors have tried to develop such a model by administering high doses of uric acid to various animal species but the potent liver uricase that converts uric acid to the more soluble compound allantoin prevented the development of sustained hyperuricemia (Stavric and Nera, 1978). Inhibition of uricase by potassium oxonate significantly elevates plasma uric acid levels and serves as a useful animal model to evaluate hypourecemic agents (Nguyen et al., 2005). However, potassium oxonate treated animal models do not actually prove that the antihyperuricemic effect produced is a result of XO inhibition. In this model, rats are treated with potassium oxonate one hour before the oral or intraperitoneal administration of the potential hypourecemic agents. Then blood samples are taken and serum uric acid level is analyzed by HPLC method to ascertain the hypourecemic action of the test compound. The resultant hypourecemic effect is a result of either a reduction in uric acid production through XO inhibition or enhancement of urinary excretion of urate (uricosuric action). Thus, treated rat livers are processed and the resultant supernatant is used to ascertain spectrophotometrically the XO inhibitory activity of the tested compound. Therefore, a more straightforward animal model is needed to screen and evaluate the XO inhibitory activity of potential agents.

In this work, we have developed a new simple animal model-bioassay procedure that measures the degree of XO inhibition indirectly by determining the blood levels of 6-mercaptopurine. 6MP is subjected to a metabolic clearance through XO which catalyzes a two-step hydroxylation reaction of 6MP; leading to formation of 6-thioxanthine first and subsequently 6-thiouric (Kalra et al., 2007) (Figure 2). Therefore, the amount of drug available in the plasma is reduced due to this metabolism.

It has been found that co-administration of allopurinol, a suicide inhibitor of XO, with 6-MP inadvertently enhanced the 6MP blood level by blocking the metabolism of 6-MP to 6-thiouric acid (Coffey et al., 1972; Zimm et al., 1983). It is a common clinically relevant drug-drug interaction in oncology that is used for dose economy and increase therapeutic effectiveness (McLeod, 1998; Ragab et al., 1974; Walker et al., 1973). In this suggested anti-XO evaluation protocol, the effects of investigational agents on XO activity is measured by the change in blood levels of 6-mercaptopurine. As a proof of concept, 6-mercaptopurine levels obtained from treatments with different concentrations of allopurinol, as a reference XO inhibitor, were evaluated. The XO inhibitory effect of allopurinol, served as the test compound in our model, was verified by appreciable elevated levels of 6-MP in the two allopurinol and 6-MP treated groups (Figures 3C and D) as compared to the control group that is treated with 6-MP only (Figure 3B). Furthermore, the degree of 6-MP elevation was proportional to the administered allopurinol dose as shown by the double increase in 6-MP area in the chromatogram of serum samples for rats treated with 50 mg/kg allopurinol (Figure 3C) as compared to 6-MP peak in the chromatogram of serum samples of rats treated with 25 mg/kg allopurinol (Figure 3D).

Conclusion

The described model represents a direct *in vivo* model for the evaluation of XO inhibitors. In this model, serum obtained from: untreated rats, rats treated with 6-MP alone and rats treated with 6-MP and the tested compound are analyzed, using HPLC-UV, for levels of 6-mercaptopurine. An elevated plasma 6-mercaptopurine level in the treated rats as compared to untreated rats is an indication of an *in vivo* inhibition of the enzyme XO.

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