

THE DEVELOPMENT OF A LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY METHOD FOR THE DETECTION OF ALLOPURINOL AND ITS METABOLITE IN HUMAN HAIR

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ABSTRACT: The preliminary development of a liquid chromatography-mass spectrometry (LC/MS) method for the detection of allopurinol, and its metabolite, oxypurinol in human hair is reported. The experimental results indicate that allopurinol is not present in incorporated hair, while oxypurinol is. Furthermore, the results indicate that the developed LC/MS method may provide a means by which clinical monitoring of oxypurinol can be achieved; an application of potential importance due to the adverse effects associated with the accumulation of oxypurinol in the body. The LC/MS method described is the first to detect these analytes in hair, and among the first methods to utilise LC/MS for the analysis of drugs in hair.

KEY WORDS: Allopurinol; Oxypurinol; Hair analysis; LC/MS.

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INTRODUCTION

Allopurinol (4-hydroxypyrazolo[3,4-d]pyrimidine) is used in the treatment of medical conditions associated with elevated levels of uric acid, such as gout. The primary metabolic pathway associated with allopurinol involves the enzyme xanthine oxidase, resulting in the major metabolite, oxypurinol (1H-pyrazolo[3,4-d]pyrimidin-4,6-diol) (Figure 1). Allopurinol is rapidly converted to oxypurinol and cleared from plasma, with a half-life of two to three hours; while oxypurinol has a considerably longer half-life of eighteen to thirty hours [4]. Both allopurinol and oxypurinol are amphoteric, a property of particular interest to us with respect to the incorporation and binding of drugs in hair.

The precise mechanism for the incorporation of drugs in hair remains unclear, although a number of mechanisms have been proposed. The potential routes of entry previously postulated include passive diffusion from the bloodstream into the growing hair follicle, passive diffusion from sweat and sebaceous gland secretions, transdermal diffusion, and via external contamination of the hair shaft [1]. Furthermore, various mecha-

nisms and sites associated with the binding of the drug analytes to the hair structure have been hypothesised. The two major potential binding sites are the protein (keratin) and pigment (melanin) components of the hair structure; although the lipid component of the cell membrane complex (CMC) has also been proposed as a potential binding site [1].

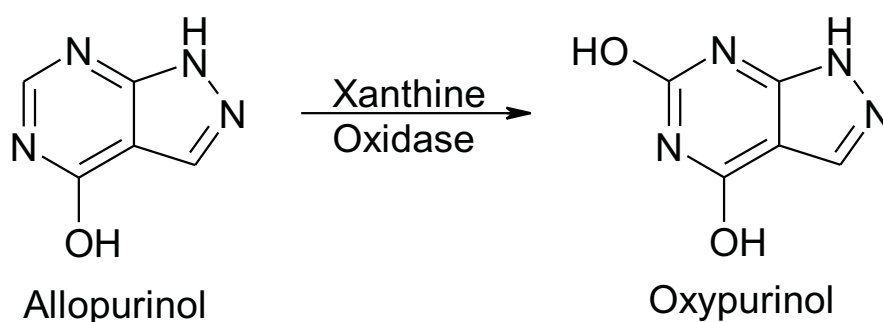


Fig. 1. The primary metabolic pathway associated with allopurinol.

EXPERIMENTAL

Chemicals

Unless stated otherwise, all reagents were of analytical grade. Allopurinol and oxypurinol were purchased from Sigma Chemical Company (Sigma-Aldrich, Australia). Ammonium acetate was obtained from Fluka BioChemika (Sigma-Aldrich, Australia), while glacial acetic acid (BDH HiPerSolv for HPLC, Crown Scientific, Australia), was used for the pH adjustment of the mobile phase. During the preparation of the hair samples, 0.45 mm 25 mm diameter Nylon HPLC syringe filters (Bonnet Equipment, Australia) were used.

Instrumentation

The HPLC method for the detection and separation of allopurinol and oxypurinol was developed using an Waters Alliance 2690 Separations Module, fitted with a Waters 996 Photodiode Array detector and column heater (Waters, Australia). Waters Millennium Software, version 3.05, was utilised. Three 5 mm columns were evaluated during the HPLC method development; a Symmetry C-18 column (3.9 x 150 mm), a Symmetry C-8 column (3.0 x 150 mm), and a SymmetryShield RP-8 column (3.0 x 150 mm) (Waters, Australia). The SymmetryShield RP-8 column was chosen for the analysis of the hair samples, using 10 mmol/l ammonium acetate (pH 4.6) as the mobile phase. Additional chromatographic conditions include: flow rate: 0.5 ml/min, column temperature:

25°C, injection volume: 50 µl and a run time of 10 minutes. Capacity factors were calculated by assuming a constant dead volume of the column.

The hair samples were analysed using a Perkin-Elmer SCIEX API 365 LC/MS-MS system, fitted with an atmospheric pressure chemical ionisation (APCI) heated nebuliser, a PE Series 200 autosampler, a micro PE Series 200 LC pump and a vacuum degasser (PE Applied Biosystems, Australia). Dry air was used as the nebulising gas (flow rate 1.8 l/min), while nitrogen was used as the curtain gas (flow rate 1.2 l/min). An orifice voltage of 15 V was used, at a temperature of 400°C. All hair samples were analysed in negative ion mode, with selective ion monitoring (SIM) of the molecular ions of allopurinol, m/z 135 ($[M-H]^-$) and oxypurinol, m/z 151 ($[M-H]^-$). Quantitation of the two analytes was achieved using MacQuan software, version 1.6.

Procedure

Four patients who were being treated with allopurinol participated in the study. A small tuft of hair, approximately 1 cm in width, was cut as close to the scalp as possible from the vertex posterior region of each individual's scalp. Approximately 30 mg of each hair sample was finely cut with scissors into lengths of roughly 2 mm, and digested under alkaline conditions (1 ml 1 mol/l NaOH, 80°C) for thirty minutes. After cooling, the pH of the resulting solution was adjusted to approximately pH 5, with 0.1 mol/l acetic acid; 1 ml of 10 mmol/l ammonium acetate pH 4.6 (mobile phase) was then added to the digested sample solution. Prior to injection, each digested sample was filtered, using a 0.45 µm nylon syringe filter.

Linearity

Allopurinol and oxypurinol standards were prepared, within a concentration range of 0 to 1 ppm ($n = 6$) and 0 to 2 ppm ($n = 7$), respectively. These standards were included in each batch of samples to produce calibration curves, with quantitation based on peak area. To assess linearity, the line of best fit was determined by least squares regression.

Precision

The precision of the LC/MS analysis was determined by performing replicate analyses ($n = 6$) on a 0.26 ppm oxypurinol standard.

Recovery

For both analytes, the recovery using the alkaline digestion procedure was determined by spiking drug-free hair samples, with a known volume of 2 ppm standard. Recovery was determined as the ratio of the mean determined amount found for each analyte to the amount added.

Limit of detection

For each analyte, the limit of detection was defined as three times the signal-to-noise for six spiked hair samples (at concentrations of 0.05 ppm and 0.1 ppm).

RESULTS

Donor samples

A control hair sample was obtained from an individual who was not receiving allopurinol treatment, who was denoted as donor 1. The limited availability of patients undergoing allopurinol treatment restricted the number of consenting donors participating in study to four. They were denoted as donor 2 through to donor 5. The retention times of allopurinol and oxypurinol were observed to be 5.8 minutes and 5.3 minutes, respectively. A typical chromatogram of a digested hair sample for donor 1 (blank) and donor 3 is given in Figure 2. The concentrations of allopurinol were below the limit of detection in all samples, while the concentrations of oxypurinol ranged from 0 to 14 ng/mg (Table I).

Linearity

All calibration curves obtained displayed good linearity, with correlation coefficients r^2 greater than 0.996, within the concentration ranges of 0–1 ppm and 0–2 ppm for allopurinol and oxypurinol, respectively.

Recovery

The recovery results suggest adequate recovery of analytes by the digestion procedure (Table II).

TABLE I. CONCENTRATIONS OF ALLOPURINOL AND OXYPURINOL FOUND IN THE HAIR OF EACH DONOR

Donor	Dosage allopurinol (per week)	Duration of treatment	Allopurinol mean conc.	Oxypurinol mean conc.
1 (blank)	0	0	BDL	BDL
2	300 mg 7 times	20 days	BDL	BDL
3	300 mg 7 times; decreased to 100 mg 7 times; during last week	> 1 year	BDL	14 ± 1 (n = 6)
4	300 mg 7 times	> 1 year	BDL	2.3 ± 0.3 (n = 4)
5	300 mg 3 times	> 1 year	BDL	3.2 ± 0.3 (n = 6)

BDL – below detection limit; concentrations given as (ng/mg of hair \pm SE_{mean}) $\times 10^{-2}$.

TABLE II. RECOVERY OF ALLOPURINOL AND OXYPURINOL SPIKED DRUG-FREE HAIR SAMPLES

Analyte	Mean recovery \pm coefficient of variation [%]
Allopurinol	88.7 \pm 14.4 (n = 3)
Oxypurinol	65.5 \pm 5.6 (n = 9)

Precision

The coefficient of variation (CV) corresponding to the replicate analyses was determined to be 3.3% (n = 6).

Limit of detection

The determined limits of detection for allopurinol and oxypurinol were (1.3 \pm 0.2) \times

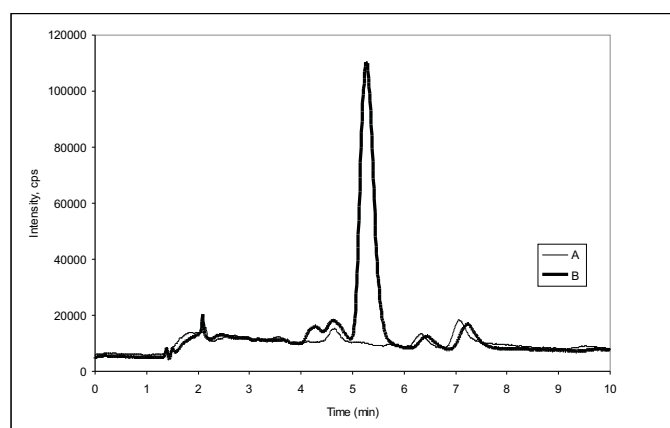


Fig. 2. An example chromatogram of a) digested donor 1 (blank) hair sample; b) digested hair sample from donor 3 (oxypurinol retention time: 5.3 min).

10^{-2} and (1.3 \pm 0.3) $\times 10^{-2}$ ppm, respectively (n = 6).

DISCUSSION

Method development – LC/MS

ElectroSpray Ionisation (ESI) and Atmospheric Pressure Chemical Ionisation (APCI) were both evaluated during the LC/MS method development. The use of ESI was examined, however a high signal to noise ratio lead to a poor limit of detection. A

number of different factors may have contributed to this observation, for example, the ions of each analyte may not be pre-formed in solution, a desirable occurrence for the effective utilisation of ESI. Furthermore, ESI generally is most effective for the analysis of analytes of high molecular mass and/or high polarity, while APCI is commonly utilised for compounds of moderate molecular mass and polarity [5]. Following the evaluation of ESI and APCI (positive and negative modes), it was concluded that APCI in negative ion mode was best suited for the ionisation of both analytes.

It should be noted that hypoxanthine and xanthine are the isosteres of allopurinol and oxypurinol, respectively, and therefore could have potentially interfered with the SIM analysis of allopurinol and oxypurinol. Hypoxanthine and xanthine are present in serum (1–2 mg/l) [3]. However, neither hypoxanthine nor xanthine standards were observed to elute from the column within the analysis time, that is the retention times were greater than 15 minutes.

Method development – HPLC

A total of three columns were evaluated during the HPLC method development for the detection and separation of allopurinol and oxypurinol; a Symmetry C-18 column, a Symmetry C-8 column, and a SymmetryShield RP-8 column. The SymmetryShield RP-8 column was found to be the most appropriate stationary phase for the separation of allopurinol and oxypurinol.

Although a 50 mmol/l ammonium acetate solution was originally used as the mobile phase, preliminary results from the direct infusion of allopurinol and oxypurinol standards into the mass spectrometer suggested that this ionic strength was too high, resulting in ion suppression. Thus, a mobile phase of 10 mmol/l ammonium acetate (pH 4.6) was subsequently utilised. The effect of the addition of an organic modifier of methanol was investigated; 10%, 5%, 2% and 1% v/v methanol with 10 mmol/l ammonium acetate (pH 4.6) were studied. However, optimum capacity and resolution is attained in the absence of the organic modifier (Table III). Furthermore, a linear gradient elution of 10 mmol/l ammonium acetate, pH 4.6 (solvent A) and methanol (solvent B) was considered: 0–100% solvent B over 50 minutes, 100% solvent B held for 10 minutes, with a flow rate 0.5 ml/min. However, little effect was observed, and thus an isocratic elution of the mobile phase was used.

TABLE III. THE INFLUENCE OF ORGANIC MODIFIER (METHANOL) CONCENTRATION ON THE RESOLUTION, R_s AND CAPACITY, k' , OF THE TWO ANALYTE PEAKS OF ALLOPURINOL AND OXYPURINOL

Organic modifier concentration [% v/v]	Resolution, R_s	Capacity, k' (allopurinol)	Capacity, k' (oxypurinol)
0	2.01	2.55	2.23
1	1.81	2.30	2.02
2	1.66	2.09	1.84

5	1.33	1.60	1.41
10	0.95	1.08	0.96

Method development – extraction procedure

For the purposes of developing an extraction procedure for the digested hair samples, spiked hair samples were digested, evaporated and the residue extracted with either acetonitrile or chloroform. Neither solvent was found to efficiently dissolve allopurinol and oxypurinol from the digested hair samples, with recoveries less than 22%. Therefore, the direct injection of the filtered digested hair samples was used for LC/MS analysis. Optimisation of the digestion and extraction procedures could be considered in future studies, with the evaluation of alternative digestion conditions, such as acidic or enzymatic digestion, and solid phase extraction (SPE).

Donor samples

Allopurinol was not detected in any of the samples tested, while oxypurinol was detectable in three out of the four samples collected from treated subjects (Table I). The absence of oxypurinol in the donor 2 hair sample was expected due to the growth rate of hair and the fact that the donor had only been receiving allopurinol for 20 days, prior to sample collection. The oxypurinol level in hair determined for donor 3 (14×10^{-2} ng/mg of hair) was approximately five times greater than the average oxypurinol concentration determined for donor 4 and donor 5 (2.75×10^{-2} ng/mg of hair). This result may reflect the allopurinol dosage regime of donor 3 (Table I), which was originally high and had recently been decreased. Therefore, it can be proposed that the current method may have applications for the therapeutic monitoring of oxypurinol, which is of importance due to the possible adverse effects associated with accumulation of oxypurinol, such as idiosyncratic bone marrow depression [2]. However, it should be noted that the experimental data is insufficient to infer that a relationship between the dose of allopurinol and the amount of metabolite (oxypurinol) found in hair. For example, donor 3 and donor 4 had previously been receiving equivalent doses of allopurinol, but the mean determined concentration of oxypurinol found in the hair samples differed by a factor of approximately 6. This variability presumably reflects individual variability in factors such as allopurinol metabolism and/or those that influence both the incorporation and subsequent binding of analytes to the hair structure. Due to the small number of donors participating in the study, no conclusive inferences can be drawn from the experimental data regarding dose-hair concentration relationships.

The data indicates that oxypurinol binds to the hair matrix, while allopurinol does not. However, the rapid clearance of allopurinol from plasma may be the factor that restricts its incorporation and binding into the hair matrix. The detection of oxypurinol in hair demonstrates that amphoteric drugs can become incorporated into hair. Therefore, the developed method may form the basis for the development of LC/MS

methods for the detection of more toxic amphoteric drugs in hair. This would include drugs not easily analysed by GC/MS, such as pyrimidines and uridines.

CONCLUSION

A LC/MS method for the detection and quantitation of allopurinol and its metabolite, oxypurinol in hair has been successfully developed. The data indicated that allopurinol does not become incorporated into hair, but its metabolite oxypurinol does, suggesting that the method may have therapeutic monitoring applications for oxypurinol. The study demonstrated that amphoteric drugs can become incorporated into hair. The present method is the first to detect these analytes in hair, and among the first methods to utilise the technique of LC/MS for the analysis of drugs in hair.

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