OPTIMISING THE EXTRACTION, SEPARATION AND DETECTION OF SMALL POLAR COMPOUNDS

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Introduction

Small polar compounds present a significant analytical challenge, particularly in an environment where fast turnaround with no compromise on quality is expected. Notably, bioanalysis of this type of compound is compromised by poor retention on generic reverse-phase LC systems and low sensitivity as a result of inefficient mass spectrometric ionisation. The challenge is intensified when the analysis involves dried blood spots (DBS), for which the generic methodology is extraction in organic solvent, an approach that is typically unsuitable for more polar compounds.

An extreme example of this phenomenon was a quantitative method developed for uric acid and oxypurinol in rat DBS, using methyluric acid as an internal standard (Figure 1).

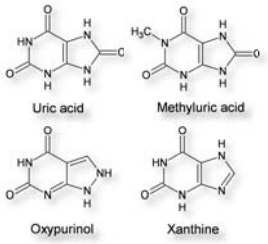

Figure 1: Uric acid, methyluric acid, oxypurinol and xanthine structures

Further examples of small polar compounds which we have successfully quantified in dried blood spots are shown Figure 2.

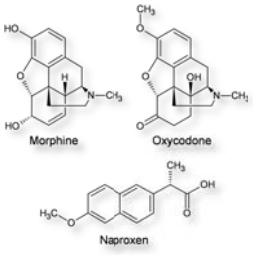

Figure 2: Morphine, oxycodone and naproxen structures

Dried Blood Spots

Dried blood spots have many advantages, particularly at the point of sampling. However, they do give the bioanalyst less analyte to work with. Where sensitivity is limited due to poor MS ionisation, poor extraction recovery and/or poor retention on the LC system, this limited sample volume becomes a real issue.

Extraction

For uric acid, its related compounds and morphine the extraction was in water, whilst for oxycodone and naproxen, the extraction was in 25% water/acetonitrile. The extraction solutions were transferred to a clean plate to remove the spot ready for assay.

Figure 3: Blood samples (20µL) were spotted onto Whatman DMPK B DBS cards and dried. 3mm punches were then taken for extraction

Endogenous Uric Acid

For the uric acid assay it was not possible to use blank rat blood as the matrix for preparation of calibration standards, since it contains significant levels of uric acid. For calibration standards and quality control samples, 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

To account for any matrix effects from components impregnated on the cards, a 3mm punch of blank DBS card was added to 20µL of standard working solution + 20µL internal standard and 500µL of water was added for extraction.

Chromatography

By making modifications to our generic 2D system[1] good chromatography was obtained for the polar compounds, involving decreasing the aqueous loading time on the trapping cartridge (Opti-Trap EXP™ packed with Halo material) to achieve retention, followed by slowing down the gradient to elute the analyte and obtain separation on the analytical column (Accucore Polar Premium, 50x3mm, 2.6µm).

Uric acid was not sufficiently retained on the trapping cartridge, so was injected directly onto a Synergi Fusion RP80A, 50x4.6mm, 4µm column in aqueous loading mobile phase, with an aqueous injection solvent to avoid peak splitting. The gradient was a slow ramp (0.1-2.0mins, 1mL/min) from 100%–60%A, where A was 2mM ammonium acetate + 0.03% formic acid in water and B was acetonitrile.

Mass Spectrometry

Uric acid and related compounds were detected in negative ion mode (Table 1) and morphine, oxycodone and naproxen in positive ion mode (Table 2) on an API5000 triple quadrupole mass spectrometer.

	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: Negative ion MS/MS parameters

	Q1 (m/z)	Q3 (m/z)	Collision energy (eV)
Morphine	286.2	165.1	65
Oxycodone	316.2	241.2	39
Naproxen	231.1	185.1	22

Table2: Positive ion MS/MS parameters

Recovery

Poor recovery of morphine from blood spots was obtained, even in water (Figure 4), most likely resulting from ionisation suppression by a component of the DBS card.

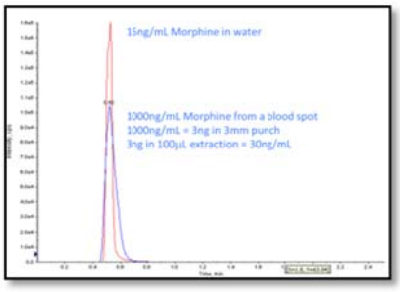

Figure 4: Morphine extracted from a 1000ng/mL DBS spot compared to a solution of morphine in water

Conclusions

Although these compounds presented significant challenges due to their polar properties, robust methods with good retention and separation were achieved, notably for uric acid, where resolution from potential interferences was critical (Figure 5).

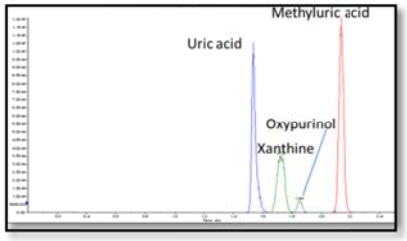

Figure 5: LC/MS/MS data for uric acid and related components

Dried blood spot analysis presented additional analytical issues, such as poor solvent extraction, whilst sample volume was limited to approximately 3µL in a 3mm punch, significantly less that standard serum/plasma extraction volumes[2] as calculated below:

3mm DBS:
100ng/mL standard = 0.3ng/3µL
Reconstituted in 200µL = 0.3ng/200µL
= 1.5ng/mL

100µL Plasma:
100ng/mL standard = 10ng/100µL
Extracted and reconstituted in 200µL = 10ng/200µL
= 50ng/mL

DBS also presented little opportunity for repeat analysis, such that a robust method was crucial. As the analysis of uric acid is typically a biomarker test, the analysis of other compounds from the same spot may be required; in the likely event that these analytes are lipophilic, a separate solvent extraction method may be required, necessitating a second 3mm punch to be taken from the spot.

For biomarker assays, matrix matching standards can be difficult; we determined that using standards in water and in the presence of a DBS blank card punch made for a good surrogate. An alternative isotope dilution method utilises $^{15}N_2$-uric acid to quantify uric acid in serum[3]. The DBS methodology could be adapted to incorporate this for quantitation or comparison.

Overall, our methods have 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: Clark G and Haynes JJ. Utilization of DBS within drug discovery: a simple 2D-LC–MS/MS system to minimize blood- and paper-based matrix effects from FTA elute™ DBS. *Bioanalysis*, **3**, 1253-1270 (2011).

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3: 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).

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