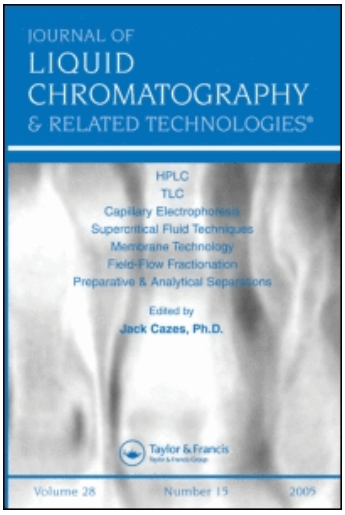

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ANALYSIS OF ALLOPURINOL AND OXIPURINOL IN PLASMA BY REVERSED PHASE HPLC

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ABSTRACT

A reversed phase HPLC assay is described for the quantitation of allopurinol and oxipurinol in human plasma. The strategies for the development of this method are discussed. The analysis is performed on a C-18 column using an acidic aqueous mobile phase. Sample preparation consists of protein precipitation with a mixture of trichloracetic acid and perchloric acid. The detection limit of the assay for both substances is in the region of 30ng/ml. This method has been applied to an investigation of the relative bioavailability of two commercial preparations of 300mg allopurinol tablets in eight healthy volunteers.

INTRODUCTION

Allopurinol (ALP) is a potent xanthine oxidase inhibitor and is widely used in the treatment of primary hyperuricemia of gout. It has also been employed as therapy for secondary hyperuricemia due to haematological disorders and cancer chemotherapy where mass cell destruction occurs and causes an over-

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production of uric acid. ALP is rapidly metabolised in the body to oxipurinol (OXP) (1), which is also a potent inhibitor of xanthine oxidase. Despite its clinical use for over twenty years, little is known about the pharmacokinetics of ALP and OXP in humans. The paucacity of information on these compounds is probably due to the lack of suitable assay procedures.

Advances in HPLC technology with its relative ease of sample preparation appears to offer the most suitable assay method for these pharmaceuticals. Quantitation of ALP and OXP in plasma with a sensitivity of 0.1 to 0.5µg/ml has been achieved using high performance ion-exchange chromatography (2,3). However the sample preparation procedures for these assays are time consuming and cumbersome.

Reversed phase HPLC has recently been employed in the analysis of these solutes in plasma and urine (4-9). The sample pretreatment in most of these studies is relatively simple involving either deproteination of the plasma with protein precipitants (4,9,10) or direct injection of the samples after appropriate dilution (7,8). These assays are usually troubled by plasma interference and column deterioration as a result of an inadequate sample clean-up step. In the present investigation, a chromatographic separation for ALP and OXP is developed, and its suitability for assaying these solutes in plasma from a bioavailability study of two commercial allopurinol tablets is assessed.

EXPERIMENTAL

Apparatus

The chromatographic system consisted of a LKB2150 pump (Bromma, Sweden) and a Waters Associate M440 UV absorbance detector, (Milford, MA, USA). The chromatographic column was 100mm x 4.6mm I.D. and slurry packed with 5µm Hypersil-ODS (Shandon, London, G.B.). Samples were injected via a Rheodyne 7125 injector (Cotati, California, USA) fitted with a 20µl loop.

Materials

ALP and OXP were obtained from Sigma Chemicals (St. Louis, Missouri, USA). Acetone, ammonium carbonate, ammonium sulphate, diethyl-ether, disodium hydrogen phosphate dihydrate, orthophosphoric acid (88%), perchloric acid (60% w/v) and trichloroacetic acid were purchased from BDH (Poole, UK). Pentane sulfonic acid sodium was supplied by Eastman-Kodak (Rochester, N.Y., U.S.A.). Acetonitrile (HPLC grade) was obtained from J.T. Baker (Phillipsburg, N.J., U.S.A.). The generic allopurinol tablets (batch 562002) were supplied by Evans Medical (Palmerston North N.Z.) and Zyloprim® tablets (batch 9583) were manufactured by Wellcome New Zealand Ltd. (Auckland, N.Z.). Both tablets contained 300mg of ALP. The internal standard floxuridine was kindly donated by Roche Products (Auckland, N.Z.). Water was glass distilled and MilliQ® filtered. All reagents were of Analar or equivalent grade.

Sample Preparation

To 1ml of plasma in a centrifuge tube, 100µl of the internal standard, floxuridine (102.7µg/ml in HPLC grade water), was added and vortexed for 10 seconds. The plasma was then deproteinated by adding 200µl of an aqueous solution containing 30% w/v trichloroacteic acid and 30% w/v perchloric acid. This mixture was vortexed for 10 seconds and placed in an ice bath for 2 minutes. The sample was then centrifuged at 3000xg for 20 minutes. Twenty microlitres of the clear supernatant was injected onto the column.

Chromatography

The mobile phase was HPLC grade water containing 20mM disodium hydrogen phosphate dihydrate and adjusted to pH 2.0 with orthophosphoric acid. A flow rate of 2ml/min was employed and detection was at a UV wavelength of 254nm.

Study Design

Eight healthy adult male volunteers, between 20 and 27 years of age participated in a randomized two way crossover study. The subjects were divided equally into two groups. Group one received Zyloprim® tablet first, followed by Allopurinol tablets. Group two received Allopurinol tablet first, followed by Zyloprim tablets. A 14 days period was allowed for complete drug washout. Ten millilitre blood samples were collected from the subjects at scheduled intervals over 8 hours after drug administration via a catheter. The heparinized blood samples were then centrifuged. The plasma samples were collected and frozen at -15°C until assayed.

RESULTS AND DISCUSSION

Reversed Phase Chromatography

The widespread popularity of HPLC is, in part, due to the analyst's ability to alter the mobile phase composition to optimize the resolution of the solutes. In this study the effects of acetonitrile concentration, pH and ionic strength of the eluent on the chromatographic behaviour of ALP and OXP have been investigated.

The acetonitrile content in the mobile phase has a profound effect on the retention and resolution of these two compounds as shown in Figure (1). At above 2.5% v/v acetonitrile, separation between ALP and OXP is inadequate. However, at acetonitrile concentrations below 5% v/v, the k' of these two compounds is very sensitive to alterations in organic modifier content. The use of organic modifiers, such as methanol or acetonitrile, will result in a very unstable chromatographic system and would inevitably give poor reproducibility of the assay. It is, therefore, not surprising that pure aqueous eluents have been used in most of the reported reversed phase HPLC assays for ALP and OXP (4,6-9).

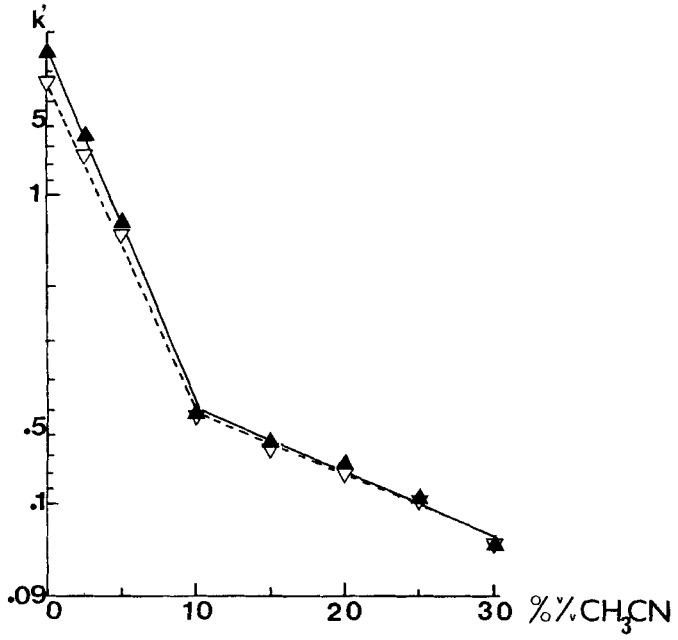

Figure 1 The effect of the mobile phase acetonitrile content on k' of ALP (▲) and OXP (▽). Chromatographic conditions: acetonitrile-buffer of 10mM Na_2HPO_4 at pH 3.

Figure (1) also demonstrates that, the log k' of ALP and OXP do not follow the generally accepted linear relationship with the volume fraction of organic solvent in the eluent (11). Instead, a bilinear relationship between log k' and volume fraction of acetonitrile is observed. This retention behaviour might well suggest that a mixed interaction mechanism is operative (12).

The ionic strength of the eluent has minimal effect on the retention and separation of ALP and OXP. It was, however, observed that at below 10mM disodium hydrogen phosphate, peak tailing became apparent. Thus, the phosphate buffer concentration of the eluent was fixed at 20mM.

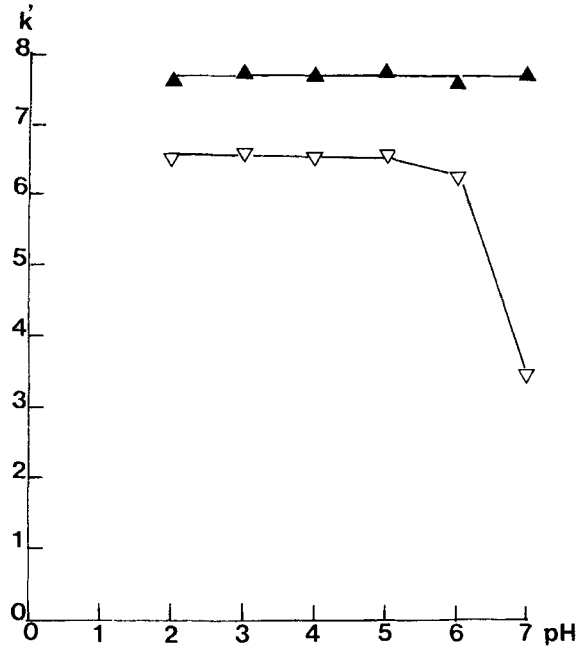

Figure 2 Influence of the eluent pH on the k´ of ALP (▲) and OXP (▽). Chromatographic conditions: aqueous buffer of 20mM Na_2HPO_4.

The effects of the mobile phase pH on the k´ of ALP and OXP are presented in Figure (2). It can be observed that the k´ of both solutes are very stable within pH2 to pH6. At pH above 6, the k´ of oxipurinol is seen to decrease drastically. The pH of the eluent was fixed at 2, because at higher pHs (>4) interference peak were observed in blank plasma samples. In addition, column efficiency decreased rapidly at higher pHs apparently due to the precipitation of plasma components in the column. Flushing the column with 50ml of 0.1% w/v orthophosphoric acid (pH2) restored its performance.

Ion Pair Chromatography

In view of the reported basic properties of ALP (pK_a 9.4) and OXP (pK_a 7.7) (13), the chromatographic behaviour of these

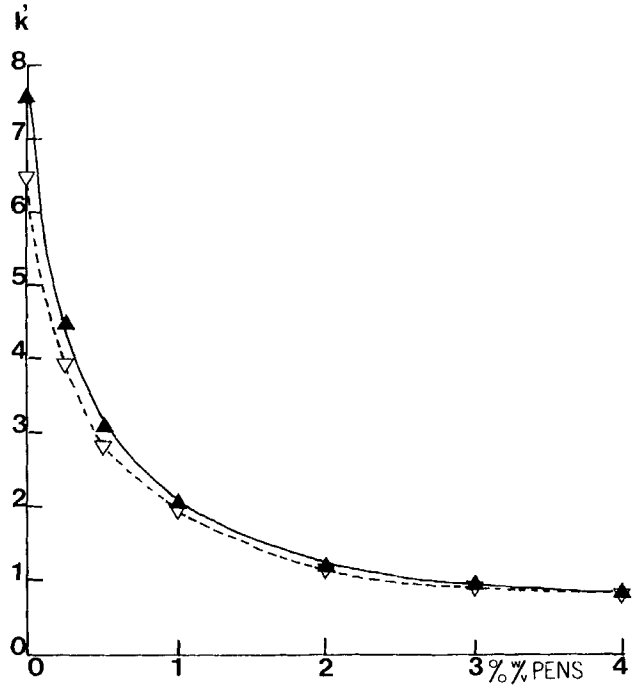

Figure 3 Variation of k´ with mobile phase PENS concentration for ALP (▲) and OXP (▽). Chromatographic conditions: aqueous buffer of 20mM Na_2HPO_4 at pH 2.

solutes as a function of the pairing ion concentration in an acidic eluent was also investigated. It has been demonstrated in our previous studies that the loading of pairing ions on the C18 stationary phase in aqueous eluents is complex (14,15). The use of very hydrophobic pairing ions, such as octanesulphonic acid sodium and sodium lauryl sulphate, may produce unacceptably high k´ values when used with solutes having appreciable retention in the absence of pairing ion. Pentanesulfonic acid (PENS) was thus chosen as the pairing ion in the present investigation. The variation in k´ of ALP and OXP as a function of eluent PENS concentration is shown in Figure (3). The k´ and the separation of both compounds are seen to decrease as the PENS

concentration increases. Such chromatographic characteristics strongly indicate that ALP and OXP are not basic in nature. The reported dissociation constants of ALP and OXP are probably due to the ionization of the hydroxyl group on the azapurine nucleus, ie they are weak acids. Addition of anionic pairing ions into the eluent will only act to deactivate the column rather than to prolong the retention of ALP and OXP (16). Ion-pair chromatographic separation of ALP and OXP using cationic pairing ion has also been reported (17). However, in this study the pH of the eluent was at around 8, and therefore, was insufficient to cause complete ionization of ALP and OXP. Furthermore, such a high mobile phase pH will cause disintegration of the silica support. Therefore, ion-pair chromatography is not an appropriate technique for the separation and quantitation of ALP and OXP when a reversed stationary phase is employed.

Sample Pretreatment

Direct injection of the plasma samples onto the column was found to be unrewarding. Rapid deterioration of the column performance and high blank readings were observed. Extraction of drugs from plasma using diethylether-propranol mixtures of various composition (10) did not provide adequate recoveries (<40%) and selectivities. The use of ammonium sulphate-(diethyl-ether-propranol mixtures) or ammonium carbonate-ethyl acetate salt-solvent pairs (18,19) did not result in any significant improvement of the assay. Injection of the samples after protein precipitation was then adopted. Diluting the plasma three-fold with either acetonitrile or acetone, followed by evaporation under reduced pressure, and reconstitution gave adequate recoveries (>60%). However, the high blank reading obtained drastically reduced the sensitivity of the assay. Removal of the plasma protein and interfering substances by the addition of excess ammonium sulphate crystals was ineffective (20). Trichloracetic acid and perchloric acid were then studied for their sample

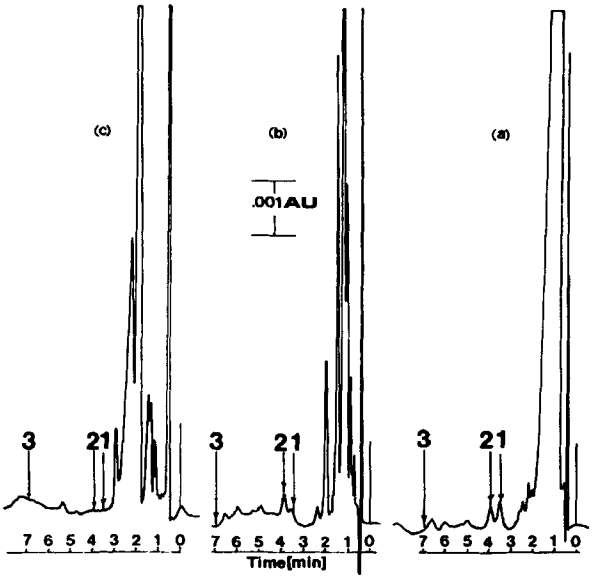

Figure 4 Representative chromatograms of blank human plasma extracts showing the relative effectiveness of different protein pprecipitants in removing interfering endogenous peaks: (a) 200µl trichloroacetic acid (60% w/v) (b) 200µl perchloric acid (60% w/v) and (c) 200µl aqueous solution of trichloroacetic acid (30% w/v) and perchloric acid (30% w/v), all added to 1ml plasma. Chromatographic conditions: see text. Peak retention identification: 1 - OXP, 2 - ALP and 3- floxuridine.

clean-up and extraction efficiency. In order to minimise the sample dilution, very concentrated solutions of the acids (60% w/v) were used. Although both chemicals were found to be quite effective in the removal of interfering substances, a combination of these two protein precipitants provided cleaner chromatograms than when either was used alone. This is demonstrated in Figure (4). This shows chromatograms of a subject's blank plasma processed by the three protein precipitant mixtures. A typical chromatogram from human plasma with known quantities of ALP, OXP and the internal standard is shown in Figure (5).

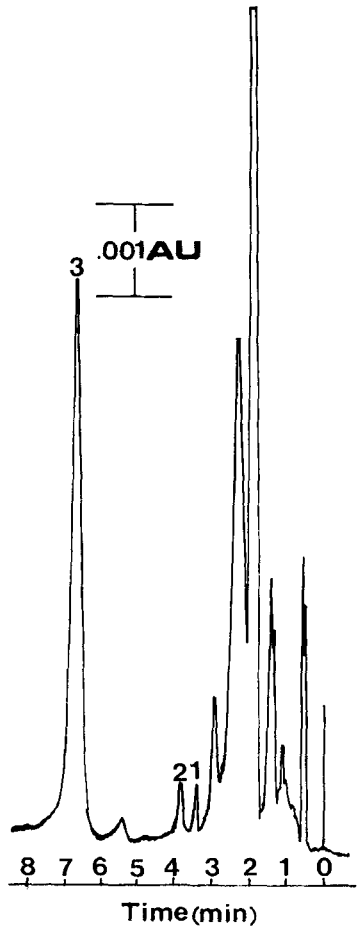

Figure 5 Representative chromatogram of human plasma spiked with 0.1μg/ml of OXP (1), ALP (2) and the internal standard floxuridine (3). Chromatographic conditions: see text.

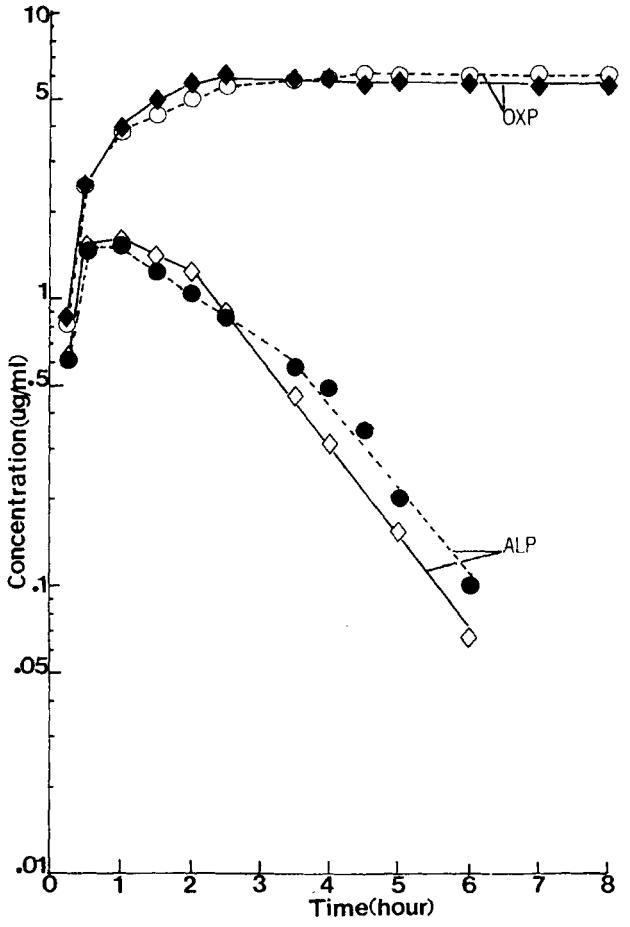

Figure 6 Mean plasma concentrations of ALP and OXP resulting from the oral administration of 300mg allopurinol tablet to eight subjects.
(◆, ◇ - Zyloprim®, ●, ○ - Generic allopurinol).

Linearity, Precision and Sensitivity

Standard curves of ALP (0.05 to 5µg/ml) were prepared in human plasma. All calibration curves were linear with coefficient of determination values greater than 0.97. The between day and within day coefficient of variation based on five determinations was less than 8% for both compounds at plasma concentrations of 0.1, 0.5 and 5µg/ml. Taking a signal to noise ratio of 3 as the criterion, the lowest quantifiable concentration of ALP and OXP was 30ng/ml.

Bioavailability Studies

Figure (6) shows the mean plasma concentration of ALP and OXP in eight volunteers following the oral ingestion of 300mg allopurinol tablets from two sources. Comparison of the bioavailability parameters of ALP, such as the peak plasma level, time to peak level, area under the plasma concentration time curve and the terminal elimination rate constant, using a paired t-test indicates no difference at 5% level. It can therefore be concluded that there is no difference in the bioavailability of ALP from these two dosage forms.

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