

Urine Stability for Metabolomic Studies: Effects of Preparation and Storage

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ABSTRACT

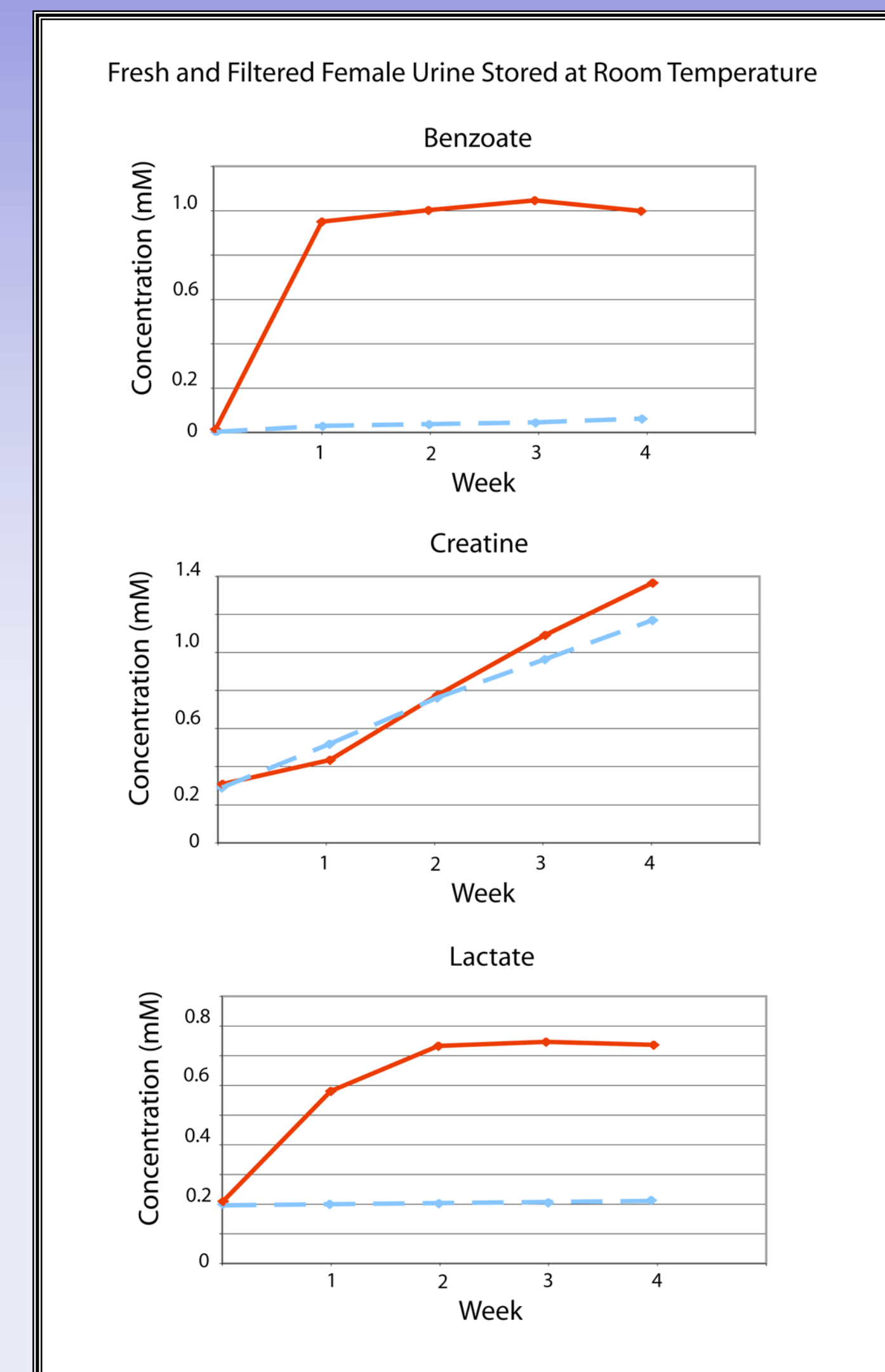
Due to the ease of collection and the metabolite-rich nature of urine, it is frequently used as a bio-fluid for human and animal metabolic studies. Urine samples were collected from a healthy male and female subject and prepared: raw, following centrifugation, filtration, or the addition of the bacteriostatic preservative sodium azide and analyzed by NMR. In addition, these samples were stored at room temperature (22°C), in a refrigerator (4°C), or in a deep-freeze (-80°C). Samples were analyzed by 1D ¹H nuclear magnetic spectroscopy every week for a month and changes in concentrations of fifty-five easily identifiable metabolites were followed. The degree of change in metabolite concentrations following storage over a four-week period were influenced by the different methods of sample preparation and storage. Significant changes in urine metabolites are likely due to bacterial contamination of the urine. Our study demonstrates that bacterial contamination of urine in normal individuals significantly alters the metabolic profile of urine over time and proper preparation and storage procedures must be followed to reduce these changes. By identifying appropriate methods of urine preparation and storage investigators will preserve the fidelity of the urine samples in order to better reflect the original metabolic state.

INTRODUCTION

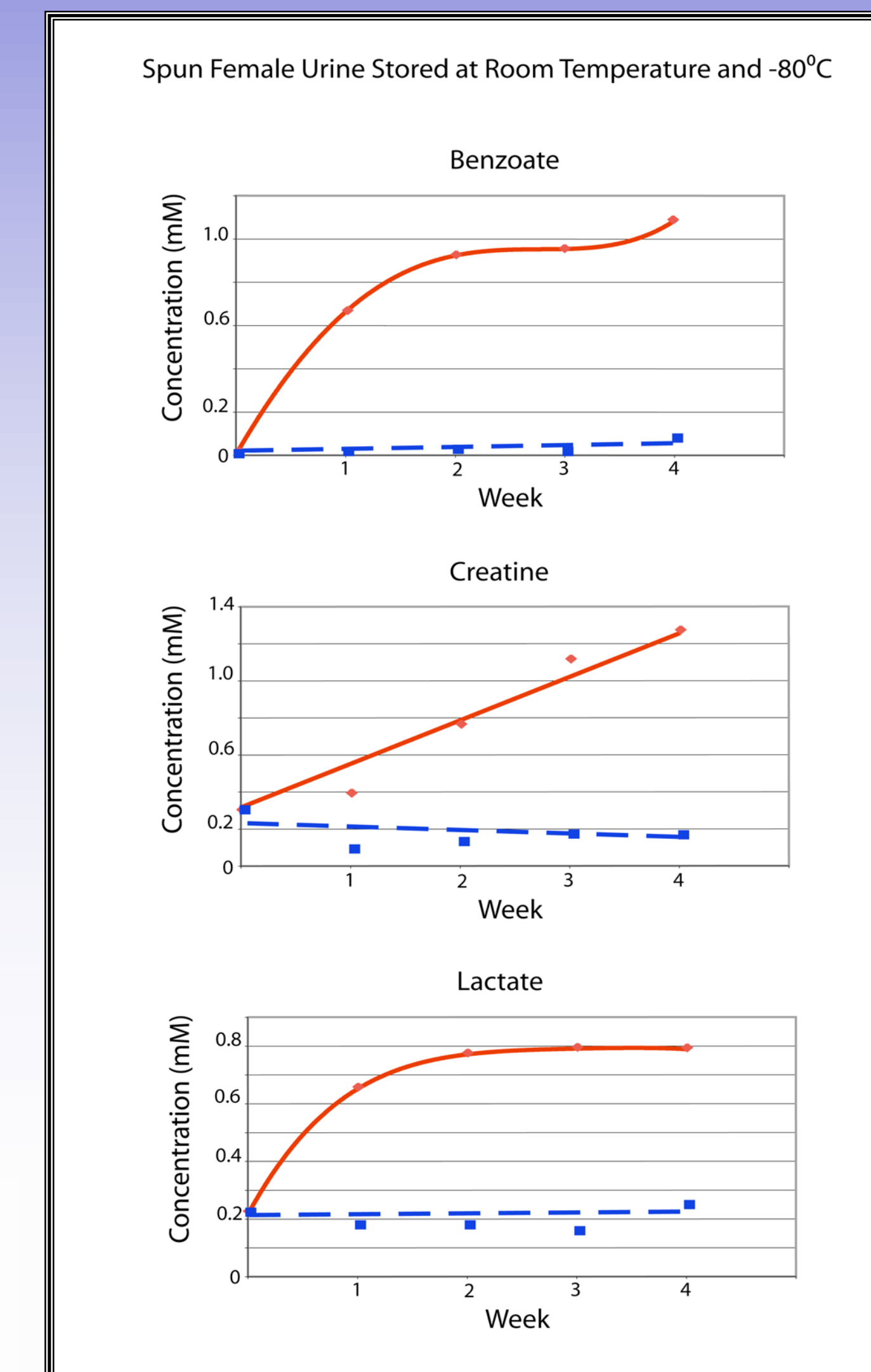
- Urine is a common biofluid for metabolomic investigations due to the ease of collection and rich metabolite profile
- Use of urine in human studies often involves collection in a clinical setting
- Spectral acquisition by ¹H-NMR may take place days or weeks following collection
- Metabolomic studies rely upon the correlation of metabolite concentration differences with the unique system or disease state being investigated
- To ensure the fidelity of the biological sample it is clear that attention must be given to how a sample is prepared, and stored prior to analysis
- Our study documents the effect sample handling, preparation, and storage has upon the metabolic make-up of urine

METHODS

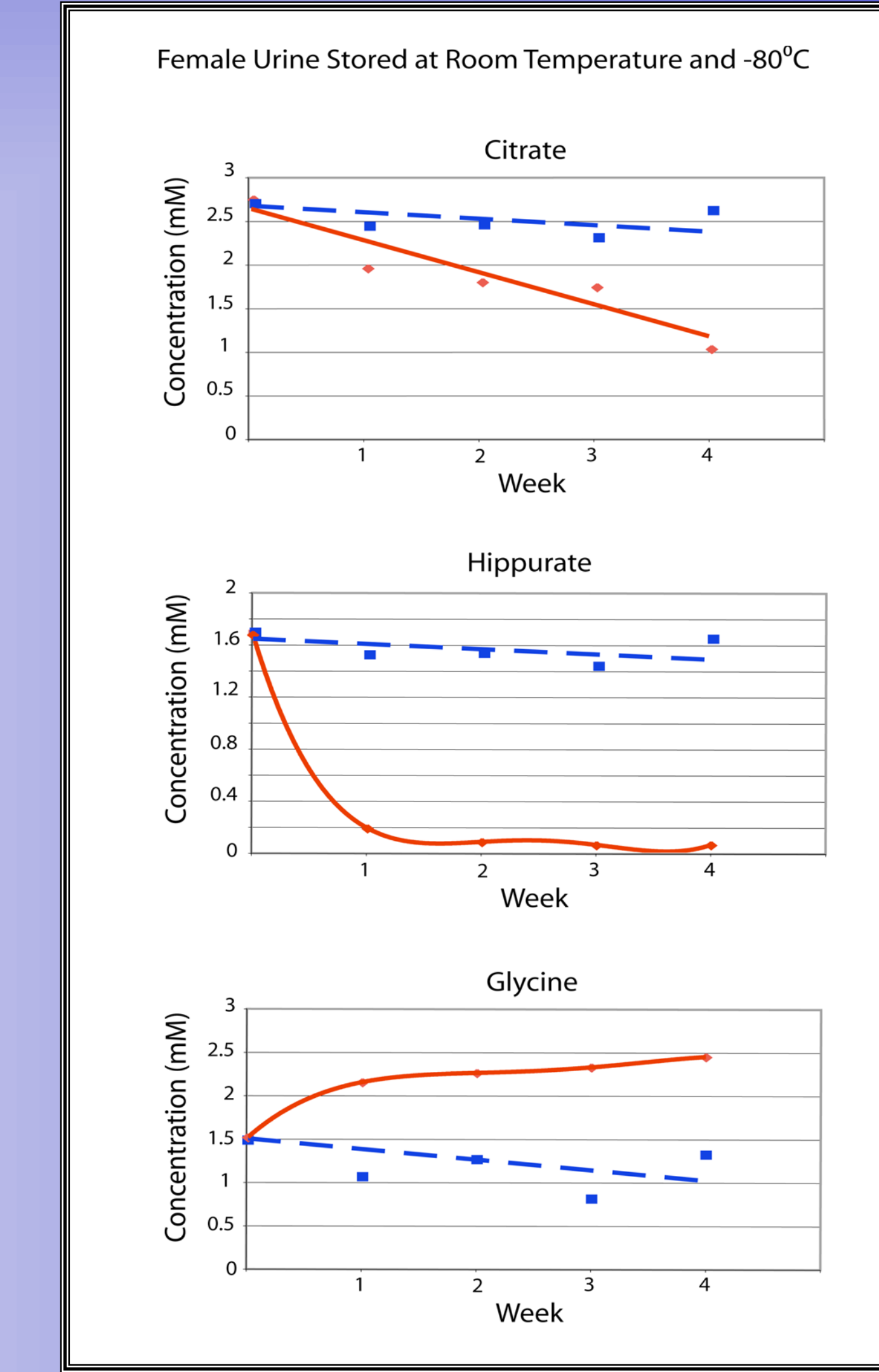
Urine Collection: One healthy female and male volunteer provided urine samples
Sample Preparation: Sample preparation was duplicated for both male and female urine samples and in triplicate for the three different methods of storage. In a biosafety fume hood fresh urine samples were prepared by transferring a 630 µl aliquot of urine to a 1.5 ml Eppendorf tube followed by the addition of 70 µl of a standard solution (4.9 mM DSS, and 100.0 mM imidazole in D₂O). Filtered urine samples were prepared by filtering 1 ml of urine through a 0.22 µm syringe filter (Millipore, Cambridge, Ont. Canada), a 630 µl aliquot of the filtered urine was transferred to a new Eppendorf tube and 70 µl of the standard solution was added (see above). Spun urine samples were prepared by the centrifugation of 1ml of urine in an Eppendorf tube at 10,000 rpm for 10 min., a 630 µl aliquot of the spun urine was placed in a new Eppendorf tube followed by the addition of 70 µl of the standard solution (see above). For urine samples with the preservative sodium azide, 1ml of raw urine was transferred to a 1.5 ml Eppendorf tube followed by the addition of a stock solution of sodium azide in order to reach a final sample concentration of 0.1, 1.0, and 10 mM sodium azide. A 630 µl aliquot of the urine and sodium azide solution was transferred to a 1.5 ml Eppendorf tube followed by the addition of 70 µl of the standard solution (see above).
Sample Storage: Urine samples for each of the preparatory methods above (in triplicate) were stored in the 5 mm NMR tubes at room temperature (22°C), a refrigerator (4°C), or a deep freeze (-80°C) for the four-week duration of the study. Once a week samples were removed from storage and allowed to equilibrate to room temperature (roughly one hour) prior to NMR data acquisition. In order to investigate the effects of freeze-thaw cycles on metabolites found in the urine additional samples were prepared from the male and female subjects. Male and female urine samples were prepared as raw urine and another with 10 mM azide (see sample preparation outlined above); both were stored at -80°C and thawed twice a week.
NMR Analysis: All ¹H-NMR spectra were acquired on a 500 MHz Inova (Varian Inc.) spectrometer equipped with a 5mm triple resonance probe with z-axis pulsed field gradients. One-dimensional ¹H-NMR spectra were collected at 25°C using a standard presaturation pulse sequence (one-dimensional, transmitter pre-saturation delay of 1 s for water suppression, followed by a 8.4 µs 90° read pulse), and a spectral width of 8000 Hz. The time-domain data points were 64k, acquisition time was 4 s, repetition time was 5 s, 4 steady state scans, and the number of acquired scans was 128. The FID was apodized with an exponential window function corresponding to a line broadening of 0.5 Hz and Fourier transformed.
Statistical Analysis: Differences between variables were completed using t-tests and analysis of variance (ANOVA). A p-value of < 0.05 was considered significant.



Absolute concentrations of three random metabolites; benzoate, creatine, and lactate, in fresh (red) and filtered (dashed) normal female urine stored at room temperature (22°C) over a four-week period.



Concentration of citrate, hippurate, and glycine in fresh female urine stored at room temperature (red, 22°C) and in a deep-freeze (dashed, -80°C) over a four-week period.



Absolute concentrations for three random metabolites; benzoate, creatine, and lactate, measured in centrifuged female urine stored at room temperature (red, 22°C) and in a deep-freeze (dashed, -80°C) over a four-week period.

Initial	Acetate	Benzoate	Citrate	Creatine	Creatinine	Formate	Glycine	Hippurate	Lactate	Malonate	Succinate	Trimethylamine	Uracil	Urea
Raw	0.00	0.00	2.74	0.30	13.1	0.22	1.49	1.69	0.21	0.07	0.02	0.07	0.18	1000
Spun	0.00	0.00	2.87	0.30	13.1	0.22	1.49	1.69	0.21	0.07	0.02	0.07	0.05	1000
Filtered	0.00	0.00	2.58	0.27	12.7	0.22	1.40	1.54	0.21	0.07	0.02	0.07	0.06	42.5
0.1mM Azide	0.00	0.00	2.66	0.30	13.1	0.22	1.49	1.69	0.21	0.07	0.02	0.07	0.08	42.7
1.0mM Azide	0.00	0.00	2.74	0.30	13.4	0.22	1.49	1.69	0.23	0.05	0.02	0.07	0.05	1000
10mM Azide	0.00	0.00	3.03	0.30	14.4	0.22	1.61	1.95	0.26	0.07	0.02	0.07	0.05	1000
Week 4														
Raw	4.66	1.02	1.00	1.38	10.1	0.76	2.42	0.06	0.75	0.15	1.03	0.57	0.26	29.9
Spun	0.82	1.08	2.47	1.27	9.26	0.49	2.08	0.09	0.79	0.09	0.13	0.06	0.06	48.5
Filtered	0.20	0.09	2.16	1.17	9.44	0.24	1.09	1.76	0.23	0.09	0.17	0.05	0.06	40.7
0.1mM Azide	0.23	1.02	2.27	1.03	9.57	0.24	1.79	0.31	0.51	0.11	0.16	0.05	0.03	40.3
1.0mM Azide	0.22	1.02	2.44	1.18	10.1	0.25	2.03	0.44	0.31	0.06	0.18	0.06	0.07	45.1
10mM Azide	0.22	0.26	2.43	1.22	10.1	0.26	1.53	1.45	0.29	0.09	0.16	0.05	0.06	47.9

Table 1: Female Urine Preparation

Female urine samples prepared by different methods and stored at room temperature
 Metabolite concentrations reported as millimolar

Initial	Acetate	Benzoate	Citrate	Creatine	Creatinine	Formate	Glycine	Hippurate	Lactate	Malonate	Succinate	Trimethylamine	Urea
Room Temp	0.00	0.00	2.74	0.30	13.1	0.22	1.49	1.69	0.21	0.07	0.02	0.07	1000
Week 4													
Room Temp	4.66	1.02	1.00	1.38	10.1	0.76	2.42	0.06	0.75	0.15	1.03	0.57	29.9
Fridge	0.17	0.11	2.55	0.29	10.9	0.23	1.35	1.73	0.25	0.12	0.09	0.05	55.9
Deep Freeze	0.13	0.09	2.45	0.22	11.4	0.24	1.31	1.64	0.23	0.13	0.09	0.06	52.0
Freeze/Thaw	0.16	0.19	2.36	0.30	11.2	0.17	1.33	1.52	0.21	0.11	0.09	0.06	47.0

Table 2: Female Urine Storage

Raw female urine samples stored under different conditions
 Metabolite concentrations reported as millimolar

CONCLUSIONS

- Differences were noted in the metabolites that changed for male and female urine samples over the four-week period
- Spinning the urine in a counter top centrifuge reduced the degree of change of metabolites over the four weeks, but filtering the urine produced a greater reduction in the degree of metabolite change.
- Addition of increasing concentrations of the preservative sodium azide (0.1, 1, and 10 mM) had a correlated rise in efficacy of inhibition of metabolite changes over a four-week period.
- Storage of the urine in a refrigerator (4°C) produced a slight reduction in the degree of metabolite change when compared to room temperature (22°C), but storage in the deep freeze (-80°C) provided urine with a metabolite profile that best reflected the original metabolite concentrations
- The raw urine samples that underwent repeated cycles of freeze/thaw over the four-weeks had an intermediate degree of metabolite change when compared with raw urine stored at room temperature and in the deep-freeze
- Investigators must take appropriate measures to remove or inhibit bacterial influence upon the metabolic profile of the urine sample.

Acknowledgements

