ENHANCING BIOMARKER ANALYSIS: QUANTITATION OF URIC ACID FROM A DRIED BLOOD SPOT

Jill Segelbacher, Julian Haynes and Angus Nedderman

Unilabs York Bioanalytical Solutions, Discovery Park, Ramsgate Road, Sandwich, Kent, CT13 9ND

Introduction

Uric acid measurement is an important tool in the diagnosis of gout or renal disease. Allopurinol and its active metabolite oxypurinol inhibit the synthesis of uric acid by action on the enzyme xanthine oxidase.

We have developed a quantitative method for uric acid and oxypurinol in rat dried blood spots (DBS), using methyluric acid as an internal standard.

Method development involved the resolution of a number of analytical issues due to the polar, diprotic nature of these sparingly soluble acids (Figure 1).

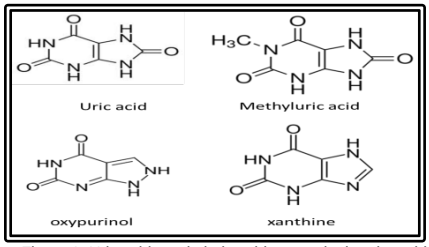

Figure 1: Uric acid, methyluric acid, oxypurinol and xanthine structures

Poor chromatographic retention was observed on reverse phase columns and subsequent additional chromatography work was performed to enable the simultaneous analysis of oxypurinol and xanthine (Figure 1). Due to the endogenous presence of uric acid in rat blood, calibration curves were prepared to assess the optimum surrogate matrix for standard preparation. The final method was suitable for the quantitation of uric acid in the range 0.01–10µg/mL.

Optimising Chromatography

In order to achieve sufficient retention of these polar analytes a Synergi Fusion RP-80-A, 50 x 4.6mm, 4µm column in aqueous loading mobile phase conditions was used. Injection solvent composition was also kept aqueous to avoid peak splitting which was apparent with injections of uric acid in increasing in organic solvent (Figure 2).

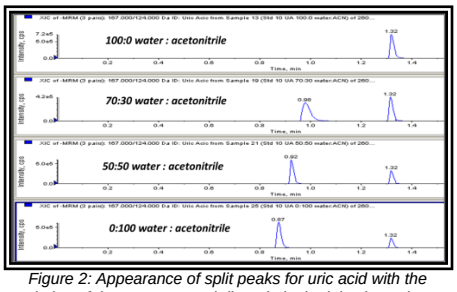

Figure 2: Appearance of split peaks for uric acid with the variation of the water:acetonitrile ratio in the injection solvent.

The gradient used for optimum retention of uric acid and oxypurinol was a slow ramp (0.1-2.0mins, 1mL/min) from 100%A – 40%B, where A was 2mM ammonium acetate + 0.03% formic acid in water and B was acetonitrile.

Method Adaptation

Oxypurinol is a xanthine oxidase inhibitor, and therefore can lead to an increase in xanthine levels. Method adaptation was required to separate oxypurinol and xanthine (Figure 1) present in blood. These compounds are isobaric and share the same MRM transition, using the existing method the peaks co-eluted.

A gradient of 100%A - 20%B at 1.8mins was employed to separate oxypurinol and xanthine (Figure 3) whilst preserving the uric acid retention. Two further endogenous components were separated on analysis of blood samples and standards.

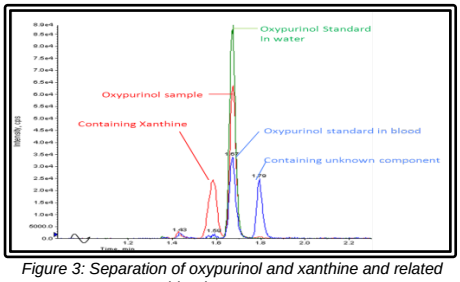

Figure 3: Separation of oxypurinol and xanthine and related blood components

Detection was in negative mode on an API5000 triple quadropole mass spectrometer (Table 1).

	Q1 (m/z)	Q3 (m/z)	Collision energy (eV)
Uric acid	167.0	124.0	-22
Methyluric acid	181.0	138.0	-22
Oxypurinol / xanthine	150.8	108.0	-22

Table1: MS/MS parameters

Sample Preparation

Blood samples (20µL) were spotted onto Whatman DMPK B DBS cards and dried (Figure 4). Extraction recovery testing revealed water to be the optimum extraction solvent for these polar analytes.

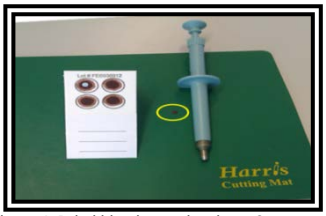

Figure 4: Dried blood spot showing a 3mm punch

Blank rat blood is not a suitable matrix for preparation of calibration standards, since it contains significant levels of uric acid. To assess and compare blank matrices, calibration lines were prepared three ways. Individual working solutions were prepared to spike 20µL for each standard at equivalent concentrations to a 3mm punch (3µL of blood).

e.g. 1000ng/mL standard = 3ng/3µL
= 3ng in 20µL spiked water
= working solution 150ng/mL

1: Dog blood 3mm DBS + 20µL standard working solution + 20µL internal standard + 500µL water.
2: Blank DBS card + 20µL standard working solution + 20µL internal standard + 500µL water.
3: 20µL standard working solution + 20µL internal standard + 500µL water.

Dog blood was chosen as an effective blank matrix for this test as dogs metabolise uric acid to allantoin, whereas rat blood contains high levels of endogenous uric acid. Calibration lines showed good correlation between the three methods (Figure 5) and it was concluded that the addition of a blank 3mm card punch (method 2) was a good matrix match for the blood samples on a dried blood spot.

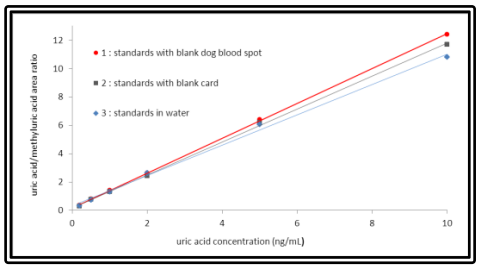

Figure 5: Calibration lines showing correlation between uric acid standards spiked into water, and in the presence of blank card and blank dog blood.

In the case of oxypurinol, which is not endogenous in rat blood, standards were shown to be equivalent when prepared in rat blood or card only.

Conclusions

Challenging chromatography presented as almost no retention on reverse phase columns, good retention was obtained using aqueous loading and injection conditions on a Synergi Fusion RP phase. Optimisation for uric acid and oxypurinol alone lead to the appearance of the closely related xanthine, which co-eluted with oxypurinol in blood samples. This was overcome with a gradient modification.

Dried blood spot analysis presented additional analytical issues, such as poor solvent extraction due to the polar nature of these analytes. Sample volume was limited to just 3µL in a 3mm punch, significantly less that standard serum / plasma extraction volumes[2]. There is also little opportunity for repeat analysis, so a robust method was crucial. As uric acid can be an additional test, there may be other analytes to measure from the same spot; with many drugs being lipophilic, a separate solvent extraction method may be required, necessitating a second 3mm punch to be taken from the blood spot. Endogenous components also need to be separated, as they may interfere with quantitation, particularly at the low end of the calibration range.

Matric matching standards can be difficult; we determined using standards in water and in the presence of a DBS blank card punch a good surrogate. An alternative isotope dilution method utilises $^{15}N_2$-Uric Acid to quantify uric acid in serum[1]. The DBS methodology could be adapted to incorporate this for quantitation or comparison.

Our method has performed well with excellent standard and QC data, and we have been able to deliver good quality data with fast turnaround in a discovery environment.

References

1: Dai X, Fang X, Zhang C, Xu R and Xu B. Determination of serum uric acid using high-performance liquid chromatography (HPLC)/isotope dilution mass spectrometry (ID-MS) as a candidate reference method. *J Chromatogr B*, **857**, 287-295 (2007).

2: Kim KM, Henderson GN, Ouyang X, Frye R, Sautin Y, Feig DI and Johnson RJ. A sensitive and specific liquid chromatography-tandem mass spectrometry method for the determination of intracellular and extracellular uric acid. *J Chromatogr B*, **877 (22)**, 2032-2038, (2009).

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