

Efficient Recoveries of Drug-Related Material from Rodent Eye Tissues for Metabolite Analysis Using Novel Adaptive Focused Acoustic Technology



Caroline Sychterz; Michael J Morris; Kitaw Negash; May Ho
Drug Metabolism and Pharmacokinetics, GlaxoSmithKline, King of Prussia, PA

Introduction

Occasionally during the course of a drug's development the levels of drug and potential metabolites in specific tissues must be determined; however, when those tissues are fibrous and small (~100 mg) efficient sample preparation where homogenization and extraction are required often proves to be a difficult task. Previous attempts to utilize standard homogenization and pulverizing techniques to prepare rodent eyes, such as Waring blenders, probe homogenizers and a mortar and pestle, were either inefficient or labor intensive and technically difficult. In each instance the chances for cross-sample contamination were high as was the time and effort spent by the analyzing scientist during multiple sample preparations.

Adaptive Focused Acoustic (AFA) technology has shown promise in enhancing sample recovery in plant and animal-based samples [1, 2, 3]; however, this technique has yet to be incorporated in rodent eye quantitative metabolite analysis. We report here a novel non-contact sample preparation method for determination of drug-related material of a proprietary compound in rat and mouse eye extracts, based on freeze fracturing accompanied by AFA technology.

Methods

Sample Source

Rat eyes were collected during a GlaxoSmithKline-sponsored study performed at Covance (Madison, WI) where 20 male Long Evans rats received a single oral dose of [¹⁴C]compound A in 1% methylcellulose at 300 mg/kg. Eyes were collected after euthanasia 8 h post-dose.

Mouse eyes were collected during an internal study performed by DMPK, GlaxoSmithKline where 30 male B6C3F1 mice received a single oral dose of [¹⁴C]compound A in 1% methylcellulose at 70 mg/kg. Eyes were collected after euthanasia 24 h post-dose.

Freeze Fracture and Homogenization of Rodent Eyes

4 rat eyes and 5 mouse eyes were used. On the day of analysis, each rat eye was placed into individual Covaris TissueTubes™ (Covaris, Inc., Woburn, MA), frozen using a dry ice/acetone bath and the extracellular matrix disrupted after 1 impact event using a Covaris Cryoprep™ CP02 (see figures below). The resulting powder from each rat eye was pooled by simply inverting the TissueTube™ such that the samples fell into a 16 mm glass tube. Approximately 3-6 volumes of water were added to the pooled eye sample and homogenization carried out by sonicating 60-80 seconds on a Covaris E210 sonicator. Mouse eyes were treated similarly, however, eyes were pooled prior to disruption by the CryoPrep™. An aliquot of the dissolved [¹⁴C]compound A dose suspension was processed similarly to monitor compound stability during the sonication process.



Extraction

Eye extraction methodology was adapted from Ono and Tanaka [4]. Resulting eye homogenates generated following freeze fracturing and AFA sonication were extracted by adding 3 volumes of 1:1 (v:v) methanol:0.5M hydrochloric acid. Samples were sonicated using the Covaris E210 sonicator and shaken for an additional 10-15 minutes prior to centrifugation at 3000-3900g at room temperature for 5 minutes. Resulting supernatants were removed. Residual rat eye pellets were extracted a further 4 times as described above with the last extraction using 10% trichloroacetic acid as the extraction solvent. Residual mouse eye pellets were extracted a further 4 times as described above using 10% trichloroacetic acid followed by 3 successive portions of 4:1 (v:v) methanol:ethyl ether. All residual pellets were dissolved by adding an aliquot of 1 M aqueous sodium hydroxide (3 times the original sample volume) at 50°C. The total volume of each extract was determined and triplicate aliquots removed for liquid scintillation counting to monitor sample recovery.

Stability samples were prepared by spiking a dissolved aliquot of [¹⁴C]compound A dose suspension into control eye homogenates and processed similarly to monitor compound stability during this extraction process.

Radio-HPLC Analysis of Eye Extracts

Relevant eye extracts were pooled and evaporated under a stream of nitrogen gas. Samples were reconstituted in 1:1 (v:v) methanol:water prior to radio-HPLC analysis.

Determination of [¹⁴C]-related Material Recovery

Recoveries of radioactivity were determined by comparing the total amount of radioactivity recovered in individual extracts to the total radioactivity summed between individual extracts and the pellet dissolution.

Results

Recoveries of Radioactivity

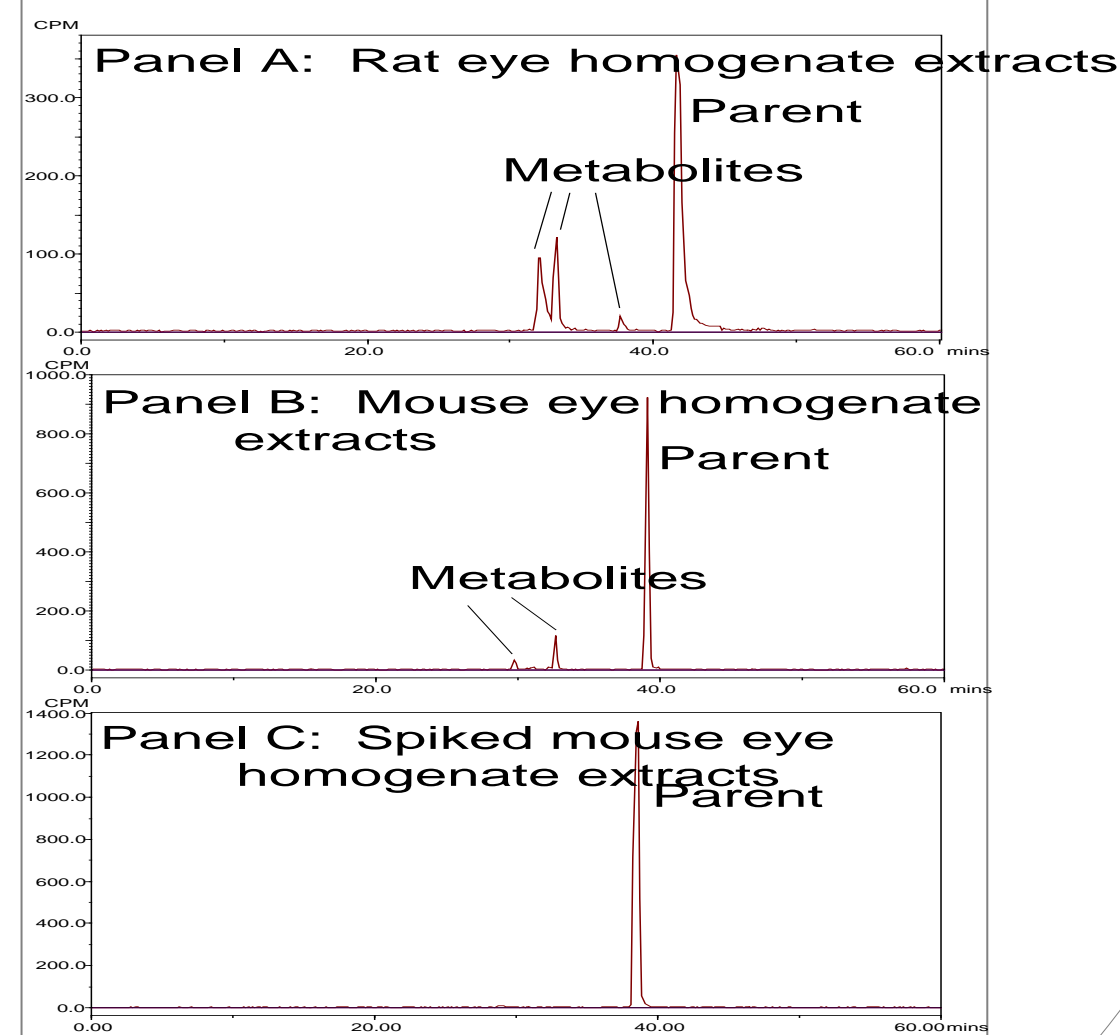
Recoveries of radioactivity of [¹⁴C]compound A following extraction of rat and mouse eyes are shown below. Using this sample preparation method >86% of [¹⁴C]compound A-related material was recovered in the rodent eye extracts.

	% Radioactivity Recovery from Rodent Eyes			
	Mouse Eyes	Mouse Eye Stability	Rat Eyes	Rat Eye Stability
Extract #1	80.3	90.4	49.7	58.9
Extract #2	2.6	1.1	23.6	25.3
Extract #3	1.4	1.0	10.0	9.5
Extract #4	0.9	0.9	5.4	3.7
Extract #5	0.9	0.2	1.2	1.1
Total Extractable	86.1	93.6	89.9	98.5
Pellet	13.9	6.4	10.1	1.5

See Extraction section for extraction solvents used for each sample.

Radio-HPLC Analysis of Eye Extracts

Radio-chromatograms of eye extracts are shown below (panels A and B). Radio-HPLC analysis of control samples spiked with [¹⁴C]compound A revealed that the parent molecule had not degraded as a result of sample preparation (panel C).



Retention time shifting was observed with this HPLC method between species. The analytical columns used for each study were of the same stationary phase and vendor but represented two separate lots of packing material.

Conclusions

- In combination with freeze fracturing, AFA is a non-contact technology that can be used to pulverize rodent eye samples with minimum cross-sample contamination for multiple sample processing.
- Successful sample processing of small (<100 mg) fibrous samples was achieved with >86% recovery of radioactivity from rodent eyes following oral dosing of [¹⁴C]compound A. Spiked control samples exhibited recoveries >93%.
- No evidence of compound degradation as a result of the extraction or high-powered sonication technique.
- Efficient multiple sample preparation can be performed quickly (~2 minutes/sample) with little effort from the analyzing scientist.

References

- [1] Toorchi M, Nouri MZ, Tsumura M, Komatsu S (2008) Acoustic Technology for High-Performance Disruption and Extraction of Plant Proteins. *J Proteome Res* 7:3035-3041.
- [2] Wenger MD, DePhillips P, Bracewell DG (2008) A Microscale Yeast Cell Disruption Technique for Integrated Process Development Strategies. *Biotechnol Prog* 24:606-614.
- [3] <http://www.covarisinc.com/publications.html> (accessed 20.03.2009)
- [4] Ono C and Tanaka M (2003) Binding Characteristics of Fluoroquinolones to Synthetic Levodopa Melanin. *J Pharm Pharmacol* 55:1127-1133.

Acknowledgements

Fangming Xia