Development of a Normal Phase HPLC Method for the Separation of Creatinine and Purine Metabolites

Merlin K. L. Bicking, Ph.D.
ACCTA, Inc.
PO Box 25602
St. Paul, MN 55125
Phone: 651-731-3670
Email: mbicking@accta.com

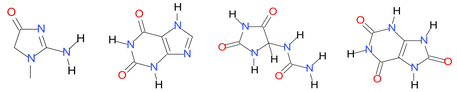
Bryan Evans
Imtakt USA
1511 Walnut St., Ste 310
Philadelphia, PA 19102
Phone: 888-456-HPLC
Email: bevans@imtaktusa.com

Abstract

The determination of creatinine and purine metabolites can be an important marker for renal function, and the concentrations of various metabolites are an indicator for certain disease states. However, this is a difficult separation problem because of the dramatic differences in chromatographic behavior of the analytes.
A novel HPLC method was developed that separates and quantifies these compounds of interest on an aminopropyl phase without the use of ion pairing additives. Important variables that were optimized for this method include stationary phase selection, sample solvent composition, mobile phase additives, and pH.

A single column, multi-wavelength method will be shown for the following analytes: creatinine, xanthine, allantoin, and uric acid.
This method is currently being used for routine urine analysis.

Structures for Creatinine and Purine Metabolites



creatinine	xanthine	allantoin	uric acid
mwt = 113	mwt = 152	mwt = 158	mwt = 168
xLogP3 = -1.3	xLogP3 = -0.7	xLogP3 = -2.2	xLogP3 = -1.9

http://pubchem.ncbi.nlm.nih.gov/

Analytical Problems

- The four analytes have significantly different chromatographic characteristics.
  - Range of polarity makes column selection difficult
- Absorbance spectra are all unique
  - Single wavelength analysis not practical
- Need a single column system with short analysis times and without the use of ion pairing agents.

Initial Chromatographic Results

•This chart summarizes initial results for initial column choices.

	Reversed Phase/C18	HILIC/Silica	PFP*
Creatinine	No retention	Good retention	Good retention
Allantoin	No retention	Some retention, not separated from uric acid	No retention
Uric Acid	Slight retention	Some retention, not separated from allantoin	No retention

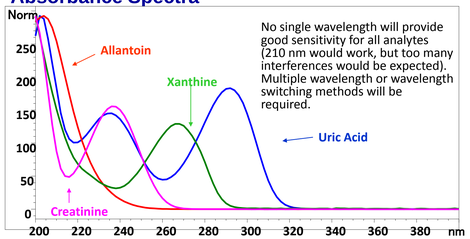
* Pentafluorophenylpropyl

Conclusion: No single phase was capable of providing adequate retention for all analytes!

Unison UK-Amino (3um)

- Experiments indicated that Unison UK-Amino (Imtakt USA), using an ammonium formate buffer (pH ~ 7), produced retention for all analytes.
- The retention was affected by buffer concentration, with less retention at higher concentrations.
- The retention patterns indicated that the separations were a combination of Aqueous Normal Phase (ANP) and anion exchange.
  - Retention decreased with an *increase* in water concentration.
  - Water is a "strong" solvent in this system.

Absorbance Spectra



Optimized Chromatographic Conditions

- Unison UK-Amino, 150 x 4.6mm (3um), 35 ºC
- Flow: 1.5 mL/min
- Injection: 5 uL

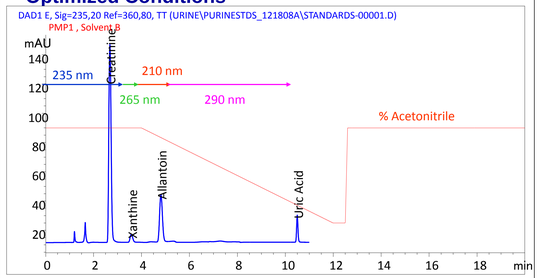
Gradient Program

Time, min.	A: 50mM Ammonium Formate	B: Acetonitrile
0	10	90
4.0	10	90
12.0	75	25
12.5	75	25
12.6	10	90
20.0	10	90

Wavelength Switching

Time, min.	Wavelength, nm
0	235
3.2	265
4.0	210
5.5	290

Optimized Conditions



Compound	Retention Time, min.	Wavelength, nm	Conc., µg/mL
Creatinine	2.66	235	100
Xanthine	3.66	265	10
Allantoin	4.79	210	100
Uric Acid	10.5	290	10

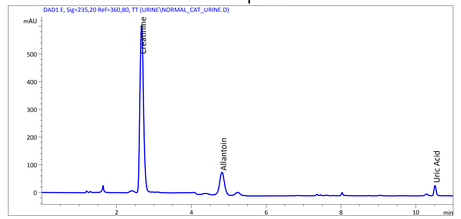
Sample Preparation

- Collect urine sample
- Dilute 1:10 with 50 mM ammonium formate
- Filter through 0.45 um filter into autosampler vial
- Inject 5.0 µL

- Note: concentrations in urine are high relative to the sensitivity of the method, so actual dilution factors can be adjusted as needed. However, some pH adjustment is needed to prevent precipitation of some analytes.

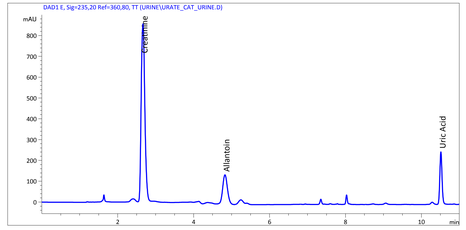
Normal Cat Urine

The normal cat has more allantoin, as allantoin is the final step in the metabolic pathway and is quite water soluble. Thus, animals producing more allantoin do not form stones composed of purine metabolites. Xanthine is not present.



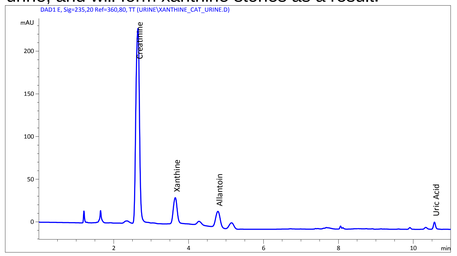
Urate Cat Urine

- This animal has elevated levels of uric acid, resulting in the formation of urate stones in the urine.



Xanthine Cat Urine

- This cat, with hereditary xanthinuria, formed xanthine stones in the urinary bladder. Xanthine is found in the urine, and will form xanthine stones as a result.



Conclusions

A method has been developed that allows rapid and complete separation of the three main purine metabolites in urine.
- A gradient program with wavelength switching is required.
- Real-world samples can be analyzed with no sample preparation. Only dilution is required. No interferences are present.

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References

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YAZAWA Itaru, TACHIKAWA Hiroshi, OKAMURA Fumiko
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